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Mathew Jacob
M.V.Sc., Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode, Wayanad, Kerala, India

S Suja Rani
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

R Shankar
M.V.Sc. Scholar, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Archana Raj
M.V.Sc. Scholar, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

S Sujith
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Corresponding Author
S Suja Rani
Assistant Professor, Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

An experiment-based approach for selecting optimal dosage of carbon tetrachloride for research studies on fatty liver disease

Mathew Jacob, S Suja Rani, R Shankar, Archana Raj and S Sujith

Abstract
The animal models for fatty liver disease are of utmost significance, particularly for the effective evaluation of the lipotropic activity of various agents. It can be induced by multiple methods, amongst which, CCl_4 treatment can effectively induce hepatic steatosis, although the diverse dose and duration of CCl_4 administration may engender various degrees of hepatic damage ranging from mild degeneration to fibrosis and/or necrosis. Thus, it necessitates an experiment-based approach for selecting optimal dosage of CCl_4 for the rapid and reproducible induction of fatty liver in rats with no overlapping of hepatic necrosis or fibrosis. Accordingly, 30 male albino Wistar rats were randomly allocated to five groups; Group I was injected with olive oil at 1.0 ml/kg body weight subcutaneously (SC) and served as vehicle control, while Groups II to V were administered SC with CCl_4 mixed with olive oil (1:1) at dose levels of 0.5, 1.0, 1.5 and 2.0 ml per kg body weight respectively for four consecutive days. The animals were sacrificed after 72 hours of the last dose and the hepatic as well as serum lipid levels were estimated. Serum biomarkers of hepatic function and histopathology of liver were also assessed at the end of experiment. Amongst the CCl_4 treated groups, CCl_4 at 1.0 ml per kg dose level has effectively elevated the hepatic and serum triglyceride and cholesterol levels with a moderate degree of centrilobular hepatic fatty infiltration, even in the absence of any advanced degree of hepatic necrosis and fibrosis. Besides, it significantly augmented the levels of serum ALT, AST and ALP, thereby indicating 1.0 ml/kg to be the optimal dosage of CCl_4 for effective induction of fatty liver in male albino Wistar rats.

Keywords: Animal model, CCl_4, Fatty liver, Hepatic fatty infiltration

Introduction
Being a vital organ, liver is responsible for the maintenance of many metabolic functions and detoxification of exogenous and endogenous compounds. Accumulation of excessive fat in the liver is the common denominator in the two most prevalent types of chronic liver diseases, alcoholic fatty liver disease (AFLD) and non-alcoholic fatty liver disease (NAFLD); both of which are global public health concerns [1]. Hepatic steatosis or fatty liver, the early pathological change associated with hepatic ailments, is the accumulation of large vacuoles of triglycerides/lipid within the cytoplasm of hepatocytes [2]. If left untreated, it may progress to steatohepatitis and more severe disease pathologies such as fibrosis and cirrhosis. Steatosis is a crucial step in the advancement of hepatic disorders as its attenuation is reversible and has been proclaimed to prevent further progression of liver damage [3].
Considering the high prevalence of liver disease worldwide and the lack of preclinical alternatives, animal models are essential to further elucidate the pathophysiology of hepatic steatosis. However, the lack of standardisation in the methods used to generate steatosis and steatohepatitis in animals is a problem in studies involving fatty liver disease. The major fatty liver animal models available includes genetically engineered mice which predispositions to obesity and fatty liver and a variety of diet-induced models using high-energy diets containing various sugars and fats as well as choline and/or methionine deficient diets that accelerated and accentuated the disease process. Although the use of these methods separately and in combination has yielded a lot of information, it's still unclear whether they are closely resembling to that of human NAFLD [4]. Moreover, with regard to the diet-induced models, long-term feeding may be warranted for the effective induction of fatty liver.
Carbon tetrachloride (CCl_4) is a chemical agent widely used for the experimental induction of liver injury and fibrosis in laboratory animals including fatty liver, especially as an acute or subacute model. However, there is a major limitation that there is no demarcation between the various stages of hepatotoxicity [5]. This is due to the lack of previous studies investigating the

ideal dose of CCl_4 for the effective induction of hepatic steatosis in laboratory animals, which has been mostly carried out based on the individualised observation of CCl_4 induced fatty liver/hepatotoxicity condition. Thus, there is a huge difference between the dose and duration of exposure of CCl_4 between various animal studies which causes a negative impact on experiments. The absence of standardisation of CCl_4 dosage for the effective induction of fatty liver can affect the quality, repeatability and reproducibility of research studies especially on lipotropes, which are the agents used to mitigate liver lipids [6]. Moreover, an acute or subacute animal model is mandatory for screening of the arsenal of potential therapeutic agents, to identify the lead compounds. The present study was, therefore, conducted with the objective of identifying and selecting an ideal dose and duration of CCl_4 exposure for the rapid and reproducible induction of fatty liver with no overlapping of hepatic necrosis and fibrosis, for the research studies on fatty liver disease.

Material and Methods

Animals

Six to eight weeks old, 30 male albino Wistar rats were procured from Small Animal Breeding Station, Mannuthy and housed in well-ventilated cages with free access to fresh water and laboratory animal feed. The animals were maintained under 12 h/12 h light/dark cycle at 25±2°C with 30-70% relative humidity and were acclimatised for two weeks before experimentation. The experimental protocol was approved by the Institutional Animal Ethics Committee, College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University.

Drugs and Chemicals

All the chemicals used in the study were of analytical grade and procured from Merck and Himedia, India. The diagnostic kits for the present study were procured from Span Diagnostics Ltd., India.

Experimental Design

After the acclimatisation period, the rats were randomly allocated to five groups of six animals each. Group I was injected with olive oil at 1 ml/kg body weight subcutaneously (SC) as vehicle control. Groups II to V were administered with CCl4 mixed with olive oil in the ratio 1:1 at the dose levels of 0.5, 1.0, 1.5 and 2.0 ml per kg body weight respectively through the SC route. The dosing was carried out for four consecutive days. The grouping of experimental animals with respective treatments is detailed in table 1.

Table 1: Group design of animals with their respective treatments

Group	Treatment
I	Vehicle control - olive oil at 1.0 ml/kg SC
II	Animals receiving CCl_4 in olive oil at 0.5 ml/kg SC
III	Animals receiving CCl_4 in olive oil at 1.0 ml/kg SC
IV	Animals receiving CCl_4 in olive oil at 1.5 ml/kg SC
V	Animals receiving CCl_4 in olive oil at 2.0 ml/kg SC

Biochemical Analyses of Serum Enzymes and Lipid Profile

Blood was collected from the retro-orbital plexus under anaesthesia at the end of the experiment i.e., after 72 hours of the last dose of CCl_4. Serum was separated for the estimation of serum biochemical parameters such as triglycerides (TG) and total cholesterol (TC), as well as enzymes like alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) as markers of liver injury using diagnostic kits.

Lipid Profile and Histopathological Examination of Liver

The animals were sacrificed at the end of the experiment and the liver was excised, cleaned of extraneous tissue and examined for gross lesions. Liver tissues were collected for the estimation of liver lipids such as liver TG and TC using diagnostic kits. Representative samples of the liver were also collected in 10% neutral buffered formol saline and processed for the histopathological examination [7].

Statistical Analysis of Data

All the results were represented as Mean ± SEM and statistical analysis were performed by Statistical Package for Social Sciences (SPSS) version 24. The data were analyzed using one-way analysis of variance (ANOVA) with Bonferroni or Dunnett T_3 method as a post hoc test. Statistical significance was set at $p \leq 0.05$.

Results

Estimation of Serum and Hepatic Lipids

All the groups with CCl_4 treatment i.e., Group II to V, showed a significant increase in the serum TG and TC in a dose dependent manner as compared to the normal control, Group I. Moreover, Group III, IV and V showed a significant increase in the liver TG and TC levels with a nonsignificant increase been observed in Group II as compared to normal control. The terminal serum lipid and hepatic lipid levels of different groups of rats are presented in table 2.

Assessment of Serum Marker Enzymes

The serum marker enzymes *viz.*, ALT, AST, ALP of different groups of rats is presented in table 2. Group III, IV and V showed a significant increase in serum ALT and AST levels in a dose dependent manner as compared to normal control. However, a non-significant increase in serum ALT and AST was observed in animals treated with CCl_4 at 0.5 ml/kg (Group II) when compared to normal control. Furthermore, the serum ALP showed a significant increase in animals treated with CCl_4 at 2.0 ml/kg, when compared to normal control group. There was a non-significant increase in serum ALP levels in animals treated with all the other tested dose levels of CCl_4 as compared to normal control.

Table 2: Effect of different dose levels of CCl_4 on serum and hepatic lipids, mg/dl

Group	Serum lipids		Hepatic lipids	
	Serum TG	Serum TC	Liver TG	Liver TC
I	95.55±1.88	41.45±0.50	256.28±11.82	180.23±17.83

II	156.42±5.46[a]	66.00±3.44[a]	300.68±10.35	200.80±25.51
III	173.00±8.94[a]	76.97±0.61[a]	322.25±5.80[a]	293.97±20.94[a]
IV	186.05±3.63[a]	105.42±1.16[a]	350.57±12.23[a]	327.87±26.29[a]
V	217.67±9.28[a]	192.78±4.86[a]	504.97±18.31[a]	487.17±15.79[a]

Values are expressed as Mean ± SEM; n=6; TG: triglycerides; TC: cholesterol;
[a] $p \leq 0.05$, CCl_4 treated groups Vs Normal control

Table 3: Effect of different dose levels of CCl_4 on serum marker enzymes, IU/L

Groups	ALT	AST	ALP
I	153.50±2.34	116.80±3.81	217.73±10.54
II	166.80±2.61	144.90±8.14	347.08±17.17
III	176.98±2.27[a]	171.67±2.66[a]	373.03±25.37
IV	181.90±5.57[a]	215.60±20.20[a]	413.40±27.33
V	203.93±4.50[a]	246.22±16.04[a]	493.42±30.55[a]

Values are expressed as Mean ± SEM; n=6; ALT: Alanine amino transferase; AST: Aspartate amino transaminase; ALP: Alkaline phosphatase; [a] $p \leq 0.05$, CCl_4 treated groups Vs Normal control

Gross pathology

No gross pathological changes were observed in the normal control group whereas, livers of CCl_4 treated animals at 0.5 ml/kg showed minimal paleness and at 1.0 ml/kg showed mild to moderate degree of paleness. Severe diffuse paleness was observed in livers of animals treated with 1.5 and 2.0 ml/kg of CCl_4 and their edges were rounded. Moreover, animals treated with 2.0 ml/kg dose level of CCl_4 showed reduction in size of the liver (Fig. 1).

Histopathology

There were no alterations in the liver of animals from normal control group showing normal hepatic morphology, normal cords of hepatocytes around central vein and portal triad area. Animals treated with CCl_4 at 0.5 ml/kg showed minimal centrilobular fatty infiltration, with hepatocytes showing cytoplasmic vacuolations of varying sizes.

Livers from animals treated with CCl_4 at 1.0 ml/kg showed mild to moderate degree of centrilobular hepatic steatosis with macrovesicular and microvesicular types of vacuolations while no necrosis was observed. On examination, diffused severe cytoplasmic vacuolation predominantly of macrovesicular type was observed in CCl_4 treated animals at 1.5 and 2.0 ml/kg dose levels with mild to moderate degree of centrilobular hepatic necrosis (Fig. 2). Fat specific stain (Oil-red O) confirmed the presence of fatty change in the representative liver samples of CCl_4 treated groups. The presence of fatty change in the liver sample of CCl_4 treated groups at 1.0 ml/kg under Oil- red O staining is represented in figure 2F.

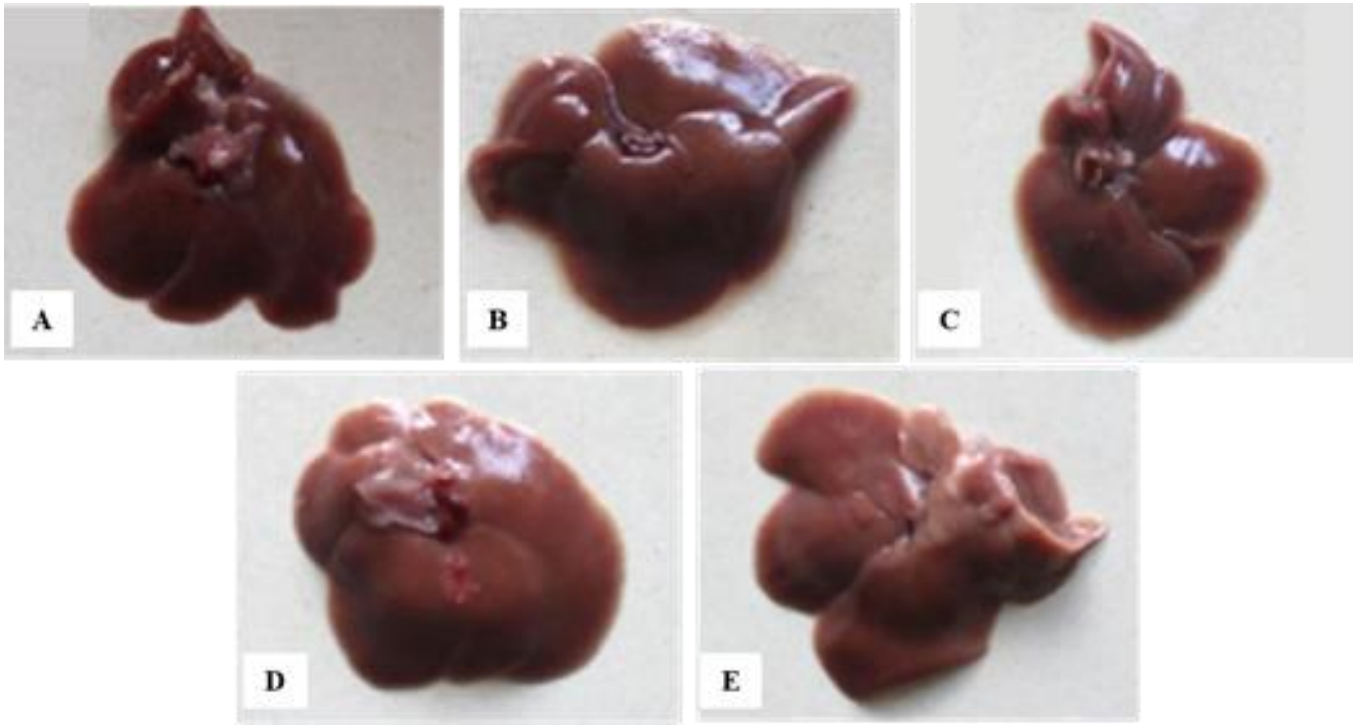

Fig 1: Gross appearance of liver of male albino Wistar rats with different doses of CCl_4 treatment A: Normal control rat; B: CCl_4 at 0.5 ml/kg; C: CCl_4 at 1.0 ml/kg; D: CCl_4 at 1.5 ml/kg; E: CCl_4 at 2.0 ml/kg

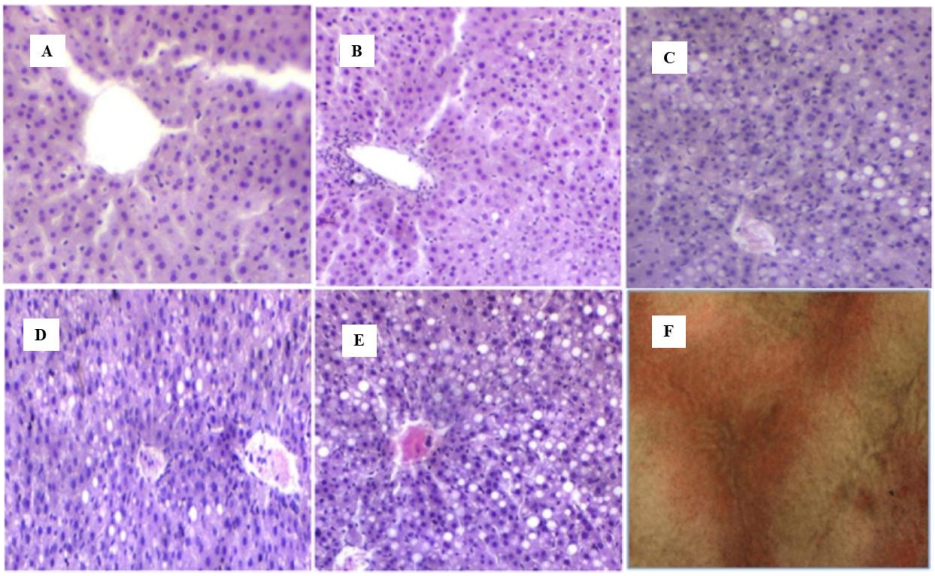

Fig 2: Section of liver of male albino Wistar rats with different doses of CCl_4 treatment A: Normal control rat; B: CCl_4 at 0.5 ml/kg; C: CCl_4 at 1.0 ml/kg; D: CCl_4 at 1.5 ml/kg; E: CCl_4 at 2.0 ml/kg; f- Oil- red O stain of liver sample of animal treated with CCl_4 at 1.0 ml/kg

Discussion

Carbon tetrachloride, a chemical used frequently to induce experimental liver toxicity engender hepatic steatosis, necrosis, fibrosis and carcinogenicity depending on the dose and duration of exposure, or time of observation [5]. At low concentrations of CCl_4, transitory effects like Ca^{2+} sequestration loss, lipid homeostasis disruption, release of noxious or beneficial cytokines and apoptosis, followed by regeneration occurs. Furthermore, consequences such as fatty degeneration and inflammatory cells infiltration followed by irreparable damages like fibrosis, cirrhosis and even cancer may be developed over chronic exposure of CCl_4. In addition, hepatic necrosis exceeds the regenerating capacity of liver at high acute toxic levels of CCl_4 and results in fatal liver failure. Nonspecific solvent toxicity, involving central nervous system depression and respiratory failure with death, can also be precipitated by increased doses of CCl_4 [5,8]. Nevertheless, almost similar stages of hepatic damage have been reported in various stages of NAFLD as well.

However, amongst the different advancing stages of fatty liver diseases, only the initial stages alone are repairable or reversible, while the damages associated with the subsequent stages are quite irreparable or irreversible [9]. Hence, for developing potential therapeutic agents that are presumed to mitigate fatty liver disease, the compounds need to be initially screened for their lipotropic activity, for which pre-clinical animal models are imperative. Although several high energy diet models are available for fatty liver induction, they are time consuming and are not that desirable especially for screening purpose as it warrants acute or subacute models of fatty liver disease.

Even though, CCl_4 is taken as a model substance to induce hepatotoxicity for a multitude of research studies, an experiment-based approach has not been carried out yet, regarding the dose and duration of CCl_4 to effectively induce hepatic steatosis alone in laboratory animals. Therefore, different dose levels of CCl_4 (0.5, 1.0, 1.5 and 2.0 ml/kg b.wt.) were tested to finalize the ideal dose for the induction of fatty liver in male albino Wistar rats. All the rats were exposed to these specific doses SC for a period of four consecutive days.

In the current study, CCl_4 treatment significantly increased not only the serum TG and TC levels in a dose dependent manner, but also the liver TG and TC levels in Group III to V alongwith a nonsignificant increase as observed in Group II. El-Hadary *et al.* [12] reported a similar increase in the total lipid, TC and TG in rats treated with CCl_4 with olive oil (1:1) at a dose of 1 ml/kg bodyweight for 8 weeks by gastric gavage.

CCl_4 induced lipotoxicity in liver develops due to the formation of active metabolite trichoromethyl radical (CCl_3^-) and its derivative trichloromethylperoxy radical (CCl_3OO^-) due to the biotransformation of CCl_4 by cytochrome P-450 enzymes in the endoplasmic reticulum [10,11]. The activated free radicals cause peroxidative degradation of membrane lipids in the endoplasmic reticulum and cause formation of lipid peroxides that pave the way to the fatty liver condition. The probable biochemical disruptions that act synergistically for the fatty liver induction on CCl_4 exposure include mitochondrial damage, augmented cytokine synthesis, diminished hepatic lipid outflow and insulin resistance [3]. Hence, the fatty liver observed in the CCl_4 treated rats in this study can be attributed to these effects even though the mechanisms have not been explored in the present study.

The liver produces several enzymes, which are normally dispersed throughout the hepatocytes. The free radical produced by the biotransformation of CCl_4 disrupts the intact lipid-bilayer structure of the membrane, allowing a detectable amount of these enzymes such as ALT, AST and ALP to escape into the extracellular fluid. The enzyme is released into circulation as a result of necrosis or membrane damage and it may thus be detected in the serum. The elevated levels of these enzymes are indicative of cellular leakage as well as loss of functional integrity of the cell membrane in liver and can be taken as the sensitive biomarkers of liver toxicity [11,13,14]. Thus, the significantly elevated levels of serum biomarkers in all the CCl_4 treated rats of this study also accords on CCl_4 induced hepatotoxicity [8,12,15].

Altogether the study indicated that all the selected doses of CCl_4 in the present work, especially 1.0, 1.5 and 2.0 ml/kg dose levels, effectively induced hepatotoxicity with lipidosis in male albino Wistar rats. On histopathological examination, livers from animals treated with CCl_4 at 1.0 ml/kg showed moderate degree of macrovesicular and microvesicular

hepatic steatosis, without any lesions of necrosis, whereas CCl_4 at 1.5 and 2.0 ml/kg dose levels showed mild to moderate degree of centrilobular hepatic necrosis along with steatotic changes. Consequently, the dose of CCl_4 for the induction of fatty liver was standardised as 1 ml/kg b.wt. in male albino Wistar rats, owing to the fact that at higher dose levels of CCl_4 even though hepatic steatosis was effectively induced, centrilobular hepatic necrosis was also been produced additionally, which was not an intended outcome. This was in accordance with the work of Bagban *et al.* [16] who reported that rats treated with CCl_4 with olive oil (1:1) at a dose 2.0 ml/kg b.wt. S.C. produced varying degree of fatty degeneration including ballooning of hepatocytes, infiltration of lymphocytes and loss of cellular boundaries.

Conclusion

The prevalence of fatty liver (hepatic steatosis) is mounting enormously in both domestic species and humans and seems to be a great challenge to modern allopathic medicine. Hence, a suitable animal model with fatty liver condition is warranted for screening various lipotropic agents. The present study provides an ideal dose of CCl_4 for the effective induction of fatty liver on a short-term period without any advanced degree of hepatotoxicity like necrosis, fibrosis and others in male albino Wistar rats. The results suggested that amongst the different dose levels of CCl_4 tested, administration of CCl_4 mixed with olive oil (1:1) at 1.0 ml per kg body weight SC for four consecutive days was the optimum dose of CCl_4 that has effectively induced significant elevation of hepatic triglyceride and cholesterol concentration with moderate degree of centrilobular hepatic infiltration as compared to normal control, while sans any histological manifestation of fibrotic and cirrhotic changes and hence was concluded as the ideal dose of CCl_4 for the effective induction of fatty liver in male albino Wistar rats.

Conflict of Interests

The authors declare no conflict of interests.

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