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Research Paper

PROTEIN AND BIOMARKER INVESTIGATION IN HEPATIC DYSFUNCTION IN CCL_4 INDUCED HEPATIC DAMAGE IN RATS

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We began this series of investigations by performing a dose response study examining for possible extra-hepatic sites of CCl_4-induced damage. In this research we defined the ideal dose level of CCl_4 for the induction of hepatotoxicity to be 2.0 mL/kg. Above this dose level results from serum and urine clinical chemistry as well as histopathological examination of kidney tissue samples revealed evidence of nephrotoxicity consistent with CCl_4 administration. Therefore, in subsequent CCl4 studies, we kept the dose level administered to the rat below this threshold value. Our comparative study of CCl_4-induced toxicity (7 and 13 weeks old respectively) revealed no significant differences in the severity of liver damage as confirmed by histopathological examination.1H NMR analysis of urine samples in this study contributed further to our knowledge of the metabolite changes seen at 24 hours post-dosing as described in Chapter 3. Taurine, citrate, 2-oxoglutarate and hippurate were considered the most sensitive urinary markers and all showed a general change in the first 18-24 hours followed by an opposing change in urinary levels during the liver regeneration phase.

Key Words: CCl_4, Histopathological examination, Taurine, 1H NMR analysis.

INTRODUCTION

Herbal drugs play an important role in healthcare programmes worldwide, mainly due to the general belief that they are without any side effects, besides being cheap and locally available. Ayurveda is a perfect science of life; it works effectively fighting against various infections and diseases and thereby gaining quick recovery. Lately there is a resurgence of interest in herbal medicines for treatment of various ailments including liver disorders.Liver, the largest organ in the vertebrate body - is the major site of intense metabolic activities such as drug and xenobiotic metabolism. Liver injury caused by toxic chemicals and certain drugs has been recognized as a crucial toxicological problem. In the present world a large number of toxins are introduced daily. So it is more important than ever to keep the liver healthy and potent. The most important metabolic function of liver is the detoxification and excretion of toxic chemicals, drugs and hormones. Liver tissue has the capacity to regenerate, so a moderate cell injury is not reflected by measurable change in its metabolic function. Due to the high tolerance of liver, liver disease is seldom detected at the early stage and once detected treatment faces a poor prognosis in most cases.

Till date, there is no effective medicine for hepatic disorders, such as hepatic fibrosis and hepatocellular carcinoma. Many plants have been reported for their antioxidant and hepatoprotective activity and are used in ayurvedic system of medicine for the treatment of liver disorders.

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Woodfordiafruticosa is a traditional medicinal plant and its flowers are used for the preparation of fermented drugs and for the treatment of various disorders such as dysentery, sprue, rheumatism, hematuria,hemorrhoids, derangement of liver, disorders of mucous membrane etc. All parts of the plant possess valuable medicinal properties vizanti inflammatory, anti tumour, hepatoprotective and free radical scavenging activity but flowers are in maximum demand. But still there is a paucity of information regarding the potential of *Woodfordiafruticosa* flowers in resisting oxidative stress, hepatic fibrosis and hepatocellular carcinoma. Hence, this study was undertaken with the following

Liver - Structure and functions

The liver is the largest gland in the body, and is situated slightly below the diaphragm and anterior to the stomach. It consists of two lobes which are wedge-shaped. Two blood vessels enter the liver, namely the hepatic portal veinwith dissolved food substances from the small intestine and the hepatic artery with oxygenated blood from the lungs. Two ducts originate in the liver and these unite to form the common hepatic duct which opens with the pancreatic duct in the hollow side of the duodenum. The gall bladder lies inside the liver, and is the storage place for bile, which is formed by the liver cells.

The right lobe of the liver is larger than the left lobe. Each lobe is further divided into many small lobules, each being about the size of a pin- head and consisting of many liver cells, with bile channels and blood channels between them. Permeating the entire liver structure is a system of blood capillaries, bile capillaries and lymph capillaries.

Aim and Objective: The aim of the current studies is to identify Protein andBiomarker Investigation in Hepatic dysfunction in CCl_4induced Liver damage in Rats. Urine was chosen for biomarker identification since it can be easily collected in relatively large volumes and without any associated pain, distress and discomfort to the animal. The identification of non-invasive markers of liver damage that could be used in the preclinical toxicology assessment and clinical safety testing of drugs would provide relevant information to the process of drug development and could be extremely useful in monitoring target organ toxicity in clinical trials.

In the present investigations animal models of liver toxicity will be developed using CCl_4 as the hepatotoxicant. Initially, a single administration of CCl_4 at a range of concentrations will be used to determine the optimal dose level for our acute hepatic injury model. CCl_4 exerts its toxic effects mainly in the liver; however, injury has also been detected in other organs, including the kidneys. Therefore, our primary goal will be to identify the dose level of CCl_4 that is below the threshold for nephrotoxicity. To reliably determine the presence of renal damage we will investigate the sensitivity of a panel of kidney injury biomarkers recently approved by the FDA to use in toxicology studies. These will be used in our hepatic injury model to confirm the absence of a CCl_4induced toxic effect in the kidneys. Confirmation of absence of renal damage will be made by means of histopathological examination of kidney samples collected at autopsy.

With the preliminary information collected from the dose

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response study regarding the most sensitive urinary markers of renal damage we will then focus on the development of a model of CCl_4 induced liver fibrosis in the male rat.

Identification of biomarkers in the urine samples collected from animals with hepatic injury in this project will be carried out using a metabonomics approach. 1H NMR combined with PCA and OPLS/OPLS-DA will be used for the identification of potential biomarkers. These biomarkers will be further assessed for sensitivity.

- The primary aim of this project was to identify biomarkers of hepatic toxicity; therefore, the first objective was to create a model of hepatotoxicity in the male Hanover-Wistar rat. Firstly, it was necessary to establish the optimal dose level of CCl4 capable of causing maximal hepatotoxicity without inducing injury to other tissues.
- Therefore, a dose response experiment was conducted to investigate the effect of a single CCl4 administration in potential target organs. There is limited data available on CCl4-induced injury to other organs.
- Histological examination would confirm the presence or absence of CCl4-induced injury in the organs.

MATERIAL AND METHODS

Animals

Wistar albino rats (150-200g) were obtained from the Ethical committee (CPCSEA MGZ – 209 dated) and were housed under standard conditions (temperature 24-28ºC relative humidity 60- 70% and 12 hrs dark light cycles),fed commercial rat feed (Lipton India Ltd, Mumbai, India) and boiled water add libitum.[1]

Carbon tetrachloride administration

CCl_4 was dissolved in corn oil and administered by gavage. Control animals (vehicle-treated) were also dosed by gavage with an equivalent volume of corn oil. Dosage volumes were adjusted according to the body weight of individual animals so that the maximum volume they received did not exceed 1.5 mL per rat.[2]

Hexacloro-1:3-diene administration

HCBD was dissolved in corn oil and administered by Intraperitoneal (I.P.) injection. Control animals were dosed in the same way with an equivalent volume of corn oil. For all animals a constant dosing volume of approximately 0.3 mL was administered.[3]

Post mortem

Animals were killed by i.p. injection of Pentobarbital sodium and exsanguinated from the abdominal aorta. At all autopsies blood was taken into SSTTM microtainer tubes for the separation of serum. After 30 minutes at room temperature serum tubes were centrifuged at 5000 rpm for 5 minutes. Serum was store at -80 ºC for future analysis.

Both clinical and internal observations of the animals were made and recorded during the autopsies. The liver was removed and weighed. A section 2-3 mm wide and 20-30 mm long was cut from the left lobe of the liver, sliced into 5/6 pieces, placed in RNALater tubes

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and stored at 4 ºC. A section was also cut from the right kidney, placed in RNALater tubes and stored at 4ºC.The remainder of the liver and kidney were placed in 10.5 % (v/v) phosphate buffered formalin fixative for later histopathological examination.

For dose ranging experiments, other tissues including the spleen, right and left adrenals, thymus and heart were removed at autopsy. Organs were weighed and placed in 10.5 % (v/v) Phosphate buffered formalin fixative.[4]

Serum clinical chemistry

ALT assay

The enzyme ALT transfers one amino group from L-alanine to 2-oxoglutarate forming pyruvate and glutamate. Pyruvate then enters a second reaction with nicotinamide adenine dinucleotide (NADH), catalysed by lactate dehydrogenase (LDH), producing lactate and NAD+. Absorbance is measured at 340 nm and the decrease due to NADH consumption is directly proportional to the ALT activity in the sample.[5,6]

$$\text{L-Alanine + 2-Oxoglutarate} \xleftrightarrow{\text{ALT}} \text{L-Glutamate + Pyruvate}$$

$$\text{Pyruvate + NADH + H}^{+} \xrightarrow{\text{LDH}} \text{D-Lactate + NAD}^{+}$$

AST assay

In this reaction AST catalyses the reversible transamination of L-aspartate and 2- oxoglutarate to oxalacetate and L-glutamate.Oxalacetate then reacts with NADH and is then further reduced to malate in the presence of malate dehydrogenase (MDH). AST activity is determined by measuring the rate of oxidation of NADH at 340 nm.

$$\text{L-Aspartate + 2-Oxoglutarate} \xleftrightarrow{\text{AST}} \text{L-Glutamate + Oxalacetate}$$

$$\text{Oxalacetate + NADH + H}^{+} \xrightarrow{\text{MDH}} \text{D-Malate + NAD}^{+}$$

ALP assay

The enzyme ALP hydrolyses p-nitrophenylphosphate to form p-nitrophenol which is a yellow chromogen. The increase in absorbance at 415 nm due to the production of p-nitrophenol is proportional to the ALP activity

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ALP

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p-Nitrophenylphosphate + H_2O ⟶ Phosphate + p-Nitrophenol

GLDH assay

Measurement of GLDH occurs according to the reaction below and was measured using the kinetic DGKC method. Ammonia and 2-oxoglutarate are converted to glutamate in a reaction catalysed by GLDH. Simultaneously, one mole of NADH is oxidised. The absorbance is read at 340 nm and the decrease in absorbance per minute is proportional to the GLDH activity.

$$\text{2-Oxoglutarate + NADH + } NH_4^+ \xrightarrow{\text{GLDH}} \text{Glutamate + } NAD^+ + H_2O$$

Glucose assay

In this reaction glucose receives a phosphate group from adenosine triphosphate (ATP) in a reaction catalysed by hexokinase (Wright et al., 1971). The resulting product, glucose-6-phosphate, is then oxidised in the presence of NAD+. This reaction is catalysed by the enzyme glucose-6-phosphate dehydrogenase (G6PDH). At the same time NAD+ is reduced to NADH. The increase in absorbance at 340 nm due to the NADH formation is directly proportional to the glucose concentration.

$$\text{Glucose + ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate + ADP}$$

$$\text{Glucose-6-phosphate + } NAD^+ \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate + NADH}$$

Urea assay

The enzyme urease catalyses the hydrolysis of urea to carbamate and ammonia.Carbamate then spontaneously decomposes to yield ammonia and carbonic acid. The ammonia then reacts with 2-oxoglutarate and NADH in a reaction catalysed by GLDH with the production of NAD+. The decrease in absorbance at 340 nm due to the decrease in NADH is proportional to the urea levels.

$$\text{Urea + 2 } H_2O \xrightarrow{\text{Urease}} 2\ NH_4^+ + 2\ HCO_3^-$$

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$$\text{2-Oxoglutarate + NADH + } NH_4^+ \xrightarrow{\text{GLDH}} \text{Glutamate + } NAD^+ + H_2O$$

Creatinine assay

This is a multi-step reaction; the first step consists of the conversion of creatinine to creatine by the enzyme creatinineamidohydrolase. Creatine is then converted to urea and sarcosine. Further reactions result in the production of a colouredchromogen and the change in absorbance is read at 545 nm.

$$\text{Creatinine + } H_2O \xrightarrow{\text{Creatinine amidohydrolase}} \text{Creatine}$$

$$\text{Creatine + } H_2O \xrightarrow{\text{Creatine amidinohydrolase}} \text{Sarcosine + Urea}$$

$$\text{Sarcosine + } H_2O + O_2 \xrightarrow{\text{Sarcosine oxidase}} \text{Glycine + Formaldehyde + } H_2O_2$$

$$2\ H_2O_2 + \text{4-Aminoantipyridine + ESPMT} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye + 4 } H_2O$$

Total protein assay

Total protein measurement based on the Biuret assay. This assay is based on the fact that peptides bonds have the ability to form a purple complex with copper salts in alkaline solution. Absorbance is read at 540 nm.

Albumin assay

Serum albumin was measured using the bromocresol green (BCG) method. BCG forms a coloured complex with albumin and the intensity of the colour, measured at 620 nm is directly proportional do the albumin concentration in the sample.

Acetoacetate and β-Hydroxybutyrate assay

The 2 assays are based on endpoint reactions catalysed by β-hydroxybutyrate dehydrogenase. The generation or consumption of NADH is measured by changes in absorbance at 340 nm.

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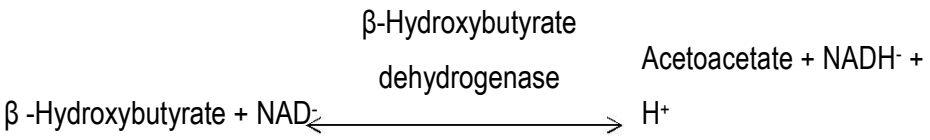
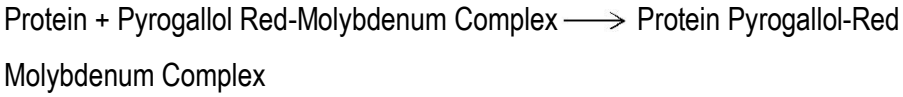


Urinary clinical chemistry

Total protein

Urinary protein concentration was measured based on the pyrogallol red procedure, which is based on the change in absorbance that occurs when pyrogallol red-molybdate complex binds to basic amino acids of proteins. Absorbance is read at 600 nm and the increase is proportional to protein concentration.



N-acetyl-β-D-glucosaminidase assay

This is a colourimetric assay using sodium *m*-cresolsulfonphthaleinyl N-acetyl-β-D-glucosaminidase (MCP-NAG). NAG hydrolyses MCP-NAG to MCP and NAG. MCP shows maximum absorption at 580 nm.

Testosterone assay

Testosterone was measured in urine samples using a competitive enzyme immunoassay. A monoclonal antibody specific for testosterone binds to the goat anti-mouse antibody coated onto the microplate. The plate is washed to remove excess monoclonal antibody and the testosterone present in the sample competes with a fixed amount of horseradish peroxidase-labelled testosterone for sites on the monoclonal antibody.

This step is followed by another wash to remove excess conjugate and unbound sample after which a substrate solution is added to the wells to determine the bound enzyme activity. Absorbance is read at 450 nm and the intensity of the colour is inversely proportional to the concentration of testosterone in the sample.

Result

Observations during the study

Throughout the study, animals were observed for signs of ill-health and clinical observations were recorded during the post-mortem procedure. The behaviour of the animals was observed to guarantee their welfare and as a first indicator of pain and distress. During the period in the metabolism cages, animals treated with CCl4 at 3.2 mL/kg and above appeared to be slightly subdued in comparison to the control animals. One animal treated with 3.6 mL/kg CCl4 had a hunched posture. At autopsy, i.e., 24 hours post-dosing, livers from CCl4-treated animals appeared paler in colour when compared to the control livers and the change in colour was more pronounced as the CCl4 dose level increased.

Body weights

Table 3.1 shows the change in body weight (for both control and CCl4-treated animals) during the 18 hour period while rats were in the metabolism cages. The

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mean change in body weight for CCl4-treated animals was compared to the change in body weight for controls. Although all animals lost weight over the 18 hour period the decrease in body weight for animals in the 1.2 mL/kg dose level group was the greatest (-19.83 g). Animals dosed at 2.8 mL/kg lost the least weight, -9.03 g (**P<0.01). However, there appears to be no clear CCl4 dose-related effect on body weight of the animals.

Table 1: Body weight change for male Hanover-Wistar rats treated with increasing doses of CCl4 during an 18 hour period in metabolism cages.

Dose level of CCl_4 (mL/kg)	Mean (SD) change in body weight (g)
0	-16.12 (2.09)
0.4	-19.25 (1.95)
0.8	-15.13 (2.89)
1.2	-19.83 (2.16)
1.6	-16.33 (1.84)
2.0	-17.15 (2.32)
2.4	-9.23** (3.01)
2.8	-9.03** (3.77)

Liver weights

Figure 5.1 A shows the mean relative liver weights at each CCl4 dose level. The liver weights for CCl4-treated animals were significantly increased over controls (**P<0.01) at 0.8 mL/kg and above, and there was a dose-related increase. At the highest dose level (3.6 mL/kg), relative liver weights were approximately 2-fold greater than control animals (***P<0.001).

Kidney weights

The mean relative kidney weights for rats treated with CCl4 were similar to control at the lower dose levels (0.4, 0.8, 1.2, 1.6 and 2.0 mL/kg CCl4). However, at 2.4 mL/kg the relative kidney weights (4.23 g/kg BW) were significantly greater than controls (3.64 mg/kg BW) (*P<0.05), and at the highest dose level (3.6 mL/kg) there was a 1.23-fold increase over control weights (**P<0.01). However, there was no significant difference in kidney weights at 2.8 and 3.2 mL/kg CCl4 when compared to controls.

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Table 2: Serum clinical chemistry parameters for male Hanover-Wistar rats treated with increasing doses of CCl_4.

Parameters	CCl_4 dose level (mL/kg)									
0	0.4	0.8	1.2	1.6	2.0	2.4	2.8	3.2	3.6	
ALP (U/L)	356.00 (45.17)	521.83** (107.76)	544.83*** (94.71)	642.17*** (169.41)	609.50*** (96.42)	759.67*** (122.07)	822.50*** (218.99)	1144.50*** (270.71)	884.17*** (133.30)	1142.17*** (195.28)
TIMP-1 (µg/L)	7.00 (1.10)	8.33 (1.86)	15.83 (5.74)	24.67** (9.63)	23.50** (5.79)	45.00*** (38.16)	39.83*** (21.45)	82.33*** (50.43)	70.33*** (19.77)	177.83*** (189.76)
MCP1 (µg/L)	8.33 (0.82)	7.67 (1.75)	14.67 (5.92)	23.83 (15.65)	28.83** (5.19)	65.17*** (69.16)	54.67*** (37.65)	138.00*** (105.46)	117.50*** (110.95)	206.67*** (123.67)
A2M (mg/L)	45.00 (11.83)	30.33 (9.87)	42.67 (13.87)	40.33 (9.69)	35.00 (10.06)	29.17 (6.05)	86.83* (43.60)	55.50 (17.52)	59.50 (24.52)	44.17 (10.83)
AGP (mg/L)	45.00 (11.42)	63.83 (9.02)	66.33 (14.77)	76.00 (36.33)	82.50 (25.91)	138.00** (86.84)	130.33* (112.33)	106.50* (39.35)	155.17** (114.08)	128.83* (89.30)
Lipocalin-2 (µg/L)	75.17 (13.12)	77.17 (14.13)	81.50 (25.24)	93.00 (25.51)	90.33 (5.72)	437.50 (704.85)	612.17 (857.02)	854.67** (748.44)	2054.50** (4185.14)	1099.50*** (748.17)
Urea (mmol/L)	6.62 (0.67)	13.12*** (0.72)	10.33*** (1.72)	10.57*** (1.02)	10.43*** (1.29)	10.82*** (1.31)	8.68* (1.03)	9.07*** (0.64)	9.90*** (1.32)	13.03*** (4.65)
Creatinine (µmol/L)	25.17 (0.75)	32.67 (4.23)	24.17 (1.60)	26.67 (1.21)	33.33 (11.57)	32.83 (11.23)	24.50 (3.78)	33.67 (16.46)	29.33 (12.21)	73.00** (55.02)
Albumin (g/L)	35.33 (0.82)	36.50 (1.05)	35.67 (1.03)	35.67 (0.82)	34.83 (1.72)	34.67 (1.03)	34.50 (0.84)	33.67 (2.16)	34.50 (1.22)	35.50 (1.87)
Glucose (mmol/L)	5.60 (1.17)	6.15 (0.40)	5.74 (0.53)	5.50 (0.62)	5.97 (0.57)	5.81 (0.65)	5.28 (0.36)	4.29 (1.35)	5.41 (0.48)	4.34 (2.22)
Total protein (g/L)	53.83 (1.17)	56.00 (1.41)	54.50 (1.05)	53.83 (1.47)	52.67 (2.80)	52.33 (1.63)	50.00** (2.68)	49.00 (4.29)	50.33 (2.66)	53.67 (2.50)

Urine clinical chemistry

Urine clinical chemistry was carried out to further assess the possibility of kidney injury. Table 5.4 shows that creatinine levels were similar to control in all CCl4-treated groups apart from at 2.8 mL/kg and 3.2 mL/kg CCl4 when a statistically significant decrease was observed (**P<0.01 and *P<0.05, respectively). Urinary total protein concentration was significantly lower than control in all CCl4-treated rats apart from at the lowest dose (0.4 mL/kg) when levels were similar to control. However, there was no evidence of a dose-related trend. Urinary albumin levels were not affected by CCl4 administration in a dose-related manner; however, at the highest dose level (3.6 mL/kg) urinary albumin was significantly increased by 4.6-fold over control

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(*P<0.05). Urinary glucose levels were increased in all CCl4-treated animals with the greatest increase occurring in animals in the 2.0 mL/kg CCl4 group. Animals in this group had mean glucose levels of 6.17 µmol/c.p. in comparison to 3.00 µmol/c.p. for the controls (***P<0.001).

Histopathology

Animals were autopsied as described in Section 4.2. Tissue samples were collected and placed in fixative for histopathological examination. In this study, kidneys, liver and nasal cavity were examined in all animals, except for those in the 2.4 or 3.2 mL/kg CCl4-treated groups. These groups were eliminated as a result of the large number of tissue samples generated and the time taken to process the samples. In addition, adrenals, heart, lungs, pancreas, spleen, testes, thyroid and thymus were only examined from control animals and animals dosed at 2.0, 2.8 and 3.6 mL/kg CCl4 as the probability of finding CCl4-treatment-related injuries increases with increasing dose level.

For each of the histopathologicalchanges described below, Table 5.5 to Table 5.8 show the number of animals in each dose group exhibiting the pathological change. Hepatocellular changes associated with CCl4 treatment were apparent at all CCl4 dose levels (Table 5.5, Figure 5.3). Centrilobular hepatocellular vacuolation was either marked or very marked and a proportion of centrilobular hepatocytes showed ballooning of the cytoplasm in all treated animals Hepatocyte necrosis was recorded in animals administered CCl4 at 0.8 mL/kg and above, the extent

Table 3: Urinary clinical chemistry parameters for male Hanover-Wistar rats treated with increasing doses of CCl_4.

Urinary parameters					**CCl4 dose level (mL/kg)**					
0	**0.4**	**0.8**	**1.2**	**1.6**	**2.0**	**2.4**	**2.8**	**3.2**	**3.6**	
Creatinine (µmol/c.p.)	34.17 (9.04)	43.83 (4.17)	31.50 (2.26)	35.00 (2.45)	34.83 (2.56)	40.50 (8.02)	26.67 (10.03)	22.17** (2.48)	23.67* (4.13)	27.67 (4.23)
Total protein (mg/c.p.)	6.13 (1.83)	6.02 (1.47)	2.25*** (0.27)	1.95*** (0.58)	2.23*** (0.60)	2.42** (0.66)	1.45*** (0.62)	1.32*** (0.24)	1.47*** (0.68)	3.90* (3.18)
Albumin (ng/c.p.)	149.98 (70.37)	216.37 (56.12)	146.42 (89.83)	125.99 (43.14)	130.83 (10.78)	225.64 (107.93)	199.49 (67.43)	154.26 (75.37)	204.26 (152.58)	688.33* (660.78)
Glucose (µmol/c.p.)	3.00 (0.63)	5.00** (0.89)	4.17 (0.98)	4.50* (0.55)	4.33 (1.03)	6.17*** (1.72)	5.17** (1.94)	4.00 (0.89)	4.50* (0.84)	5.67*** (1.51)
Volume (mL)	10.48 (4.90)	11.77 (5.74)	19.90 (7.07)	28.73** (8.43)	20.05 (10.14)	18.13 (15.07)	12.97 (5.65)	15.28 (9.41)	9.70 (3.79)	11.87 (4.11)

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Table 4 Histopathological findings in the liver of male Hanover-Wistar rats treated with increasing doses of CCl_4.

				CCl_4 dose level (mL/kg)				
	0	**0.4**	**0.8**	**1.2**	**1.6**	**2.0**	**2.8**	**3.6**
No abnormality detected								
	6	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Centrilobularvacuolation								
Marked	0	6	6	5	0	0	1	0
Very marked	0	0	0	1	6	6	5	6
Centrilobular cytoplasmic								
ballooning								
Minimal	0	5	0	0	0	0	0	0
Mild	0	1	2	1	0	1	1	5
Moderate	0	0	2	4	4	1	4	1
Marked	0	0	2	1	2	4	0	0
Very marked	0	0	0	0	0	0	1	0
Focal and mixed inflammatory								
cell infiltrate								
Mild	0	6	4	2	1	0	0	0
Moderate	0	0	2	4	5	5	6	6
Marked	0	0	0	0	0	1	0	0
Centrilobular hepatocyte necrosis								
Minimal	0	0	2	2	0	0	0	0
Mild	0	0	0	1	1	0	0	0
Moderate	0	0	0	1	5	4	0	0
Marked	0	0	0	0	0	2	6	2
Very marked	0	0	0	0	0	0	0	4

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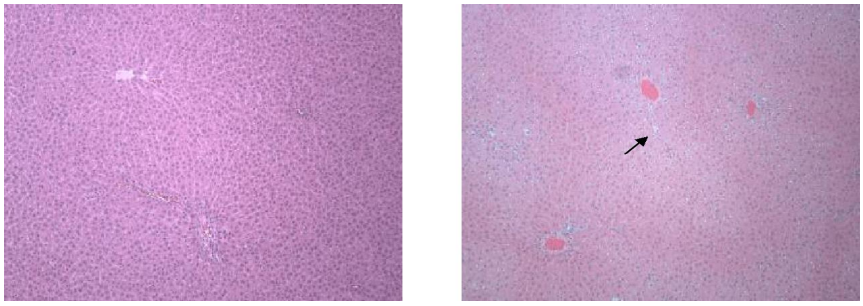
(A) (B)



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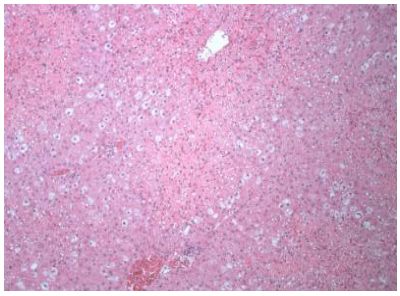

Figure 1 Histology of liver sections from male Hanover-Wistar rats treated with different doses of CCl4.

Table 5: Histopathological findings in the kidneys of male Hanover-Wistar rats treated with increasing doses of CCl_4.

				CCl_4 dose level (mL/kg)					
		0	0.4	0.8	1.2	1.6	2.0	2.8	3.6
	No abnormality detected								
		0	4	5	5	5	0	0	0
	Proximal tubule vacuolation								

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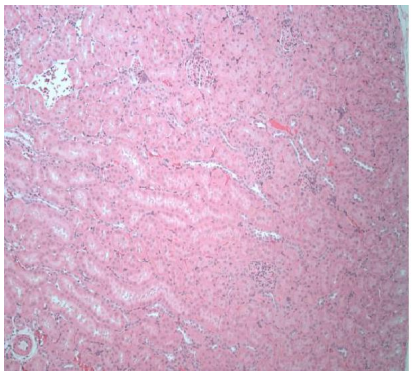
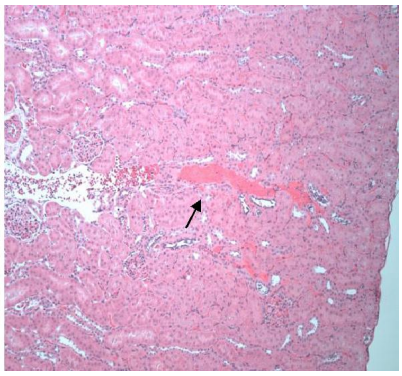
	Minimal	4	0	0	0	0	4	2	0
	Mild	0	0	0	0	0	0	4	4
	Focal tubular basophilia								
	Minimal	1	0	0	0	0	1	2	0
	Granular casts in papillary								
	collecting ducts								
	Mild	0	0	0	0	0	0	0	2
	Granular casts in collecting tubules								
	Minimal	0	0	0	0	0	0	1	1
	Mild	0	0	0	0	0	0	0	1
	Distal and collecting tubule								
	dilatation								
	Mild	0	0	0	0	0	0	0	2
	Hyaline droplets in proximal tubule								
	Minimal	5	2	1	1	1	5	0	2
	Proximal tubule necrosis								
	Minimal	0	0	0	0	0	0	0	2

(A) **(B)**





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(C)

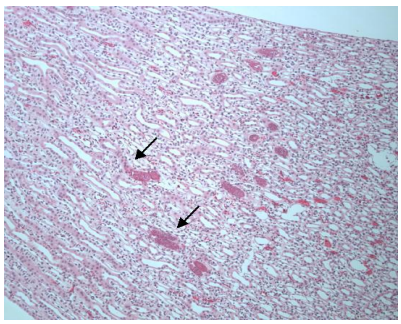

Figure 2: Histology of the kidney cortex from male Hanover-Wistar rats treated with different doses of CCl4.

Table 6: Histopathological findings in the nasal cavity of male Hanover-Wistar rats treated with increasing doses of CCl_4.

				CCl_4 dose level (mL/kg)					
		0	**0.4**	**0.8**	**1.2**	**1.6**	**2.0**	**2.8**	**3.6**
	No abnormality detected								
		6	**0**	**0**	**0**	**0**	**0**	**0**	**1**
	Olfactory epithelium vacuolation								
	Minimal	0	0	0	0	1	0	0	0
	Mild	0	5	2	1	4	1	2	2
	Moderate	0	1	4	5	1	3	3	1
	Olfactory epithelium degeneration								
	Minimal	0	0	1	0	4	1	0	1
	Mild	0	2	2	3	0	2	4	0
	Moderate	0	3	1	3	0	3	1	2
	Marked	0	0	0	0	0	0	0	2
	Olfactory epithelium ulceration								
		0	0	0	0	0	2	3	3
	Respiratory epithelium ulceration								
		0	0	0	0	0	0	0	2

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B

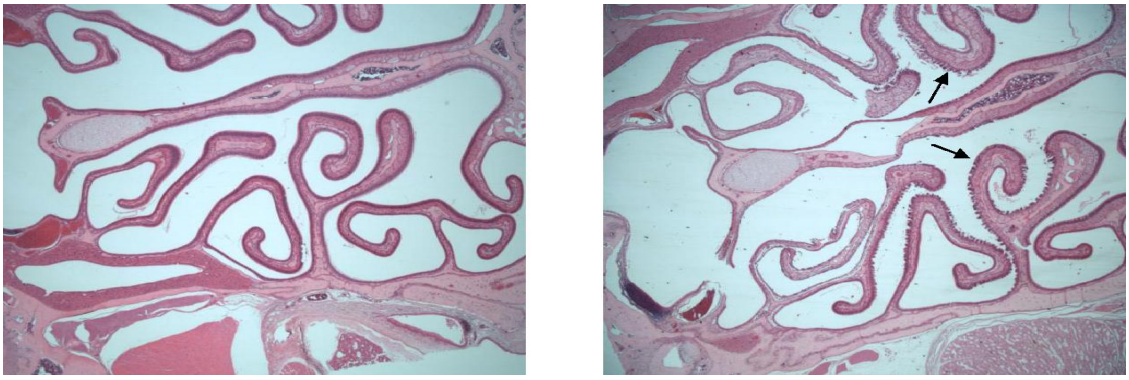

Figure 3: Histology of the nasal cavity from male Hanover-Wistar rats treated with different doses of CCl_4.

Table 7: Histopathological findings in the testes of male Hanover-Wistar rats treated with increasing doses of CCl_4.

	CCl_4 dose level (mL/kg)			
	0	**2.0**	**2.8**	**3.6**
No abnormality detected				
	5	**6**	**6**	**5**
Focal unilateral atrophy of the seminiferous tubules				
Minimal	1	0	0	0
Multinucleate spermatid giant cells				
Minimal	0	0	0	1

of which became more marked with increasing dose level. There was also a focal mixed inflammatory cell infiltrate present in all CCl4-treated animals.In the kidneys, pathological findings considered to be related to CCl4 treatment were observed in a small number of the animals at dose levels of 2.8 mL/kg and above. These changes included granular casts in collecting ducts, dilatation of distal and/or collecting tubules, and minimal proximal tubular necrosis (Table 5.6, Figure 5.3).

Sections of the nasal cavity were prepared to include the ethmoidturbinates. Turbinate bone refers to the thin and curved bone structures that project from the walls of the nasal cavity into the respiratory passage in vertebrates. The ethmoidturbinates extend from the ethmoid bone. Degeneration of the olfactory epithelium,

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characterised by necrosis and vacuolation, was present at all CCl4 dose levels. The severity and extent of nasal cavity injury varied between animals in a group and there was no clear dose response relationship, except for the findings of ulceration of the olfactory epithelium which only occurred in some animals administered CCl4 at 2.0 mL/kg and above. Additional histology sections of the nasal cavity were examined to evaluate the extent of the lesion and in the 3.6 mL/kg CCl4 group 2 animals showed evidence of respiratory epithelial ulceration.

Histological examination revealed that there was no evidence of CCl4-related toxicity in any of the other organs examined (spleen, pancreas, adrenals, thymus, thyroid, heart and lungs) apart from the testes where the presence of multinucleate spermatid giant cells was found in 1 animal in the 3.6 mL/kg CCl4 group. Atrophy of the seminiferous tubules was seen in 1 control animal. In summary there were CCl4-treatment-related findings in the liver and nasal cavity at all CCl4 dose levels and in the kidneys at dose levels of 2.8 mL/kg CCl4 and above.

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