ORIGINAL ARTICLE

Comprehensive characterization of serum clinical chemistry parameters and the identification of urinary superoxide dismutase in a carbon tetrachloride–induced model of hepatic fibrosis in the female Hanover Wistar rat

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Summary

Carbon tetrachloride (CCl_4) was used to induce liver fibrosis in the rat. Using this model, we have identified changes in serum and urinary clinical chemistry parameters, and characterized histopathological lesions in the liver. Two experiments were conducted. In Experiment 1, rats were dosed at six levels of CCl_4 (0.06–0.36 ml/kg) twice weekly for 6 weeks, followed by a 6-week non-dosing recovery period (week 12). Livers were removed for histology at 6 and 12 weeks and serum parameters analysed. In Experiment 2, rats were given seven dose levels of CCl_4 (0.4–1.0 ml/kg) twice weekly for 6 weeks, followed by a 6-week recovery period (week 12); urine samples were analysed at 3, 6, 9 and 12 weeks using one-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Liver fibrosis was evident at 6 weeks in Experiments 1 and 2, and the activity of serum enzymes (including alanine aminotransferase, aspartate aminotransferase and glutamate dehydrogenase) was increased. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis analysis (Experiment 2) revealed a protein band at 18.4 kDa in urine from rats treated with CCl_4, not present in control urine, which was identified as copper/zinc superoxide dismutase (Cu/Zn SOD). Western blotting revealed that SOD was increased in urine from rats treated with CCl_4 at 3 and 6 weeks, but not at 9 and 12 weeks. We conclude that Cu/Zn SOD is a urinary marker of hepatic necrosis, but not hepatic fibrosis.

Keywords

carbon tetrachloride, hepatic fibrosis, rat, serum, superoxide dismutase, toxicity, urine

Introduction

In man, fibrosis is a common response of the liver to many types of chronic injury, for example alcohol consumption and viral hepatitis (Friedman 1993). The early stages of fibrosis in man consist of necrosis and inflammation, which may, in turn, initiate hepatocyte regeneration and repair. But, with continuing repeated injury, this response leads to an accumulation of matrix proteins, e.g. collagen I, III and IV, proteoglycans, and fibronectin and hyaluronic acid (Friedman 1993; Benyon *et al.* 1996; Kauser *et al.* 1998; Li & Friedman 1999), and the deposition of excess collagen fibres, resulting in the disruption of normal liver structure and function. With further continuing injury, cirrhosis may eventually develop.

Several rodent models of liver fibrosis/cirrhosis exist, including the repeat administration of carbon tetrachloride (CCl_4), allyl alcohol, thioacetamide and dimethylnitrosamine (Fontana *et al.* 1996; Sanz *et al.* 1997; Ueki *et al.* 1999; Al-Bader *et al.* 2000; Jung *et al.* 2000; Sato *et al.* 2000; George *et al.* 2001). In the present studies, the rat CCl_4 model was used and although CCl_4 causes injury to a number of organs (e.g. the kidney and lungs) the major damage occurs in the liver. Carbon tetrachloride is metabolized in the liver endoplasmic reticulum (Slater 1966, 1978; Recknagel 1967; Castro *et al.* 1973; Recknagel & Glende 1973) particularly by the ethanol inducible cytochrome P-4502E1 (Edwards *et al.* 1993; de Zwart *et al.* 1998). Carbon tetrachloride induces liver injury progressing from steatosis to centrilobular necrosis, and following repeated administration, fibrosis and cirrhosis may develop (Luckey & Petersen 2001). Although liver fibrosis and cirrhosis were thought to be irreversible, evidence is accumulating to suggest that these conditions may be reversible following a period of non-exposure (Benyon & Iredale 2000; Arthur 2002,). The current studies therefore investigated the effect of a 6-week recovery period at the end of CCl_4 dosing, during which time the rats received no treatment.

In a clinical setting, the current methods for assessing liver fibrosis are biopsy and histopathology, serum enzyme analysis and the assessment of other serum clinical chemistry parameters. Examples of serum markers include hyaluronic acid, procollagen III propeptide, collagen-IV, metalloproteinase, tissue inhibitors of metalloproteinase and plasma prolidase (Savolainen *et al.* 1988; Guéchot *et al.* 1994; Abraham *et al.* 2000; Olga & Nikolai 2003). Serum enzymes are also measured as markers of organ toxicity (Plaa & Charbonneau 2001), and for the determination of hepatic injury the enzymes commonly investigated include alanine aminotransferase (ALT), asparate aminotransferase (AST), glutamate dehydrogenase (GLDH) and sorbital dehydrogenase. In the present studies in the rat, a comprehensive range of serum parameters routinely used in toxicology was investigated. Unfortunately, many current markers are non-specific and fail to detect fibrosis until the later stages of the disease (Friedman 1999). There is also a need for non-invasive markers, which could be detected at an early stage of fibrosis development (Friedman 2003). Therefore, the present studies investigated the possibility of identifying a non- invasive marker for hepatic fibrosis by analysing the urine samples.

Urine analysis is a quick and effective means of identifying target organ toxicity, or the progression of a disease, and many biomarkers are measured in urine, for example urinary aldehydes, catecholamines, nitric oxide and isoprostane (Hermanns *et al.* 1998; Draper *et al.* 2000; Obata *et al.* 2000; Smith & Lassmann 2002; Banerjee *et al.* 2003). However, in general, these biomarkers are not organ-specific and there is a need for urinary markers that reflect a change in a specific organ or disease state. Studies have been carried out to identify urinary markers of bladder cancer (Kageyama *et al.* 2004), and Dare *et al.* (2002) identified parvalbumin as a urinary marker of skeletal muscle toxicity in rats.

In experiments reported here in the rat, two main studies were carried out. The first (Experiment 1) involved the repeat administration of relatively low-dose levels of CCl_4 (0.06–0.36 ml/kg). A brief report of the results of this study is presented. In Experiment 2, higher dose levels of the agent were used (0.4–1.0 ml/kg). However, it was important that in both experiments there should be no evidence of an adverse effect on the kidney, and that the urinary system should be structurally normal. Therefore, as CCl_4 in the rat is known to exert nephrotoxicity (Doi *et al.* 1991; Abraham *et al.* 1999; Ozturk *et al.* 2003), a preliminary experiment was conducted involving the administration of a single dose of CCl_4, at dose levels from 0.4 to 2.8 ml/kg, with animals necropsied at 24 h post-dosing. The objective was to identify CCl_4 dose levels that caused nephrotoxicity, but below which the kidney was structurally normal. In this way, any protein detected in the urine would be the result of hepatotoxicity, and not injury to the kidney. A brief account of the preliminary experiment is presented. Reports of the present studies have been presented in abstract form (Smyth *et al.* 2002, 2004).

Materials and methods

Animals, administration of CCl_4 and collection of urine

Female Hanover Wistar rats (B and K Universal Ltd, Grimston, Aldbrough, Hull, UK) of 160–220 g were used.

Animals were acclimatized for 7 days before each experiment, in communal cages, with diet (SDS Ltd, Wiltham, Essex, UK) and water *ad libitum*. The temperature was controlled at 19–21 °C with relative humidity of 45–65% and lighting on a 12-h light/dark cycle (lights on 7 AM). Animals were weighed before dosing and at appropriate times, and observed daily for signs of ill health. Animal procedures were carried out under local Ethical Committee guidelines and approval and followed the Home Office (1989) 'Code of Practice for the Housing and Care of Animals Used in Scientific Procedures'. Carbon tetrachloride (Fluka Chemicals, New Road, Gillingham, Dorset, UK) was dissolved in vegetable oil and administered by gavage; the concentration of CCl_4 was adjusted to give a maximum volume of 2 ml. Control rats received 2 ml of vegetable oil (vehicle). For urine collection, animals were placed individually in metabolism cages (Techniplast, Kettering, Northants, UK.), with water *ad libitum*, but not diet. Urine was collected for 24 h over ice and stored at −80 °C.

Necropsy procedure, serum and urine analysis

Animals were killed by an i.p. injection of pentobarbitone sodium (Sagatal; Rhone Merieux, Harlow, Essex, UK) and exsanguinated from the abdominal aorta. Blood was placed in Microtainers (Becton Dickinson and Co, Rutherford, NJ, USA) for separation of serum. After 2–3 h, microtainers were centrifuged at 3030 *g* for 5 min at room temperature. Serum was removed and stored at −80 °C. Liver and kidneys were removed and weighed. Sections of liver lobes and both kidneys were placed in 10.5% phosphate-buffered formalin, processed, sectioned and stained with haematoxylin and eosin (H&E) or Sirius red. Serum and urine samples were analysed spectrophotometrically at 37 °C on a Boehringer-Hitachi 917 automated clinical chemistry analyser according to manufacturer's instructions (Roche Diagnostics Ltd, Lewes, Sussex, UK). The following serum parameters were measured; ALT, AST, alkaline phosphatase (ALP), GLDH, lactate dehydrogenase (LDH), creatine kinase (CK), total protein (TP), total-bilirubin (T-BIL), bile acids (BA), albumin (ALB), creatinine (CREA) and urea, triglycerides (TRIG) and cholesterol (CHOL), calcium (Ca) and glucose (GLU). Reagents were supplied by Roche Diagnostics Ltd. Superoxide dismutase (SOD) activity was determined in serum and urine using a Ransod assay kit (Randox Laboratories, Antrim, N. Ireland). Glutamate dehydrogenase activity was also assayed in serum and urine using the kinetic Deutsche Gesellschaft für Klinische Chemie method.

One-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Sodium dodecyl sulphate gels were poured according to Laemmli (1970). A constant voltage of 200 V was applied for 1 h or until the dye front had reached the bottom of the resolving gel. Gels were further processed for Western blotting; gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer Life Sciences Inc, Boston, MA, USA) using the semi-dry transfer method. Following transfer, the PVDF membrane was stored in blocking solution: 1% Marvel in high salt tween buffer (HST; 20 mM Tris–HCl pH 7.4; 0.5 M NaCl; 0.5% tween 20) overnight at 4 °C. A polyclonal rabbit SOD-1 antibody (Santa Cruz Biotechnology Inc, supplied by Autogen Bioclear UK Ltd, Wiltshire, UK) was diluted 1:300 in 1% Marvel in HST. The secondary antibody was a goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Bio-Rad Laboratories, Hemel Hempstead, Hertfordshire, UK), diluted 1:3000 in 1% Marvel in HST buffer. Protein bands were detected by using enhanced chemiluminescence detection reagents (Amersham BioSciences, Amersham, Buckinghamshire, UK). Images were recorded at 1-min intervals on a Synergy Gene-Gnome detector, the final image recorded was that taken after 5-min exposure time.

Statistical analysis

Treated and control groups were compared by using Student's *t*-test for unpaired samples using MICROSOFT EXCEL (Microsoft Ltd, Microsoft UK, Reading, UK).

Experimental design

Preliminary experiment. Forty rats (mean body weight 201.9 g) were divided into eight groups, *n* = 5 animals per group. Rats were gavaged with a single dose of vegetable oil (vehicle control) or CCl_4 at 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 and 2.8 ml/kg. Animals were autopsied at 24-h post-dosing; livers and kidneys removed for histological investigation and serum prepared for analysis.

Experiment 1. Eighty-four rats (mean weight 206.2 g) were divided into seven groups of 12 animals, and dosed by gavage with vegetable oil (vehicle control) or CCl_4 in vegetable oil at 0.06, 0.12, 0.18, 0.24, 0.30 and 0.36 ml/kg. Rats were treated twice weekly (Monday and Thursday) for 6 weeks. At the end of dosing (week 6), six rats from each dose level group were killed and samples taken; the

remaining six rats per group were left untreated for a further 6-week recovery period (to week 12).

Experiment 2. Ninety-six rats (mean weight 185.2 g) were divided into eight groups of 12 animals and gavaged with vegetable oil (vehicle control) or CCl_4 at 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml/kg. Animals were treated twice weekly (Monday and Thursday) for 6 weeks. At the end of dosing (week 6), six rats from each dose level group were killed, the remaining six rats per group were left untreated for a further 6-week recovery period (to week 12). After 3 weeks of CCl_4 dosing, six rats from the control group, six from the 0.7 ml/kg group and six from the 1.0 ml/kg group were placed in metabolism cages and urine collected for 24 h; they were then returned to communal cages. At the end of the 6-week dosing period, the same rats were placed in metabolism cages and urine again collected for 24 h. Urine was collected from the same six rats at 3 weeks into the non-dosing recovery period (i.e. week 9) and at the end of the 6-week recovery period (i.e. week 12).

Results

Preliminary experiment

CCl_4-induced nephrotoxicity. Rats were given a single administration of CCl_4 at dose levels from 0.4 to 2.8 ml/kg. Briefly, organ weights, serum clinical chemistry analysis and histopathology demonstrated that liver injury was induced at all dose levels of CCl_4. For the kidney, histopathology showed that CCl_4 at 0.4 and 0.8 ml/kg did not cause nephrotoxicity, but at 1.2 ml/kg and above, dose-related changes, including degeneration and necrosis of the proximal-convoluted tubules, were evident.

Experiment 1

Clinical observations, body weight changes, and liver and kidney weights. Throughout the 6-week period of CCl_4 administration, at dose levels from 0.06 to 0.36 ml/kg, there were no clinical signs of CCl_4 toxicity. However, animals in the CCl_4-treated groups did not gain as much weight as the control (vehicle-treated) rats. At 12 weeks (i.e. after the 6-week non-dosing recovery period), the mean body weights of all groups of CCl_4-treated rats were similar to the controls.

At the 6-week necropsy, the mean relative liver weights showed significant dose-related increases with the highest CCl_4 dose group (0.36 ml/kg CCl_4) having the heaviest liver weight. At the 12-week necropsy, livers from CCl_4-treated

animals were heavier (relative weights) than the controls and the increases were significant at the 0.18 ($P < 0.05$) and 0.24 ($P < 0.05$) ml/kg dose levels. The mean relative kidney weights of CCl_4-treated rats at the necropsies at 6 and 12 weeks were directly comparable to the mean concurrent control kidney weights.

Serum clinical chemistry: sampling at 6 weeks. At the 6-week necropsy, at the end of CCl_4 dosing, mean serum ALT, aspartate aminotransferase (AST) and GLDH levels were increased in all groups of CCl_4-treated rats; the increases were greater in the higher-dose level groups. At the lowest CCl_4 dose administered (0.06 ml/kg), there were significant increases for ALT ($P < 0.001$) and GLDH ($P < 0.05$); AST levels were significantly raised at 0.18 ml/kg CCl_4 ($P < 0.01$) and at all higher CCl_4 levels. Other serum parameters that were increased at 6 weeks were ALP, BA and T-BIL. Mean ALP activity was significantly raised at the lowest dose level (0.06 ml/kg) of CCl_4 ($P < 0.05$) and enzyme activity increased with increasing dose levels of CCl_4. Mean BA levels were significantly increased at 0.06 ml/kg CCl_4, and in all the other CCl_4-treated dose groups. Total-bilirubin was raised in all CCl_4-treated groups and the increases were statistically significant at 0.12 ml/kg CCl_4 and above. Serum ALB was decreased in rats treated with CCl_4 at all dose levels, when compared with the mean control level, and this reduction was significant at 0.30 ($P < 0.05$) and 0.36 ml/kg ($P < 0.05$).

Sampling at 12 weeks. At the 12-week necropsy, at the end of the 6-week recovery period, serum enzyme analysis revealed that, in general, the mean levels of ALT, AST and GLDH in CCl_4-treated rats were similar to control levels. However, mean AST levels were significantly lower than the controls in the 0.24, 0.30 and 0.36 ml/kg CCl_4 groups. ALP activity and levels of BA, T-BIL and ALB in the serum at 12 weeks were similar in CCl_4-treated rats and in the controls.

Histology. After 6 weeks of dosing with CCl_4, the livers of treated rats showed hepatocellular degeneration and necrosis at all dose levels from 0.06 ml/kg and above (Figure 1). Hepatocytes in centrilobular regions showed cytoplasmic swelling and vacuolation, with cytoplasmic and nuclear degeneration and necrosis. Inflammatory cells were sometimes present. Around the centrilobular veins and in the periportal regions, immature and mature fibrosis was present, which extended into the surrounding parenchyma along the sinusoidal tracks. The amount of fibrosis showed considerable variation between CCl_4 dose level groups, and also between individual rats in each dose level group.
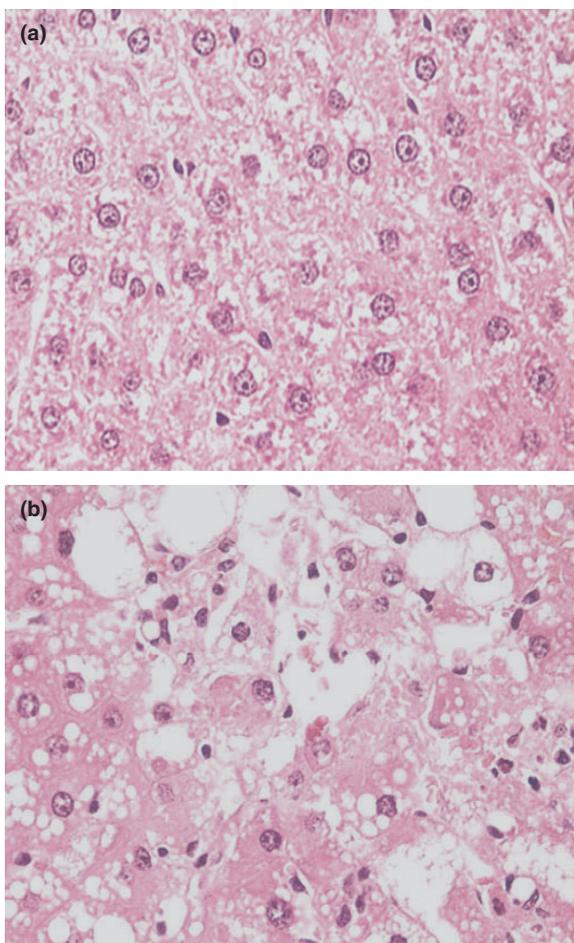
Figure 1 Histology of the liver from a rat treated with carbon tetrachloride (CCl$_4$) twice a week for 6 weeks and sampled at 6 weeks. (a) Liver from a control rat to show the normal arrangement and appearance of hepatocytes. (b) Centrilobular region in the liver of a rat treated with CCl$_4$ at 0.36 ml/kg twice weekly for 6 weeks showing diffuse, marked, hepatocellular vacuolation and degeneration, with necrotic cells. Original magnification of images; ×400 (haematoxylin and eosin).

However, there was a trend for the degree of fibrosis to increase with increasing CCl$_4$ dose level. Liver sections were chosen at random for Sirius red staining. This revealed the presence of collagen fibres in some liver sections. The fibrosis was often perivascular in distribution and sometimes included delicate 'bridging' between the centrilobular and periportal areas. In some sections, the capsular region was also involved. However, in general, the induced fibrosis was relatively mild. At week 12, after the 6-week recovery period, the livers appeared to have returned to normal; there was no clear or consistent evidence of fibrosis on Sirius red staining. Histological examination of kidney sections demonstrated no CCl$_4$-treatment-related findings at 6 weeks, and

no evidence of CCl$_4$-induced changes at the end of the 6-week period of recovery (week 12).

Experiment 2

Clinical observations, body weight changes, and liver and kidney weights. Throughout the 6-week period of CCl$_4$ dosing at levels from 0.4 to 1.0 ml/kg, all animals appeared healthy and there were no clinical signs of CCl$_4$ toxicity. However, during this period, the animals in CCl$_4$-treated groups did not gain as much weight as control rats (Table 1). Nevertheless at 12 weeks, after the 6-week non-dosing recovery period, body weights of CCl$_4$-treated rats were, in general, approximately similar to control animals. The percentage increases in body weight from the beginning of the experiment to week 12 were, for example, 30.87%, 31.77%, 30.60%, 30.02% and 25.82% for the groups treated with CCl$_4$ at 0 (vehicle control), 0.4, 0.7, 0.9 and 1.0 ml/kg respectively.

Mean relative liver weights for rats treated with CCl$_4$ twice a week for 6 weeks were significantly heavier than those from control animals at all CCl$_4$ dose levels ($P < 0.001$ for all groups) (Table 1). At the lowest dose (0.4 ml/kg), the relative liver weights from CCl$_4$-treated rats were 24.49% heavier than the controls, and there was a 52.24% increase in relative liver weight in rats treated with 1.0 ml/kg CCl$_4$ compared to the controls. After the 6-week recovery period, at 12-week post-dosing, the mean relative liver weights for CCl$_4$-treated rats were still heavier than the controls (Table 1), but the only statistically significant increases were in the 0.7 and 0.8 ml/kg groups ($P < 0.05$ for both groups).

The mean relative kidney weights from CCl$_4$-treated rats at the 6-week necropsy were slightly but significantly heavier than the controls at all dose levels (Table 1); the increase in rats treated at 1.0 ml/kg CCl$_4$, for example, was 9.38% greater than the control animals. However, at the 12-week necropsy, there were no statistically significant differences between the mean relative kidney weights of control and CCl$_4$-treated rats (Table 1), although the weights at all dose levels were equal to, or greater than, the controls.

Serum clinical chemistry: Sampling at 6 weeks. After 6 weeks of dosing, serum ALT, AST and GLDH activities were significantly increased in many CCl$_4$-treated groups compared to the controls (Table 2). The mean ALT activity in control rats was 39.50 U/l and this increased 4.71-fold in the 0.4 ml/kg CCl$_4$ group to 225.67 U/l (not significant). Alanine aminotransferase activity then increased as the dose level of CCl$_4$ rose until at 1.0 ml/kg activity had risen

Table 1 Mean (±SD) body weight changes, and relative liver and kidney weights of female Hanover Wistar rats treated with carbon tetrachloride (CCl₄) at dose levels from 0.4 to 1.0 ml/kg twice weekly for 6 weeks, and at week 12 following a 6-week recovery period without dosing†

| | | Dose of CCl₄ (ml/kg) | | | | | | | |
	Week	0 (control)	0.4	0.5	0.6	0.7	0.8	0.9	1.0
Body weight§ (g)	0‡	185.17 ± 17.68	164.67 ± 10.67	174.67 ± 10.67	166.50 ± 6.98	172.67 ± 5.39	177.00 ± 14.87	177.67 ± 9.31	178.83 ± 7.88
	6	234.00 ± 23.19	194.67 ± 14.90	203.67 ± 26.79	193.83 ± 11.11	200.33 ± 5.82	202.83 ± 16.25	200.83 ± 12.53	201.50 ± 9.14
	12	242.33 ± 21.87	216.33 ± 16.81	228.83 ± 34.20	218.67 ± 9.99	225.50 ± 5.79	232.50 ± 17.63	231.00 ± 14.67	225.00 ± 7.51
Relative liver weight (g/kg body weight)¶	6	35.89 ± 1.93	44.68*** ± 2.17	44.57*** ± 3.70	46.91*** ± 3.10	51.33*** ± 4.63	50.12*** ± 5.09	46.95*** ± 2.93	54.64*** ± 5.78
	12	24.30 ± 2.82	26.03 ± 0.79	26.93 ± 1.47	27.06 ± 1.30	27.38* ± 1.31	28.02* ± 1.80	27.34 ± 2.52	26.59 ± 1.24
Relative kidney weight¶ (g/kg body weight)	6	2.88 ± 0.15	3.15** ± 0.14	3.08* ± 0.15	3.26* ± 0.29	3.36** ± 0.31	3.2* ± 0.22	3.15* ± 0.25	3.50*** ± 0.24
	12	2.67 ± 0.16	2.67 ± 0.16	2.71 ± 0.15	2.77 ± 0.14	2.82 ± 0.17	2.75 ± 0.17	2.73 ± 0.08	2.71 ± 0.18

†Rats were treated with CCl₄ by gavage. *Significantly different from control at the same time point $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $n = 6$ rats per dose level group at each time point.
‡Body weight at the time of administration of the first dose of CCl₄.
§Body weight data were not analysed statistically.
¶Mean weight of left and right kidney.

38.10-fold to 1544.33 U/l. Asparate aminotransferase activity was significantly increased in all CCl₄-treated groups except at 0.7 ml/kg (Table 2). Mean AST activity in control rats was 123.17 U/l and this increased 1.64-fold in the 0.4 ml/kg group to 325.50 U/l. Asparate aminotransferase activity then rose as the dose of CCl₄ increased until at 1.0 ml/kg activity had risen 14.53-fold to 1912.33 U/l. The mean control GLDH activity was 4.63 U/l, this increased 22.90-fold in the 0.4 ml/kg group to 110.65 U/l. Activity then increased as the CCl₄ dose level rose until at 1.0 ml/kg, activity had risen 155.00-fold to 722.25 U/l.

At the 6-week autopsy, mean serum ALP activity was increased in all CCl₄-treated groups (Table 2). At the lowest dose administered (0.4 ml/kg), ALP activity was 0.55-fold greater than the control group ($P < 0.05$). Alkaline phosphatase activity tended to rise in the serum as the CCl₄ dose increased up to the 0.7 ml/kg dose level. At 1.0 ml/kg CCl₄, mean ALP activity was 1.72-fold greater than in the controls. Total-bilirubin and BA also tended to increase in CCl₄-treated rats compared to control levels. Bile acid measurements in the serum revealed that at the lowest dose of CCl₄ (0.4 ml/kg), the value was significantly higher ($P < 0.001$) in the CCl₄-treated rats than in the controls. At the highest CCl₄ dose level (1.0 ml/kg), the mean BA value was 2.84-fold greater in the CCl₄-treated rats than in the controls. The mean TP levels in serum from rats treated with CCl₄ were decreased at all dose levels; however, the decrease was not significant in the 0.9 ml/kg group. The serum ALB level in the serum of rats treated with CCl₄ was also decreased in all treatment groups compared to the control value; this decrease was significant at 0.5, 0.7, 0.8 and 1.0 ml/kg CCl₄. Serum superoxide dismutase activity was significantly increased in all CCl₄-treated groups. Creatine kinase levels were significantly decreased in the higher dose CCl₄ groups (i.e. 0.6 to 1.0 ml/kg) and there was some evidence of a general dose–response relationship; at the highest CCl₄ dose (1.0 ml/kg), the mean CK level was reduced to 40.76% of the control value. The LDH activity in CCl₄-treated rats was decreased in all groups (except at 0.5 and 0.7 ml/kg), but was only significantly reduced in the 0.8 ml/kg group. There was no difference in serum Ca levels between the control and CCl₄-treated rats at any dose level. Cholesterol measurements were decreased in all CCl₄-treated groups, but this was not statistically significant in the 0.7, 0.8 and 1.0 ml/kg groups. Triglyceride levels were significantly decreased in all CCl₄-treated groups except at 0.4 and 0.7 ml/kg. Glucose values were decreased to statistically significant levels in all CCl₄-treated groups. Serum urea values were significantly increased in all CCl₄-treated groups, but the levels did not clearly increase with increasing

Table 2 Mean (±SD) of serum clinical chemistry parameters of female Hanover Wistar rats treated with carbon tetrachloride (CCl$_4$) at dose levels from 0.4 to 1.0 ml/kg twice weekly for 6 weeks[†]

Serum chemistry parameter	Dose of CCl$_4$ (ml/kg)							
	0 (control)	0.4	0.5	0.6	0.7	0.8	0.9	1.0
ALT	39.50 ± 9.35	225.67 ± 222.97	576.17* ± 416.95	502.40*** ± 158.41	1088.67 ± 1280.35	995.83* ± 770.51	789.83* ± 705.70	1544.33** ± 948.40
AST	123.17 ± 15.20	325.50* ± 192.29	704.17* ± 590.27	557.20** ± 228.61	1095.17 ± 1316.29	1019.33* ± 724.55	867.50* ± 805.10	1912.33* ± 1509.21
GLDH	4.63 ± 0.98	110.65 ± 145.51	296.53 ± 409.14	279.26* ± 213.69	714.38 ± 1140.30	502.20* ± 444.16	433.07* ± 387.32	722.25* ± 574.37
LDH	3035.67 ± 841.80	2380.33 ± 687.20	3927.50 ± 2133.18	2945.00 ± 2006.42	3448.50 ± 2286.28	1944.67* ± 622.36	1825.17 ± 111.63	2644.17 ± 969.37
CK	1150.33 ± 304.01	830.50 ± 237.27	1026.67 ± 531.30	658.40* ± 293.53	660.00* ± 356.85	573.83** ± 204.11	575.67* ± 342.04	468.83*** ± 134.72
ALP	204.67 ± 28.19	316.33* ± 103.57	393.83*** ± 60.17	421.20*** ± 77.55	676.17* ± 411.27	592.00*** ± 167.64	526.00** ± 205.41	555.83** ± 281.13
T-BIL	3.28 ± 0.17	4.73** ± 0.92	4.40* ± 0.98	4.94*** ± 0.72	7.05 ± 4.44	5.08 ± 2.35	5.33* ± 0.73	6.23* ± 2.40
BA	35.28 ± 15.06	84.68*** ± 18.36	128.60** ± 56.31	148.24*** ± 56.91	127.28** ± 55.82	133.67** ± 60.17	110.28** ± 44.81	135.60** ± 57.47
ALB	38.02 ± 0.97	36.77 ± 1.72	35.62* ± 1.75	36.80 ± 1.04	35.67* ± 1.74	35.18*** ± 0.93	36.43 ± 1.65	34.80* ± 2.57
UREA	7.43 ± 0.37	9.63** ± 1.17	9.79*** ± 0.91	9.84*** ± 0.80	9.42** ± 1.08	9.24** ± 1.40	9.19** ± 1.17	9.15*** ± 0.78
CREA	57.83 ± 2.71	56.67 ± 1.97	56.50 ± 3.94	54.50* ± 0.89	51.17* ± 4.45	52.17* ± 3.54	56.17 ± 3.43	51.67 ± 5.13
SOD	386.67 ± 55.72	593.50* ± 179.42	772.50*** ± 175.77	739.20*** ± 34.10	612.33* ± 237.89	701.67*** ± 170.36	760.50*** ± 147.87	726.50*** ± 98.64
TP	64.42 ± 2.20	60.38** ± 1.01	59.18** ± 1.96	61.22* ± 1.63	58.37** ± 2.76	57.83*** ± 2.44	55.63 ± 14.21	57.33** ± 4.45
Ca	2.52 ± 0.05	2.51 ± 0.08	2.53 ± 0.08	2.54 ± 0.10	2.52 ± 0.10	2.47 ± 0.12	2.52 ± 0.09	2.54 ± 0.15
CHOL	1.56 ± 0.25	1.24* ± 0.23	1.16* ± 0.20	1.14** ± 0.14	1.35 ± 0.40	1.30 ± 0.21	1.28* ± 0.09	1.35 ± 0.46
TRIG	0.79 ± 0.27	0.52 ± 0.29	0.38* ± 0.11	0.29** ± 0.06	0.65 ± 0.58	0.35** ± 0.04	0.31** ± 0.04	0.34** ± 0.08
GLU	8.42 ± 0.81	6.78** ± 0.66	5.69*** ± 0.52	6.25*** ± 0.45	6.02** ± 1.50	5.87*** ± 0.73	6.31** ± 0.74	5.40*** ± 0.89

ALT, alanine aminotransferase (U/l); AST, asparate aminotransferase (U/l); GLDH, glutamate dehydrogenase (U/l); LDH, lactate dehydrogenase (U/l); CK, creatine kinase (U/l); ALP, alkaline phosphatase (U/l); T-BIL, total bilirubin (μmol/l); BA, bile acids (mmol/l); ALB, serum albumin (g/l); Urea (mmol/l); CREA, creatinine (μmol/l); SOD, superoxide dismutase (U/l); TP, total protein (g/l); Ca, calcium (mmol/l); CHOL, cholesterol (mmol/l); TRIG, triglyerides (mmol/l); GLU, glucose (mmol/l). *Significantly different from control, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $n = 6$ rats per dose level group.

[†]Rats were treated with CCl$_4$ by gavage.

Table 3 Mean (±SD) of serum clinical chemistry parameters of female Hanover Wistar rats treated with carbon tetrachloride (CCl₄) at dose levels from 0.4 to 1.0 ml/kg twice weekly for 6 weeks followed by a 6-week recovery period without dosing (week 12)[†]

Serum chemistry parameter	Dose of CCl₄ (ml/kg)							
	0 (control)	0.4	0.5	0.6	0.7	0.8	0.9	1.0
ALT	45.50 ± 7.53	43.33 ± 9.69	43.67 ± 8.59	56.17 ± 26.42	41.67 ± 8.14	35.67 ± 11.33	40.00 ± 7.38	42.00 ± 7.01
AST	144.50 ± 14.01	133.67 ± 18.03	134.00 ± 18.89	126.67 ± 26.61	117.17** ± 15.33	99.67* ± 37.53	102.33*** ± 10.33	123.50* ± 14.63
GLDH	5.97 ± 6.60	4.60 ± 4.95	2.88 ± 0.69	4.13 ± 2.92	2.13 ± 1.03	2.02 ± 1.24	3.03 ± 2.63	6.17 ± 7.80
LDH	3298.83 ± 242.74	2896.33 ± 492.15	2939.83 ± 725.70	2578.67** ± 343.31	2506.33** ± 347.69	1849.17** ± 781.07	1759.83*** ± 633.69	2217.83* ± 905.5
CK	1416.83 ± 130.17	1225.83 ± 202.97	1233.17 ± 321.34	1046.33** ± 153.52	959.33*** ± 125.70	775.00*** ± 316.59	720.17*** ± 233.58	795.50*** ± 240.55
ALP	65.33 ± 5.92	66.67 ± 4.37	65.67 ± 6.35	69.17 ± 25.10	68.83 ± 20.85	75.83 ± 27.55	77.33 ± 28.39	76.83 ± 8.26
T-BIL	3.68 ± 0.36	3.37 ± 0.27	3.77 ± 0.36	3.97 ± 0.39	4.33 ± 0.65	4.25 ± 1.37	4.28 ± 0.57	3.87 ± 0.69
BA	29.65 ± 6.04	41.67 ± 16.57	51.18* ± 19.84	54.12* ± 23.95	42.45* ± 8.96	34.35 ± 10.92	49.27 ± 21.61	50.07* ± 19.49
ALB	38.13 ± 1.77	37.50 ± 1.44	36.88 ± 1.64	38.47 ± 1.54	38.03 ± 1.47	37.22 ± 1.24	36.55 ± 1.26	35.63* ± 1.13
UREA	7.48 ± 0.36	8.17** ± 0.24	8.65 ± 1.38	9.09** ± 1.13	7.20 ± 1.36	6.80 ± 1.37	6.93 ± 0.87	6.40* ± 1.02
CREA	57.50 ± 13.17	63.33 ± 5.79	63.83 ± 4.54	64.50 ± 3.27	60.67 ± 5.43	55.67 ± 11.47	56.00 ± 3.74	58.17 ± 3.76
SOD	622.67 ± 139.03	513.83 ± 113.99	444.33 ± 143.27	494.50 ± 201.06	471.67* ± 48.50	473.17* ± 76.49	455.17* ± 85.92	616.67 ± 113.05
TP	66.05 ± 2.69	64.05 ± 2.35	63.80 ± 3.33	65.73 ± 2.72	66.75 ± 2.70	63.63 ± 2.54	64.07 ± 1.93	62.92 ± 3.41
Ca	2.45 ± 0.08	2.39 ± 0.07	2.42 ± 0.10	2.44 ± 0.03	2.46 ± 0.09	2.45 ± 0.06	2.47 ± 0.08	2.44 ± 0.06
CHOL	1.03 ± 0.22	1.69** ± 0.34	1.56* ± 0.40	1.52** ± 0.29	1.62** ± 0.26	1.65*** ± 0.13	1.78*** ± 0.19	1.19 ± 0.20
TRIG	0.56 ± 0.19	0.52 ± 0.23	0.78 ± 0.40	0.66 ± 0.39	0.44 ± 0.07	0.47 ± 0.23	0.60 ± 0.49	0.43 ± 0.10
GLU	4.68 ± 2.00	3.85 ± 1.21	4.31 ± 1.26	4.52 ± 2.09	5.36 ± 1.08	4.43 ± 1.44	4.48 ± 1.01	4.54 ± 1.23

ALT, alanine aminotransferase (U/l); AST, asparate aminotransferase (U/l); GLDH, glutamate dehydrogenase (U/l); LDH, lactate dehydrogenase (U/l); CK, creatine kinase (U/l); ALP, alkaline phosphatase (U/l); T-BIL, total bilirubin (μmol/l); BA, bile acids (mmol/l); ALB, serum albumin (g/l); UREA, Urea (mmol/l); CREA, creatinine (μmol/l); SOD, superoxide dismutase (U/l); TP, total protein (g/l); Ca, calcium (mmol/l); CHOL, cholesterol (mmol/l); TRIG, triglyerides (mmol/l); GLU, glucose (mmol/l).

[†]Rats were treated with CCl₄ by gavage. *Significantly different from control, $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. $n = 6$ rats per dose level group.

dose levels of CCl$_4$ (Table 2). Creatinine levels were significantly decreased in rats treated with CCl$_4$ at 0.6, 0.7, 0.8 and 1.0 ml/kg.

Sampling at 12 weeks. Serum analysis at 12 weeks (i.e. after the 6-week recovery period) revealed that mean ALT and GLDH activities were similar to the controls at all CCl$_4$

dose levels (Table 3). However, rats that had received doses of 0.7 ml/kg CCl$_4$ or higher showed a significantly lower serum AST activity after the 6-week period of recovery; serum ALP and T-BIL levels, in general, showed a return to control values. Serum BA levels after the 6-week recovery period were highly variable in the different CCl$_4$ dose groups and showed significant increases in CCl$_4$-treated
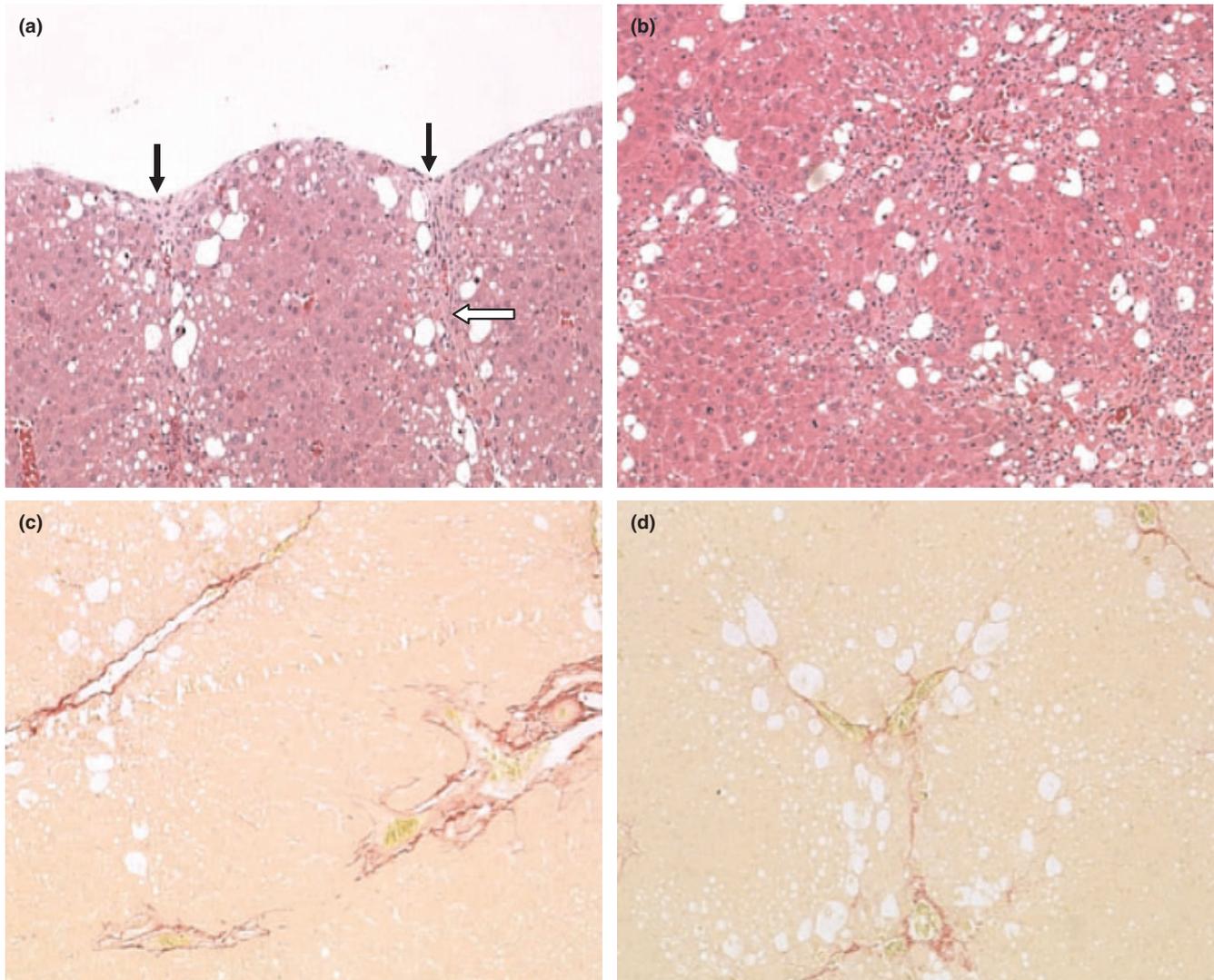

Figure 2 Histopathology of the liver in rats treated with carbon tetrachloride (CCl$_4$) twice a week for 6 weeks and sampled at that time. (a) Liver from a rat treated with CCl$_4$ at 0.6 ml/kg twice weekly for 6 weeks. In the subcapsular region, there is hepatocellular vacuolation, degeneration and necrosis, with capsular pitting or contraction (black arrows). There is collagen formation in the capsular area which extends downwards into the liver parenchyma (open arrow). Original magnification of image; ×100 [haematoxylin and eosin (H&E)]. (b) Liver from a rat treated with CCl$_4$ twice weekly for 6 weeks at 1.0 ml/kg. There is significant fibrosis which is tending to link centrilobular and periportal areas. Hepatocytes are vacuolated with evidence of degeneration. Original magnification of image; ×100 (H&E). (c) Liver from a rat treated with CCl$_4$ twice weekly for 6 weeks at 0.7 ml/kg; there is perivascular fibrosis in the centrilobular and periportal areas, with some evidence of bridging along the sinusoidal tracts. Original magnification of image; ×100 (Sirius red). (d) Liver from a rat treated for 6 weeks, twice weekly at 1.0 ml/kg CCl$_4$. Perivascular fibrosis is evident with bridging along sinusoidal tracts within the liver parenchyma. Original magnification of image; ×100 (Sirius red).
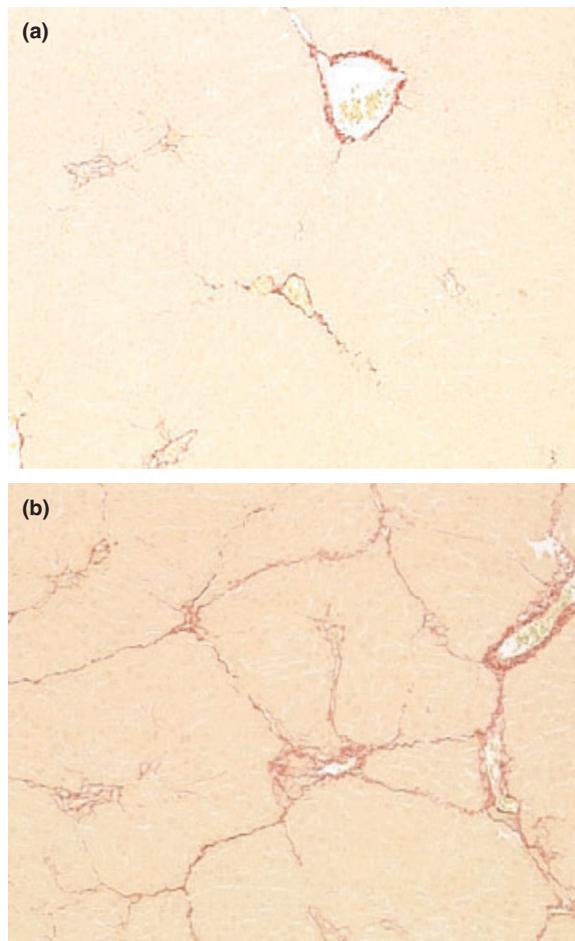
Figure 3 Histopathology of the liver from rats treated with carbon tetrachloride (CCl₄) twice a week for 6 weeks, then left untreated for a 6-week period of recovery. (a) Liver from a rat treated with CCl₄ for 6 weeks at 0.4 ml/kg and left untreated for a 6-week recovery period. There is perivascular fibrosis with some evidence of delicate partial bridging within the liver parenchyma. (b) Liver from a rat treated with CCl₄ for 6 weeks at 1.0 ml/kg and left untreated for a 6-week recovery period. There is perivascular fibrosis in centrilobular and portal regions, with delicate bridging of fibrosis between these areas. Original magnification of both images; ×100 (Sirius red).
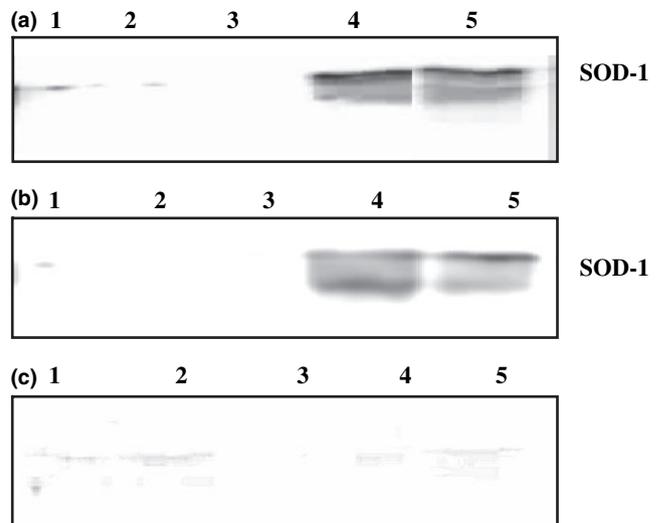

Figure 4 Western blots with a superoxide dismutase (SOD-1) antibody of rat urine samples collected during the induction of liver fibrosis with carbon tetrachloride (CCl₄) repeatedly dosed at 0.7 ml/kg. Samples for sodium dodecyl sulphate–polyacrylamide gel electrophoresis were prepared, with each sample containing 14 μl of urine. (a) Urine collected after 3 weeks of dosing twice a week with CCl₄. (b) Urine collected after 6 weeks of dosing twice a week with CCl₄. (c) Urine collected at the end of the 6-week non-dosing recovery period. In all blots: lane 1, 2, 3 = control urine from rats treated with vehicle, and lanes 4 and 5 = urine from rats treated with CCl₄ at 0.7 ml/kg.

animals at 0.5, 0.6, 0.7 and 1.0 ml/kg. Following the 6-week recovery period, mean TP and ALB levels in general showed a return to control values, as did TRIG and GLU values. Superoxide dismutase activity was significantly decreased in the 0.7, 0.8 and 0.9 ml/kg CCl₄ groups ($P < 0.05$). Lactate dehydrogenase and CK activities were significantly lower than the controls in all CCl₄-treated groups except at 0.4 and 0.5 ml/kg. Mean CHOL levels were significantly increased above control values in all CCl₄-treated groups except at 1.0 ml/kg. Urea levels in rats treated with CCl₄ were greater than the control level at 0.4 and 0.6 ml/kg. However, in the higher dose CCl₄ groups, urea levels were lower than the controls, and the mean value was significantly decreased at 1.0 ml/kg CCl₄. Control and CCl₄-treated serum CREA levels at 12 weeks were similar.

Histology. At the 6- and 12-week necropsies, livers and kidneys were removed for histopathological assessment. Tissues were stained with H&E and Sirius red. Haematoxylin and eosin staining of liver tissues at 6 weeks revealed in the CCl₄-treated animals hepatocellular vacuolation in the centrilobular region and fibrosis with capsular pitting (Figure 2). Haematoxylin and eosin staining of kidney sections at 6 weeks did not reveal treatment-related changes.

Liver tissues at 6 weeks were randomly selected for Sirius red staining; this showed evidence of fibrosis in all selected animals in all CCl₄-treated groups (Figure 2). In all rats, there was perivascular fibrosis and delicate 'bridging' among centrilobular, periportal and capsular areas. There was no evidence of fibrosis in any kidney sections stained with Sirius red.
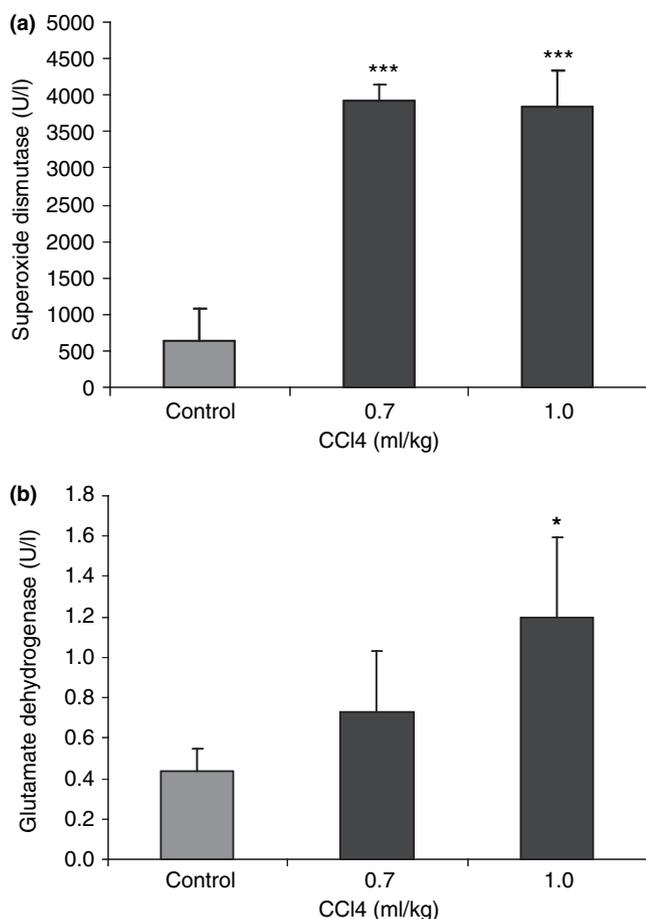
Figure 5 Mean superoxide dismutase (SOD) (a) and glutamate dehydrogenase (GLDH) (b) levels in the urine of rats treated with carbon tetrachloride at 0.7 and 1.0 ml/kg twice a week for 6 weeks. Superoxide dismutase activity was determined by using a Ransod assay kit; GLDH activity was measured by using the kinetic Deutsche Gesellschaft für Klinische Chemie method. Values are means (error bars represent SD) of six animals. Values that differ from control by Student's *t*-test are shown, *$P < 0.05$, ***$P < 0.001$.

At the 12-week necropsy, after the 6-week recovery period, H&E staining of liver sections identified fibrosis in some animals. Also, in some animals, mature fibrosis was present in subcapsular regions associated with capsular pitting or contraction. Some liver sections also showed parenchymal fibrosis with fibrous tissue present around the centrilobular veins, the periportal regions and extending into the parenchyma along the sinusoidal tracks. In the kidney, at the 12-week necropsy, there were no treatment-related changes identified with H&E staining. Sirius red staining of liver sections from rats treated with CCl₄ showed evidence of fibrosis in all randomly selected animals, although there was some intra-group variation in relation to the degree of fibrosis detected (Figure 3). The fibrotic response did not therefore return to normal during the 6-week recovery period.

Analysis of urine for novel protein markers of hepatic fibrosis. SDS–PAGE. Urine samples from control and 0.7 and 1.0 ml/kg CCl₄ groups were collected during Experiment 2 at week 3 and 6 of the dosing period and at week 3 and 6 of the non-dosing recovery period (i.e. week 9 and 12 respectively). Samples were analysed by SDS–PAGE. The only difference identified between control and CCl₄-treated rat urine was the presence of a band at 18.4 kDa in the CCl₄-treated urine samples at week 3 and 6 of the dosing period. Previous studies (Smyth *et al.* 2004) had shown that following the administration of a single dose of CCl₄ this same protein band had appeared in the urine of CCl₄-treated rats and was identified by nano-scale reversed-phase liquid chromatography (nano-LC) combined with electrospray ionization tandem mass spectrometry (ESI-MS/MS) as copper/zinc superoxide dismutase (Cu/Zn SOD or SOD-1). In the present studies, the appearance of the SOD-1 band in the urine samples from rats repeatedly dosed with CCl₄ at week 3 and 6 of dosing was confirmed by Western blotting with an SOD-1 antibody.

Western blotting with SOD-1 antibody. Urine samples collected after 3 weeks (i.e. 6 doses of CCl₄) and 6 weeks (i.e. 12 doses of CCl₄) at 0 (vehicle control) and the 0.7 ml/kg CCl₄ dose level were Western blotted with SOD-1 antibody. This revealed that SOD-1 was greatly increased after both 3 and 6 weeks of dosing in the CCl₄-treated rat urine samples compared with control animals (Figure 4a,b). However, when the urine samples collected from rats dosed with CCl₄ at 0.7 ml/kg at 3 weeks into the non-dosing recovery period (i.e. week 9) and at the end of the 6-week period of recovery (i.e. week 12) were Western blotted with the SOD-1 antibody, SOD-1 could no longer be detected (Figure 4c).

SOD and GLDH activity measurements. Urine samples collected after 6 weeks of dosing twice a week with CCl₄ at 0, 0.7 and 1.0 ml/kg were assayed for SOD activity. Superoxide dismutase activity was increased in the CCl₄-treated groups. The mean control SOD activity was 633.25 U/l; this was increased to 3911.83 and 3835.33 U/l in the 0.7 and 1.0 ml/kg groups (increases of 5.18-fold and 5.06-fold) respectively ($P < 0.001$ at both CCl₄ dose levels) (Figure 5a). However, SOD activity did not appear to be related to the dose level of CCl₄ administered. Glutamate dehydrogenase activity was also measured in the urine samples collected after 6 weeks of CCl₄ dosing at 0.7 and 1.0 ml/kg. The

GLDH activity in control samples was 0.43 U/l and this was increased in the 0.7 ml/kg group to 0.73 U/l (0.70-fold greater than control; NS). However, GLDH activity was increased to 1.20 U/l (1.79-fold greater than control; $P < 0.05$) in the urine samples from rats treated with CCl_4 at 1.0 ml/kg (Figure 5b).

Discussion

In the present studies to induce hepatic fibrosis, CCl_4 was administered using a repeat dose regimen at a series of dose levels and a comprehensive range of serum enzymes and other clinical chemistry parameters measured at each dose level. Urine samples were also analysed in an attempt to identify a non-invasive marker of hepatic fibrosis. A preliminary experiment was carried out to identify the lowest dose level of CCl_4 which, when administered as a single dose (0.4–2.8 ml/kg), caused liver but not kidney injury. Carbon tetrachloride induced histopathological and clinical chemistry evidence of hepatotoxicity at 0.4 ml/kg and above, whereas kidney injury was only evident at 1.2 ml/kg and above. It was therefore decided that in Experiments 1 and 2, rats would be dosed with CCl_4 at levels below 1.2 ml/kg, and thus any proteins detected in the urine were likely to result from liver injury, not kidney injury. However, as CCl_4 in Experiments 1 and 2 was to be given repeatedly, twice a week for 6 weeks, and as the effect of summating CCl_4 doses on the liver and kidney were unclear, it was decided to err towards using lower doses of CCl_4; for this reason, dose levels in Experiment 1 were set at levels of 0.06–0.36 ml/kg. Nevertheless, in Experiment 1, only mild fibrosis was induced, and therefore in Experiment 2, higher dose levels of CCl_4 were used, between 0.4 and 1.0 ml/kg. However, Experiment 1 produced results of interest, demonstrating that the very low-dose level of 0.06 ml/kg CCl_4 induced significant hepatotoxicity, and was above the 'no toxic effect' or 'threshold' level for CCl_4 administration using the present dosing regimen.

In Experiment 1, liver weights were increased at all CCl_4 dose levels from 0.06 to 0.36 ml/kg and the increases in liver weight were closely related to CCl_4 dose. Serum chemistry results showed significant increases in ALT, AST and GLDH and as the dose level of CCl_4 increased, the serum levels of the three enzymes increased. Park *et al.* (2000) found significant increases in serum ALT and AST after 4 weeks of dosing rats with 1 ml/kg CCl_4 twice a week. However, studies by Jiang *et al.* (1992) in which rats were dosed i.p. with 1 ml/kg CCl_4 only demonstrated a two-fold increase in serum ALT activity. In Experiment 1, serum ALP

and T-BIL levels were also increased in rats treated with CCl_4. These results suggest an impairment of bile flow, possibly because of the deposition of collagen fibres disrupting normal liver structure and function (Zimmerman 1978; Gaudio *et al.* 1997).

The amount of serum ALB in rats treated with CCl_4 twice a week for 6 weeks (Experiment 1) was reduced in relation to the control animals at all dose levels, and this decrease was significant in the 0.30 and 0.36 ml/kg CCl_4 groups. The reduction in ALB levels finds a parallel in reports of other authors. Serum ALB levels tend to be reduced in chronic liver injury as a result of the impaired ability of liver cells to synthesize protein (Horne *et al.* 1973). Giménez *et al.* (1994) reported a decrease in serum ALB following 16 weeks of treatment with CCl_4 by inhalation. Similarly, studies by Hernandez-Munoz *et al.* (2001) in which liver cirrhosis was induced in rats demonstrated that after 10 weeks of repeated dosing with CCl_4, serum ALB levels were significantly reduced.

Histopathological examination of liver tissues in Experiment 1 after 6 weeks of CCl_4 dosing showed the presence of fibrosis, but this was not a consistent response throughout the CCl_4-treated rats. Furthermore, even at the highest dose (0.36 ml/kg), the fibrosis induced was mild. Sirius red staining of randomly selected liver samples showed that collagen fibres were present, but again there was variation in the degree of fibrosis induced. Examination of the literature demonstrated that CCl_4-induced hepatic fibrosis has been induced in several studies by the administration of larger doses of CCl_4, or by dosing for longer periods. For example, Luckey and Petersen (2001) induced fibrotic changes after 4 weeks of dosing rats with 1.0 ml/kg CCl_4 twice a week; however, the injury was more evident after 6 weeks of dosing, with greater deposition of extracellular matrix and the appearance of bridging fibrosis. Similarly, Park *et al.* (2000) administered CCl_4 orally at 1.0 ml/kg twice a week for 4 weeks; this regimen induced the extensive accumulation of connective tissue resulting in the destruction of the lobular architecture and the formation of septa. Paradis *et al.* (1999) induced fibrosis by dosing 2.0 ml/kg CCl_4 twice a week for 3 weeks, whereas Iredale *et al.* (1998) administered 2.0 ml/kg CCl_4 to male rats twice a week for 4 weeks and observed nodular fibrosis with the deposition of fibrotic septa. In the present Experiment 1, following the 6-week non-dosing recovery period (i.e. at 12 weeks), liver weights remained heavier than in the control animals and the increases were statistically significant at 0.18 and 0.24 ml/kg CCl_4. At the 12-week necropsy, serum clinical chemistry results showed ALT and GLDH activities similar to the controls in all dose level groups. However, AST activities

were significantly reduced compared to the controls in the 0.24, 0.30 and 0.36 ml/kg groups, but comparable to control values at all the lower dose levels of CCl₄. In general, there were no significant differences between the control and CCl₄-treated groups for the other serum parameters measured (e.g. ALP, ALB and T-BIL). These results suggest that in the 6-week recovery period the liver underwent a period of repair. Histology results confirmed that following the 6-week recovery period the livers had returned to a normal structure with no evidence of fibrosis.

The findings of Experiment 2 at 6 weeks, in general, compared with Experiment 1; increases in liver weights (Table 1) and serum clinical chemistry results (Table 2) tended to follow similar patterns of change. Elevations of relative liver weights, as a result of CCl₄ administration, are generally considered to be related to hepatocyte cytoplasmic swelling as a result of cellular injury and degeneration, and the infiltration of inflammatory cells; also, with repeated CCl₄-induced cellular damage, the deposition of collagen in the development of the fibrotic response would also play a part. Thus, the general overall change is an increase in hepatocyte size, an increase in infiltrating inflammatory cell numbers, and an increase in matrix tissue deposition. Alanine aminotransferase, AST and GLDH were also increased in Experiment 2 at all dose levels of CCl₄ (Table 2) and dose–response relationships were apparent. At all CCl₄ dose levels, AST activity was greater than ALT indicating the induction of chronic injury. Alkaline phosphatase activity was significantly elevated in all CCl₄ groups, as were BA measurements. Total-bilirubin values were also greater than the controls at all levels of CCl₄. Vollmar *et al.* (1999) reported that after administering 0.15 ml CCl₄/100 g rat body weight (i.e. 1.5 ml/kg) twice a week for 8 weeks, marked increases were observed in serum ALT and AST. Wang *et al.* (1996) found ALT activity in female rats treated with CCl₄ for 6 weeks was four times greater than in controls. In these two studies, serum T-BIL was also measured and was significantly greater in CCl₄-treated rats than in control animals. Serum CREA measurements at 6 weeks were reduced in all CCl₄-treated groups, with significant decreases at 0.6, 0.7, 0.8 and 1.0 ml/kg CCl₄ (Table 2). Reductions of serum CREA generally result from decreases in muscle mass, or from a diet that is inadequate in protein (York, pers. comm.). Lopez-Lirola *et al.* (2003) reported muscle atrophy in CCl₄-induced cirrhosis; however, this response was due to protein deficiency and not the result of induced cirrhosis. At 12 weeks, after the 6-week non-dosing recovery period, most serum parameters had returned to control levels (Table 3), indicating that no new hepatocellular injury

was occurring and therefore enzymes and other biomarkers were at normal blood levels.

Histopathological study of the CCl₄-treated livers from Experiment 2 revealed that fibrosis had been induced, with capsular pitting (Figure 2). Sirius red staining revealed perivascular fibrosis and delicate bridging between the centrilobular veins, periportal and capsular areas. However, even at the higher dose levels of CCl₄ used in this experiment, there was still variation between, and within, treatment groups. Histopathological examination of livers at 12 weeks (Figure 3) revealed the presence of fibrosis in the subcapsular regions, which extended into the parenchyma along the sinusoids. Therefore, Experiment 2 demonstrated that the fibrosis had not reversed during the 6-week recovery period. Other workers have reported a significant fibrotic response after dosing rats with CCl₄ twice a week for 8 and 12 weeks at 0.2 ml/kg by i.p. injection (Ohishi *et al.* 2001). Here, after 8 weeks of dosing, assessment of the livers demonstrated periportal fibrosis. By 12 weeks, the fibrosis had extended along the sinusoids with bridging between portal tracts. Ferré *et al.* (1999) induced cirrhosis in rats by the i.p. injection of 0.5 ml/kg CCl₄ twice a week for 9 weeks. In another study (Rivera *et al.* 2001), rats were dosed intragastrically with CCl₄ once a week for 9 weeks. The outcome was extensive collagen deposition and bridging between portal regions. Cabré *et al.* (2000) administered 0.5 ml CCl₄ by i.p. injection to rats twice a week for 9 weeks. By 2 weeks after the commencement of dosing, fibrosis was observed in all animals. In a similar study by Varela-Moreiras *et al.* (1995), steatosis was evident after 3 weeks of CCl₄ dosing and cirrhosis after 9 weeks. It is therefore considered that in the present studies more significant fibrosis may have been achieved by dosing for a longer period or with higher CCl₄ doses.

Urine samples collected during Experiment 2 were analysed by 1D SDS–PAGE. This revealed that the only protein band apparent in the urine of CCl₄-treated rats, not detected in control urine, was an 18.4-kDa band (Figure 4). Previous studies (Smyth *et al.*, 2004) had demonstrated the same 18.4-kDa band in rat urine in response to a single dose of CCl₄. This protein had been identified using nano-electrospray tandem mass spectrometry (nano-ESI-MS/MS) as Cu/Zn SOD (SOD-1) (Smyth *et al.* 2004). In the present studies, the identification of Cu/Zn SOD in urine was confirmed, but in this case in repeat-dosed CCl₄ rats by Western blotting with SOD-1 antibody.

Superoxide dismutase belongs to a group of metalloenzymes that are responsible for catalysing the dismutation of superoxide anions ($O_2^{\bullet-}$) into oxygen and hydrogen peroxide (McCord & Fridovich 1969). Therefore, SOD forms

part of the antioxidant defence system (McCord *et al.* 1971). Carbon tetrachloride is metabolized by the cytochrome P450 oxidase system to produce the trichloromethyl free radical. This free radical initiates lipid peroxidation, whereby a number of reactive oxygen species and free radicals are generated (de Zwart *et al.* 1998).

Superoxide dismutase-1 was identified in urine from rats treated with CCl_4 after both 3 and 6 weeks of dosing (Figure 4). However, SDS–PAGE analysis did not detect SOD-1 in urine samples collected at 3 weeks into the recovery period (i.e. week 9) nor at 6 weeks of the recovery period (week 12). Western blotting with SOD-1 antibody showed SOD-1 was greatly increased in the urine from rats treated with CCl_4, but was barely visible in control urine samples (Figure 4). However, after 6 weeks of no CCl_4 treatment, Western blotting demonstrated that SOD-1 levels were similar to the control animals in all urine samples from CCl_4-treated rats.

The activity of SOD was also measured in urine from rats treated with CCl_4 twice a week for 6 weeks (Figure 5a). Superoxide dismutase activity was approximately 5-fold greater in the urine from rats treated with CCl_4 at 0.7 ml/kg than in control urine. However, SOD activity was not further increased in rat urine at the higher CCl_4 dose level of 1.0 ml/kg (Figure 5a). The activity of GLDH was also measured in urine samples from rats after dosing with CCl_4 twice a week for 6 weeks (Figure 5b). A clear dose–response relationship is seen, with GLDH activity in urine increasing with an increasing dose of CCl_4. Glutamate dehydrogenase activity was approximately 1.8 times greater in urine from rats treated with CCl_4 at 1.0 ml/kg than in the controls. However, although GLDH is present at high concentrations in the liver, it can also be identified in the kidney and skeletal muscle. Therefore, GLDH is not a liver-specific biomarker. The activity of GLDH is higher in the centrilobular area of the hepatic lobule than in other regions and the enzyme therefore appears to be a good marker of centrilobular necrosis. Urinary SOD activity values showed greater fold increases in rat urine following CCl_4 treatment, compared with the fold increases for GLDH levels; however, GLDH activity may be a more sensitive urinary marker than SOD activity as GLDH demonstrated a dose–response relationship to the dose levels of CCl_4 administered.

Superoxide dismutase-1, as well as showing raised levels in urine, was also increased in the serum from rats treated with CCl_4 for 6 weeks (Table 2). However, although the mean levels of the enzyme in serum were significantly greater than in the controls at all CCl_4 dose levels, SOD-1 appears to be a less sensitive serum biomarker in comparison with other conventional biomarkers of liver injury such as ALT, AST and GLDH.

The present studies show that at week 12, on removal of the stimulus for fibrosis (i.e. CCl_4 administration), hepatocytes were not being injured, and therefore SOD-1 levels and levels of other biomarker enzymes were, in general, at base-line (control) levels in the blood (Table 3), and SOD-1 was also absent from the urine (Figure 4c). Nevertheless, at this time (week 12), fibrosis remained clearly evident (Figure 3). Therefore, it is concluded that SOD-1 is a urinary marker of hepatocyte injury (degeneration and necrosis), but not a marker of the established fibrotic state. However, as SOD-1 is found in tissues other than the liver, e.g. the kidney and heart (Frederiks & Bosch 1997), the enzyme cannot be considered as a liver-specific urinary biomarker.

Acknowledgements

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