THE EFFECTS OF CHRONIC SUBCUTANEOUS EXPOSURE TO LOW DOSE OF CHLORPYRIFOS ON THE RAT KIDNEYS

BY

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ABSTRACT

Introduction: Chlorpyrifos (CPF) is an organophosphate (OP) that is widely used as pesticide in agriculture. Epidemiological studies reported that the incidence of chronic kidney disease (CKD) of unknown cause was increasing among agricultural workers who were exposed to OPs during their working life through dermal contact. However, there is little information on the effects of chronic subcutaneous low dose of OP CPF on kidney in experimental animals. To date, the mechanism of OP-induced kidney damage is not fully elucidated. **Objective:** The aim of this study was to assess the effects of chronic subcutaneous low dose of OP CPF exposure on the kidney by investigating the structure and function of kidney and to assess the possible mechanisms of OP-induced kidney damage. **Methodology:** Eighteen male Sprague Dawley rats were randomly divided into three groups, with six rats in each group. Group 1 served as control group, while groups 2 and 3 received subcutaneous vehicle (3% dimethyl sulfoxide + 97% v/v soy oil) and CPF (18.0 mg/kg) respectively, every other day for 180 days. Blood was taken for biochemical analysis while kidney tissues were harvested for histology, immunohistochemistry (IHC) and selective gene expression. Cystatin C, acetylcholinesterase (AChE), advanced glycation end products (AGEs) and malondialdehyde (MDA) levels were measured using quantitative sandwich enzyme immunoassay. **Results:** Biochemical parameters (urea, creatinine, uric acid, glucose), cystatin C, AGEs and MDA levels were significantly increased ($p < 0.05$), while AChE activity and electrolytes levels were significantly decreased ($p < 0.05$) in the CPF-exposed rats. Structural damage of kidney, including diffuse global glomerular hypercellularity and diffuse necrosis of proximal tubular cells were observed in CPF-exposed kidney. IHC revealed strong immunostaining of polyclonal anti-AGEs in glomeruli and polyclonal anti-MDA in the proximal tubular cells of CPF-exposed rats. The expression of genes involved in glucose metabolism (*Ager*), oxidative stress (*Sod3, Cat, Gsr, Pon1 and Nos2*) and cell death pathways (*Cycs, Casp3, Casp, 8, Casp, 9, Ripk1, Ripk3, Cst3, Havcr1 and Lcn2*) showed downregulation trend. **Conclusion:** Chronic subcutaneous low dose of CPF caused renal dysfunction as evidence by increased endogenous glomerular filtration markers and reduced electrolytes levels and structural kidney damage. The downregulation of these genes could be due to selective exhaustion of pathways that were persistently activated during the prolonged chronic OP-mediated injury. In brief, chronic subcutaneous low dose of CPF caused nephrotoxicity. The process could be facilitated by the effects of CPF on glucose metabolism and oxidative stress.

خلاصة البحث

المقدمة: الكلوربيريفوس (CPF) هي فوسفات عضوية (OP) تستخدم على نطاق واسع كمبيد للآفات في الزراعة. أفادت الدراسات الوبائية أن معدل الإصابة بأمراض الكلى المزمنة (CKD) لسبب غير معروف يتزايد في أوساط العمال الزراعيين الذين تعرضوا للفوسفات العضوية أثناء حياتهم العملية من خلال ملامسة الجلد. و مع ذلك ، هناك القليل من المعلومات حول آثار التعرض لجرعة منخفضة مزمنة تحت الجلد من الفوسفات العضوية من نوع الكلوربيريفوس على الكلى في الحيوانات التجريبية. حتى الآن ، لم يتم توضيح آلية تلف الكلى الناتج عن الفوسفات العضوية. الهدف: كان الغرض من هذه الدراسة هو تقييم آثار التعرض لجرعة منخفضة مزمنة تحت الجلد من الفوسفات العضوية من نوع الكلوربيريفوس على الكلى من خلال فحص هيكل و وظيفة الكلى وتقييم الآليات المحتملة للتلف الكلوي الناجم عن الفوسفات العضوية. المنهجية: تم تقسيم ثمانية عشر ذكراً من فئران سبراغ داولي بشكل عشوائي إلى ثلاث مجموعات ، بستة فئران في كل مجموعة. عملت المجموعة الأولى كمجموعة تحكم ، في حين تلقت المجموعتان 2 و 3 حامل تحت جلدي من (3٪ داي ميثيل سلفوكسيد +97% حجم / حجم من زيت فول الصويا) و الكلوربيريفوس (18.0 ملغم / كغم) على التوالي ،كل ثاني يوم لمدة 180 يوماً. ثم تم أخذ الدم للتحليل الكيميائي الحيوي في حين تم حصاد أنسجة الكلى من أجل اختبارات الأنسجة ، و كيمياء الأنسجة المناعية (IHC) و التعبير الجيني الانتقائي. تم قياس مستوى السيستاتين C و الأسيتيل كولين إستريز (AChE) والمنتجات النهائية المتقدمة للجلوكوز (AGEs) و مستويات المالون داي ألدهايد (MDA) باستخدام التحليل الكمي المناعي الأنزيمي المنشطر. النتائج: كان هناك زيادة ذات دلالة ($p<0.05$) في مستويات معاملات الكيميائية الحيوية (اليوريا، الكرياتينين ، حمض اليوريك ، الجلوكوز) و مستويات السيستاتين C والمنتجات النهائية المتقدمة للجلوكوز (AGEs) مستويات المالون داي ألدهايد (MDA) في حين انخفض مستوى الأسيتيل كولين إستريز (AChE) و مستويات الشوارد بمستويات ذات دلالة ($P <0.05$) في الفئران المعرضة للكلوربيريفوس.وقد لوحظت الأضرار الهيكلية للكلية ، بما في ذلك الزيادة الخلوية الكبيبية الشاملة المنتشرة و نخر منتشر في الخلايا الأنبوبية القريبة في الكلى المعرضة للكلوربيريفوس .كشفت كيمياء الأنسجة المناعية (IHC) تصبغات مناعية قوية في الجسم المضاد متعدد النسيلة للمنتجات النهائية المتقدمة للجلوكوز (AGEs) في الكبيبات و في الجسم المضاد متعدد النسيلة للمالون داي ألدهايد (MDA) في الخلايا الأنبوبية القريبة في الفئران المعرضة للكلوربيريفوس. أظهر التعبير عن الجينات المشاركة في استقلاب الجلوكوز (Ager) و التوتر التأكسدي (Sod3و Cat و Gsr و Pon1 و Nos2) و مسارات موت الخلايا (Cycs و Casp3 و Casp8 و Casp9 و Ripk1 و Ripk3 و Cst3 و Havcr1 و Lcn2) اتجاه إنخفاضي. الخاتمة: تسببت الجرعة المنخفضة المزمنة تحت الجلد من الكلوربيريفوس في اختلال وظيفي كلوي بدليل زيادة علامات الترشيح الكبيبي الداخلية وانخفاض مستويات الشوارد وتلف الكلى الهيكلي. يمكن أن يكون سبب انخفاض هذه الجينات هو الاستنفاد الانتقائي للمسارات التي تم تنشيطها باستمرار أثناء الإصابات المزمنة عن طريق التعرض للفوسفات العضوية لفترة طويلة. باختصار ، تسببت الجرعة المنخفضة المزمنة تحت الجلد من الكلوربيريفوس في السمية الكلوية. يمكن أن العملية قد تم تسهيلها من خلال تأثيرات الكلوربيريفوس على استقلاب الجلوكوز و على التوتر التأكسدي.

APPROVAL PAGE

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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Signature Date

This thesis is dedicated to my parents for laying the foundation of what I turned out to be in life.

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LIST OF ABBREVIATIONS

$(NH_4)_2 MoO_4$	Ammonium molybdate
$(NH_4)_3 [PO_4 (MoO_3)_{12}]$	Phosphomolybdate complex
ACh	Acetylcholine
AChE	Acetylcholinesterase
Actb	Actin, beta
ADP	Adenosine dinucleotide
Ager	Advanced glycosylation end product-specific receptor
AGEs	Advanced glycation end products
AKI	Acute kidney injury
BuChE	Butyrylcholinesterase
Ca^{2+}	Calcium
Casp3	Caspase 3
Casp8	Caspase 8
Casp9	Caspase 9
Cat	Catalase
CAT	Catalase
Ccl2	Chemokine (C-C motif) ligand 2
Ccl2	Chemokine (C-C motif) ligand 2
CKD	Chronic kidney disease
CPF	Chlorpyrifos
CRF	Chronic renal failure
Cst3	Cystatin C

Cycs	Cytochrome C, somatic
ELISA	Enzyme-linked immunoabsorbent assay
esRAGE	Endogenously secretory RAGE
G6PD	Glucose-6-phosphate dehydrogenase
GFR	Glomerular filtration rate
GITC	Guanidine isothiocyanate
GLDH	Glutamate dehydrogenase
GPX	Glutathione peroxidase
Gpx3	Glutathione peroxidase 3
GR	Glutathione reductase
GSH	Reduced glutathione
Gsr	Glutathione reductase
H&E	Haematoxylin and eosin stain
H^+	Hydrogen ions
H_2O	Water
H_2O_2	Hydrogen peroxide
Havcr1	Hepatitis A virus cellular receptor 1
Havcr1	Hepatitis A virus cellular receptor 1
HCO_3	Bicarbonate ions
HK	Hexokinase
HKG	Housekeeping genes
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IMS	Intermediate syndrome
IQR	Interquartile range

KIM-1	Kidney injury molecule-1
Lcn2	Lipocalin 2
Lcn2	Lipocalin 2
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
Mg^{2+}	Magnesium ions
NAD	Nicotinamide adenine dinucleotide.
NADH	The reduced form of nicotinamide adenine dinucleotide
NADP	Nicotinamide dinucleotide phosphate
NADPH	The reduced form of nicotinamide dinucleotide phosphate
NGAL	Neutrophil gelatinase-associated lipocalin
$NH4^{+}$	Ammonium
Nos2	Nitric oxide synthase 2, inducible
O_2	Oxygen
$O_2^{-\circ}$	Superoxide
OCPC	O-cresolphthalein complexone
OD	Optical density
OP	Organophosphate
OPIDPN	OP-induced delayed polyneuropathy
PAS	Periodic acid Schiff
Pgk1	Phosphoglycerate kinase 1
PO_4^{3-}	Inorganic phosphate
Pon1	Paraoxonase-1
PON-1	Paraoxonase-1
PPE	Personal protective equipment

RAGE	AGEs receptor
RBC	Red blood cell
Ring1	Ring finger protein 1
RIPK-1	Receptor associated kinase-1
Ripk1	Receptor-interacting serine-threonine protein kinase 1
RIPK-3	Receptor associated kinase-3
Ripk3	Receptor-interacting serine-threonine protein kinase 3
RT-PCR	Real time polymerase chain reaction
SOD	Superoxide dismutase
Sod3	Superoxide dismutase 3, extracellular
sRAGE	Secretory RAGE
TCO_2	Total carbon dioxide
TCP	3,5,6-trichloro-2-pyridinol
TMB	3, 3’, 5, 5’ tetramethylbenzidine
TNF	Tumour necrosis factor
Tp53	Tumor protein p53

CHAPTER ONE

INTRODUCTION

Organophosphorous (OP) compound is commonly used as a pesticide in the environment, public health and agriculture industry, particularly in developing countries. It is also used as chemical warfare nerve agents, therapeutic drugs, fire retardants, solvents and plasticizers (Balali-Mood, 2014). Due to its widespread use worldwide, OP poisoning was estimated to be between 750,000 to 3,000,000 cases globally per year (Soltaninejad & Shadnia, 2014).

Chlorpyrifos (CPF) is an OP which was first introduced in the U.S in 1965 (Christensen, Harper, Luukinen, Buhl, & Stone, 2009). It is used as insecticide, acaricide, and miticide (Koshlukova & Reed, 2014).

CPF is oxidized by cytochrome P450 in the liver to form CPF-oxon, which is a strong acetylcholinesterase (AChE) inhibitor. CPF-oxon is metabolized by hepatic and extra-hepatic esterases (Eleršek & Filipič, 2011) to form water soluble 3,5,6-trichloro-2-pyridinol (TCP) and diethylphosphate (Timchalk, 2006). Both are excreted in urine (Tang, Rose, & Chambers, 2006).

Although, CPF is classified as moderately hazardous (Class II) pesticide (Organization, 2010), it is widely used in the agrochemical industry in Malaysia (Farina, Abdullah, Bibi, & Khalik, 2016; Ismail, Halimah, Tan, & Tayeb, 2017; Zaidon, Ho, Hashim, Saari, & Praveena, 2018) because of its potency, broad-spectrum activity against a wide range of pests, short residuality on foliar surfaces and moderate residuality in soil, as well as low mammalian toxicity (Science, 2017).

The routes of CPF exposure include inhalation, ingestion, dermal contact and conjunctival route (Christensen et al., 2009). Dermal contact is the main route of entry in agrochemical chemistry (Costa, 2013; Soltaninejad & Shadnia, 2014).

Pest control, agricultural and industrial workers are exposed to low dose of OP throughout their lifetime, mostly through dermal contact (Costa, 2013). Agriculture related OP exposure may involve 25.96 % and 10.67% of all OP exposures in the world and Malaysia, respectively (ILOSTAT, 2019). In Malaysia, Indian has the highest prevalence of pesticide poisoning and death. Due to poor education, many of the farmers are often lack of adequate knowledge on safety practices and use of personal protective equipment (PPE) (Ali & Shaari, 2015; Rajasuriar, Awang, Hashim, & Rahmat, 2007).

The primary target organ for CPF toxicity is the central and peripheral nervous system because the accumulated acetylcholine (ACh) neurotransmitter in nerve endings causing sustained cholinergic hyperstimulation in central and peripheral nervous systems (Eleršek & Filipič, 2011). However, the previous animal studies showed that CPF may also cause tissue damage to literally any tissue, including kidneys (Tripathi & Srivastav, 2010), liver, spleen, lymph node, adrenal gland, epididymis, and testis (Akhtar, Srivastava, & Raizada, 2009).

Epidemiological studies have reported that there is an association between chronic kidney disease (CKD) and long-term occupational exposure to OP (Johnson, Wesseling, & Newman, 2019). However, there is limited evidence of chronic exposure to low dose OP effects on the kidney in animal models.

As CPF is regarded as a neurotoxin, the studies on the effects of subclinical or subthreshold dose of CPF on the nervous system in animal models were done (Rush, Liu, Hjelmhaug, & Lobner, 2010; Speed et al., 2012; Terry et al., 2007). However, the effects on other major organs, such as the kidneys were not mentioned. Based on few available studies, the possible mechanism of nephrotoxicity in chronic low dose of CPF exposure could be through CPF induction of oxidative damage to the kidney (Heikal, Mossa, Marei, & Rasoul, 2012; Nasr, El-Demerdash, & El-Nagar, 2016). Hence, this study aimed to assess the effects of chronic subcutaneous low dose of CPF exposure on the rat kidneys.

1.1 JUSTIFICATION OF THE STUDY

i. OPs are widely used in agriculture and agrochemical industry. The incidence of CKD of unknown aetiology increases in agriculture workers who are routinely exposed to low dose OPs throughout their life via dermal contact may give rise to OP-induced nephrotoxicity.
ii. CPF is widely used as an agrochemical in Malaysia.
iii. There is limited study on the effects of chronic subcutaneous exposure to low dose CPF on the kidney.
iv. Understanding the toxic mechanisms for nephrotoxicity provides useful information on the management and prevention of the complications.

1.2 GENERAL OBJECTIVE

To assess the effects of chronic subcutaneous low dose of CPF exposure on the kidney

1.3 SPECIFIC OBJECTIVES

i. To assess the renal function parameters (urea, creatinine, uric acid, cystatin C and electrolytes) in rats exposed to chronic low dose CPF