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Immunohistochemical and Histopathological Evaluation of 2,4-Dichlorophenoxyacetic Acid-Induced Changes in Rat Kidney Cortex

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Abstract This study aims to investigate the effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on rat kidney cortex histology. Oral exposure of rats to 2,4-D for 28 days resulted in decreases in body weight gain and kidney weight. Histological examination showed degeneration in renal corpuscles and podocytes; vacuolization in the glomerulus with disintegration of the basal membrane; tissue edema; vacuolization, cystic dilation and invagination of the basal laminae in the tubular structures; dilation and congestion in renal corpuscular vessels and marked decrease in glomerular and stromal fibronectin reaction; suggesting that subacute 2,4-D administration induces dose-dependent histopathological degenerative effects in rat kidney cortex.

Keywords Herbicide · 2,4-D · Histopathology · Kidney · Rat

Chlorophenoxy herbicides are used for pest control with their higher insecticidal activity and considerably lower mammalian toxicity compared to other pesticides. 2,4-dichlorophenoxyacetic acid (2,4-D; CAS No. 94-75-7) is a member of the chlorophenoxy herbicides. 2,4-D is use as a foliar herbicide in the post emergence control of unwanted broad-leaved plants, especially in agricultural areas growing commodity crops such as wheat, rice, oats, corn; in aquatic areas such as lakes and ponds; and in noncrop areas, namely golf courses and parks (Donald et al. 1999). It is also accepted as WHO Class II "moderately hazardous" pesticide (WHO 2005). Various 2,4-D formulations (aqueous salts or esters) are available on the market for use in indoor pest control, carrying potential human health risk.

Early studies have reported that phenoxyacetic acid derivatives are rapidly excreted unchanged in animals (St. John et al. 1965). 96% of an orally administered dose of radioactive labeled-2,4-D was recovered from the urine of a sheep within 24 h of ingestion (Clark et al. 1964). A previous study evaluating tissue residues of some chlorophenoxy acids in sheep and cattle following oral doses has reported that chlorophenoxy acids and the related phenolic metabolites are found in muscle, fat, liver, and kidney tissues of sheep and cattle; with the highest residues in the kidney (Clark et al. 1975). Metabolism of 2,4-D in laying hens and lactating goats has recently been studied and the most abundant tissue residue of 2,4-D was found in the kidneys (Barnekow et al. 2001).

Although it has been well-documented that 2,4-D is accumulated abundantly in the kidney tissue, there is limited research in the literature investigating the potential effects of 2,4-D directly on the histology of kidney. Therefore, this study aimed to analyze the effect of the oral 2,4-D administration on the histology of kidney in rats. Animal toxicity tests mostly use rodents; therefore, Wistar albino male rats were selected as the test animal in the current study and subacute effects of 2,4-D on rat kidney tissue was investigated using the draft protocol of the "OECD Enhanced Test Guideline 407" (Enhanced TG 407, OECD 2000). Due to this protocol, 2,4-D was administered for 28 consecutive days in repeated oral doses and potential effects on the kidney tissue were assessed

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using histopathological, histochemical and immunohistochemical methods.

Materials and Methods

Commercially available form of 2,4-D (Koruma Weed Killer D, Koruma Agriculture, Turkey) was used. This formulation contains 500 g/L 2,4-D as diethylamine salt (80% active ingredient). Test concentrations of 2,4-D (20, 40, 80 mg/kg) were calculated from the percentage of the active ingredient and final dilutions were prepared freshly in distilled water.

The protocol was approved by the Animal Ethical Committee of Ege University, Faculty of Medicine and conformed to the "Guide for the Care and Use of Laboratory Animals" (NIH Publication no: 85-23, revised 1996). Experiments were conducted on adult male Wistar Albino rats (170–180 g). After 10 days of acclimation, the rats were randomly assigned to either the experimental groups (receiving 20, 40 or 80 mg/kg 2,4-D) or the control group. 2,4-D was administered orally to rats using gastric gavage at 13:30 p.m. every day for a standart 28-day period to comply with OECD guideline 407 (OECD 2000), control rats received distilled water by gastric lavage. The number of rats in each group was kept constant throughout the experimental period so that each group contained 10 male rats.

At the end of 28-day exposure period; kidneys were removed under ketamine + xylasine anaesthesia after intracardiac fixation with 4% paraformaldehyde, weighed, post-fixed for 24 h and processed for paraffin embedding. Paraffin sections were cut into 3 μm thick slices in microtome (Leica RM 2145) and stained with routine Haematoxylin and Eosin (H&E), Mallory-Azan and Alcian Blue & Periodic Acid–Schiff (Alcian Blue & PAS) histostains. Immunohistochemical expression was analyzed using anti-fibronectin antibodies. Briefly, paraffin sections were immersed in xylene overnight and incubated in methanol containing 1% H_2O_2 to reduce endogenous peroxidase activity. Sections were kept in sodium citrate solution in the microwave oven at 90 Watt for 5 min and at 360 W for 15 min (Galbavý et al. 2002). After washing in 0.2 M Tris–HCl including 0.5% Triton X, the sections were exposed to the mouse anti-fibronectin primary antibody (Dako A0245). Sections were then incubated with mouse monoclonal PAP complex (DAKO Corporation, Carpenteria, CA, USA; 1:200 dilutions) and reacted with 0.05% diaminobenzidine (Zymed Histostain Plus Ref/Cat No: 859643 San Francisco, CA, USA) and 0.01% H_2O_2. Immunoreaction was assessed by light microscopy (Olympus BX-51 light microscope, Olympus C-5050 digital camera) at a magnification of X40.

Data are presented as mean ± standard error mean. Analysis of variance (ANOVA) for repeated measures was used for the statistical analysis of the body weight differences between first and 28th day of the experiments and between groups. One way ANOVA was performed for the comparison of kidney weight differences between groups. $p < 0.05$ was considered significant and when significance was observed in the initial ANOVA test, post hoc analysis with Bonferroni test was carried out for further between-group analysis. Pearson's correlation coefficient was calculated with its significance level as a measure of linear correlation between kidney and body weights at sacrifice.

Results and Discussion

Mean body weights and mean wet kidney weights of rats are presented in Table 1. Rats in the control group significantly gained weight during the 28-day experimental period ($p < 0.001$), while rats in the experimental group lost weight in a dose-dependent manner. Mean kidney

Table 1 Mean body weight of rats in control group and experimental groups before and after exposure to 2,4-D for 28 days and mean kidney weight of rats at sacrifice

	Body weight (g)		Kidney weight (g)
	Before	After	
Control	174.81 ± 1.44	180.10 ± 1.48***	2.03 ± 0.04
20 mg/kg 2,4-D	174.60 ± 1.74	171.77 ± 1.57*	1.90 ± 0.01$^{\#\#}$
40 mg/kg 2,4-D	174.44 ± 1.72	166.57 ± 2.71**.$^{\#}$	1.77 ± 0.01$^{\#\#\#,\&}$
80 mg/kg 2,4-D	176.04 ± 1.70	159.92 ± 1.86***.$^{\#\#}$	1,67 ± 0.01$^{\#\#\#,\&,\$}$

* $p < 0.05$ ** $p < 0.01$, and *** $p < 0.001$, after vs. before

$^{\#}$ $p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, when compared to control group

$^{\&}$ $p < 0.01$, when compared to 20 mg/kg 2,4-D group

$^{\$}$ $p < 0.05$, when compared to 40 mg/kg 2,4-D dose group

n = 10

weights of rats in the 2,4-D exposure groups were significantly less than the control group at sacrifice ($p < 0.01$ for 20 and $p < 0.001$ for 40 and 80 mg/kg groups respectively, Table 1) and the decrease in kidney weight was dose-dependent. There was a significant positive correlation between the body weights and kidney weights at sacrifice ($p < 0.01$, $r = 0.7207$, Fig. 1).

The lower no-observable-adverse-effect level (NOAEL) for 2,4-D in rats subchronically exposed to the herbicide for 13 weeks was reported as 15 mg/kg/day (Gorzinski et al. 1987; Charles et al. 1996); therefore, a slightly higher dose of NOAEL (20 mg/kg) and its multiples were chosen as the test doses in this study. Apart from a dose-dependent decrease in body and kidney weights, changes in the kidney morphology at light microscopical level (Table 2) was observed distinctly in this dose range, indicating the congruity of the dose range selected. Morover, a recent study has evaluated 2,4-D exposure in farm family members in Minnesota and South Carolina of United States on their farm and the geometric mean systemic doses were found 2.46, 0.8 and 0.22 μg/kg in applicators, spouses and children, respectively (Alexander et al. 2007). This study has revealed a considerable heterogeneity of 2,4-D exposure among farm family members and showed that exposure to the spouses and children is primarily determined by direct contact with the application process and the number of acres treated. When compared to the estimated systemic doses in potential human exposure, the dose range in our study is considerably low; suggesting the requirement of a critical evaluation of the developing exposure and risk characterizations in 2,4-D-exposed agricultural populations.

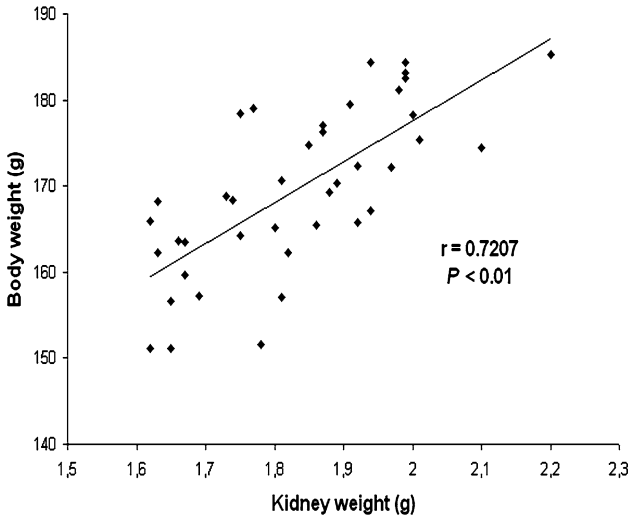

Fig. 1 Scatter graph of the correlation between kidney and body weights of rats at sacrifice. Pearson's correlation coefficient is 0.7207

H&E staining of kidney sections in the control group showed renal corpuscles in the cortex with adjacent proximal and distal convoluted tubules (Fig. 2). Renal corpuscles were more distinguishable at the higher magnification, each consisting of a glomerulus and an intact Bowman's capsule (Fig. 2). Numerous proximal convoluted tubules were noticed with small, uneven lumen and composed of large cuboidal cells with granular cytoplasm; distal convoluted tubules were observed with larger and regular lumen surrounded by smaller and more distinctly cuboidal cells having cytoplasms stained less with eosin. Mallory-Azan staining of the control kidney confirmed the integrity of the connective tissue between the renal corpuscles and convoluted tubules and showed the evenness of the blue-stained basement membrane (Fig. 3). Mallory-Azan staining also revealed erytrocytes with orange–yellowish color in the glomerular arteriole; the integrity of red colored brush borders of proximal tubules was noticed clearly. PAS staining confirmed the presence of regular brush borders of the proximal convoluted tubules and the basement membranes evenly (Fig. 4). Immunostaining with fibronectin antibody elicited the integrity of the extracellular matrix in the nephron (Fig. 5).

Contrarily, histology of the experimental groups revealed renal corpuscular degeneration showing a dose-dependent increase in severity (Table 2). Podocytes surrounding the glomerular capillaries showed a dose-dependent degeneration and tissue edema was noticed (Fig. 2). Marked vacuolization and cystic dilation were observed in the tubular structures,

Table 2 Histopathological findings in the kidney of the control group and experimental groups exposed to 2,4-D

Histopathological findings	Control	20 (mg/kg)	40 (mg/kg)	80 (mg/kg)
Renal corpuscular degeneration	–	+	++	+++
Podocyte degeneration and edema	N	+	++	+++
Glomerulary fibronectin immunoreactivity	N	↓	↓↓	↓↓↓
Cystic dilatation in kidney glomerulus	–	+	++	++
Peritubular vessels dilatation and congestion	–	+	++	++
Vacuolization of proximal tubulus	–	+	+	++
Epitelial change in distal tubulus	N	+	++	+++
PAS(+) granules in tubulus	–	+	++	+++
Stromal fibronectin immunoreactivity	N	↓	↓↓	↓↓↓

N, normal; –, none; +, mild; ++, moderate; +++, severe; ↓, mild decrease; ↓↓, moderate decrease; ↓↓↓, severe decrease

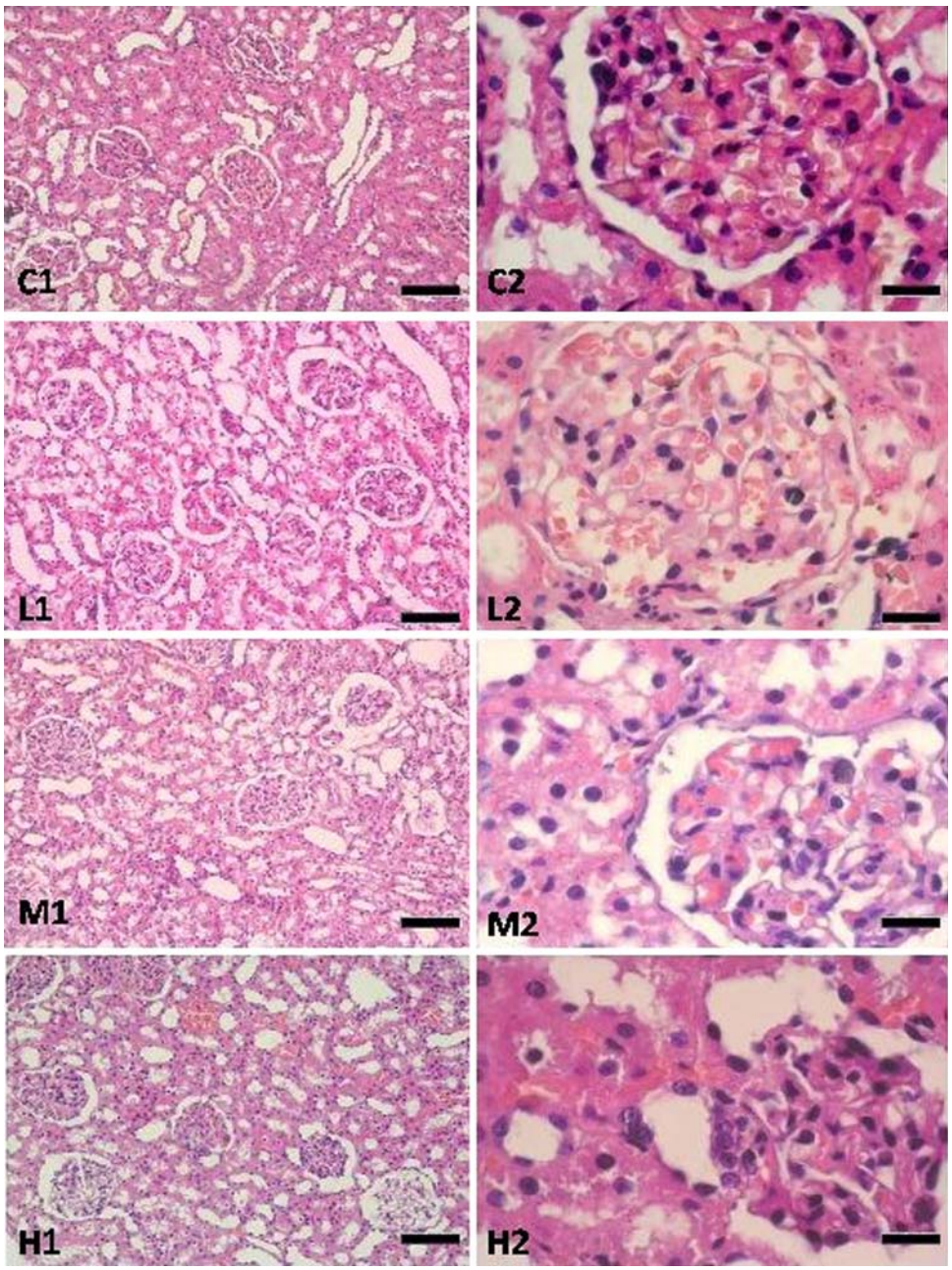

Fig. 2 Representative photomicrographs of histopathologic findings in rat kidney cortex from the subacute study of 2,4-D. Staining was performed on kidney sections with Hematoxylin and Eosin (H&E). C1–C2 are from nontreated control rats; L1–L2, M1–M2 and H1–H2 are from rats exposed to 20, 40 and 80 mg/kg 2,4-D, respectively (Series number 1 and number 2 are ×10 and ×40 magnification, respectively; *scale bar* is 500 μM for ×10 and 125 μM for ×40 magnification)

becoming more distinguishable with the increasing dose of 2,4-D. Mallory-Azan stain revealed vacuolization in the glomerulus with the disintegration of the basal membrane and the disappearance of tubular structures (Fig. 3). PAS (+) stained granules were seen in the proximal and distal tubular epithelium and the amount of these granules increased dose-dependently (Fig. 4). PAS histosatin showed invagination in the basal laminae of proxymal and distal tubules; confirming degeneration in the tubular structures. It was noticed that peritubular capillaries were not well preserved in the sections of 2,4-D-exposed groups; exhibiting a prominent and dose-dependent increase in dilation and congestion in the vessels (Figs. 2, 3). Anti-fibronectin immunostaining showed a marked and dose-dependent decrease in both glomerular and stromal fibronectin reaction; revealing disruption in the extracellular matrix integrity (Fig. 5).

The phenoxyacetic acid herbicides have previously been shown to accumulate at high levels in liver and kidney via the organic acid transport system and this accumulation depresses oxygen consumption by renal cortical slices and results in uncoupling of renal mitochondria raising the possibility of organ-specific toxicity secondary to transport (Pritchard et al. 1982). These changes may accompany the alterations in the histology of the renal cortex. Indeed, the deleterious effects of 2,4-D and related chlorophenoxy compounds on kidney have been investigated in different

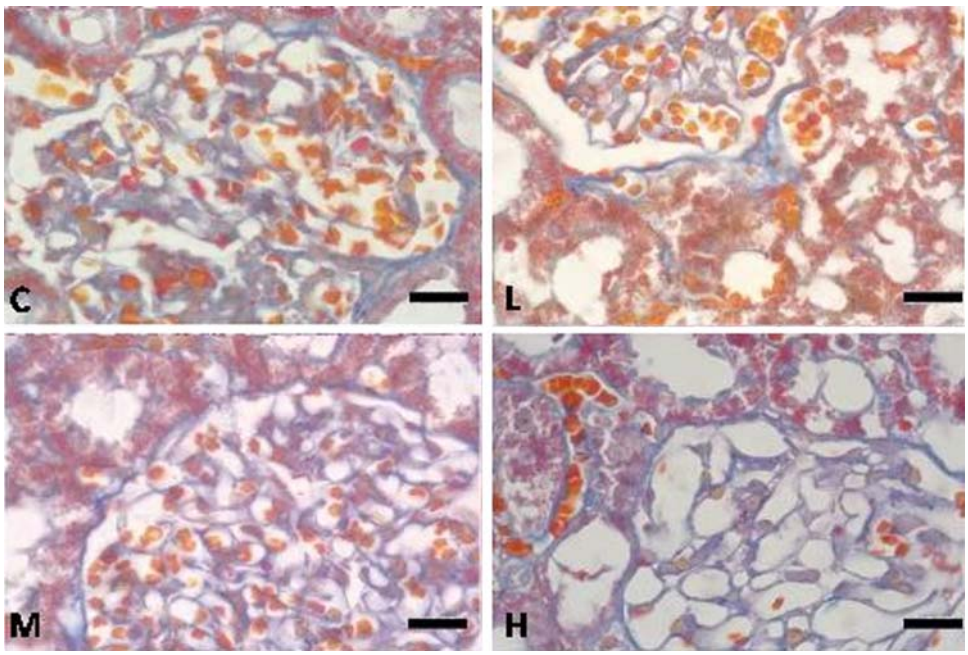

Fig. 3 Representative photomicrographs of glomerular corpuscle in kidney cortex of rats exposed to subacute 2,4-D. Staining was performed on kidney sections with Mallory-Azan histostain. *C*, control group; *L*, 20 mg/kg, *M*, 40 mg/kg and *H*, 80 mg/kg 2,4-D exposure groups (×40 magnification, *scale bar* 125 μM)

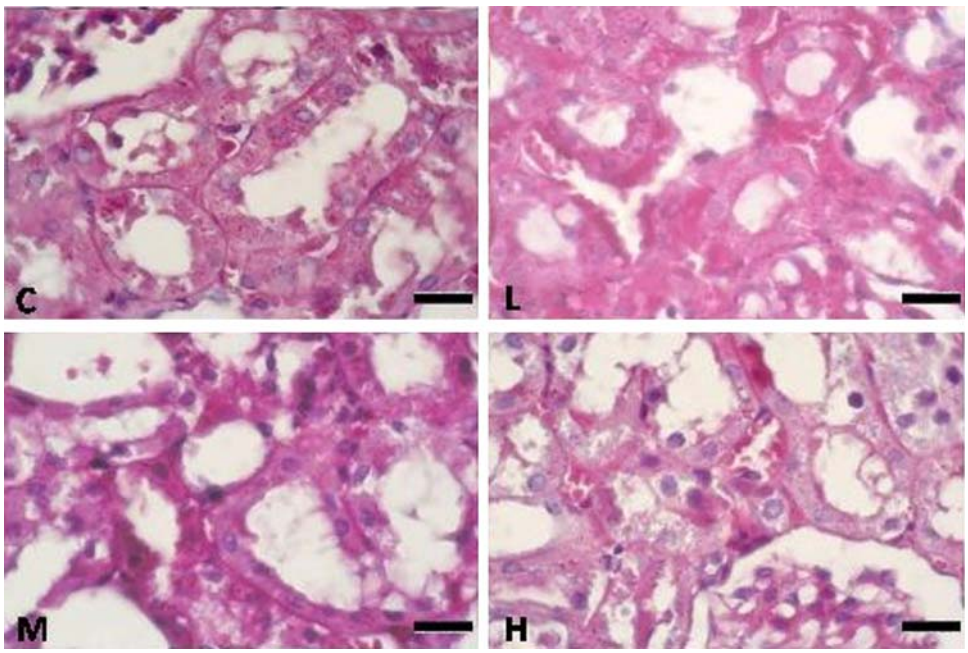

Fig. 4 Representative photomicrographs of renal tubules in kidney cortex stained with Alcian Blue & Periodic Acid–Schiff (Alcian Blue & PAS) histostain. *C*, control group; *L*, 20 mg/kg, *M*, 40 mg/kg and *H*, 80 mg/kg 2,4-D exposure groups (×40 magnification, *scale bar* 125 μM)

mammalian species. A recent study performed on rats, mice and hamster exposed to increasing concentrations of 2,4-D for 3 months has revealed that subchronic exposure to 2,4-D results in a distinctive morphologic alterations diagnosed as simple hyperplasia in the renal tubules of rats and the severity and incidence of renal tubular damage increases in a dose-dependent manner (Ozaki et al. 2001). In this study, the morphological alteration was restricted to the outer stripe of the outer medulla and consisted of a few scattered foci of tubules with prominent basophilia due to high nuclear density and decreased cytoplasmic volume of the epithelial cells. Electron microscopy in this study has shown that the height of these cells was diminished, the cytoplasm of the altered cells was markedly reduced in volume, and there was a prominent decrease in the number and volume of mitochondria (Ozaki et al. 2001). Treatment-related cytoplasmic alterations (described as increased homogenicity) were observed in the renal proximal tubules of rats given 60 mg/kg/day and higher of 2,4-D for 13 weeks (Gorzinski et al. 1987). Furthermore, subchronic exposure of male and female rats to 300 mg/kg/day 2,4-D for 2 years has resulted in brush border loss and

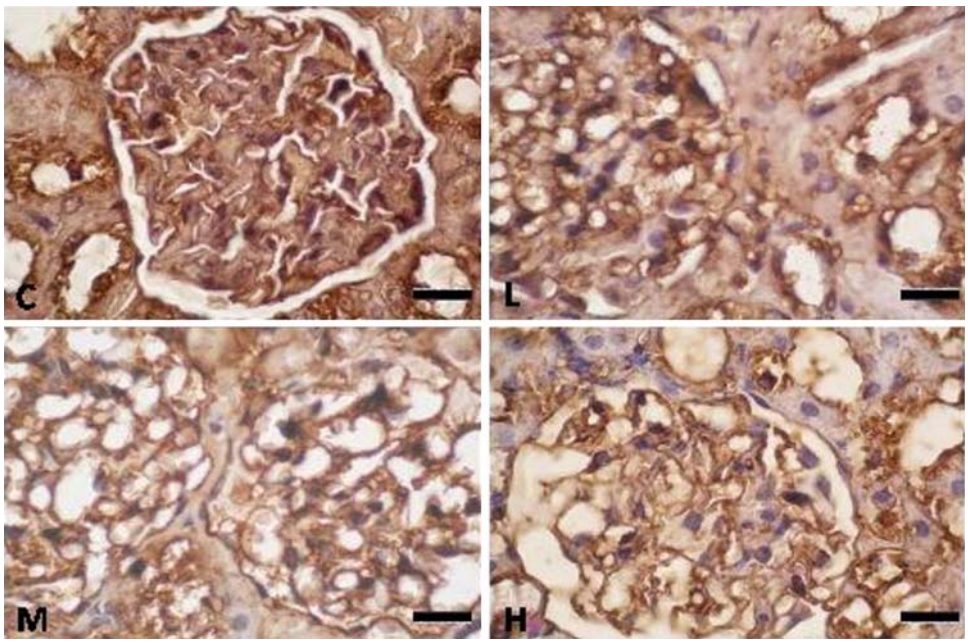

Fig. 5 Representative photomicrographs demonstrating immunohistochemical staining of renal corpuscle with anti-fibronectin antibody. Note relatively faint staining intensity and shrinkage of the glomerular structures and vacuolization. *C*, control group; *L*, 20 mg/kg, *M*, 40 mg/kg and *H*, 80 mg/kg 2,4-D exposure groups (×40 magnification, *scale bar* 125 μM)

vacuolization of proximal tubular cells in the kidney (Charles et al. 1996).

Current available studies on the renal toxicity of 2,4-D mentioned above have mostly focused on the carcinogenicity induced by subchronic, long-term exposure to 2,4-D. So far, no study investigating the kidney morphology has been performed in vivo to examine the effects of subacute, considerably shorter exposure of rats to 2,4-D. Therefore, it is not possible to compare our results with the previous data. However, similar histopathological features have been observed in a fetotoxicity study performed on female, pregnant rats using 250 mg/kg 2,4-D for 2 months before fertilization and during pregnancy and lactation (Sulik et al. 2002). This study has revealed that intrauterine exposure to 2,4-D results in renal toxicity in newborn rats and chronic intoxication with probably smaller amounts of 2,4-D leads to vacuoles in cytoplasm and necrosis of renal tubular epithelial cells; with varying degrees of isometric vacuolization of proximal tubular epithelium, tubular microfocal calcification, tubular epithelial inclusion bodies and peritubular capillary congestion (Sulik et al. 2002). Additionally; a recent, 4-week oral rat toxicity study of some other chlorophenoxy herbicides based on the microscopic examination of kidney has shown an increased severity of basophilic tubules and calcification at the outer/inner medulla transition in the kidneys (van Ravenzwaay et al. 2005).

2,4-D is known to be a weak peroxisome proliferator in rodents and the rat is the species most sensitive to the nephrotoxic effects of peroxisome proliferators (Kawashima et al. 1984). There is a site specificity to this toxicity related to areas of peroxisome proliferator-related enzyme induction (namely proximal tubule cells) and 2,4-D is the most effective peroxisome proliferator at inducing renal lesions (Ozaki et al. 2001). It is well-documented that peroxisome proliferator-activated receptor-alpha (PPAR-alpha) is highly expressed in the proximal tubule cells of kidney (Braissant et al. 1996). It is worth noting that renal damage observed in the available oncogenicity, toxicity and fetotoxicity studies is limited to the tubular level. However, light microscopy of kidney in our study has shown not only marked vacuolization and cystic dilation in the tubular structures, but also degeneration in renal corpuscles and podocytes surrounding the glomerular capillaries, as well as vacuolization in the glomerulus with disintegration of the basal membrane. These morphological changes in the glomerular structures may probably lead to functional abnormalities in glomerular filtration (i.e. decreased/increased glomerular filtration rate). These findings also suggest that mechanisms other than the activation of peroxisome proliferator-related enzymes may mediate the nephrotoxicity induced by subacute 2,4-D exposure. Moreover, anti-fibronectin immunostaining showed a marked decrease in both glomerular and stromal fibronectin reaction in a dose-dependent manner; revealing disruption in the extracellular matrix integrity and this "shrinkage" in the kidney at the microscopic level resulted in a significant decrease in kidney weight at organ level.

The overall results of this study showed that oral exposure to 2,4-D induced significant alteration in the cortex of rat kidney tissue. Based on our observations and literature data, we conclude that use of 2,4-D may cause hazardous effects in various levels to non-target organisms, including humans and further studies are required to clarify

the level of kidney toxicity and its potential impact on the maintenance of renal function.

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