UNIVERSITI PUTRA MALAYSIA

THE EFFECTS OF SELECTED PESTICIDES ON CYTOCHROME P450 SYSTEM AND ACETYLCHOLINESTERASE ACTIVITY IN HARUAN, CHANNA STRIATUS

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By

TIAN YU

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Abstract of the thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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Chairman: Associate Professor Dr. Abdul Manan Mat Jais

Faculty: Medicine and Health Science

Distribution of microsomal cytochrome P450 was detected in organs of haruan (*Channa Striatus*), a tropical freshwater fish. Microsomal isolation was performed based on conventional ultra-centrifugation at 105,000 g for 60 minutes, high-speed centrifugation at 40,000 g for 90 minutes and calcium aggregation technique respectively. Cytochrome P450 was present only in liver and gut microsomes, but not in the other organs: namely heart, kidney, spleen, gill and muscle. The CO-reduced cytochrome P450 showed the spectrum of absorption maximum at 453 nm in liver microsome and 451 nm in gut microsome isolated by both ultra- and high- speed centrifugation. High-speed centrifugation was the most suitable method to be used for microsome isolation in this study since it gave the highest level of cytochrome P450 amounting to 3.23 nmol/g hepatic protein and 2.24 nmol/g intestinal protein.

The effects of three insecticides namely deltamethrin, malathion and endosulfan, and a model inducer of cytochrome P450 -- phenobarbitone (PB) on the content of cytochrome P450 and its dependent 7-ethoxyresorufin O-demethylase

(EROD) activity were examined in liver and gut of haruan, *Channa striatus*. The 48 hours exposure to a series of concentrations of PB (0.25, 5, 15 ppm) had produced a dose-effect induction of hepatic cytochrome P450 content and CYP1A dependent EROD activity in *C. striatus*. While in gut microsome, EROD activity was significantly enhanced, $P \leq 0.01$, but without significant increased in cytochrome P450 amount. On the other hand, endosulfan pre-treatment has resulted in significant induction of P450 content at $P \leq 0.01$compared to control level of P450 content at 0.189 ± 0.0161 nmol/mg microsomal protein (mgmic. Prot.). Similarly, EROD activities of hepatic and extra-hepatic microsomes increased on exposure to a series of endosulfan concentrations. Deltamethrin (DM) has enhanced hepatic P450 content and EROD activity only on the first two days, although intestinal P450 amount was not induced by the exposure. EROD activity was markedly enhanced by all treated concentrations 0.2, 2, 20 ppb of DM. Malathion administration led to a slight induction of P450 system.

Endosulfan (0.5 and 5 ppb) did not affect AChE activity throughout the 7 days of exposure. However, malathion at 50 ppb and 5 ppm inhibited AChE activity in haruan brain tissue. Deltamethrin (2 and 20 ppb) reduced the AChE activity after 1 day of exposure, after which it increased gradually. Hepatic cytochrome P450 dependent ρ- nitroanisole (PNA) O- demethylase activity increased linearly with dose and time of exposure to endosulfan. But only malathion at 5 ppm and deltamethrin 20 ppb increased the activity of PNA O-demethylase. This is the first study to be conducted to observe the effects of the three insecticides on cytochrome P450 system in Haruan, *C. Striatus*. The results suggested that the haruan could be a candidate as a tool for monitoring pesticide pollution in tropical freshwater system.

However, haruan P450 system and its interaction with mixed pesticides or other xenobiotics should be further studied.

Abstrak tesis dikemukakan Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

KESAN BEBERAPA PESTISID TERHADAP SISTEM SITOKROM P-450 DAN AKTIVITI ASETILKOLINESTERASE DI DALAM HARUAN, *CHANNA STRIATUS*

Oleh

TIAN YU

Oktober 2000

Pengerusi: Profesor Madya Dr. Abdul Manan Mat Jais

Fakulti: Perubatan dan Sains Kesihatan

Sebaran Sitokrom P-450 mikrosomal dikesan dalam organ haruan (*Channa striatus*), ikan air tawar tropika. Pengasingan mikrosom telah dilakukan berdasarkan tiga kaedah iaitu pengemparan-ultra pada 105,000 g selama 60 minit, pengemparan kelajuan-tinggi pada 40,000 g selama 90 minit dan teknik agregasi kalsium secara berturutan. Sitokrom P-450 hanya terdapat di dalam hati dan mikrosom usus, tetapi tidak di dalam organ lain seperti jantung, ginjal, limpa, insang dan otot. Karbon monoksida (CO) terturun sitokrom P-450 di dalam mikrosom hati menunjukkan penyerapan maksima spektrum pada 453 nm manakala di dalam usus mikrosom adalah pada 451 nm dalam mikrosom usus aman dengan menggunakan kedua – dua kaedah pengemparan-ultra dan kelajuan-tinggi. Pengemparan kelajuan-tinggi merupakan kaedah paling sesuai untuk digunakan di dalam pengasingan mikrosom kerana ia memberikan paras sitokrom p-450 yang paling tinggi iaitu sehingga 3.23 nm/g protein hepatik dan 2.24 nmol/g protein intestinal.

Kesan tiga racun serangga perosak iaitu deltametrin, malation dan endosulfan, dan satu model peransang sitokrom p-450, iaitu fenobarbiton (PB) terhadap kuantiti sitokrom p-450 dan aktiviti 7-etoksiresorufin O-demetilase (EROD) diperiksa di dalam hati dan usus haruan, *C. striatus*. Pendedahan 48 jam kepada beberapa siri kepekatan PB (0.25, 5.0, 15.0 mg/l) telah menghasilkan perangsangan kesan-dos ke atas sitokrom p-450 hepatik dan aktiviti CYP IA tanggungan EROD di dalam *C. striatus*. Manakala di dalam mikrosom usus, aktiviti EROD meningkat secara bererti, $P \leq 0.001$, tetapi tanpa peningkatan bererti dalam jumlah sitokrom p-450. Sebaliknya, pra-pendedahan endosulfan menghasilkan peningkatan bererti kandungan sitokrom p-450 pada $P \leq 0.01$ berbanding dengan paras kawalan pada 0.189 ± 0.016 nmol/mg protein. Begitu juga, aktiviti EROD mikrosom hepatik dan ekstra-hepatik meningkat kepada pendedahan siri kepekatan endosulfan. Deltametrin telah meningkatkan kandungan sitokrom p-450 hepatik dan aktiviti EROD hanya pada dua hari pertama, sedangkan kandungan P450 di dalam intestinal tidak diransangkan oleh pendedahan. Aktiviti EROD telah meningkat dengan ketara oleh semua siri kepekatan (0.2, 2.0, 20.0 ppb) DM. Pemberian malation hanya menunjukkan sedikit induksi pada sitokrom P450.

Endosulfan (0.5 dan 5 ppb) tidak memberi kesan kepada aktiviti ACHE sepanjang 7 hari pendedahan. Walau bagaimanapun, malation pada 50 ppb dan 5 ppm telah merencatkan aktiviti ACHE di dalam tisu otak haruan. Deltametrin 2 dan 20 ppb menurunkan aktiviti ACHE selepas 1 hari pendedahan, kemudiannya meningkat secara beransur-ansur. Sitokrom p-450 hepatik dependen ρ - nitroanisole (PNA) O – demetilase meningkat secara selari dengan dos dan masa pendedahan endosulfan Tetapi hanya malation pada 5 ppm dan deltametrin 20 ppb

meningkatkan aktiviti PNA O – demetilase. In adalah kajian pertama yang dilakukan untuk melihat kesan 3 insektisid ke atas system sitokrom p-450 di dalam haruan, *C. striatus*. Keputusan ini mencadangkan bahawa haruan boleh dijadikan calun alat memantau pencemaran pestisid di dalam system airtawar tropika. Walau bagaimanapun, sistem p-450 haruan dan interaksinya dengan pestisid campuran dan xenobiotik perlu dikaji selanjut.

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I certify that an Examination Committee met on 20th October 2000 to conduct the final examination of Tian Yu on her Master of Science thesis entitled "The Effects of Selected Pesticides on Cytochrome P450 System and Acetylcholinesterase Activity in Haruan, *Channa Striatus*" in accordance with Universiti Pertanian Malaysia (High Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and was accepted as fulfilment of the requirements for the degree of Master of Science.

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Date: **14 DEC 2000**

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Tian Yu

Candidate
Tian Yu

Date: 1st, Nov. 2000

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AChE	acetylcholinesterase
AE	aldrin epoxidase
AHH	aryl hydrocarbon hydroxylation
AhR	aryl hydrocarbon receptor
ANF	α- naphthoflavone
ARNT	aryl hydrocarbon nuclear transferase
ATPase	adenosine triphosphatase
BNF	β- naphthoflavone
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CDNB	1- chloro- 2,4- dinitrobenzene
ClA50	clophen A50
CO	carbon monooxide
CYP	cytochrome P450
DCNB	1,2- dichloro- 4- nitrobenzene
DM	deltamethrin
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
EMND	ethylmorphine N- demethylation
ER	endoplasmic reticulum
EROD	7- ethoxyresorufin O- demethylation
GR	glutathione reductase
GST	glutathione S- transferases
HSP90	heat-shock protein 90
MACA	Malaysian Agriculture Chemical Association
MARDI	Malaysian Agricultural Research and Development Institute
MC	3- methylacholanthrene
MO	monooxygenase
MRNA	messenger ribonucleic acid
N.D.	not detected
NADH	nicotinamide adenine dinuclotide reduced form
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
PAH	poly aromatic hydrocarbon
PB	phenobarbital
PCBs	polychlorinated biphenyls
PCN	pregnenolone 16α- carbonitrile
PNA	p- nitroanisole
ppb	parts per billion
ppm	parts per million
PROD	pentoxyresorufin O- dealkylation
TCDD	2,3,7,8- tetrachlorodibenzo-ρ-dioxin
UPM	Universiti Putra Malaysia
XRE	xenobiotic regulatory element

CHAPTER I

INTRODUCTION

The extensive use of pesticides has increased environmental pollution. Pesticides are among the most dangerous agents of water contamination, since they are used on or near the soil and in many instances in water for aquatic pests or weed control. On the other hand runoff from areas where pesticides are used for intensive agriculture may create areas of non-point source pollution. Such pesticides impacts are often only part of a more complex environmental problem involving solid particle runoff, agroindustrial effluent and river flowing regulation, which can be harmful to aquatic animals especially leading to a decrease in fish population.

The cytochrome P450 dependent monooxygenase (MO) system among the most widely studied enzyme systems plays a determinant role in the initial stage of the metabolism of xenobiotics such as pesticides, drugs, chemical carcinogens, and various industrial chemicals. This enzyme system is involved also in the metabolism of endogenous compounds. One of the basic features of this system is its substrate inducibility: xenobiotic compounds actively stimulate the synthesis of new function protein (Payne *et al.*, 1987). The quantitative analysis of the haemoprotein cytochrome P450 and the measurement of the induction of ethoxyresorufin -O-deethylase (EROD) activity have been proposed as an "early warning system" for the

biological monitoring of environment contamination particularly aquatic, by micropollutant (Payne *et al.*, 1987; Bucheli and Fent, 1995).

Thus, various chemicals have different effects on the quantity or activity of this system and these measurements usually can be used to characterize the inducing agent into one of two classes. In fish, the induction of P450 system has generally been considered a phenomenon associated with PAH-type inducers of the P4501A subfamily whereas induction by PB-type inducers seems to be absent (Goksøyr *et al.*, 1987; Gooch *et al.*, 1989), although the corresponding genes (P4502B) apparently are present and expressed (Stegeman, 1989).

Pesticides are common pollutants in the aquatic environment and its impacts are usually very difficult to detect by means of analytical chemistry in the areas of non-point source pollution. Therefore, the use of the cytochrome P450 system as a biochemical indicator for assessing potential exposure to trace levels of complex mixtures of pesticides in the aquatic environment has been gradually valued. Until now, various classes of pesticides involving different categories were intensively studied on their effects on fish cytochrome P450 system by laboratory experiments and a few field trials using the induction of fish

to monitor aquatic pesticide pollution. (Bucheli & Fern, 1995; Vindimian, 1993). It is known that some pesticides are not inducers. Organophosphate compounds, for example, were inhibitors of EROD activity (Simon et al., 1984; Flammarion et al., 1996), deltamethrin, a pyrethroid, had inhibited cytochrome P450 detoxification system in aquatic species (Banka *et al.*, 1997; Deér *et al.*, 1996). Permethrin appeared

to be a weak inducer of the mixed function oxidase system in rats (Carlson & Schoeing, 1980). Endosulfan, an organochlorine insecticide, had induced the EROD activity and the cytochrome P450 1A1 content in rainbow trout liver (Jensen *et al.*, 1991). Therefore, the nature of the interaction of the chemicals and P450 dependent EROD activity remains unclear. Furthermore, research in tropical aquatic environmental toxicology and contamination, especially by pesticides, is still very limited and there is no report on the study of induction of cytochrome P450 monooxyganese system as a biomarker particularly in freshwater species.

Therefore, the main objective of this study is to investigate the effects on haruan microsomal cytochrome P450 and its dependent enzyme activities and compare with haruan brain acetylcholinesterase activity by treatment with three pesticides: deltamethrin (DM), malathion, and endosulfan which represent different groups of pyrethroid, organophosphate, and organochlorine insecticides respectively. In this study, haruan, *Channa Striatus*, a tropical freshwater fish was chosen. The choice of the fish in this study was based on its wide distribution in tropical and sub-tropical areas and the availability of the culture species, which is never being exposed to any pesticide contamination.

CHAPTER II

LITERATURE REVIEW

Recognition that cytochrome P450 play key roles in pharmacology, toxicology, carcinogenesis and endocrinology, stimulates the drive to understand the diversity of forms, functions and regulation of these enzymes. Subsequently, the study of cytochrome P450 forms in fish is regarded as very important from evolutionary, ecological and toxicological standpoints. There are about 20,000 species of fish known to exist, representing nearly one-half of all known vertebrate species. The fishes present extraordinary diversity, inhabit virtually every niche within the world's fresh and marine waters and are a vital source of protein for humans. Therefore, the health and protection of these organisms are of pivotal important.

Adamson (1967) first

several fish species. And then, driven by environmental concerns, the search for animal models especially fish

continued so that now there are over 800 publications concerning monooxygenase activity or cytochrome P450 in fish.

Cytochrome P450 Monooxygenase System in Fish

Organ Distribution

In fish, as in other vertebrates, cytochrome P450 dependent monooxygenases are mainly localized in the endoplasmic reticulum (ER) and mitochondria of liver, kidney, brain, and small intestine, as well as in several other organs (Stegeman and Hahn, 1994). Fish liver microsomes exhibit typical reduced-CO absorption spectra with a peak near 450 nm, and electron paramagnetic resonance characteristics with low-spin δ values near 2.41, 2.25 and 1.91 typical for cytochrome P450. The levels of total microsomal P450 in fish

2.0 nmol/mg protein, in untreated fish.

characterize different species. At least 10-fold differences in content can occur with a single species, depending on strain, sex or chemical treatment. In general, however, the levels of hepatic microsomal P450 from untreated fish

nmol/mg (Stegeman *et al*., 1981), lower than the values generally appeared in untreated mammals.

Cytochrome P450 Enzyme System in Fish

The overall cytochrome P450 enzyme system and characteristics in fish

to be very similar with mammals. Basically, it consists of two linked enzymes, NADPH-cytochrome P450 reductase and cytochrome P450. Connected with these are cytochrome b5 and its reductase, NADH-cytochrome b5 reductase. They comprise one family of the phase I enzymes, which oxidize, reduce, or hydrolyze xenobiotic substances. Their primary task is add or expose functional groups, which