ORIGINAL ARTICLE

The Effects of Chronic Low Dose Exposure of Chlorpyrifos on the Rat Kidney

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ABSTRACT

Aim. An increased incidence of chronic kidney disease (CKD) was reported among agricultural workers who were exposed to organophosphates (OP). There is little information on the effects of chronic low OP exposure on kidney in experimental animals. Chlorpyrifos (CPF) is a common type of OP used in agriculture. Thus, the aim of this study was to assess the effects of chronic low subcutaneous exposure of CPF on the kidney. **Methods.** Eighteen male Sprague Dawley rats were divided into three groups, with six rats in each group. Group 1 served as a control group, and groups 2 and 3 received a subcutaneous vehicle (3% dimethyl sulfoxide + 97% v/v soy oil) or CPF (18.0 mg/kg) respectively, every other day for 180 days. Serum urea, creatinine, uric acid, cystatin C, electrolyte levels, acetylcholinesterase (AChE), and arylesterase (ArE) activities were measured. Histopathological changes in the kidney tissues were examined. **Results.** Urea, creatinine, uric acid, and cystatin C levels were significantly increased ($p < 0.05$), while electrolytes were reduced ($p < 0.05$) in the CPF-treated rats. Both AChE and ArE activities were depressed in the CPF group ($p < 0.01$ and $p < 0.001$, respectively). Diffuse necrosis of proximal tubules and glomerular hypercellularity were observed in the kidney in the CPF group. **Conclusion.** A chronic low dose of CPF via subcutaneous exposure caused considerable renal tubular necrosis and derangement of glomerular function. These findings suggest that chronic occupational OPs exposure can cause kidney damage.

KEYWORDS: Organophosphates, chlorpyrifos, kidney damage, biochemistry, histology

INTRODUCTION

Chlorpyrifos (CPF) (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate), a broad-spectrum chlorinated OP insecticide that was first introduced in 1965 in the U.S., is widely used in agriculture to control pests and increase crop production. CPF is mainly metabolized in the liver by the action of cytochrome P450 and forms CPF-oxon, which is a strong (AChE) inhibitor. The latter is metabolized by hepatic and extra-hepatic esterases to form water soluble 3,5,6-trichloro-2-pyridinol and diethylphosphate, which are excreted in urine.[1]

The mechanism of OP neurotoxicity involves an

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accumulation of acetylcholine neurotransmitter, leading to hyper-stimulation of post-synaptic cholinergic receptors at neuromuscular junctions and in the autonomic and central nervous systems. Although OP toxicity was mainly thought to affect the nervous system,[2] many studies showed that at an accepted susceptible limit, OPs can cause tissue damage to literally any tissues,[3] including the kidney.[4]

The chronic low dosage of CPF needs to be considered when investigating the effects of CPF in humans, as this dosage mimics actual exposure. The sub-clinical or sub-threshold dose of CPF refers to the CPF dosage that is not sufficient to induce overt signs of cholinergic toxicity.[5] Pest control, agricultural and industrial workers are exposed to low doses of OP throughout their lifetime, mostly through dermal contact. Such workers also often lack adequate knowledge of safety practices.[6]

CKD is known attributes to common medical illness, such as diabetes mellitus and hypertension. Interestingly, a significant proportion of 5 to 20% of CKD are of unknown aetiology.[7] CKD is diagnosed based on the evidence of kidney damage or decreased glomerular filtration rate (GFR) (less than 60 mL/ min/1.73 m^2) for at least 3 months duration. Kidney damage refers to pathological abnormalities of the kidney or presence of kidney damage markers, by the evidence of increased glomerular filtration markers, such as urea, creatinine and cystatin C.[8]

In Malaysia, a high proportion of the population is subjected to OP exposure with 10.67% of total employment is related to agriculture.[9] Although epidemiological evidence for an association between CKD and long-term OP occupational exposure exists,[10] evidence based on direct effects in animal models is lacking.

A previous study demonstrated effects of a low dose of OP on the nervous system.[11] However, data are lacking on the effects of chronic low exposure on other major organs, such as the kidney. Hence, this study aimed to assess the effects of chronic low subcutaneous exposure to CPF on the kidney of male Sprague Dawley rats.

MATERIALS AND METHODS

Ethics approval

All study protocols were reviewed and approved by the ethics committee of the International Islamic University Malaysia (reference no. IIUM/305/20/4/10). Measures were taken to minimize pain or discomfort, according to guidelines for the care and use of laboratory animals.[12]

Experimental animals

Eighteen male Sprague Dawley rats aged 2–3 months and weighing 200–250 g were purchased from an animal house, University of Putra Malaysia, Selangor. The rats were housed in pairs in elevated polypropylene cages containing wood bedding under the laboratory conditions of 25°C ± 2 and 60-90% relative humidity with light-controlled 12-hour light/ dark cycle. They were fed rat chow (Gold Coin Feedmills, Malaysia) and had access to tap water ad libitum.

Experimental design

After 1 week of adaptation, the rats (n =18) were randomly divided into three groups. The groups were as follows:

1. A control group: The rats were fed a standard pellet diet.
2. A vehicle group: The rats were injected subcutaneously with 3% dimethyl sulfoxide and 97% v/v soy oil (vehicle of CPF) on alternate day for 180 days.
3. A CPF-exposed group: The rats were injected subcutaneously with CPF (Moon Trading Company, Malaysia) in a concentration of 18.0

mg/kg dissolved in the vehicle in a volume of 0.7 mL/kg every other day for 180 days. The purity of CPF was 38.7%.

All the rats were weighed and monitored throughout the study for visible cholinergic symptoms or other signs of distress. The treatment regimen and duration were based on previous published data that fulfilled the criteria for a chronic sub-threshold dose of CPF.[5, 13] As stipulated by the guidelines of the Organization for Economic Co-Operation and Development, 6-month is the minimum duration for the study of chronic toxicity in an experimental animal.[14] In adulthood, every day of the rat is approximately equivalent to 34.8 human days (i.e., one rat month is comparable to three human years).[15] Hence, the CPF exposure of the rats in the current study was equal to almost 18 human years.

Sample collection

For biochemical analysis, blood was taken from the orbital sinus at the end of the exposure period after the rats had fasted overnight. The blood was centrifuged, and the serum was aliquoted into microtubes and stored at -70°C prior to the analysis. For the histopathological examination, following standard animal sacrification, the kidney was quickly harvested, perfused, rinsed with 0.9% NaCl solution and fixed in 10% formalin.

AChE activity

Serum AChE was measured using a quantitative sandwich ELISA protocol in accordance with the manufacturer's instruction manual (Cusabio, China).

ArE activity

Serum ArE activity was determined by measuring the liberation of phenol using a spectrophotometer at 270 nm, with phenylacetate (Sigma Aldrich, Germany) as the substrate solution. The method was as described previously.[16]

Renal biochemical markers analysis

Serum creatinine concentration was determined using the Jaffé alkaline picrate reaction. The concentration of urea was measured by a kinetic assay using glutamate dehydrogenase and urease.[17]

A commercial Cobas Integra 400 Plus Chemistry Analyzer (Roche, USA) was used for both assays. Serum uric acid and calcium (Ca^{2+}) were determined using a modification of the uricase method and a modification of the calcium o-cresolphthalein complexone (OCPC) reaction, respectively. Serum phosphate (PO_4^-) level was measured using a modification of the classical phosphomolybdate method. Serum sodium (Na^+), potassium (K^+) and chloride (Cl^-) levels were measured by ion selective electrode method.[17]

A commercial chemistry analyser (Siemens Dimension RXL, USA) was used to measure the concentrations of serum uric acid and electrolytes (Na^+, K^+, Cl^-, Ca^{2+} and PO_4^-). Cystatin C concentration was measured using a quantitative sandwich ELISA method following the protocols of the manufacturer (Cusabio, China).

Histopathological examination

Kidney preserved in 10% formalin was subjected to standard tissue processing, including steps of dehydration, with ascending grades of alcohol and xylene clearance. The tissues were embedded in paraffin, and 5-µm-thick sections were cut from the paraffin block using a rotary microtome (Leica, Germany) and stained with Haematoxylin & Eosin, Masson's trichrome and periodic Acid Schiff, following the manufacturer's protocol for each (Clin-Tech Limited, UK). A pathologist blinded to the study groups performed the morphological assessments.

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science (SPSS for Windows, version 25). The non-parametric Kruskal –Wallis H test and Mann–Whitney U test with Post-hoc Bonferroni were used to compare the groups. Histopathological changes were assessed descriptively.

RESULTS

AChE activity

The serum AChE activity was significantly decreased in the CPF-exposed group as compared with that in the control groups ($p = 0.05$) (Table 1).

ArE activity

ArE activity was significantly decreased in the CPF-exposed group as compared with that in the control groups ($p < 0.001$) (Table 1).

Table 1: Effects of chronic low dose of CPF on serum AChE and ArE activities of male rats

Parameters	Control Mean ± SE	Vehicle Mean ± SE	CPF Mean ± SE
AChE (pg/mL)	113.22 ± 4.59	112.10 ± 3.66	70.62 ± 8.32*
Arylestearse (U/mL)	48.2 ± 4.2	38.5 ± 13.3	27.8 ± 4.4*

Values are expressed as mean ± standard error (SE); n = 6. AChE, acetylcholinesterase; ArE, arylesterase; CPF, chlorpyrifos; *p<0.05 for group comparison of CPF vs control and vehicle respectively. *Kruskal-Wallis test and Mann–Whitney *U* test with Post-hoc Bonferroni adjustment.

Renal biochemical markers

The concentrations of serum urea, creatinine, uric acid and cystatin C were significantly ($p < 0.05$) higher in the rats exposed to CPF as compared with these parameters in the control groups. In contrast, serum electrolytes (Na^+, K^+, Cl^-, Ca^{2+} and PO_4^-) ($p < 0.05$) were significantly lower in the CPF-treated group as compared with the concentrations in the controls (Table 2).

Histopathological findings

The histological structure (glomeruli, renal tubules, blood vessels and interstitium) of the kidney of the control rats (groups 1 and 2) was normal. In contrast, the histological structure of the kidney of the CPF-treated rats was characterized by diffuse global glomerular hypercellularity. In many areas of proximal tubules, hydropic degeneration of tubular cells was apparent. Also, there were some areas where dilated tubules were lined with flat epithelium, with an absent brush border, exfoliated tubular cells crowding the lumen and scattered apoptotic bodies. Eosinophilic tubular casts were present in focal areas of the distal tubules (Figure 1).

DISCUSSION AND CONCLUSION

OPs are widely used in agriculture and household applications. OP exposure among workers is often chronic and most likely at a very low dose. Although the effects of subchronic OP exposure are well reported,[4, 18-21] data on the effects of chronic low dose of OPs on major organs, such as the kidney, are very scarce. Thus, the purpose of this study is to determine the effects of chronic subcutaneous low-dose of CPF exposure on the kidney of male Sprague Dawley rats. The model used in the present study mimics human occupational exposure of 18 years to CPF. The kidney is the target organ of interest in the present study as its function is to remove exogenous toxicants and drugs. Thus, the kidney is susceptible to toxicants.[22]

The results demonstrated that chronic exposure, even at a low dose, significantly affected AChE activity. This finding can be attributed to the inhibitory activity of OP-oxon, which is an OP

Table 2: Effects of chronic low dose of CPF on the renal biochemical markers of male rats

Parameters	Control	Vehicle	CPF
	Mean ± SE	Mean ± SE	Mean ± SE
Urea (mmol/L)	5.91 ± 0.06	5.95 ± 0.12	7.00 ± 0.13*
Creatinine (mmol/L)	26.50 ± 0.96	28.00 ± 0.63	49.33 ± 3.09*
Cystatin C (ng/mL)	124.89 ± 2.10	126.76 ± 2.60	145.20 ± 4.34*
Uric acid (mmol/L)	1.38 ± 0.60	1.43 ± 0.11	2.57 ± 0.67*
Na (mmol/L)	147.50 ± 0.56	147.00 ± 0.37	142.33 ± 0.49*
K (mmol/L)	7.05 ± 0.14	6.97 ± 0.12	6.10 ± 0.06*
Cl (mmol/L)	111.17 ± 0.95	110.17 ± 0.31	106.67 ± 0.21*
Ca (mmol/L)	2.69 ± 0.03	2.67 ± 0.02	2.52 ± 0.03*
PO_4 (mmol/L)	3.04 ± 0.18	2.74 ± 0.05	2.29 ± 0.04*

Values are expressed as mean ± standard error (SE); n = 6. CPF, chlorpyrifos; Na, sodium; K, potassium; Cl, chloride; Ca, calcium; PO_4, phosphate; $p<0.05$ for group comparison of CPF vs control and vehicle. *Kruskal-Wallis test and Mann–Whitney *U* test with Post-hoc Bonferroni adjustment.

metabolite that phosphorylates the serine hydroxyl group at the active site of AChE.[1]

The marked reduction of the ArE enzyme in the CPF group indicated that this hydrolysing enzyme had been utilised to counter the oxidative effect of OP-oxon. Alternatively, the production of pro-oxidants may have exceeded the ability of antioxidants to hydrolyse them.[23] The current study showed a significant increase of glomerular filtration markers (urea, creatinine, uric acid and cystatin C) as compared with the controls. This finding indicates the reduction of GFR in the treated rats. Physiologically, cystatin C is completely filtered by the glomerulus and is not secreted but reabsorbed and catabolised by the proximal tubular cells. Thus, this marker is superior as compared with the urea, creatinine and uric acid in estimating GFR. The increase of cystatin C in this study suggests the dysfunction of glomerular filtration capacity.[8] As the glomeruli filter approximately 180 L of plasma each day, they are vulnerable to toxins, such as OPs, that can damage their structure and function.[22]

Serum electrolyte (Na^+, K^+, Cl^-, Ca^{2+} and PO_4^-) concentrations were reduced in the CPF group as compared with those in the control group. As 67% of Na^+, Cl^- and K^+ and 60%–80% of Ca^{2+} and PO_4^{-} [24] are reabsorbed in the proximal convoluted tubule, any reduction in these electrolytes may indicate a proximal tubular cell dysfunction in rats exposed to OPs. In a study on the effect of OPs on renal function at susceptible dosages, Tizhe et al. (2014) [21] suggested that a reduction in electrolyte concentrations in OP-treated animals was due to a direct neurotoxic effect of the OPs on the proximal tubules or dysregulation of the renal nerve activity as a result of the action of OP-oxon on AChE. Moreover, Desai and Desai (2008) [25] stated that reduction in the concentrations of electrolytes, such as Na^+, were due to increased Na^+ excretion via a natriuretic effect of acetylcholine. In extensive kidney failure, Ca^{2+} reabsorption decreases due to the reduced conversion of 1,25-dihydroxycholecalciferol.[24]

In the present study, diffuse global glomerular hypercellularity was observed in the kidney of the CPF group. However, no evidence of glomerular atrophy or other degenerative changes in glomeruli, such as sclerosis and fibrosis, was found. The

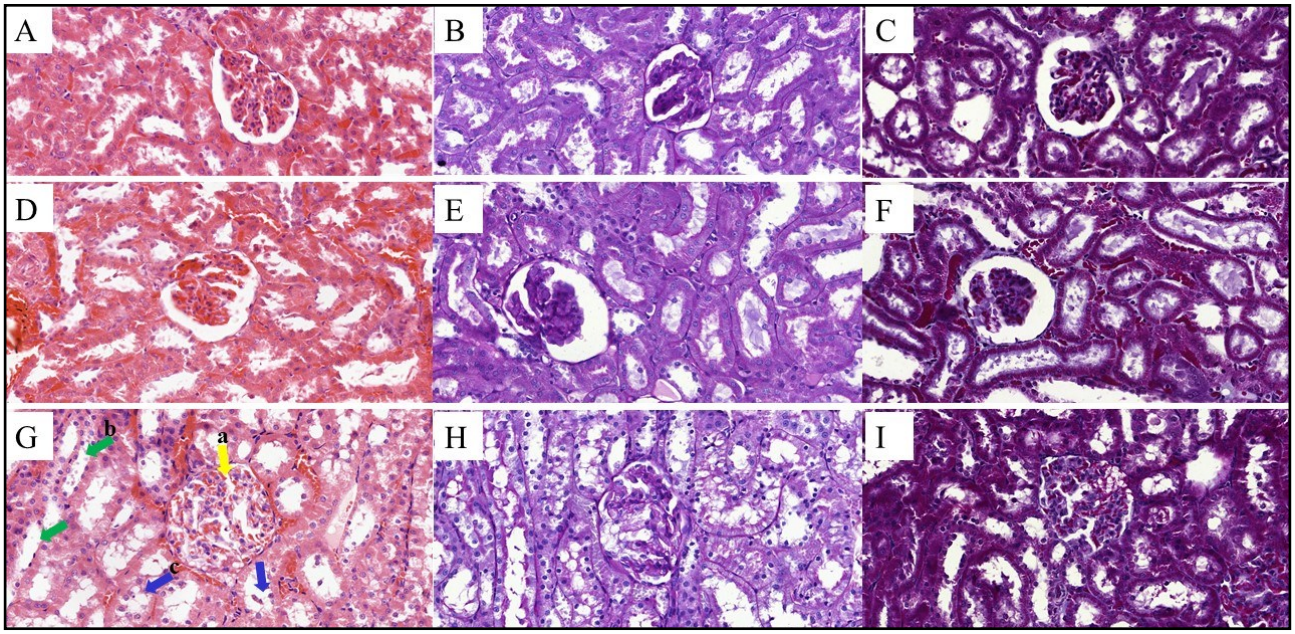

Figure I: Histopathological findings of kidney of rats treated with CPF chronic low dose (18.0 mg/kg). A-C: representative sections of a control group showed normal glomeruli and tubules. D-F: representative sections of a vehicle group showed normal glomeruli and tubules. G-H: Global glomerular hypercellularity (arrow a). Dilated tubules were lined with flat epithelium, with an absent brush border (arrow b). Exfoliated tubular cells crowding the lumen (arrow c). (A, D & G: haematoxylin & eosin stain, X400; B, E & H: periodic acid-Schiff stain, X400; C, F & I: Masson's trichrome stain, X400)

observed glomerular changes were minimal as compared with those in the previous studies that utilised higher dosages of oral OPs but at shorter durations of exposure.[26, 27] Glomerular hypercellularity has been described in various inflammatory conditions.[28] A chronic low-OP dosage is thought to induce oxidative damage, which leads to inflammation and subsequent hypercellularity of glomerular cellular components. As in any chemical causes of intra-renal inflammation, glomerular function may be disrupted, leading to a decrease in the GFR.[22]

The histological assessment of the tubular system of the CPF-exposed rats revealed extensive tubular damage and necrosis. Although scattered apoptotic bodies were found in the proximal tubular cells, the overall picture was tubular cell necrosis. The appearance of prominent pyknotic nuclei, absent nuclei and flat proximal tubular epithelium, with loss of the brush border and exfoliated necrotic debris, are all features of tubular cell necrosis, as described by Pirani (1994). [29]

The histomorphological assessment showed that the tubular cells in the kidney were more susceptible to OP-induced damage than the glomerular component. Based on this finding, at a low dosage of CPF exposure to rats aged 2–3 months (equivalent to 18 human years) for 180 days, despite diffuse and extensive tubular damage, the glomerular filtration system appeared to be resilient, with only a reactive glomeruli hypercellularity observed.

The physiological function of the structure of the proximal convoluted tubule explains its susceptibility to toxic injury, with the structure reabsorbing approximately 60%–80% of filtered solutes and water-using transporters.[24] As a consequence, even at non-toxic concentrations, chemicals in plasma become concentrated and act as potential toxicants, with passive diffusion to tubular cells. If a toxin directly damages tubular cells, then the GFR may decrease due to back-leakage of glomerular filtrate and increased tubular pressure secondary to the obstruction of tubular casts.[22] The mechanism of tubular damage may be due to OP-oxon metabolites

(oxygen radicals) that disrupt the cell membrane via lipid peroxidation.[30]

In comparison with previous studies on OP toxicity, unequivocal evidence of renal injury due to OP toxicants was found. However, such evidence was either from acute, subacute or subchronic exposure of OP [18, 21, 31, 32]. As for chronic low-dose of OP exposure, no previous data support the OP-induced renal damage as shown in this study. As reported, in different settings of OP exposure, either acute, subacute or subchronic, evidence of glomerular dysfunction and tubular injury was found. Elevation of the urea and creatinine suggested glomerular dysfunction in acute (oral 1/4 LD_{50} dimethoate) [31], subacute (oral 1/20 LD_{50} CPF) [32] and subchronic (oral 1/10 LD_{50} glyphosate [21] and oral 1/10 LD_{50} CPF-ethyl, CPF-methyl and methomyl[18]) OPs exposure.

Histologically, glomerular atrophy was observed in acute oral exposure to 10 mg/kg of CPF. Meanwhile, glomeruli atrophy, hypercellularity and degeneration were observed in subacute and subchronic oral exposure to low-dose of CPF (5 mg/kg).[4] By contrast, Akhtar et al. (2009) found no similar changes in glomeruli at different CPF dosages (3, 6 and 9 mg/kg) in subchronic oral exposure.[3]

Many studies (acute [19], subacute [32, 33] and subchronic [19, 20]) have documented various histological abnormalities with the renal tubules, such as tubular dilatation, atrophy, degeneration, tubular cell hypertrophy, vacuolation, necrosis and tubular cast formation. Contrasting reports on the minimum dosage of subchronic CPF exposure that lead to renal tubular injury are also available. For instance, Tripathi et al. (2010)[4] observed renal tubular damage at 5 mg/kg of CPF dosage, and Akhtar et al. (2009)[3] found normal tubular histology at a CPF dosage ranging from 3 to 9 mg/kg.

As these studies demonstrated renal damage by oral exposure, the current study experimented on a subcutaneous route of low-dose OP to mimic dermal contact in the agricultural industry.

In conclusion, a chronic low dose of CPF subcutaneous exposure caused considerable renal tubular necrosis and derangement of the glomerular function. The findings in the present study on OP exposure are equivalent to 18 years of human occupational OP exposure. Therefore, exposure to OPs must be minimised, and alternative less toxic pesticides should be sought. Furthermore, enforcement of personal protective equipment must be advocated.

Conflict of interest

There is no conflict of interest.

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AUTHORS' CONTRIBUTIONS

A.T.N., and N.Z.A. conceptualized and designed the methodology. S.A performed the experiment, collected and analysed the data and wrote the initial draft. Z.M.Z, and N.H. provided resources. T.A.R., and N.M.N. supervised and administrated the project. All co-authors provided critical feedback on the manuscript, suggested additional analyses and critical revisions, and edited the manuscript for clarity and precision. All authors contributed to and approved the final manuscript.

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