HEPATOTOXIC AND GONADOTOXIC EFFECTS OF LOW DOSE OF INSECTICIDE DIAZINON IN MALE RATS

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DECLARATION

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ABSTRACT

Over the decades, more than hundred thousands of synthetic pesticides have been invented as a helpful source to eradicate nuisance pests and to maintain adequate food supply. Diazinon [*O,O*-diethyl-0-(2-isopropyl-6-methyl-pyrimidine-4-yl) phosphorothiote], an organophosphate insecticide has been widely used in agriculture and domestic scale. It is rapidly degraded by microbes in the environment and to a small extent by exposure to sunlight. However, its ubiquitous and negligible amount in the environment presents a continuing health hazard to exposed farmers. Human beings can be potentially exposed to this semi-volatile insecticide through inhalation, ingestion and dermal contacts. Diazinon toxicity was proposed via lipid oxidative damage and relatively was less studied in male reproductive system. Hence the current work aimed to investigate the dose dependant adverse effects of diazinon on few physiological, biochemical and histopathological changes in liver and testis of adult male Sprague-Dawley rats based on LD_{50} as a measure of the lowest possible exposure in agricultural environment. The rats were gavaged with diazinon of 10, 15 and 30 mg/kg body weight (LD_{50}>5000 mg/kg body weight) in 3 durations of 1, 2 and 8 weeks. Activity of liver aspartate aminotransferase, alkaline aminotransferase, alkaline phosphatase, and lipid peroxidation exhibited an increase with consensus inhibition of reduced glutathione and catalase levels. Parallel with the biochemical changes, diazinon treatment significantly enhanced the damage in testis. The levels of lipid peroxidation was significantly increased in 30 mg/kg dose in 1 week's treatment and all doses in 2 and 8 weeks' treatment, along with significant decrement in reduced glutathione levels in all doses of 2 and 8 weeks' and catalase showed diminishing trend. Diazinon significantly decreased serum testosterone levels in 8 weeks' treatment and was accompanied with an increased incidence of sperm abnormality in testis. Increase in serum lactate dehydrogenase activity with a qualitative derangement in liver and testis histology support cytotoxic effects. The toxicity of diazinon with low dose exposure study revealed a dose and time dependant response in the parameters of this study. Different antioxidants and enzymes showed significant alterations with an increase in lipid peroxidation. Diazinon at low doses is cytotoxic to liver and germ cell lines.

ABSTRAK

Kesan-kesan Hepatotoksik dan Gonadotoksik Akibat daripada Pendedahan Racun Serangga Diazinon yang Berdos Rendah pada Tikus Jantan

Peningkatan jumlah penghasilan racun serangga sintetik sejak beberapa dekad lalu menunjukkan keperluan global dalam menangani serangga perosak demi memantau keperluan sumber makanan. Diazinon [0,0-diethyl-0-(2-isopropyl-6-methyl-pyrimidine-4-yl) phosphorothiote] ialah sejenis insektisid organofosforus yang telah digunakan secara meluas dalam industri pertanian dan secara domestik. Diazinon mudah terurai di alam sekitar melalui tindakan mikroorganisma, malah turut dipengaruhi terik matahari. Namun demikian, penyebarannya yang meluas dalam kuantiti yang sangat kecil telah diabaikan. Justeru, diazinon wujud sebagai ancaman pada kesihatan yang berterusan di kalangan para petani. Manusia berpotensi terdedah pada insektisid separa meruap ini melalui respirasi, oral dan dermal. Ketoksikan diazinon berpunca daripada stres oksidatif yang dijana oleh proses oksidasi lipid. Sesunguhnya, kajian ini masih kurang dijalankan ke atas sistem pembiakan jantan. Oleh yang demikian, dos dipilih berlandaskan skala sukatan LD_{50} bagi menyerupai pendedahan terendah yang mungkin di kawasan pertanian. Kajian ini bertujuan untuk menyiasat kesan negatif diazinon ke atas perubahan fisiologi, biokimia dan histopatologi hepar dan testis tikus spesies Sprague-Dawley jantan. Tikus dirawat pada dos 10, 15 dan 30 mg/kg (LD_{50}>5000 mg/kg berat badan) diazinon dalam 3 kategori jangka masa iaitu 1, 2 dan 8 minggu. Aktiviti aspartat aminotransferase, alkali aminotransferase dan alkalin fosfatase serta oksidasi lipid menunjukkan peningkatan diikuti penurunan reduced glutathione dan katalase. Sejajar dengan perubahan biokimia, rawatan diazinon mengakibatkan kerosakan di testis. Oksidasi lipid ketara meningkat pada dos 30 mg/kg dalam rawatan seminggu, dan semua dos dalam rawatan 2 dan 8 minggu. Ini diikuti penuruan ketara paras reduced glutathione pada semua dos dalam rawatan 2 dan 8 minggu serta penurunan katalase. Diazinon menurunkan paras testosteron serum dengan jelas dalam rawatan 8 minggu selaras dengan peningkatan insiden kecacatan sperma. Peningkatan aktiviti laktat dehidrogenase serum seiring dengan kemerosotan kualitatif di hepar dan testis menganjak kemungkinan kesan sitotosik diazinon. Kajian diazinon pada dos rendah menyingkap pekaitan hubungan kesan toksik dengan dos dan tempoh pendedahan adalah saling berkaitan. Antioksida dan enzim jelas dipengaruhi oleh peningkatan oksidasi lipid. Pendedahan diazinon tampak bertindak sebagai agen sitotoksik pada hepar dan testis walaupun dalam dos yang rendah.

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LIST OF ABBREVIATIONS

ABS	-	Absorbance
Ach	-	Acetylcholine
AChE	-	Acetylcholinesterase
ALP	-	Alkaline phosphatase
ALT	-	Alanine aminotransferase
AST	-	Aspartate aminotransferase
ASTDR	-	Agency for Toxic Substances and Disease Registry
BSA	-	Bovine serum albumin
b. w.	-	Body weight
CAT	-	Catalase
Cm	-	Centimeter
D	-	Diameter
DEP	-	Diethylphosphate
DETP	-	Diethylthiophosphate
DPX	-	Di-N-butyle phthalate in xylene
DNA	-	Deoxyribonucleic acid
DTNB	-	5,5'-Dithiobis (2-nitrobenzoic acid)
DZN	-	Diazinon
EP	-	Eppendorf tube
EPA	-	Environmental Protection Agency
G	-	Gram
GGT	-	Gamma-glutamyl transpeptidase
GIT	-	Gastrointestinal tract
GOD	-	L-glutamate oxidase
GPX	-	Glutathione peroxidase
GSH	-	Reduced glutathione
H & E	-	Haematoxylin and eosin
HDL	-	High density lipoprotein
HRP	-	Horseradish peroxidase
H_2O	-	Water
H_2O_2	-	Hydrogen peroxide
IMHP	-	2-isopropyl-4 methyl-6-hydroxypyrimidine

IU/L	-	International units per litre
Kg	-	Kilogram
LCAT	-	Lecithin-cholesterol acetyltransferase
LD_{50}	-	Lethal dose, 50% kill
LDH	-	Lactate dehydrogenase
LDL	-	Low density lipoprotein
LPO	-	Lipid peroxidation
M	-	Meter
MDA	-	Malondialdehyde
Min(s)	-	Minute(s)
Mg	-	Milligram
Ml	-	Mililiter
mm^2	-	Milimeter square
NAD	-	Nicotinamide adenine dinucleotide
N	-	Number
NADH	-	Reduced form of NAD
Nm	-	Nanometer
Nmol	-	Nanomole
O_2	-	Oxygen
OD	-	Optical density
OP	-	Organophosphate
OPIDN	-	Organophosphate-induced delayed neuropathy
PBS	-	Phosphate buffer saline
PMS	-	Post mitochondrial supernatant
POD	-	Peroxidase
PyOD	-	Pyruvate oxidase
ppm	-	Parts per million
RBC	-	Red blood cell
ROS	-	Reactive oxygen species
Rpm	-	Revolution per minute
Sec(s)	-	Second(s)
SD	-	Standard deviation
SGOT	-	Serum glutamic oxalate transferase
SGPT	-	Serum glutamate pyruvate transferase

SOD	-	Superoxide dismutase
SPSS	-	Statistical Package of Social Science
SSA	-	5-Sulfosalicyclic acid dehydrate
STD	-	Diameter of seminiferous tubule
ST	-	Seminiferous tubule
TBARS	-	Thiobarbituric acid reactive substance
TC	-	Total cholesterol
TCA	-	Trichloroacetic acid
TMB	-	3,3', 5,5"-tetramethylbenzidine
UMS	-	Universiti Malaysia Sabah
VLDL	-	Very-low-density lipoprotein
WBC	-	White blood cell
WHO	-	World Health Organization
w/v	-	Weight/volume
µg	-	Microgram
µl	-	Microliter
µm	-	Micrometer
µmol	-	Micromol
°C	-	Degree Celcius
<	-	Less than
>	-	More than
%	-	Percent

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The inventions of synthetic pesticides (Azmi *et al.*, 2006) have stalked themselves by its importance in the new globally changed community. Pesticide use is pervasive and growing fast, and serious adverse health effects on animal and human populations have become widespread and common (Windham, 2010). Its usage is extensive due to the needs to control invasive pest in agricultural and horticultural activities (Gawish, 2010; Patil, *et al.*, 2009). The progressive use of pesticides parallel to the large amount of application (Kalender *et. al.,* 2005) is alarmed. Its great concern (Azmi *et al.,* 2006; Kalender *et al.,* 2005) in its hazardous effects on environment and human health. Malpractice and illegal usage (Azmi *et al.,* 2006), in addition, have enhanced the negative effects in the ecosystem.

There are many types of pesticides manufactured globally. Organophosphates, organochlorine and pyrethroid are examples of pesticides available in the market. Though, the use of pesticides has risen significantly and crop lost to pests have not declined correspondingly. Despite its beneficial purposes, pesticide residues contamination in food and drinking water has been increasing globally (Patil *et. al.,* 2009). In fact, pesticide poisoning incidents have become more than doubled in the last 10 years. More than 300,000 farm workers become sick every year through exposure to the pesticides (Windham, 2010). This condition is critical and immediate actions are needed.

Organophosphates (OP) are the common pesticides available in the market. There are different types of OP such as Chlorpyrifos, Malathion, Parathion, Dimethoate Temephos and Fenthion and each of them contains the OP group. Organophosphate pesticides have been used since the World War II as nerve agents (Abdelsalam, 1987). Today, OP has become the pesticide of choice because

it is inexpensive and effective in pest control (Saxena and Garg, 2010; Abdelsalam, 1987). However, all OP pesticides share a similar mode of toxicity as acetylcholinesterase (AChE) inhibitor in exposed organisms (Stone *et al.*, 2009; Buyukokuroglu *et al.*, 2008; Xavier *et al.*, 2004). It causes endogenous accumulation of AChE at the nerve endings and gradually affects the peripheral (muscarinic and nicotinic) (Kalipci *et al.*, 2010) and central nervous systems (ATSDR, 2006).

Among the OPs, Diazinon [O,O-diethyl-O-(2-isopropyl-6-methyl-pyrimidine-4-yl) phosphorothiote] is one of them being studied extensively. It is known by numerous commercial names such as Knoxout, Basudin, Gardentox, Diaphos and Nemazone. It is broadly applied in agricultural, commercial and household purposes. Household uses predominate with 75 million applications in the United States annually totalling over 5 million pounds. Like the other OP pesticides, Diazinon (DZN) is toxic to the nervous system (Bouchard *et al.,* 2006; Cox, 2000). To a greater extent, it induces oxidative stress via lipid peroxidation (Ogutcu *et al.,* 2006).

Over the decades, reproductive health is greatly affected by pesticides. Factors contributing to this are taken seriously. It has been demonstrated that pesticides can interfere in reproduction (Sarabia *et al.*, 2009). Diazinon is hazardous to human, fish, wildlife and invertebrates. Its adverse effects have been observed in laboratory tests with increasing reproductive problems of pregnant animals. The problems include the delay in sexual development, stillbirths, death of newborn offsprings and birth defects (Cox, 2000). In male animal model, DZN disrupts spermatogenesis with the incidence of increasing sperm abnormality and sperm DNA structure alteration (Sarabia *et al.*, 2009).

Occupational and environmental influences on male fertility are the global concern (Bustos-Obregón and Hartley, 2008). Only after 5 decades of exposing to the pesticides, the sperm count in men has halved. This statistical observation has put male fertility index at red light and need more attention. However, studies on DZN adverse effects on male reproduction of various animal models are limited.

Narayana *et al.* (2008a and 2006) reported that OP pesticide induces histopathological changes in testis. The cytotoxic effects include the decrease in seminiferous tubule diameter (STD), epithelial height (SE), sperm quality (concentration, normality and motility), and epithelial sloughing. These effects in testis are analogous to decrease testosterone level indicating Leydig cell dysfunction. These incidences, as a whole, infer that DZN disturbs spermatogenesis in male.

Cytotoxicity of DZN involving lipid peroxidation and antioxidant status in testis is yet to be elucidated. Therefore, this study is crucial to investigate DZN cytotoxicity in testis with the status of lipid peroxidation and antioxidant as a possible mechanism contributing to spermatogenesis disruption.

1.2 Objectives

The aim of this study is to investigate the acute and chronic toxicity of low dose (below LD_{50}) diazinon exposure in liver and testis of male rats.

1.3 Specific Objectives

a. To evaluate the acute and chronic hepatotoxic effects of low diazinon doses (below LD_{50}). The investigation includes the following parameters in liver.

i. body and organ weight
ii. lipid peroxidation (LPO)
iii. reduced glutathione (GSH)
iv. catalase (CAT)
v. alkaline phosphatase (ALP)
vi. alanine aminotransferase (ALT)/serum glutamic-pyruvic transaminase (SGPT)
vii. aspartate aminotransferase (AST)/serum glutamic-oxaloacetic transaminase (SGOT)
viii. qualitative histopathological changes

b. To evaluate the acute and chronice gonadotoxic effects of low diazinon doses (below LD_{50}). The investigation includes the following parameters in testis.

i. body and organ weight
ii. lipid peroxidation (LPO)
iii. reduced glutathione (GSH)
iv. catalase (CAT)
v. testosterone
vi. sperm quality (concentration, normality and motility)
vii. semineferous tubule diameter (STD)
viii. qualitative histopathological changes

c. The investigation of low dose DZN also includes serum lactate dehydrogenase (LDH).

1.4 Hypothesis

Acute and chronic exposures to low dose of diazinon lead to hepatotoxicity and gonadotoxicity. The gonadotoxic effects may result in a decline in fertility index.

CHAPTER 2

LITERATURE REVIEWS

2.1 The Studies of Toxicology and Toxicity

Toxicology is the study of the adverse effects of a toxicant on living organisms (Casarez, 2001). It is also defined as the study of poisonous effects of drugs and other chemicals, on household, environmental pollutant, industrial, agricultural and homicidal type of chemicals, emphasis on the detection, prevention and treatment of poisonings (Tripathi, 2003). Toxicology is a broad study of scientific application that incorporates biology, chemistry, physiology, pathology, physics, statistics and sometimes immunology and ecology (Casarez, 2001). Adverse effect is any kind of undesirable or unwanted consequence of drug administration (Tripathi, 2003) and it reflects the toxicity in toxicology studies.

Toxicity refers to the degree of harmfulness of a toxicant (s). It is complex and unpredictable as it is influenced by many factors. The most important factor is the amount or frequency of exposure. It determines the degree of toxicity. Different chemicals pose different ways of toxicity. Some chemicals are toxic by themselves (parent compound). Some chemicals need to undergo metabolization in the body to become metabolite to cause toxic effects. Toxicity is influenced by broad factors - the physical form and innate chemical activity, dosage and dose-time relationship, exposure route, species, age, sex, absorption ability, metabolism and distribution within the body, excretion and the presence of other chemicals (National Library of Medical, 2007).

Toxicology and toxicity knowledge is helpful to solve problems in many scientific fields such as forensic, medicine, clinical treatments, pharmacy and pharmacology, public health, industrial hygiene, veterinary science, agricultural and lots more. Toxicology studies enable researchers to have basic insight of how

organisms function. Mainly, there are two levels of exposure - acute and chronic levels. Both have specific criteria for defining purpose.

2.1.1 Exposure

Exposure is how an animal gets into the risk of toxicity. The physical properties and chemical concentration in the environment are crucial to determine the extent of exposure and developmental effects of the exposed subject.

2.1.2 Route and Site of Exposure

Generally, the route of exposure can be divided to four types, which are:

1. Ingestion
2. Inhalation
3. Dermal
4. Parenteral (intravenous, intramuscular, intraperitoneal)

Effective route of exposure begins with intravenous, inhalation, intraperitoneal, intramuscular, ingestion and finally topical. The effectiveness is determined based on the rate of absorption, distribution, metabolism and excretion of the body.

2.1.3 Duration of Exposure

a. Acute Exposure

Casarez (2001) defines acute exposure is an exposure of the organism to the toxican

t within or less than 24 hours or a series of exposure (dose) within 24 hours. The effects occur almost immediately, hours or days after an exposure. This type of exposure is usually well described in most cases of committing suicide or death. However, acute exposure is also an exposure with a substance that occurs once or twice or only for a short time, such as a week or less (Kemple, 2001). Agency for Toxic Substances and Disease Registry (2009) also defines that acute exposure can last up to 14 days. However, it can be reconstructed base on the length and dose concentration of exposure (National Library of Medicine, 2007).

b. Subacute and Subchronic Exposure

An extent exposure of acute stage is called subacute stage. Subacute stage takes longer duration with repeated doses. When it is up to certain duration, it reaches subchronic stage before the chronic stage (National Library of Medicine, 2007).

c. Chronic Exposure

Chronic exposure is defined as a long term or lifetime exposure and spans at least 10% of a life time (Kemple, 2001) with organism exposed to the substance (often at lower levels) repeatedly or continuously. It takes months, at least 3 months (Toxicology Source, 2011) or years (ATSDR, 2009) to yield the clinical manifestation that is recognizable as a disease. Chronic exposure derives significantly from subclinical exposure. The cumulative damages slowly form until it exceeded the threshold. Eventually, it becomes very severe until the organs cannot function normally (National Library of Medicine, 2007).