DEVELOPMENT OF CARBON TETRACHLORIDE-INDUCED CHRONIC HEPATOTOXICITY MODEL IN RATS AND ITS APPLICATION IN EVALUATION OF HEPATOPROTECTIVE ACTIVITY OF SILYMARIN

GOPI H SHAH¹, BHARAT G PATEL², GAURANG B SHAH³

¹Department of Pharmacology and Toxicology, Ramanbhai Patel College of Pharmacy, Charotar University of Science and Technology, CHARIUSAT – Campus, Changa, Petlad, Anand - 388 421, Gujarat, India. ²Department of Pharmacology, Charotar University of Science and Technology, CHARIUSAT – Campus, Changa, Petlad, Anand - 388 421, Gujarat, India. ³Department of Pharmacology and Toxicology, K.B.Institute of Pharmaceutical Education and Research, Sector-23, GH-6, Gandhinagar, Gujarat, India. Email: ghshah17213@gmail.com

INTRODUCTION

The liver is the largest organ within the body, involved in metabolism of drugs. Among total liver mass, hepatocytes are involved in detoxification of a variety of drugs, vitamins, hormones, and environmental toxins and the metabolism of amino acids and ammonia, biochemical oxidation reactions. Kupffer cells are a reservoir of fixed macrophages, which play a protective role against gut-derived toxins that have escaped into the portal circulation. Endotoxins are mainly responsible for the production of cytokines. Other specific liver cell types also perform the unique function. The liver is the main organ in the first line of defense; it appears to be the most common target organ damaged by industrial chemicals [1]. Occupational and environmental liver diseases may present with a wide clinical spectrum ranging from asymptomatic liver enzyme elevation to acute liver failure, cirrhosis, and cancer. Hepatocellular necrosis may occur due to most industrial chemicals, including solvents, which in turn used to analyze liver function tests such as serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphate (ALP), direct bilirubin, total protein (TP), and albumin (Alb) levels. Along with liver functional tests, tests to check cholesterol, glucose, and malondialdehyde (MDA) were also performed. Liver fibrosis and cirrhosis was quantified by histopathological studies of small portion of the excised liver. Model was validated by repetition of the experiment. Intermediate dissection was carried out to measure an extent of liver damage.

RESULT:

Serum SGOT, SGPT, ALP, and direct bilirubin were found to be significantly higher in CCl₄ intoxicated rats. TP and Alb were decreased, and MDA was found to be significantly higher in CCl₄ intoxicated rats, which is the main end product of lipid peroxidation. Whereas in the treatment group silymarin improved the liver functions in CCl₄ intoxicated drug.

CONCLUSION:

We conclude that protein oxidation may play a role in the pathogenesis of CCl₄ induced liver injury. The accumulation of oxidized proteins may be an early indication of CCl₄ induced liver damage and silymarin found to be effective in liver injury by inhibiting protein oxidation.

Keywords: Liver fibrosis, Free radicals, Lipid peroxidation, Oxidative stress, Carbon tetrachloride, Liver biomarkers.

© 2017 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/) DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10i8.18701
recent years due to their diverse pharmacological properties, including antioxidant and hepatoprotective activity [8].

Silyburm or milk thistle was the most commonly used herbal treatment of liver diseases. Silymarin is the main active constituent. It has been reported that silymarin protects liver cells from a wide variety of toxins, mainly acetaminophen, ethanol, CCl₄, and D-galactosamine [9]. Silymarin produced its hepatoprotective effects through several mechanisms including antioxidation [10], inhibition of lipid peroxidation [11], enhanced liver detoxification via inhibition of Phase I detoxification and enhanced glucuronidation [12], and protection of glutathione (GSH) depletion [13].

Silymarin elicited a significant hepatoprotective activity by lowering the levels of serum marker enzymes and lipid peroxidation and elevated the levels of GSH, superoxide dismutase, catalase, albumin (Ab) and total protein (TP) in a dose dependant manner, which was confirmed by the decrease in the total weight of the liver and histopathological examination [14]. Silymarin increases hepatocyte protein synthesis and thus promoting hepatic tissue regeneration [15]. From the animal study, it was proven that silybin reduces the conversion of hepatic stellate cells into myofibroblasts, slowing or even reversing fibrosis [16].

The aim of the proposed work is to develop hepatotoxicity model (chronic) in rats and define chronicity of the disease by measuring various liver parameters and to evaluate hepatoprotective activity of silymarin.

MATERIALS AND METHODS

Materials

CCl₄ and Arachis oil were purchased from Loba Chemie Pvt Ltd., Mumbai. Silymarin suspension was from Microlabs Ltd., Bengaluru, Karnataka. Serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), bilirubin, TP, Alb, glucose, cholesterol, and Kits were purchased from Autospan (Span Diagnostic Pvt., Limited Surat).

EXPERIMENTAL PROTOCOL AND PROCEDURE

Animals

The experiment was performed after approval from the Institutional Animal Ethics Committee (IAEC). The approved protocol number is RPCP/IAEC/2013-2014/R-32. Healthy Wistar rats of either sex, weighing 200-250 g was taken during the experiment. The animals were housed in the polypropylene cage at 25°C; 12 hrs dark-light cycle, and thus promoting hepatic tissue regeneration [15]. From the animal study, it was proven that silybin reduces the conversion of hepatic stellate cells into myofibroblasts, slowing or even reversing fibrosis [16].

The controlled rats show normal liver architecture. They show normal hepatocytes, portal triads vasculature, and bile duct. Whereas rats which were given CCl₄ up to 35 days and 49 days show extensive liver damage (Figs. 1 and 2), respectively. Silymarin treated rats show normal liver architecture.

DISCUSSION

The liver is largest organ and is target for toxicity because of its role in clearing and metabolizing chemicals through the process called detoxification [18]. The most commonly used approach to induce toxin-mediated experimental liver fibrosis is the periodic administration of CCl₄ in mice or rats [19]. It was used extensively in animal models of acute hepatic failure in the seventies and early eighties. More recently, it has been used in the development of cirrhosis animal models following its gastric and intraperitoneal administration [20]. The most studies rely on the CCl₄-model to induce toxic liver fibrosis in mice due to the good comparability, excellent reproducibility, and moderate burden for the animals [19]. Histopathological sectioning of the liver tissues indicated that CCl₄ can induce fibrosis, cirrhosis, and hepatocarcinoma [21]. The toxic effect of CCl₄ was due to trichloromethyl radical produced during oxidative stress [22]. The liver injury induced by CCl₄ increases the number of infiltrated neutrophils, macrophages, Kupffer cells, lymphocytes and natural killer cells which further induced activation of liver resident macrophages and/or chemo attraction of extra hepatic cells (e.g., neutrophils and lymphocytes) [23]. Liver fibrosis, inflammation, and injury were induced by release of activated macrophages [24].
**Table 1: Change in various serum parameters in rats exposed to CCl₄ daily and CCl₄ alternate (for 35 days)**

| Group                                      | ALP         | ALT          | AST          | Bilirubin    | TP            |
|--------------------------------------------|-------------|--------------|--------------|--------------|---------------|
| CCl₄ treated (daily for 35 days)           | p=0.8941,   | p=0.9603,    | p=0.9812,    | p=0.8774,    | p=0.9653,     |
|                                            | 243.9+4.5   | 294.7±6.69   | 338±6.13     | 4.056±1.1    | 4.51±0.3090   |
| CCl₄ treated (alternate for 35 days)       | p=0.8941,   | p=0.9603     | p=0.9093     | p=0.0127     | p=0.9653,     |
|                                            | 253.9±5.6   | 280.9±6.68   | 336.2±6.12   | 0.4319±0.107 | 4.33±0.335    |
| CCl₄ treated (daily for 35 days) and normal control | p=0.0101,   | p=0.0030,    | p=0.0019,    | p=0.0043,    | p=0.0002,     |
|                                            | 97.89±7.44  | 341.3±3.81   | 81.03±4.467  | 0.0463±0.0093| 6.61±0.2105   |
| CCl₄ treated (alternate for 35 days) and normal control | p=0.0215,   | p=0.0034,    | p=0.0020,    | p=0.050,     | p=0.004,      |
|                                            | 97.89±7.44  | 341.3±3.81   | 81.03±4.467  | 0.0463±0.0093| 6.61±0.2105   |

**Table 2: Change in various serum parameters in rats exposed to CCl₄ daily and CCl₄ alternate (for 49 days)**

| Group                                      | ALP         | ALT          | AST          | Bilirubin    | TP            |
|--------------------------------------------|-------------|--------------|--------------|--------------|---------------|
| CCl₄ treated (daily for 49 days)           | p=0.9621,   | p=0.9686,    | p=0.2968,    | p=0.5220,    | p=0.981,      |
|                                            | 191.5±3.1   | 453.7±8.6   | 427.1±95.2   | 0.4391±0.083 | 4.91±0.4662   |
| CCl₄ treated (alternate for 49 days)       | p=0.9621,   | p=0.0014,    | p=0.0093,    | p=0.5220,    | p=0.981,      |
|                                            | 189±3.09    | 445.2±8.81   | 571.5±91.9   | 0.3671±0.070 | 4.90±0.3874   |
| CCl₄ treated (daily for 49 days) and normal control | p=0.0130,   | p=0.0005,    | p=0.0005,    | p=0.0005,    | p=0.0046,     |
|                                            | 98.9±6.378  | 33.86±3.231  | 83.67±4.611  | 0.0486±0.008 | 6.65±0.1812   |
| CCl₄ treated (alternate for 49 days) and normal control | p=0.0143,   | p=0.00057,   | p=0.0002,    | p=0.0008,    | p=0.0015,     |
|                                            | 98.9±6.378  | 33.86±3.231  | 83.67±4.611  | 0.0486±0.008 | 6.65±0.1812   |

**Table 3: Effect of CCl₄ and Silymarin on various biochemical parameters on wistar rats**

| Group                                      | ALP         | ALT          | AST          | Bilirubin    | TP            |
|--------------------------------------------|-------------|--------------|--------------|--------------|---------------|
| CCl₄ treated                               | p=0.9621,   | p=0.9686,    | p=0.2968,    | p=0.5220,    | p=0.981,      |
|                                            | 191.5±3.1   | 453.7±8.6   | 427.1±95.2   | 0.4391±0.083 | 4.91±0.4662   |
| Silymarin treated                          | p=0.0143,   | p=0.00057,   | p=0.0002,    | p=0.0008,    | p=0.0015,     |
|                                            | 98.9±6.378  | 33.86±3.231  | 83.67±4.611  | 0.0486±0.008 | 6.65±0.1812   |

**Data were expressed as mean±SEM (n=6). Significant difference was indicated by p<0.5 compared with normal control group. Here, p value between CCl₄ daily and CCl₄ alternate group is not<0.5, which indicates no significant difference in between this group. ALP: Alkaline phosphate, ALT: Alanine transaminase, AST: Aspartate transaminase, TP: Total protein, MDA: Malondialdehyde, CCl₄: Carbon tetrachloride, SEM: Standard error of the mean.**

**Table 3: Effect of CCl₄ and Silymarin on various biochemical parameters on wistar rats**

| Group                                      | ALP         | ALT          | AST          | Bilirubin    | TP            |
|--------------------------------------------|-------------|--------------|--------------|--------------|---------------|
| CCl₄ treated                               | p=0.9621,   | p=0.9686,    | p=0.2968,    | p=0.5220,    | p=0.981,      |
|                                            | 191.5±3.1   | 453.7±8.6   | 427.1±95.2   | 0.4391±0.083 | 4.91±0.4662   |
| Silymarin treated                          | p=0.0143,   | p=0.00057,   | p=0.0002,    | p=0.0008,    | p=0.0015,     |
|                                            | 98.9±6.378  | 33.86±3.231  | 83.67±4.611  | 0.0486±0.008 | 6.65±0.1812   |

**Data were expressed as mean±SEM (n=6). Significant difference was indicated by p<0.5 compared with normal control group. Here, p value between normal control and CCl₄ treated group, CCl₄ treated group and silymarin treated group is <0.5, so they were statistically significant. ALP: Alkaline phosphate, ALT: Alanine transaminase, AST: Aspartate transaminase, TP: Total protein, MDA: Malondialdehyde, CCl₄: Carbon tetrachloride, SEM: Standard error of the mean.**
For acute liver injury rats were administered a single dose of CCl₄ 2 ml/kg of body weight which leads to liver fibrosis. The chronic dose of CCl₄ was 2 ml/kg of body weight twice per week which result in liver fibrosis [25]. In this study, the development of chronic model rats was administered 1.0 ml/kg of body weight of CCl₄ i.p. for 10 days daily and then after 1 ml/kg twice a week up to 7-8 weeks.

**Serum aminotransferase enzymes**

Upon cellular membrane damage and leakage serum activities were increased [26]. In all types of liver injury, serum aminotransferase activities were increased. Serum estimation of SGPT was specific for the liver tissue so in liver cell injury serum SGPT was of greater value, whereas SGOT level may increase in acute necrosis or ischemia of other organs such as the myocardium, besides the liver cell injury.

In this study, serum AST, ALT, ALP, and bilirubin activities were greatly increased (p<0.05) in rats exposed to CCl₄ as compare to normal control. The increased serum levels of hepatic markers have been attributed to the liver injury because these enzymes are placed in the cytoplasmic area of the cell and are released into circulation in case of cellular damage [27].

Zimmerman et al. stated that serum level of ALP, AST, ALT, and bilirubin was increased by mitochondrial damage in liver cells this damage was induced by CCl₄ [28]. The activities of liver enzymes were significantly elevated after CCl₄ treatment this was reported by many authors [29,30].

Binita et al. reported that chronic liver fibrosis was induced in Wistar rats by oral administration of CCl₄ for 7 weeks [31], whereas in the present study liver fibrosis was induced within 5 weeks. The proposed animal model in this study fulfilled the criteria described by Terblanche and Hickman [32].

It has been reported that silymarin stabilizes the membrane of hepatocytes, preventing toxins from entering the cell through enterohepatic recirculation, regenerates liver by stimulating nucleolar polymerase A and increasing ribosomal protein synthesis [33]. Silymarin comprises a strong antioxidant and anti-inflammatory potential [33]. Silymarin inhibits hepatic lipid peroxidation, thus it protects against membrane damage. Toxicity effect of CCl₄ was controlled by restoration of serum bilirubin, proteins and enzymes [34].

In the present study, silymarin improves liver function. It decreases liver SGPT, SGOT, ALP, Bilirubin, MDA, and globulin level. Silymarin also controls TP and Alb level. Silymarin helps in regenerating liver tissue, controls inflammation, enhances glucuronidation, and protects against GSH depletion. The non-traditional use of silymarin protects other organs along with the liver. Silymarin also prevents the absorption of toxins into hepatocytes by occupying to the binding site and also inhibits the transport of protein into the membranes [35].

**Histopathological investigations**

The histopathological evaluation of CCl₄-induced toxicity in all groups at the different time intervals was examined. The normal control group (Fig. 3) shows no abnormalities, whereas in CCl₄ treated group (both day 35 and day 49) shows liver abnormalities. On day 35, liver fibrosis (Fig. 1) was seen and on day 49, liver cirrhosis was seen (Fig. 2). Silymarin treated animals show normal liver architecture (Fig. 4).

**CONCLUSION**

From the results, it was concluded that CCl₄ has potential to cause chronic liver injury (fibrosis and cirrhosis). The proposed animal model of chronic liver injury might be suitable for screening of drugs used to treat liver injury and associated disorders. Here, silymarin as a standard drug shows hepatoprotective effect in the developed model.

**ACKNOWLEDGMENTS**

The author is thankful to Dr. B.G. Patel for his scientific advice. The author wants to thank Ms. Gopi Shah for her writing assistance.
Fig. 4: Did not reveal any specific changes (carbon tetrachloride [CCl4]+silymarin) (CCl4 1 ml/kg+silymarin suspension 3 ml/animal)

REFERENCES

1. Banrida W, Juliane IB, Heather BC, Bellis JH, Cameron FK, Craig JM, et al. Toxicant-associated steatohepatitis. Toxicol Pathol 2013;41:343-60.
2. Cave M, Falkner KC, McClain CJ. Occupational and environmental liver disease. In: Zakim and Boyer’s Hepatology: A Textbook of Liver Disease. Philadelphia, PA: Elsevier Saunders; 2011. p. 476-92.
3. Friedman SL. Liver fibrosis. J Hepatol 2003;38(1):S38-53.
4. Bigoniya P, Singh CS, Shukla A. A comprehensive review of different hepatotoxicity by a protein isolated from the leaves of the herb Cajanus Cajanus. Int J Pharm Sci 2011;3:151-4.
5. Renner H. The limited relevance of models used for testing human hepatic diseases and their prevention. In: Kepler E, Popper H, Reinhard E, editors. Mechanisms of Hepatocyte Injury and Death. Lancaster: MTP Press Ltd.; 1985. p. 491-60.
6. Cotran RS, Kumar V, Robbins SL. Genetic disorders. In: Cotran RS, Kumar V, Robbins SL, editors. Pathologic Basis of Disease. Vol. 5. Philadelphia, PA: WB Saunders Co.; 1994. p. 433-43.
7. Kaplowitz N, Aw TY, Simon FR, Stolz A. Drug-induced hepatotoxicity. Ann Intern Med 1986;106(6):826-39.
8. Ranganathan R, de la Calle M, Sanyal AJ. Impact of cytokines on liver fibrosis - Role of cytokines and different cell types. Z Gastroenterol 2013;6(2):171-80.
9. Shah et al. Protective effect of Panax ginseng against serum biochemical changes and apoptosis in liver of rats treated with carbon tetrachloride (CCL4). J Hazard Mater 2011;195:208-13.
10. Stoyanovsky DA, Cederbaum AI. Metabolism of carbon tetrachloride to trichloromethyl radical: An ESR and HPLC-ECD study. Chem Res Toxicol 1999;12(8):730-6.
11. Ramadori G, Saile B. Portal tract fibrogenesis in the liver. Lab Invest 2004;84:154-9.
12. Saile B, Ramadori G. Inflammation, damage repair and liver fibrosis - Role of cytokines and different cell types. Z Gastroenterol 2007;45(1):77-86.
13. Yu C, Wang F, Jin C, Wu X, Chan WK, McKeehan WL. Increased carbon tetrachloride-induced liver injury and fibrosis in FGFFR4-deficient mice. Am J Pathol 2002;161(6):2003-10.
14. Kaplan MM. Laboratory tests. In: Schiff L, Schiff ER, editors. Diseases of the Liver. Vol. 7. Philadelphia, PA: JB Lippincott; 1993. p. 108-44.
15. Brent JA, Rummack BH. Role of free radicals in toxic hepatic injury. II. Are free radicals the cause of toxin-induced liver injury? J Toxicol Clin Toxicol 1993;31(1):173-96.
16. Zimmerman HJ, Kedora Y, West M. Effects of carbon tetrachloride poisoning on the plasma levels of cytoplasmic and mitochondrial enzymes in animals with nutritional fatty metamorphosis. J Lab Clin Med 1965;66:324-33.
17. Mehmetçik G, Ozdemirler G, Kockak-Toker N, Cevikbas U, Uysal M. Role of carnosine in preventing thiocarbamide-induced liver injury in rats. Peptides 2008;29(3):425-9.
18. Saile B, Ramadori G. Inflammation, damage repair and liver fibrosis - Role of cytokines and different cell types. Z Gastroenterol 2013;6(2):171-80.
19. Madiwala R, Reddy KM, Rao VR, Patil G. Acute and chronic effects of a synthetic analogue on isolated rat hepatic stellate cells and myofibroblasts. Arzneimittel-Forschung 1997;46:643-9.
20. Khan AA, Alzhohairy M. Hepatoprotective effects of camel milk against CCl4-induced hepatotoxicity in Rats. Asian J Biochem 2011;6(2):171-80.
21. Kuzma DM, Alzohairy M. Hepatoprotective effects of camel milk against CCl4-induced hepatotoxicity in rats. Asian J Biochem 2011;6(2):171-80.
22. Craig JM, et al. Protective effect of Panax ginseng against serum biochemical changes and apoptosis in liver of rats treated with carbon tetrachloride (CCL4). J Hazard Mater 2011;195:208-13.
23. Stoyanovsky DA, Cederbaum AI. Metabolism of carbon tetrachloride to trichloromethyl radical: An ESR and HPLC-ECD study. Chem Res Toxicol 1999;12(8):730-6.
24. Ramadori G, Saile B. Portal tract fibrogenesis in the liver. Lab Invest 2004;84:154-9.
25. Saile B, Ramadori G. Inflammation, damage repair and liver fibrosis - Role of cytokines and different cell types. Z Gastroenterol 2007;45(1):77-86.
26. Yu C, Wang F, Jin C, Wu X, Chan WK, McKeehan WL. Increased carbon tetrachloride-induced liver injury and fibrosis in FGFFR4-deficient mice. Am J Pathol 2002;161(6):2003-10.
27. Kaplan MM. Laboratory tests. In: Schiff L, Schiff ER, editors. Diseases of the Liver. Vol. 7. Philadelphia, PA: JB Lippincott; 1993. p. 108-44.
28. Brent JA, Rummack BH. Role of free radicals in toxic hepatic injury. II. Are free radicals the cause of toxin-induced liver injury? J Toxicol Clin Toxicol 1993;31(1):173-96.
29. Zimmermann HJ, Kedora Y, West M. Effects of carbon tetrachloride poisoning on the plasma levels of cytoplasmic and mitochondrial enzymes in animals with nutritional fatty metamorphosis. J Lab Clin Med 1965;66:324-33.
30. Mehmetçik G, Ozdemirler G, Kockak-Toker N, Cevikbas U, Uysal M. Role of carnosine in preventing thiocarbamide-induced liver injury in rats. Peptides 2008;29(3):425-9.
31. Arsic OF, Cetin N. Protective role of ghrelin against carbon tetrachloride (CCl4) induced coagulation disturbances in rats. Regul Pept 2011;166:139-42.
32. Binita S, Mehub C, Vantid T. Anti-fibrotic effect of heparin, silymarin and its combination on liver fibrosis model in rats. J Pharm Res Opin 2011;6:180-6.
33. Terblanche J, Hickman R. Animal models of fulminant hepatic failure. Dig Dis Sci 1991;36:770-4.
34. Mayer KE, Myers RP, Lee SS. Silymarin treatment of viral hepatitis: A systematic review. J Viral Hepat 2005;12:559-67.
35. Zaitoun Y. Protective effect of Panax ginseng against serum biochemical changes and apoptosis in liver of rats treated with carbon tetrachloride (CCL4). J Hazard Mater 2011;195:208-13.
36. Sonnenbichler J, Zell I. Biochemical effects of the flavonolignane silybin on RNA, protein and DNA synthesis in rat livers. In: Cody V, Middleton E, Harbourne JB, editors. Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological, and Structure-Activity Relationships. New York, NY: Alan R. Liss; 1986. p. 319-31.