ORIGINAL ARTICLE

Mechanism of Chlorpyrifos Induced Chronic Nephrotoxicity

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ABSTRACT

INTRODUCTION Chlorpyrifos is one of the most commonly used organophosphates in agriculture. Reports on animal models of chronically exposed chlorpyrifos showed significant histo-structural renal damage. Moreover, evidence of high incidence of diabetes mellitus was observed among agricultural workers exposed to organophosphates. Available evidence linking derangement of glucose metabolism pathway with renal damage in this chronically exposed chlorpyrifos is lacking. The aim of this study was to determine the involvement of advanced glycation end-products formation in the development of nephrotoxicity in animals with chronic subcutaneous exposure to chlorpyrifos. **MATERIALS AND METHODS:** Eighteen rats were divided into three groups, with six rats in each group. Group 1 served as a control group, while Groups 2 and 3 received subcutaneous 3% dimethyl sulfoxide and 97% volume per volume soy oil as a vehicle or chlorpyrifos, respectively, on alternate day for 180 days. Blood samples were taken for biochemical analysis. Kidney tissues were examined for immunohistochemistry and gene expression of pathways related to oxidative stress and advanced glycation end-products. **RESULTS:** Serum fasting glucose, creatinine, advanced glycation end-products and malondialdehyde levels were significantly increased ($p<0.05$), whereas paraoxonase-1 level was decreased in chlorpyrifos-treated rats compared with the control groups ($p<0.05$). The proximal tubular cells and the glomeruli of chlorpyrifos-exposed kidney showed strong malondialdehyde and advanced glycation end-products expression respectively. Gene expression of catalase and glutathione reductase were downregulated in the chlorpyrifos-treated rats. **CONCLUSION**: Glucose derangement and oxidative stress are the possible mechanisms of organophosphate-induced kidney damage.

Keywords
Organophosphates, nephrotoxicity, oxidative stress, advanced glycation end-products

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INTRODUCTION

Increased incidence of chronic kidney disease of unknown cause has been reported among agricultural workers in developing countries who have had lifetime exposure of agrochemicals.[1] Organophosphates (OP) are widely used as agricultural pesticides in developing countries,[2] including Malaysia.[3-5] Dermal contact is the main route of entry in agricultural workers[6,7] since the skin is the most exposed organ during spraying and handling of the pesticides.[8,9] In addition, inadequate knowledge of safety practices and use of personal protective equipment further contribute to the dermal exposure.[10,11]

Chlorpyrifos (CPF) is a commonly used OP pesticide due to its broad-spectrum activity against a wide range of pests. It has short residuality on foliar surfaces, moderate residuality in soil, and low mammalian toxicity.[12] CPF is mainly metabolised in the liver through the action of cytochrome P450 and forms CPF-oxon, which is hydrolysed by paraoxonase-1 (PON-1),[13] a high-density lipoprotein-associated A-esterase. PON-1 is present in various tissues but is mainly synthesized in the liver and is released into the plasma. PON-1 acts as an antioxidant as it hydrolyses lipid peroxide and hydrogen peroxide, but its activity is inhibited by oxidative stress.[14]

To translate this epidemiological observation into an objective experiment, the tested animals should be chronically exposed to OP, which mimics the actual occupational exposure in humans. Our previous study on rats chronically exposed to subcutaneous CPF exhibited renal tubular necrosis, glomerular hypercellularity, and derangement of glomerular function.[15] However, the mechanisms to explain these observations are not clear.

In view of previous findings on the increased incidence of type 2 diabetes mellitus in agricultural workers[16] and the direct observation of glucose homeostasis derangement in animals exposed to acute[17,18], subacute[17], and subchronic[19] OP exposure, it has been postulated that the mechanisms of renal damage begin with the derangement of glucose metabolism that may build up oxidative stress and cause renal damage. The aim of the present study was to determine the involvement of advanced glycation end-products (AGEs) formation in the development of nephrotoxicity in animals with chronic subcutaneous exposure to CPF.

MATERIALS AND METHODS

Experimental animals and design

Eighteen male Sprague Dawley rats from 2 – 3 months of age (body weight: 200 – 250 g) were purchased from an animal house at University Putra Malaysia in Selangor. The rats were housed in pairs in polypropylene cages containing wood shaving bedding in a room at 25 °C ± 2 and 60 –90 % relative humidity with a 12-hour light/dark cycle. The animals were randomly divided into three groups. The control group animals were fed a standard pellet diet (Gold Coin Feedmills, Malaysia), whereas the vehicle control group animals received 3% dimethyl sulfoxide (QRëCTM, Malaysia) and 97% v/v soya bean oil (Socma Trading, Malaysia) (0.7 ml of the vehicle of CPF per body weight) subcutaneously every other day for 180 days. The exposed group received a subcutaneous injection of CPF (Moon Trading, Malaysia) 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 animals were fed rat chow (Gold Coin Feedmills, Malaysia) and had access to tap water *ad libitum*.

All animals were monitored for visible cholinergic symptoms or other signs of distress such as convulsion, salivation, diarrhoea, sleep and coma. The treatment regimen and duration were based on previous published data that fulfilled the criteria for a sub-threshold dose of CPF via a subcutaneous route of administration.[20]

Sample collection

Blood samples were taken from the orbital sinus at the end of the exposure period after the rats were fasted overnight, then centrifuged and the serum was aliquoted into 0.5 mL microcentrifuge tubes (Eppendorf, Germany) and stored at -70 °C. Following standard animal sacrifice procedure[21], the right kidney was quickly excised, perfused, and rinsed with 0.9% NaCl solution, and fixed in 10% formalin for histology and immunohistochemistry (IHC). One-fourth of the left kidney was stabilized in 0.2 mL RNAlater solution (Qiagen, Germany) and stored at -80°C until RNA extraction.

Creatinine concentration and blood glucose

The serum creatinine concentration and serum glucose level were determined using a Cobas Integra 400 Plus chemistry analyser (Roche, USA) using the Jaffé reaction[22,23] and glucose hexokinase method,[24,25] respectively.

Serum Level of advanced glycation end-products

The serum level of AGEs was measured using a rat AGE enzyme-linked immunosorbent assay (ELISA) kit (Cusabio, China) following the manufacturer's instructions.

Serum malondialdehyde level

The serum malondialdehyde (MDA) level was obtained using a rat MDA ELISA kit (Cusabio, China) following the manufacturer's instructions.

Serum paraoxonase-1 activity

The serum PON-1 activity was determined as described previously.[26]

RNA purification and Real-Time Quantitative Polymerase Chain Reaction

The total RNA was extracted from 15 mg of the left kidney cortex of both the control and CPF-exposed rats using the RNeasy Mini Kit (Qiagen, Germany) in accordance with the manufacturer's protocol. The RNA purity and integrity were assessed by the ratio of the sample's absorbance at 260 and 280 nm. RNA samples with a ratio of 1.9 or more were considered acceptable purity.[27] First-strand cDNA was synthesized from 0.5 µg of the extracted RNA from each sample using the RT² First Strand Kit (Qiagen, Germany). Eighteen genes involved in various pathways of renal damage and cell death were selected for polymerase chain reaction array assay (Table II). For 25 µL of the total reaction of the RT² Profiler PCR Array (Qiagen, Germany), the PCR mixture consisted of 0.9 µL of cDNA (synthesized from 0.5 µg of RNA), 11.6 µL of RT² SYBR® Green qPCR Mastermix (Qiagen, Germany), and 12.5 µL of RNase-free water. As per the protocol, 48 reactions were prepared for each sample. Thus, two samples that contributed to 96 reactions were run per assay using the 96-well plate on a CFX96 real-time analyser (Bio-Rad, USA). The cycling conditions were 95°C for 10 min and 40 cycles at 95 °C for 15 s. The C_T value was deduced from the CFX96 manager software. Phosphoglycerate kinase 1, actin, beta, and ring finger protein 1 were used as the reference genes to normalize the relative quantification of each target gene expression. The fold change in the mRNA expression relative to the control group was determined using the comparative C_T ($2^{-\Delta\Delta Ct}$) method. All samples were performed and analysed in duplicate.

Immunohistochemistry

The kidney tissues were fixed and processed as per standard procedure. The initial assessment of the histomorphology was based on the haematoxylin and eosin slides. For the IHC, 5-µm paraffin-embedded tissue sections were subjected to deparaffinization and unmasking of tissue antigens using a pressure cooker (Biocare Medical, USA). The target antigens were retrieved using a retrieval solution consisting of a Tris-EDTA buffer (pH: 9; Dako, Santa Clara, USA) and a citrate buffer (pH: 6; Dako, Santa Clara, USA) for AGEs and MDA, respectively. In the subsequent steps, the slides were incubated for 15 minutes with a peroxidase solution (Dako, Santa Clara, USA) to block nonspecific background staining. The primary antibodies used for immunostaining were polyclonal anti-AGEs and polyclonal anti-MDA (Abcam, USA). For the immunostaining, the tissue slides were introduced to the primary antibodies and incubated for one hour at room temperature. The concentrations of the primary antibodies were 1:1000 and 1:200 for AGEs and MDA, respectively. After labelling with anti-rabbit polymer-horseradish peroxidase (Dako, USA), the slides were incubated in a substrate of 3,3'diaminobenzidine chromogen solution (Dako, USA) and counterstained with Harris haematoxylin solution (Leica, USA). The positive control tissues used were atheromatous plaque from the rat aorta and adenocarcinoma of the human breast for anti-AGEs and anti-MDA, respectively. The immunostaining grades (trace, weak, or strong) were independently evaluated by two observers.

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 in accordance with current guidelines on the care and use of laboratory animals.[21]

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences 25 for Windows. Non-parametric Kruskal-Wallis H test and Mann-Whitney *U* test with post-hoc Bonferroni were used for the analysis

of the biochemical parameters. Immunohistochemical expressions were descriptively assessed.

The data were submitted to the Qiagen Data Analysis Center for the gene expression analysis. The web portal calculated the fold change using the DDC_T method. The fold change was determined based on the 2^ ($-DDC_T$) formula. The p-value was calculated based on the Student's t-test of the replicate 2^ ($-DC_T$) values for each gene in the control and CPF-treated groups.

RESULTS

Biochemical results

Serum creatinine, glucose, and AGEs levels were significantly increased in the rats exposed to CPF as compared with the control groups ($p<0.05$). The serum MDA level was higher in the CPF-exposed group than in the control groups (control group: $p=0.001$; vehicle control group: $p=0.08$), whereas the serum PON-1 activity was significantly decreased in the CPF-exposed group as compared with that in the control groups ($p< 0.001$) (Table I).

Table I. Biochemical results in **chlorpyrifos-exposed and control groups**

Parameters	Control Mean ± SE	Vehicle Mean ± SE	CPF Mean ± SE
Creatinine (mmol/L)	26.5 ± 0.96	28.0 ± 0.63	49.33 ± 3.09*,**
Glucose (mmol/L)	5.57 ± 0.14	6.85 ± 0.81	12.90 ± 0.71*,**
AGEs (mg/mL)	8.38 ± 0.53	8.98 ± 0.46	15.47 ± 2.88*,**
MDA (pmol/mL)	656.25 ± 7.41	688.04 ± 10.41	776.39 ± 12.11*
PON-1 (U/ml)	90.6 ± 18.7	64.0 ± 26.9	45.8 ± 9.3*,**

Values are expressed as mean ± standard error (SE); n = 6.
*$p<0.05$ for group comparison of CPF vs control.
**$p<0.05$ for group comparison of CPF vs vehicle.
Kruskal-Wallis test and Mann–Whitney *U* test with Post-hoc Bonferroni adjustment.
CPF, chlorpyrifos; AGEs, advanced glycation end-products; MDA, malondialdehyde; PON-1, paraoxonase-1.

mRNA expressions of the selected genes

The expressions of catalase *(CAT),* glutathione reductase *(GSR),* advanced glycation end-products specific receptor *(AGER),* and receptor interacting serine-threonine kinase 3 *(RIPK3)* genes were significantly downregulated more than 1.5-fold above the control group expression. The expressions of other genes [superoxide dismutase 3 *(SOD3), PON1, nitric oxide synthase 2 (NOS2), cytochrome C (CYCS), caspase 3 (CASP3), caspase 8 (CASP8), caspase 9 (CASP9),* receptor interacting serine-threonine kinase 1 *(RIPK1),* cystatin C *(Cst3),* hepatitis A virus cellular receptor 1 *(HAVCR1),* and lipocalin 2 *(LCN2)*] showed a downregulation trend but were not statistically significant. The upregulation expressions of tumour protein p53 *(TP53),* glutathione peroxidase 3 *(GPX3),* and chemokine (C-C motif) ligand 2 *(CCL2)* were also not significant (Table II).

Table II. Selected genes expression in kidneys of chronic chlorpyrifos-treated rats

Gene name	Function	Fold change	p value
SOD3	Dismutation of superoxide anion into hydrogen peroxide	-1.52	0.114
CAT	Decomposition of hydrogen peroxide	-1.62	0.015*
GSR	Reduction of GSSG to GSH	-1.87	0.032*
GPX3	Reduction of hydrogen peroxide	1.49	0.068
PON1	Hydrolysis of CPF-oxon.	-1.29	0.568
AGER	Bind AGEs	-3.06	0.001*
NOS2	Production of nitric oxide	-2.08	0.060
TP53	Tumour suppressor gene	1.77	0.533
CYCS	Involves in mitochondrial pathway of apoptosis	-1.27	0.330
CASP9	The initiator of mitochondrial pathway of apoptosis	-1.80	0.059
CASP8	The initiator of death receptor pathway of apoptosis	-1.04	0.627
CASP3	Execution phase of apoptosis	-1.28	0.513
RIPK1	Mediating necroptosis	-1.24	0.205
RIPK3	Mediating necroptosis	-1.89	0.017*
CST3	Biomarker of proximal tubular cell injury	-1.39	0.273
HAVCR1	Involve in proximal tubular cell injury	-1.13	0.490
LCN2	Intracellular signalling and expression in chronic kidney disease	-1.86	0.156
CCL2	Chemotaxis of monocytes	1.39	0.177

*Student t-test showed significant downregulation ($p<0.05$) between CPF-exposed and control groups.

SOD3, superoxide dismutase 3, extracellular; *CAT*, catalase; *GSR*, glutathione reductase; *GPX3*, glutathione peroxidase 3; *PON1,* paraoxonase-1; *NOS2,* nitric oxide synthase 2, inducible; AGER, advanced glycosylation end-product specific receptor; *TP53,* tumour protein *p53*; *CYCS*, cytochrome C; *CASP9*, caspase 9; *CASP8,* caspase 8; CASP3, caspase 3; *RIPK1,* receptor-interacting serine-threonine protein kinase 1; *RIPK3,* receptor-interacting serine-threonine protein kinase 3; *CST3,* cystatin C; *HAVCR1*, hepatitis A virus cellular receptor 1; *LCN2,* lipocalin 2; CCL2, chemokine (C-C motif) ligand 2.

Immunoexpression of AGEs and MDA

The AGEs were strongly expressed in the glomeruli of the CPF-treated rats as compared with the control group. Meanwhile, the MDA was more strongly expressed in the tubular cells of the CPF-treated rats than in the control group (Figure 1).

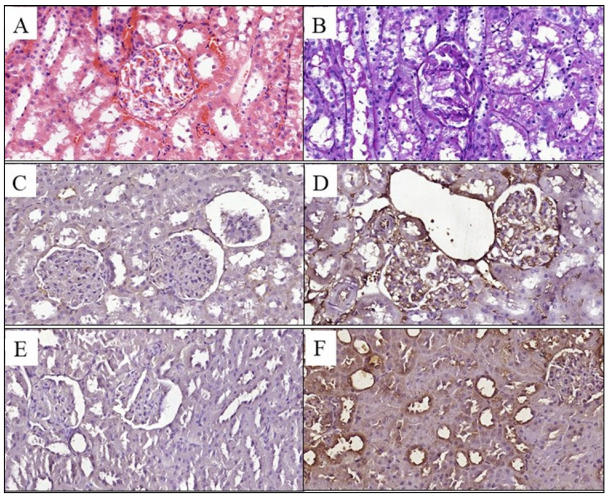

Figure 1. A and B: Histopathological changes of CPF-treated kidney showed significant renal damage with the evidence of glomerular hypercellularity and tubular cell necrosis *(A: haematoxylin & eosin stain, X400; B: periodic acid-Schiff stain, X400).* Immunohistochemical expression of AGEs and MDA (400 X). D and F: Immunostaining results for AGEs and MDA respectively for the control group. C: Strong expression of AGEs at the mesangial matrix and cell membrane of the glomeruli in the CPF-treated rats. E: Strong expression of MDA in the cytoplasm of the tubular cells in the CPF-treated rats. CPF, chlorpyrifos; AGEs, advanced glycation end-products; MDA, malondialdehyde.

DISCUSSION

The present study found a significantly higher blood glucose level in CPF-exposed animals compared with the control groups. As the pancreas is prone to OP-induced damage,[17,19] the current findings on hyperglycaemia were most likely due to the decreased insulin release from a damaged pancreas. Another alternative explanation to the finding on hyperglycaemia is the possibility of insulin resistance in animals chronically exposed to OP through alterations in the gut microbiota[28] and changes in carbohydrate metabolism with different complex mechanisms.[29]

Renal damage as a result of a hyperglycaemic state can be explained through different mechanisms. Most of the filtered glucose is reabsorbed by the proximal tubular cells.[30] Since the kidney is an insulin-independent tissue involved in gluconeogenesis, the resultant increase of intracellular glucose affects the sorbitol-aldose reductase pathway.[31] This gives rise to the accumulation of osmotic sorbitol and the depletion of nicotinamide adenine dinucleotide phosphate (NADPH).[31] Both conditions may aggravate kidney damage through osmotic lysis and oxidative stress, respectively.[32]

In the present study, the elevation of serum creatinine and AGEs in the CPF-exposed group with tell-tale signs of nephrotoxicity in the histological findings suggested the likeliness of glomerular filtration rate derangement. The IHC findings of the strong expression of AGEs in the glomeruli indicated the inability of the glomeruli to filter AGEs.

Chronic hyperglycaemia induces the accumulation of AGEs in circulation and tissues.[33] AGEs are the end-products of nonenzymatic glycation between glucose and amino acids of both intracellular and extracellular proteins. They are easily filtered by the glomerulus due to their low molecular weight. The reabsorbed AGEs are catabolized by the proximal tubular cells.[34]

Here, AGEs can cross-link with protein, and this combination is resistant to proteolysis and may further alter the structure and function of the kidney.[35] However, the study could not determine whether nephrotoxicity is caused by the direct effect of OP or that of AGEs as a consequence of hyperglycaemia.

One of the most established mechanisms of OP-induced renal damage in subacute[36,37] and subchronic[38] OP exposure is through the effect of oxidative stress on the kidney. MDA, a product of lipid peroxidation, is considered an oxidative marker. The current study found a significant event of oxidative damage for the establishment of the nephrotoxicity since the serum MDA was elevated and strongly immunoexpressed at the renal tubular cells in CPF-exposed animals. In addition, the low PON-1 activity in the CPF-treated animals further supported the presence of ongoing oxidative damage as the enzyme was reduced due to its utilization during the hydrolysis process of OP-oxon.[13]

This study proposed a mechanism for the oxidative damage in the renal tissues, which could be due to the direct effect of the OP-oxon or an indirect effect of the hyperglycaemia-AGE axis. In the latter condition, NADPH oxidase is activated, initiating the production of free oxygen radicals, superoxides in the renal cortex.39

Regarding the gene expression study, all genes studied were downregulated (*SOD3, CAT, GSR, PON1, AGER, NOS2, CYCS, CASP3, CASP8, CASP9, RIPK1, RIPK3, CST3, HAVCR1, and LCN2)* except for GPX3, TP53, and CCL2. Among them, only the expression of CAT*, GSR, AGER,* and *RIPK3* were statistically significant. Previous examples offer evidence of antioxidant-related gene downregulation in the tissues of animals chronically exposed to toxic agents, such as arsenic40 and cadmium.[41] In addition, reports have identified antioxidant-related gene downregulation in human cell lines chronically exposed to cigarette smoke[42] and iron.[43] The overall downregulation of the genes (*except GPX3, TP53, and CCL2)* could be due to the selective exhaustion of pathways that were persistently activated during the prolonged chronic OP-mediated injury.

Both *CAT* and *GSR* were genes for oxidative stress. CAT prevents oxidative damage by decomposition of hydrogen peroxide (H_2O_2) which is a source of hydroxyl radicals in the Fenton reaction.[44] GSR reduces oxidized glutathione to reduced glutathione which reduces H_2O_2 and organic peroxide.[45]

In chronic hyperglycaemia, AGEs produced bind to AGER which is present in podocytes and endothelial cells in the kidney.[46] Interaction between AGEs and AGER triggers cellular oxidative stress and inflammatory processes.[34] RIPK-3 is a critical regulator of necroptosis.[47] Interaction of RIPK-1 and RIPK-3 triggers necroptotic cell death.[48]

CONCLUSION

Based on the laboratory results of this study, a chronic CPF treatment leads to hyperglycaemia, oxidative damage in the proximal tubular cells and non-enzymatic glycation in the glomeruli. All these conditions contributed to the renal damage indicated by the elevated creatinine level and the presence of nephrotoxic changes in the renal tissues. Hence, this study concludes that prolonged exposure of OP is potentially nephrotoxic and may cause the disruption of glucose metabolism and oxidant metabolism. This combination induces oxidative damage.

CONFLICT OF INTEREST

There is no conflict of interest.

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