THE AMELIORATIVE EFFECTS OF VITAMINS C AND E ON NEUROTOXIC, HAEMATOLOGIC AND BIOCHEMICAL CHANGES INDUCED BY CHRONIC CHLORPYRIFOS IN ADULT WISTAR RATS

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FEBRUARY 2009

DECLARATION

I declare that the work in the dissertation, entitled "The Ameliorative Effects of Vitamins C and E on Neurotoxic, Haematologic and Biochemical Changes induced by Chronic Chlorpyrifos in Adult Wistar Rats" has been performed by me in the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria under the supervision of Professors J.O. Ayo, S.A. Ojo and K.A.N. Esievo. The information derived from the literature has been duely acknowledged in the text and in the list of references provided. No part of this dissertation was previously presented for another degree or diploma at any university.

Ambali, Suleiman Folorunsho	Signature	16/02/09 Date

CERTIFICATION

This dissertation, entitled "THE AMELIORATIVE EFFECTS OF VITAMINS C AND E ON NEUROTOXIC, HAEMATOLOGIC AND BIOCHEMICAL CHANGES INDUCED BY CHRONIC CHLORPYRIFOS IN ADULT WISTAR RATS" by Ambali, Suleiman Folorunsho meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello Univeristy, Zaria, and is approved for its contribution to knowledge and literary presentation

Professor J.O. Ayo — 16/02/09
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Professor S.A. Ojo — 16/02/2009
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DEDICATION

This dissertation is dedicated to Almighty Allah, the All-knowing, the All-wise; and to the leader of mankind, Sayidina Muhammed Rasulullahi (SAW) and my spiritual master, Sheikh Ibrahim Inyass Alkhaolaq

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ABSTRACT

Experiments were performed with the aim of evaluating the effect of chronic exposure to chlorpyrifos (CPF) on neurotoxieological, clinicopathological, haematologieal and biochemical changes, and the effect of antioxidant vitamins C and/or E on these changes in Wistar rats. Fifty adult Wistar rats divided into 5 groups of 10 animals each (5 males and 5 females in each group) served as subjects for this study. Rats in group I were given soya oil (S/oil) only at a dose of 2ml/kg daily. Rats in group II (CPF only) were exposed to CPF only at 10.6 mg/kg (1/8th of LD50), while those in group 111 were pretreated with vitamin C at a dose of 100 mg/kg before exposure to CPF (10.6 mg/kg) (VC+CPF) 30 minutes later. Rats in group IV were pretreated with vitamin E at a dose of 75 mg/kg before being administered with CPF (10.6 mg/kg) (VEiCPF) 30 minutes later, while those in group V were pretreated with a combination of vitamins C+E followed by exposure to CPF (10.6 mg/kg) (VC+VE+CPF) 30 minutes later. The regimens were orally administered to each animal in all the groups once daily for 17 weeks. During this period, the rats were examined for clinical signs, weekly body weight changes and death. The effect of the different regimens on motor activity, anxiety, habituation learning, sensorimotor reflex, motor strength, learning and memory were assessed using appropriate ncurobehavioural asessment protocols. At the end of the study period, the rats in each group were sacrificed for haematologieal, serum biochemical and tissue pathological examinations. The haematologieal parameters analysed were packed cell volume (PCV), haemoglobin (lib) concentration, red blood cell (RBC) count, absolute and differential white blood cell (WBC) count, and *in-vitro* erthrocyte osmotic fragility. The scrum biochemical parameters analysed included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AFP), y-glutamyl transferase (GGT), creatine kinase (CK), Na^{1}, K', CI" and HCO3 Other parameters evaluated included total proteins albumin, globulin, urea, uric acid, creatinine.

glucose, thyroid stimulating hormone (TSH), triiodothyronine (T3), thyroxine (T_4), triglycerides (TG), cholesterol (TC), high density lipoprotein-cholestcrol (HDL-c) and low-density lipoprotein-cholesterol (LDL-c). Gross examination of tissues and organs were carried out, while histopathological examinations on sections of the brain, liver, stomach, intestine, pancreas, kidneys, lungs and spleen were evaluated. In addition, the effect of the regimen on lipid peroxidation was analysed by evaluating the concentration of thiobarbituric acid reactive substances (TBARS), malonaldehyde (MDA) in the liver, brain and the serum. The results showed that rats exposed to CPF only manifested toxic signs, reduce body weight gain, increased anxiety; caused impairment of motor activity, coordinated gait, neuromuscular coordination, motor strength, sensorimotor reflex, learning and memory. Pretreatment with vitamins C and/or E ameliorated the CPF-induccd neurobchavioural and cognitive changes. Exposure to CPF resulted in reduction in the values of PCV, RBC, Hb and WBC, uric acid, IP, albumin, CK, TSH, T_3, T_4,TC, TG and IIDL-c. Exposure to CPF also caused increased erythrocyte osmotic fragility, globulin, glucose, creatinine, AST, ALT, AEP, GGT, LDL-c, serum and tissue MDA. No difference in the electrolytes level was observed in all the groups. Pretreatment with vitamins C and E but not the combination of both vitamins caused further increase in LDL-c. On the whole, pretreatment with vitamin C and/or E ameliorated CPF-induced haematological and biochemical changes. Severe degenerative changes involving neuronal and glial cells of the cerebrum, hepatocytes, myocardial cells, glomeruli and renal tubules, glandular and non-glandular portion of the stomach, intestinal villi, submucosal and muscular layer of the intestine; pancreas and spleen were observed in rats exposed to CPF only. Pretreatment with antioxidant vitamins C and/or E generally resulted in milder pathological lesions in the organs compared to those exposed to CPF only. The study has shown that chronic exposure to CPF induces clinicopathological, neurobchavioural, cognitive, haematological and serum biochemical changes in Wistar rats,

and that pretreatment with antioxidant vitamins C and/or E ameliorated the changes. The study has further shown that induction of increased lipid peroxidation by chronic CPF exposure plays an important role in the molecular mechanisms of these changes.

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AA- Ascorbic acid

ACh- Acetylcholine

AChE- Acetylcholinesterase

ALP-Alkaline phosphatase

ALT- Alanine aminotransferase

ANOVA- Analysis of variance

AST-Aspartate aminotransferase

ATP- Adenosine triphosphates

ATSDR- Agency for Toxic Substance and Developmental Research

CAT- Catalase

CCl_3- Carbon tetrachloride

CDC- Centre for Disease Control

CK- Creatine kinase

Cl- Chloride

CNS-Central nervous system

CO-Carbon monoxide

CPF- Chlorpyrifos

CSF- Cerebrospinal fluid

Cu^{2+}- Cuprous ion

CYP- Cytochrome P

DETP-Diethylthiophosphate

DHA-Dehydroascorbic acid

DNA- Deoxyribonucleic acid

EEG- Electroencephalograph

ELISA-Enzyme linked immunosorbent assay

ERK- Extracellular signal-regulated protein kinase

FAAH- Fatty acid amide hydrolase

FAO- Food and Agricultural Organisation

Fe^{2+}- Ferrous ion

FR- Free radical

GABA- Gamma-aminobutyric acid

GGT- Gamma-glutamyl transferase

GLUT Glucose transporter

GSH- Glutathione

GSHPx- Glutathione peroxidase

GSH-Px- Glutathione peroxidase

GSSG- Oxidised glutathione

GST-Glutathione sulphur transferase

H_2O_2- Hydrogen peroxide

H_2O_2- Hydrogen peroxide

Hb- Haemoglobin

HCO_3- Bicarbonates

HDL-c- High density lipoprotein cholesterol

HNO_2- Nitrous acid

HNO_2- Nitrous acid

HOCl- Hypochlorous acid

HO°- Hydroxyl radicals

HPA- Hypothalamic-pituitary-adrenal axis

IO_2- Singlet oxygen

JNK- c-jun NH_2 terminal protein kinase

K- Pottasium

LCAT- Lecithin cholesterol acetyltransferase

LD_{50}- Median lethal dose

LDL-c-Low density lipoprotein cholesterol

MAPK- Mitogen-activating protein kinase

MDA- Malonaldehyde

N_2O_3- Dinitrogen trioxide

Na- Sodium

NaCl- Sodium chloride

NADH- Nicotine adenyl dinucleotide

NADPH- Nicotinamide adenine dinucleotide phosphate Hydrogenase

NMDA- N-methyl-D,L-aspartate

NO^- - Nitric oxide

NO_2 - Nitrogen dioxide

NTE-Neuropathy target esterase

O_3- Ozone

O_3- Ozone

OFR- Oxygen free radical

$OONO^-$- Peroxynitrite

OP- Organophosphate

OPICN- Organophosphate-induced chronic neurotoxicity

OPIDP- Organophosphate-induced delayed polyneuropathy

PCV- Packed cell volume

PKC- Protein kinase C

PON- Paraoxonase

PUFA- Polyunsaturated fatty acids

PUFA-OOH- hydroxyperoxide of PUFA

RBC-Red blood cell

RNS-Reactive nitrogen species

$ROO^{\cdot}$- peroxyl radicals

ROS- Reactive oxygen species

SEM- Standard error of mean

SOD- Superoxide dismutase

SVCT- Sodium-dependent vitamin C transporter

T_3- Triiodothyronine

T_4- Thyroxine

TBA- Thiobarbituric acid

TBARS-Thiobarbturic acid reactive substance

TCA-Trichloroacetic acid

TCP-3,5,6-trichloro-2-pyridinol

TC-Total cholesterol

TG- Triglycerides

TGF- Transforming growth factor

TocH- Tocopherol

TP- Total proteins

TSH- Thyroid stimulating hormone

UNEP- United Nations Environmental Programme

UQH2- Ubiquinone

US EPA- United States Environmental Protection Agency

USA- Unites States of America

CHAPTER ONE

1.0 INTRODUCTION

1.1 Statement of research problems

The history of man is the record of a hungry creature in search of food (Stakman, 1959). Man competes with various elements including pests to obtain adequate supply of food, which reduce the quantity and quality of output of food (crops and livestock) through physical damage and disease. Apart from their effects on food production and quality, pests cause and enhance the spread of various forms of diseases to man and animals, and constitute nuisance. Therefore, to combat the danger posed by pests, man has developed pesticides. The use of pesticides to combat the menace of pests dates back to ancient times, with the use of sulphur by the ancient Summarians at around 2500 BC to control mites and other insects (Price, 1973).

Pesticides such as insecticides, acaricides, miticides, fungicides, herbicides, rodenticides and avicides have been of increased use, especially following the development of more potent synthetic pesticides. This development has become crucial due to the compelling necessity to feed the ever-growing human and animal population, and to reduce the incidence of food and vector-borne diseases (Gubler, 1998; CDC, 2002a and b; Weiss *et al.*, 2004). The use of pesticides has helped to improve human and animal nutrition, and lower costs of food. It has also reduced human labour requirements and the attendant risks of injury (Weiss *et al.*, 2004). Agricultural pesticides prevent a monetary loss of about $9 billion each year in USA, and for every $1 invested in pesticides, the American farmer gets $4 in return (Repetto and Baliga, 1996). Although the figure in Nigeria farmers is not readily available, it is most likely that the profit yield in Naira equivalent from pesticide use may be similar to what is obtained in USA.

It has been estimated that a total of 2.04 billion kilogram (4.5 billion pounds) of chemicals are applied annually as pesticides (Aspelin and Grube, 1999). Pesticides are unique contaminants in that they are intentionally released into the environment to elicit toxicity in certain 'pest' species. However, their lack of specificity often leads to problems in humans and other non-target species (Tuzmen *et al.*, 2008) such as man, animals, wildlife and even the environment. It is estimated that about 0.1% of the pesticides used reached the target pests (Pimentel, 1995). Although different mechanisms have been proposed as being responsible for the toxic manifestation of pesticides, various studies from different parts of the world revealed that toxic effects of pesticides are partly mediated via free radical (FR) mechanisms (Patil *et al.*, 2003, Abdollahi *et al.*, 2004c).

Organophosphates (OPs) constitute diverse group of highly toxic chemicals originally developed as chemical weapon during the World War II. They are widely used as agricultural and horticultural pesticides in human and veterinary medicines, in various public health hygiene products and as insecticides in homes and gardens. Exposure to OP poisoning in man may occur during manufacture, formulation and application (Zhu *et al.*, 2001). In farm animals, exposures to OP poisoning may occur from farm left-overs, contamination of feeds, water and feeding utensils (Hatch, 1982) or during acaricide bath.

Prevention of OP-induced acute and chronic toxic sequelae remains a challenging field to researchers and scientists throughout the world. Although the main mechanism of acute OP poisoning involves inhibition of acetylcholinesterase, other mechanisms have been reported, especially with regards to its long-term toxicity. One of the strong mechanisms implicated in both acute and chronic OP poisoning is oxidative stress. Several studies have established the

role of oxidative stress in OP-induced poisoning in rats (Poovala *et al.*, 1999; Gultekin *et al.*, 2000; Gupta *et al.*, 2001; Verma, 2001; Ambali *et al.*, 2007) and humans (Banerjee *et al.*, 1999; Altuntas *et al.*, 2002b; Ranjbar *et al.*, 2002, Abdollahi *et al.*, 2004b and c; Vidyasagar *et al.*, 2004).

Chlorpyrifos (CPF) (0-0-diethyl-0-3,4,6-trichloro-2-pyridyl phosphorothionate) is a chlorinated organophosphate pesticide used in both agricultural and pest control (Steenland *et al.*, 2000). Some of the common names under which chlorpyrifos are marketed include Termicot®, Britermicot®, Dursban®, Lorsban®, Pagent® F, among others. It was first marketed in USA in 1965, and its use has increased rapidly, in part due to banning of chlordane for termite application in 1988 (Steenland *et al.*, 2000). It is one of the most widely used OP insecticides in USA, with an annual usage of 3.6- 4.5 million kilogram (8-10 million pounds) in agricultural sector in 1999 (Donaldson *et al.*, 2002). In the United States, about 800 registered pesticide products on the market contain CPF (Smegal, 2002). These products have been used for pest control in a variety of food crops, turf or ornamental plants, green houses, and sod; indoors pest control, structural pest control and pet collars (Smegal, 2002). Long-term neurobehavioural and cognitive deficits have been reported in animals treated with OPs such as CPF (Duffy and Burchfiel, 1980; Stamper *et al.*, 1988; Sánchez-Santed *et al.*, 2004). However, the deficits cannot be related to the well-known neurochemical changes associated with acetylcholinesterase inhibition as well as muscarinic receptor binding, which recovered long before the neurobehavioural assessments were carried out (Pope *et al.*, 1992; Chakraborti *et al.*, 1993). Therefore, other mechanisms such as induction of oxidative stress may be playing some roles in these neurobehavioural and cognitive impairments induced by low-dose

CPF exposure. Furthermore, studies have shown that the combination of antioxidant vitamins C and E reduced lipid peroxidation induced by CPF (Gultekin *et al.* 2001; Oncu *et al.*, 2002a and b). Ambali *et al.* (2007) have demonstrated the ameliorative effect of vitamin C on clinical, haematological and biochemical changes induced by repeated subchronic administration of CPF in mice. There is paucity of information in the available literature on the protective effect of antioxidant vitamins on neurobehavioural, cognitive, biochemical and/or neuro-pathological changes induced by repeated chronic administration of CPF.

1.2 Justification of the study

Pesticides are used to combat the menace of pests that affect plants and animals. However, their continuous uses have been associated with adverse health effects in man and animals. Most of the deaths associated with acute pesticide poisoning occur in developing countries, which utilize only $1/4^{th}$ of the world production (Jeyatranam, 1990). Some of the reasons advanced for this widespread exposure and the attendant mortality include lack of or less strictly enforced pesticide usage regulations, porous country borders, which have turned the developing countries to dumping ground for banned or restricted hazardous chemicals (Mbakaya *et al.*, 1994; Smith, 2001). In many developing countries including Nigeria, there has been lack of political will to address the menace caused by pesticide use. In addition, the instructions that accompany many of these pesticides are written in foreign language, which always makes interpretation difficult. This problem is further compounded by the fact that most arable and livestock farmers, who are the end-users of the products, are not literate enough and/or are not aware of the sequelae associated with long-term exposure to pesticides. Furthermore, many livestock farmers who use pesticides, particularly OPs do not strictly observe the withdrawal period before turning pesticide-preserved food crops to the market or

slaughtering of treated animals for consumption, thereby leading to pesticide residue which further endangers public health. Other prevailing factors such as protein deficiency and water deprivation common among the rural poor in developing countries can increase the toxic effects of pesticides (WHO, 1989). Therefore, exposure to pesticides will continue to be a problem in developing countries for a long time to come. Association between acute pesticide exposure and neurotoxicity are well known (Lotti, 2000). On the other hand, the effects of chronic low-level pesticide exposure are not well established (Alavanja *et al.*, 2004).

Organophosphates remain the most widely used insecticides worldwide, with chlorpyrifos (CPF) responsible for more than 50% of the globally used insecticides (Casida and Quistad, 2004), hence toxicity associated with this group of OP insecticides. It should be noted, however, that while a large number of human and animal studies have focused on the long-term consequences of acute OP exposure, relatively little attention has been paid to the subject of chronic, "low-level" OP exposures that are not associated with acute cholinergic symptoms (Ray and Richards, 2001; Rothlein *et al.*, 2006). This may be of particular concern given the widespread use of OP insecticides (and consequent human and animal exposure) in household, agricultural, and commercial environments worldwide (Terry *et al.*, 2007). The issue of repeated, subthreshold exposures to OPs is very important, since detectable levels of OPs including CPF can remain in the environment (particularly in-door environments) for extended periods after application (Krieger *et al.*, 2001), thereby posing risk for low-level exposure (Terry *et al.*, 2003). Studies in humans showed neurological, cognitive and psychomotor impairments following cumulative exposure to OPs and organochlorines in people from agricultural communities, without history of acute poisoning (Kamel and Hoppin, 2004; Kamel *et al.*, 2007). Neurobehavioural changes following low-dose OP exposure have been

reported in sheep farmers (Stephens *et al*., 1995), greenhouse workers (Bazylewicz-Walczak *et al*., 1999), tree-fruit workers (Fiedler *et al*., 1997), and farm workers in Florida (Kamel *et al*., 2003). These studies have found deficits in measures of sustained attention, information processing, and motor speed and coordination. Chronic low-dose exposure to pesticide has also been associated with neurological impairments (Moser, 2007).

Maintenance of sound neurological and cognitive functions in animals is becoming increasingly important in veterinary medicine. This is because many animals especially dogs and cats used for security purposes, and even some captive wild animals that are used in circus show worldwide need to maintain adequate cognitive functions. These groups of animals are more frequently exposed to insecticide bath, with potentially deleterious consequence of long-term impairment on their neurological and cognitive functions.

Many studies have also found alteration in haematological and biochemical composition following low-dose exposure to pesticides (Yoshida *et al*., 1985; Barna-Lloyd *et al*., 1990; Patil *et al*., 2003; Kalender *et al*. 2006; Ambali *et al*., 2007). Alteration of these biochemical parameters due to low-dose CPF exposure may be clinically insignificant initially, but can be life threatening if it occurs concurently with other clinical problems. This exacerbates the alteration to the point of clinical manifestations. In addition, it is possible to use alteration in some haematological and biochemical indices as biomarkers to detect the level of exposure to pesticides, especially in individuals that are constantly exposed. These haematological and biochemical biomarkers may be used to take precautionary measures or advise individuals or livestock or pet owners. Furthermore, the effect of antioxidant vitamins on amelioration of

these haematological and biochem

prophylaxis of individuals continual

It is difficult or seemingly impra

pesticide usage in many developin

at reducing the impact of pesticides

fact that the developing countries s

pesticide applicators, farmers, workers in pesticide manufacturing industries and pets and other valuable animals who are constantly being exposed to the danger of low-dose OP may benefit from prophylaxis with cheap and readily available antioxidants vitamins C and E.

1.4 General aim of the study

The aim of the study was to evaluate the effect of prolonged exposure to CPF on neurological, haematological, biochemical and pathological changes, and the ameliorative effect of antioxidant vitamins C and/or E on the changes.

1.5 Specific objectives of the study

The objectives of the present study were to evaluate the:

1. Neurotoxicological, haematological, biochemical and tissue pathological changes associated with repeated chronic administration of low-dose CPF in rats.

2. Role of lipid peroxidation in neurotoxicological, hematological, biochemical and tissue pathological changes induced by repeated chronic exposure to low-dose CPF in rats.

3. Ameliorative effect of vitamins C and/or E on the above-named changes induced by repeated exposure to low-dose CPF.

1.6 Statement of research hypotheses

The null (Ho) hypotheses in which these studies were based are:

1. Repeated chronic CPF exposure is not capable of causing neurotoxicological, haematological, serum biochemical and tissue pathological changes in rats

2. Lipid peroxidation does not play important role in CPF-induced neurotoxicological, biochemical and tissue pathological changes in rats

3. Prophylaxis with antioxidant vitamins C and E, either singly or in combination cannot ameliorate the neurotoxicological, haematological, serum biochemical and tissue pathological changes induced by chronic repeated CPF exposure in rats

On the other hand, the alternate hypotheses (Ha) for this study are:

1. Repeated chronic CPF exposure is capable of causing neurotoxicological, haematological, serum biochemical and tissue pathological changes in rats.

2. Lipid peroxidation plays an important role in CPF-induced neuro-toxicological, biochemical and tissue pathological changes in rats.

3. Prophylaxis with antioxidant vitamins C and E, either singly or in combination, is capable of ameliorating the neurotoxicological, haematological, serum biochemical and tissue pathological changes induced by chronic repeated CPF exposure in rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Organophosphates

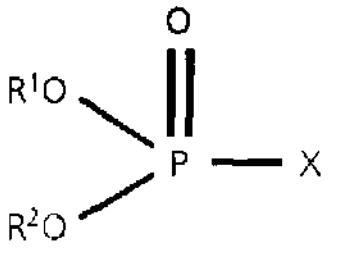
Organophosphates (OPs) are a group of insecticides that were first recognized in 1854, but their toxicity was not established until 1938 when a group of German chemists produced tetra ethyl pyrophosphate as a by-product of nerve poisons or chemical warfare agents during the World War II (Minton and Murray, 1988). They were later developed into less potent chemicals (Sullivan and Blose, 1992; Echobichon, 1996). They are chemical compounds in which a phosphate group, or phosphate derivative, is part of an organic (carbon-containing) molecule (Wood, 1999). They are normally esters, amides, or thiols derivatives of phosphoric, phosphonic, phosphorothioic, or phosphonothioic acids (WHO, 1986). In practice, OPs are those organophosphorous compounds which inhibit the enzyme acetylcholinesterase (WHO, 1986). About 50,000 OP compounds are known to exist (Koller and Klawans, 1999) with about 100 reviewed by WHO (WHO, 1986)

Organophosphates have gained popularity worldwide in preference to organochlorines, whose use was banned because of their persistence and greater damage to the environment. The OPs break down more rapidly than do organochlorines in the tissues and the environment (Schenker *et al.*, 1992; Sullivan and Blose, 1992; Jirachayabhas *et al.*, 2004). They are used to destroy insects such as fleas, lice, flies and mosquitoes (Schenker *et al.*, 1992), control pests on crops in agriculture and livestock, and for other commercial and domestic purposes (Schenker *et al.*, 1992; Sullivan and Blose, 1992). OPs are also used as industrial fluids, flame retardans, polymer additives, radiodiagnostic agents, metal extraction agents, corrosion

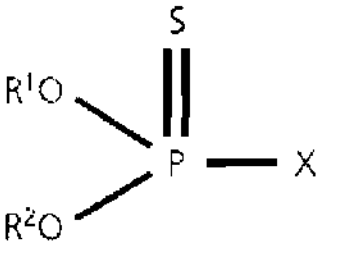
inhibitors, emulsifiers, antistatic agents, complexing agents, phase transfer and regular catalysts, ligand modifiers of catalysts and as nerve gases (Edmundsson, 1988; Abou-Donia, 2003). They have been recommended for a variety of disorders, but their acceptability has only been established for few conditions. For example, ecothiophate is used for the treatment of glaucoma either alone or in combination with other drugs (Taylor, 1990), while trichlorfon has been successfully used for the treatment of schistosomosis (Nordgren *et al.*, 1980). Despite their uses, OP compounds are not ideal pesticides because of their lack of target vector selectivity, and severe toxicity leading to death in humans and domestic animals (Balali-Mood and Balali-Mood, 2008).

2.1.1 Chemical structure

In general, organophosphorous compounds consist of a phosphate atom attached to three carbon groups and double bonded to an oxygen or sulfur atoms. Generally, OPs are classified into two main groups based on the general formula: (i) Phosphates and (ii) phosphorothioates, represented by the following general formulae (Figure 2.1).



(i) Phosphate compound



(ii) Phosphorothioate compound

Figure 2.1 General classifications of organophosphate compounds

The P=O containing structure is sometimes referred to as oxon and the P=S structure as a thion. The R^1 and R^2 moieties are usually alkyl or aryl groups, which may be bonded directly

or through oxygen or sulphur atoms (when bonded via sulphur, the compound is called a phosphorothioate. In phosphoroamidates, a carbon atom is linked to the phosphorous atom through an NH group. X represents one of a wide range of substituted or branched aliphatic, aromatic or heterocyclic groups linked to the phosphorous atom through a labile bond. During the process of inhibition of the target enzyme, acetylcholinesterase (AChE), the phosphorus atom binds to an amino acid on the enzyme with X being eliminated; the group X is thus often referred to as the 'leaving group'. The lability of the linkage between X and the phosphorus atom is critical with regard to the reactivity of the OP with the enzyme AChE (WHO, 1986; Wood, 1999).

Organophosphates are very reactive chemicals, but vary in their biological activities and potency as acetylcholinesterase (AChE) inhibitors. This variation depends largely on the nature of R^1, R^2, and X. Thions, such as parathion and chlorpyrifos, show lower toxicity in mammals, and usually require metabolism to the corresponding oxygen-containing compound (oxon) in order to inhibit AChE (Wood, 1999).

2.1.2 Sources of exposure and poisoning incidence

In view of its widespread use and contact, poisoning by OP compounds is common both in humans and animals worldwide. OP exposure is a major public health issue in terms of health, morbidity, health care, and general safety from toxicity (Fenske *et al.*, 2002b). For example, Poison Centres in USA recorded more than 13,000 cases of OP poisoning in 1999, and there were more than 3,000 cases seen in emergency departments, with 83 fatalities (Riegel, 2002). According to the data from the Toxic Exposure Surveillance System of the American Association of Poison Control Centres, there were 10,073 cases of OP exposure reported in

adults in USA, with 9,609 of them being unintentional and the rest 276 intentional in 2000 (Litovitz *et al.*, 2001). OP exposure may either be from occupational or non-occupational (environmental sources) (Muldoon and Hodgson, 1992; Sullivan and Blose, 1992). Occupational exposure due to OP occurs among people who come in contact with the chemical as a result of their occupation (Schenker *et al.*, 1992; He, 2000). Exposure to OP pesticides occurs predominantly on farms (Schenker *et al.*, 1992; Sullivan and Blose, 1992) or in orchards (Engel *et al.*, 2001), where the insecticides are used for pest control. Pesticide related illnesses, especially those due to OP pesticides, are common among field workers on farms than among agricultural workers who handle pesticides (Das *et al.*, 2001), perhaps due to inadequate protection and lack of direct contact with the pesticide among the former group. However, the farmers and their families may also be exposed to pesticide poisoning through "paraoccupational" means (Hoppin *et al.*, 2006); that is, by living close to where pesticides are used or when workers bring home contaminated articles and thus expose their family members (Fenske *et al.*, 2002a; Jaga and Dharmanni, 2003). OPs settle on various objects and items in the home, thereby increasing the risk of exposure. They are present in house dust and on floors, carpets, toys, dinnerware, tables and other furniture (Lemus and Abdelghani, 2000; Fenske *al.*, 2002b). Exposure occurs by inhalation or dermal contact (Jaga and Dharmanni, 2003).

Industrial exposure to OPs also occurs in chemical plants that produce OP pesticides (He, 2000; Sanborn *et al.*, 2002; Fox and Castilon, 2001). However, the general population is exposed by the entry and spread of the chemicals into the environment (Schenker *et al.*, 1992; Echobichon, 1996) in what has been termed environmental or non-occupational exposure. Unintentional, accidental or suicidal poisoning may occur in occupational and non-

occupational exposures to OP (Goozner *et al.*, 2002; Langley and Sumner, 2002). Homicidal poisoning would include suicide poisoning as well as intentional poisoning of one individual by another (Langley and Sumner, 2002). Chemical warfare with OPs is a form of terrorism with homicidal intent targeted to affect a large population (Goozner *et al.*, 2002), as observed during the first gulf war in the 1990s.

2.1.3 Absorption and metabolism of organophosphate compounds

Organophosphates are rapidly absorbed following inhalation or ingestion (WHO, 1986; Crinnion, 2000). Dermal absorption of active ingredients in powdered or granulated form is slower, but enhanced absorption and toxicity have been reported for some OP formulations in organic solvents as compared with the pure compounds or in aqueous preparations (WHO, 1986).

Although the actual exposure of respiratory system via inhalation may not be as high as that through dermal exposure in unprotected individuals, the efficiency of absorption might be high (WHO, 1986). Most OPs used as insecticides are liquid at room temperature and have a low vapour pressure, therefore only small amount of the compounds are present in the air in gaseous form. The air-borne concentration depends on the volatility of the OP, and also the concentration and partial pressure of the formulation. Since OP pesticides have low volatility, inhalation of aerosols such as sprays and dusts is generally more important than that of vapours. Absorption of inhaled OPs deposited in the lungs is rapid and almost complete, and limited metabolism occurs before they gain access to the general circulation (Wood, 1999). Dermal absorption tends to be slow because of the mechanical barrier posed by the skin, but lipid soluble compounds may produce a depot in the skin from which there can be slow release

into the circulation (Wood, 1999). The extent of transdermal absorption depends on a number of factors such as chemical nature of the formulation, and those factors influencing skin permeability including temperature and degree of hydration of the skin (Wood, 1999). The gastrointestinal tract has a large surface area, hence rapid and complete absorption occur in the tract. OP may be metabolized in the gastrointestinal wall with greater amounts metabolized in the liver before reaching general circulation (Wood, 1999).

Generally, metabolism of OP compounds occurs principally by oxidation, hydrolysis by esterases and gluthatione transferase reaction (WHO, 1986). Oxidation of OP compounds may result in more or less toxic products. In general, phosphorothioates are not directly toxic but require oxidative metabolism to the proximal toxin (WHO, 1986). Metabolic oxidation of OPs may result in desulfuration (such as conversion of CPF to CPF oxon) (Figure 2.6); N dealkylation of substituted amide side chain; O-dealkylation such as replacement of R^1 and R^2 by hydrogen atom; dearylation, side chain oxidation of alkyl-, aryl-, and thioether substituents (WHO, 1986; Wood, 1999), demethylation and oxidative deethylation (Mosha, 1993; Ecobichon, 2001).

Hydrolysis resulting in the removal of the more labile leaving group is the most common pathway for the metabolism of OP compounds (Wood, 1999). Since there is difference in the structure of the leaving group among the OP insecticides, the rate of hydrolysis differs from compound to compound, despite similarity in some of the urinary metabolites. The enzymes involved in the hydrolysis and inactivation of OPs are present in many mammalian tissues, but predominantly in the liver, intestine and plasma (Wood, 1999). The principal enzymes are the microsomal enzymes and the A-esterases (Wood, 1999), which are group of enzymes capable

of hydrolysing a broad range of substrates, including OPs (WHO, 1986). Most mammals have more efficient hydrolytic enzymes than insects, therefore they are often more efficient in their detoxification processes (WHO, 1986). Birds usually have lower esterase activity due to their less abundance, but may be present in some insects (Brealey *et al.*, 1980; WHO, 1986). The A-esterase enzymes include paraoxonase whose activity has been shown to have wide differences within the Caucasians (Mutch *et al.*, 1992) and in other ethnic groups (Player *et al.*, 1976). This same inter-individual variability has also been observed in some other A-esterases such as serum cholinesterase and lymphocyte neuropathy target esterase (Wood, 1999), but not erythrocyte AChE activity (Mutch *et al.*, 1992). This inter-individual variability in hydrolytic inactivation may be a major source of differences in the response of individuals to OP intoxication (Wood, 1999), and to variability in oxidative metabolism (Mutch *et al.*, 1992). Hydrolysis of carboxylic acid ester bonds and carboxyl-amide bonds in OP insecticides may be catalysed by carboxylesterases (or B-esterases), which again occurs widely in mammalian tissues. Malathion, which contains two carboxylic ester bonds, is the best known OP pesticide hydrolysed in this way. This pathway is an important detoxification pathway as shown by the reduction of the oral LD_{50} of malathion in rats from 10,000 mg/kg to 100 mg/kg following carboxyesterase inhibition (WHO, 1986)

The glutathione transferase reaction which involves transfer of the OP molecule to glutathione produces products that are in most cases of low toxicity. Hydrolytic and transferase reactions affect the thioates and their oxons (WHO, 1986). There is no evidence of prolonged storage of OP compounds in the body, but the process of elimination can be subdivided roughly according to the speed of the reactions involved. Numerous conjugation reactions follow the primary metabolic products, and elimination of the phosphorous-containing residue may be

via the urine, with lesser amount in the faeces and expired air (WHO, 1986). The rate of excretion usually reaches a peak within 2 days and decline quite rapidly. The levels of OP in the body do not almost immediately fall to zero due to storage in fat and covalent binding by tissue esterases other than AChE, which for some OPs is considered as another form of detoxification pathway depending on the number of binding sites. Some OPs bound to proteins primarily, therefore their turnover appears to be related to the half-life of these proteins (WHO, 1986).

2.1.4 Mechanisms of toxicity of organophosphates

It has been established that most OPs are highly lipid soluble agents, and are well absorbed from the skin, oral mucous membranes, conjunctiva, gastro-intestinal and respiratory routes (Ecobichon, 1992; Sullivan and Blose, 1992; Marrs, 2001; Kamanyire and Karalliedde, 2004). The onset, severity and duration of poisoning are determined by the dose, route and frequency of exposure, physico-chemical properties of OPs (such as lipid solubility), rate of metabolism, whether transformation in the liver is required before the compound becomes toxic, whether the organophosphorylated cholinesterase ages rapidly, and combined exposure to other chemicals (Abou-Donia, 2003).

Exposure to OP results in inhibition of the cholinesterase enzymes, which forms the basis of neurotransmission (WHO, 1986). In view of the similarity of the physiology and chemistry of neurotransmission across the animal kingdom, except fish (Bear *et al.*, 1986), toxic effects are observed albeit at varying doses in target species such as insects and non-target species such as mammals and other animals.

Different types of cholinesterase enzymes have been described in mammals, and they differ in tissue locations, substrate affinity and physiological function (Jeyaratnam and Maroni, 1994). The principal cholinesterase enzymes are AChE found in nervous tissues and red blood cells (RBC), and plasma cholinesterase (butyrylcholinesterase or pseudocholinesterase), a group of enzymes seen in the glial cells, plasma and liver (Jeyaratnam and Maroni, 1994). Most of the effects observed in acute OP exposure are due to inhibition of AChE, though butyrylcholinesterase is also inhibited without apparent functional deficits manifested (Jeyaratnam and Maroni, 1994). In mammals, the OP affects the function of the peripheral and central nervous system (CNS) by inhibiting the activity of AChE (Civen *et al*., 1977; Briggs and Simons, 1986), the enzyme which under physiological conditions hydrolyses the neurotransmitter acetylcholine (ACh). ACh is the chemical transmitter of somatic motor neurones to skeletal muscle, post-ganglionic parasympathetic nerve fibres, pre-ganglionic fibres of both sympathetic and parasympathetic nerves, adrenal medulla and certain regions of the CNS (Mosha, 1993; Chambers and Oppenheimer, 2004; Miranda *et al*., 2004). ACh causes excitation in some tissues such as those of the smooth muscle of the gastro-intestinal tract, but causes inhibitory responses in other tissues such as the myocardium (Adams, 1988). The excitatory effects have been ascribed to depolarization of the synaptic membrane, characterized by altered permeability to Na^+ and K^+ ions. On the contrary, the inhibitory effects are due to hyperpolarization resulting from selective increase in membrane permeability to K^+ but not Na^+ (Adams, 1988). ACh synthesized by specialized organelles within the presynaptic axon (Miranda *et al*., 2004) is able to combine with esterophilic and anionic sites of both muscarinic and nicotinic receptors due to its molecular structure (Adams, 1988).

Acetylcholinesterase synthesized within the post-synaptic membranes regulates the ACh concentration within the synapse by hydrolyzing ACh to acetic acid and choline (Haddad, 1983). ACh binds to two sites on the AChE with the quantenary nitrogen of ACh anionic site and the carbonyl of the ester binding at the serine residue in the esteratic site, with subsequent loss of choline followed by rapid hydrolysis of the acetate from the serine residue (Mosha, 1993) (Figure 2.2).

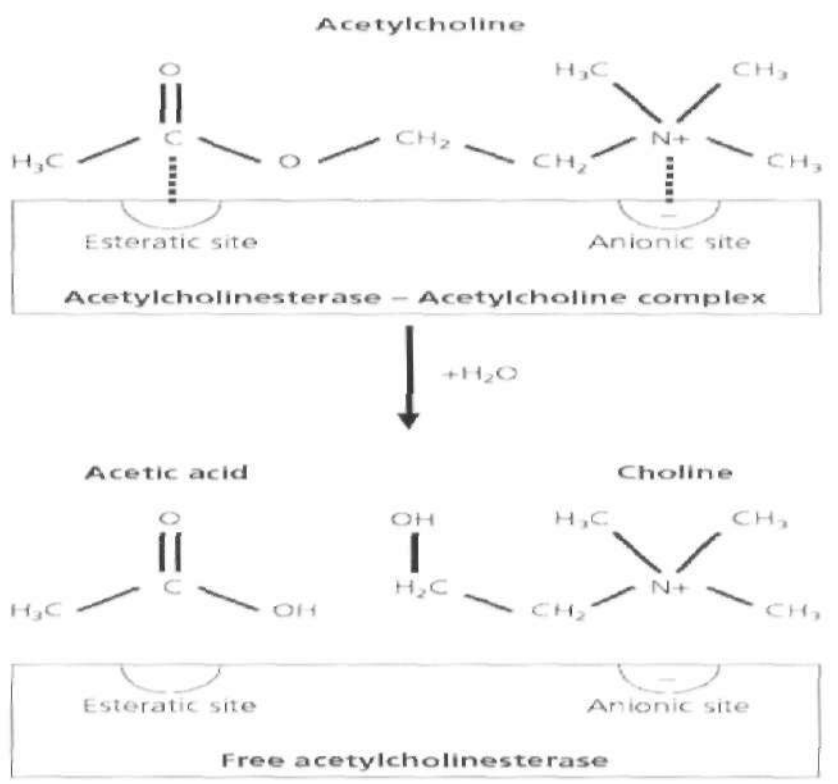
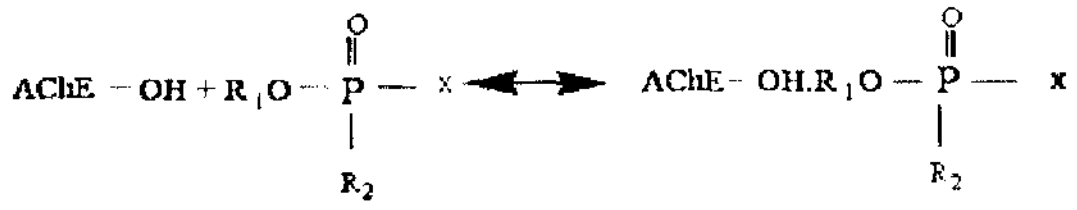


Figure 2.2: Schematic representation of acetylcholine hydrolysis by acetylcholinesterase

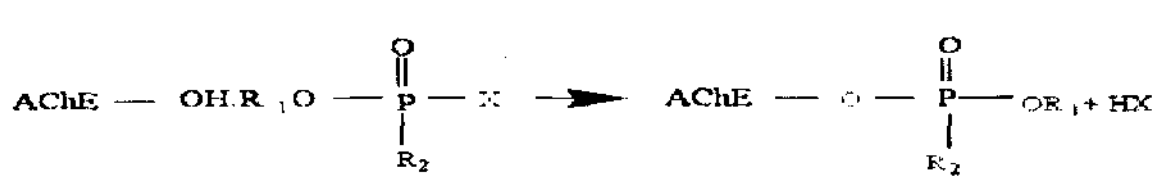
The OP insecticides bind to the active esteratic site (the serine hydroxyl group) of the AChE (Haddad, 1983; Stevens and Breckenridge, 2001), phosphorylating it and thereby leading to inhibition of its normal action (Figure 2.3). This binding is considered irreversible after a period of time due to ageing of the phosphorylated enzyme. Ageing of the inhibited AChE

involves the loss of an alkyl substituent from the phosphorylated enzyme, so that a methoxy (CH_3O^-) or ethoxy ($C_2H_5O^-$) group joined to the phosphorus is converted to an oxygen anion (O^-), which then stabilises the product and prevents reactivation of the enzyme (Wood, 1999). The steps involved in organophosphate interaction with AChE and its subsequent ageing is shown in Figure 2.3.

Step 1- Formation of a reversible enzyme-inhibitor complex.



Step 2- Phosphorylation and inactivation of the enzyme molecule



Step 3- Ageing reaction involving formation of a monophosphoric acid residue bound to the enzyme

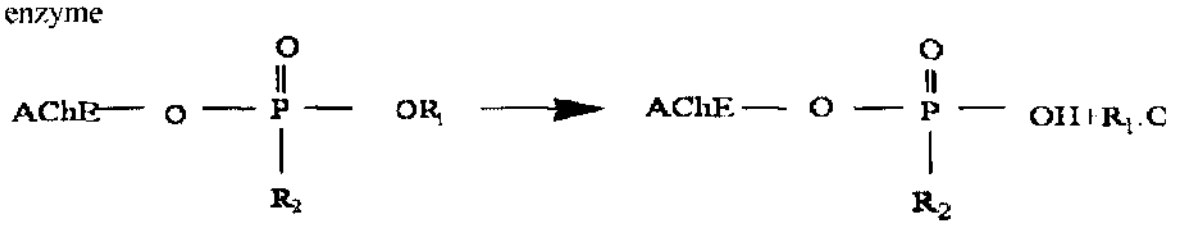


Figure 2.3: Steps involved in orgagnophosphate interaction with acetylcholinesterase and subsequent ageing (Adopted from Balali-Mood and Balali-Mood, 2008)

Phosphorylated enzymes are inactive and unable to hydrolyze ACh at the synaptic and myoneural junctions (Haddad, 1983). Therefore, in the presence of this AChE inhibitor, synaptic ACh increases to abnormally high level, which may precipitate cholinergic crisis that can be debilitating and fatal (Ecobichon, 1991; Padilla *et al.*, 1994). The continued

accumulation of ACh at the neuronal junctions results in the continued stimulation and then suppression of neurotransmission, thereby preventing the passage of new messages and eventually causing death (Ecobichon, 1991; Cooper *et al.*, 1996). Plasma cholinesterase level takes 4-6 weeks to return to the base level, while erythrocyte AChE may take 5-7 weeks following its inhibition by OP (Aaron and Howland, 1998).

Differential sensitivity to AChE inhibition has been demonstrated in OP toxicity as observed in adult rats and young pups (Liu *et al.*, 1999; Beyrouty *et al.*, 2001). This may be related to down-regulation of the muscarinic receptors (Liu *et al.*, 1999; Zheng *et al.*, 2000), and toxicokinetic differences, leading to metabolic and detoxification processes (Ginsberg *et al.*, 2002). The accumulation of ACh at the motor nerves results in weakness, fatigue, muscle cramps, fasciculations, muscular weakness and paralysis, which when it affects respiratory muscles may result in respiratory failure and subsequent death (Ecobichon, 1994; Sherman, 1995; Singh and Sharma, 2000). The mechanism of muscular paralysis observed is due to depolarization and desensitization blocks induced by ACh at the neuromuscular junction (Singh and Sharma, 2000).

The accumulation of ACh at the autonomic ganglia results in increased heartbeat and blood pressure, palor and hypoglycaemia. Accumulation of ACh at muscarinic receptors results in visual disturbances, tightness in the chest and wheezing due to bronchoconstriction, increased bronchosecretion and increased salivation, lacrimation, sweating, increased peristalsis (resulting in nausea, vomiting, cramps and diarrhoea) and increased frequency of urination. The CNS effects due to ACh accumulation include anxiety, headache, confusion, convulsive

ataxia, depression of respiration and circulation, slurred speech, tremor and generalized weakness (Echobichon, 1994; Sherman, 1995).

Several reports from experimental studies have confirmed that exposure to OPs may produce effects such as changes in some behavioural measures (Ehrich *et al.*, 1993), a decrease in muscarinic receptor function (Tandon *et al.*, 1994) and elevated responsiveness to muscarinic cholinolytics (Pope *et al.*, 1992) detectable after restitution of AChE activity. It has been suggested that such a change represents the persistence of acute OP effects, that is, elevation in cholinergic activity (Duffy *et al.*, 1979).

Apart from inhibiting AChE and butyrylcholinesterase, OP insecticides also form covalent binding with secondary targets such as other serine esterases, including butyrylcholinesterase, carboxyl esterases and/or A-esterases, and other acylpeptide hydrolase, neurotoxic esterase, fatty acid amide hydrolase, arylformamidase, cannabinoid (CB1) receptor and muscarinic acetylcholine receptor, an action which detoxifies them (US EPA, 2002; Peeples *et al.*, 2005). A-esterases such as chlorpyrifos oxonase, paraoxonase or PON 1 detoxify some OP pesticides such as chlorpyrifos (CPF), parathion and diazinon by hydrolysis (Figure 2.4). In animals, there is evidence that serum paraoxonase is protective against poisoning by OPs. Animals with low PON1 levels were more sensitive to specific OP compounds than animals with high enzyme levels. For example, birds, which have very low to undetectable PON1 activity, are more sensitive than various mammals to the acute toxicity of oxons than for other OPs (paraoxon, diazinon-oxon, CPF-oxon and pirimiphos-oxon). Furthermore, rabbits, which have a sevenfold higher serum PON1 activity than rats, are more resistant to the acute toxicity of CPF (approximately 9 and 25 fold for acute oral and dermal toxicity, respectively). Rabbit

paraoxonase hydrolyzes CPF oxon with a much higher turnover number than does rat paraoxonase (Li *et al.*, 1993; Costa *et al.*, 1999).

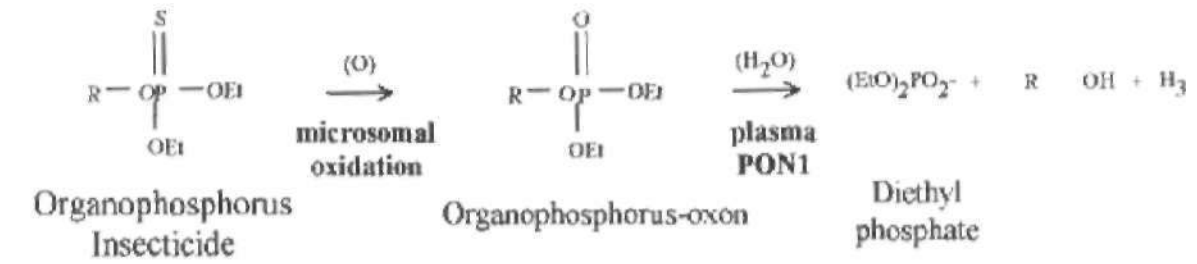


Figure 2.4 Chemical representation of the activity of A-esterase such as paraoxonase (PON I) on organophorous compounds

Carboxylesterases on the other hand binds to OP and, therefore, lessen their availability for inhibition of AChE. With the exception of neurotoxic esterase, whose inhibition is responsible for delayed neuropathy, the toxicological relevance of inhibition of these secondary targets is not yet understood (Ray and Richards, 2001; Casida and Quistad, 2004) as the search for the effect of inactivation or changes in other physiological system continues (Kamanyire and Karalliedde, 2004).

Young animals are more susceptible to the toxic effects of OPs as a result of lowered activity of detoxifying enzymes such as paraoxonase that deactivates active OP metabolites (such as paraoxon and CPF-oxon) coupled with the increased sensitivity of their developing organ systems to OPs (Benke and Murphy, 1975; National Academy of Sciences, 1993; Mortensen *et al.*, 1996; Atterberry *et al.*, 1997). According to the National Academy of Science, exposure to neurotoxic compounds at levels believed to be safe for adults could result in permanent loss of brain function, if it occurred during the perinatal and early childhood period of brain development (National Academy of Sciences, 1993).

Organophosphates (OPs) have been shown also to induce changes characteristic of oxidative stress (Malkovics, 1995; Vidyasagar *et al.*, 2004), which may contribute to the severity of symptoms observed in their poisoning. OPs have also been shown to exert the following effects, demonstrated in animal models, and are theoretically possible in man (Karalliedde, 1999; Kamanyire and Karalliedde, 2004). The effects include:

(a) Inactivation by phosphorylation of other β-esterases

(b) Alteration in the release of neurotransmitters, eg γ-aminobutyric acid (GABA) and glutamate.

(c) Increase in the number of GABA and dopamine receptors.

(d) Action as agonists at M_2/M_4 muscarinic receptors.

(e) Inhibition of mitochondrial enzymes, respiration and ATP generation.

(f) Induction of mast cell degranulation, probably causing the release of histamine or histamine-like compounds.

(g) Inhibition of nitric oxide.

(h) Interference with surfactant in the lungs.

(i) Inhibition of phopholipase A_2.

(j) Interference with humoural and cellular immunity, such as the function of T-lymphocytes.

2.1.5. Health effects of organophosphates

Acute exposure to OP compounds causes sequential triphasic illnesses, which are the initial acute cholinergic crisis, the intermediate syndrome and a delayed neuropathy referred to as organophosphate induced delayed polyneuropathy (OPIDP) (Karalliedde and Senanayake,

1989; Kamanyire and Karalliedde, 2004). The earliest cholinergic phase may only be observed. This cholinergic phase progresses to the intermediate syndrome in about 20% of the subjects (Kamanyire and Karalliedde, 2004). Both the acute cholinergic and the intermediate syndrome are associated with a high risk of mortality that may require intensive care management. The third phase (OPIDP), which sets in at about 7-12 days post-exposure does not carry the risk of death, and may not be preceded by either the cholinergic phase or the intermediate syndrome (Kamanyire and Karalliedde, 2004). In addition to these three syndromes, chronic exposure to low-doses OP compounds affect several physiologic systems in both man and animals such as the CNS, neuromuscular junction, cardiovascular system, metabolic and endocrine systems, including reproduction (Karalliedde, 1999; Singh and Sharma, 2000). The chronic effect of OP compounds on the nervous system, which has been differently termed by various authors, has now been named as organophosphate-induced chronic neurotoxicity (Abou-Donia, 2003).

2.1.5.1 Acute cholinergic syndrome

This results from inactivation of AChE by alkyl phosphorylation of aserine hydroxyl group at the esteratic site of this enzyme, leading to accumulation of ACh at the nerve endings and neuromuscular junction, and resulting in initial stimulation and eventual exhaustion of cholinergic synapses (Singh and Sharma, 2000; Karalliedde and Henry, 2001). The paralysis of cholinergic transmission arising from this affects the nicotinic receptors situated at the cholinergic junction in skeletal neuromuscular junctions, at muscarinic receptors in the autonomic ganglia and receptors in the CNS (Sullivan and Blose, 1992; Karalliedde and Henry, 2001; Kwong, 2002).

The effects of OP inhibition of AChE on muscarinic receptors result in muscarinic symptoms, which include increase in secretions (bronchorrhoea, salivation, lacrimation and sweating), bronchospasm (tightness in the chest and wheezing), bradycardia, vomiting,, increased gastrointestinal motility (abdominal tightness, cramps and diarrhoea) and increased frequency of urination (Singh and Sharma, 2000; Karalliedde and Henry, 2001). OPs also cause diagnostic miosis in the eye, which eventually results in blurring of vision (Singh and Sharma, 2000; Karalliedde and Henry, 2001; Kamanyire and Karalliedde, 2004). Hypertension and tachycardia may be observed depending on the balance between muscarinic and nicotinic effects (Singh and Sharma, 2000). Nicotinic signs resulting from activation of nicotinic receptors include muscle fasciculation and a flaccid paralysis in severe exposures and hypertension (Singh and Sharma, 2000; Karalliedde and Henry, 2001).

The clinical signs associated with the activation of cholinergic receptors within the CNS include emotional irritability, mental obtundation, headaches, insomnia, giddiness, confusion, drowsiness, and in severe exposures, slurred speech, convulsions, coma and respiratory depression (Singh and Sharma, 2000; Karalliedde and Henry, 2001; Kamanyire and Karalliedde, 2004). Death from acute OP exposure is associated with respiratory impairment resulting from massive respiratory tract secretions, bronchiolar constriction, intercostals and diaphragm muscle paralysis, and respiratory paralysis from CNS effects in the medulla (Blodgett, 2001).

Acute exposure to OP episode poisoning has resulted in long-term and potentially permanent neurological impairments, including memory loss, cognitive difficulties, mood disturbances, abnormality in a range of motor responses, and peripheral sensory neuropathies (Rosenstock *et*

al., 1991; Kaplan *et al.*, 1993) such as decreased vibration sensitivities and impaired nerve conduction (Kamel and Hoppin, 2004). Anoxia or hypoxia resulting from convulsion or respiratory impairments following moderate to severe acute toxicity may lead to death of nerve cells and irreversible neurological dysfunctions through hypoxic brain damage (Wood, 1999).

Acute dermal exposure in cats resulted in anorexia, diarrhoea, generalized weakness, miosis/mydriasis, muscle tremor, abnormal behaviour, cervical ventroflexion, depression but clonic-tonic convulsion are observed in severely affected animals and finally resulting in death (Fike, 1992; Levy *et al.*, 1993).

2.1.5.2 Intermediate syndrome

Intermediate syndrome (IS) is a neurotoxic syndrome that is observed 24-96 hours after exposure to certain compounds such as diazinon, dimethoate, methylparathion (Singh and Sharma, 2000). The term was first coined by Senanayake and Karalliedde (1987) because the syndrome arises between the period of early cholinergic syndrome and late onset peripheral neuropathy. However, the syndrome was first described by Wadia *et al.* (1974) as a type II paralysis. The syndrome which can last for up to 18 days (De Bleecker *et al.*, 1993) is characterized by muscular weakness in the ocular, neck, bulbar, proximal limbs, respiratory muscle, cranial nerve palsies, depressed tendon reflex and transient extrapyramidal symptoms (Senanayake and Karalliedde, 1987; Reigart and Roberts, 1999; Singh and Sharma, 2000; Guadarrama-Naveda *et al.*, 2001; Shahar and Andraws, 2001). Difficulty in breathing may progress to respiratory failure, following paralysis of the diaphragm and other muscles of respiration (Kamanyire and Karalliedde, 2004). The syndrome which is mainly observed in

man has recently been described in dogs (Hooper *et al.*, 2002). The common cranial nerves involved are those supplying the extraocular muscle, although cranial nerves VII and X are infrequently affected (Cherian *et al.*, 2005).

The exact pathogenesis of IS is unknown at present, but some authors have suggested various reasons. Some workers have attributed the syndrome to inadequate or delayed oxime therapy (Benson *et al.*, 1992). Pathogenesis has also been speculated to involve a combined pre- and post-synaptic dysfunction of neuromuscular transmission as a result of prolonged AChE inhibition due to slow release of OPs from deep tissues (De Bleeker *et al.*, 1993). This may impair the function and activity of nicotinic receptors at the neuromuscular junctions (Kamanyire and Karalliedde, 2004), causing functional paralysis (Besser *et al.*, 1989), followed by local necrotic damage at the motor end-plate (Dettbarn, 1984) and skeletal muscle (Bright *et al.*, 1991; Karalliedde and Henry, 1993).

The syndrome has also been attributed to desensitization of ACh receptors (Avasthi and Singh, 2000). Anterior cell or toxin-induced muscular instability is, apparently, involved in this syndrome (Cherian *et al.*, 2005). Dandapani *et al.* (2003) have shown that oxidative stress in OP poisoning resulting from severe and prolonged AChE inhibition might contribute to the development and severity of IS. Once established, the syndrome does not respond to treatment with atropine or oximes. Experimental animal studies indicate that the muscle necrosis observed following OP poisoning can be reduced or prevented by treatment with AChE reactivators (Wecker and Dettbarn, 1976) or ACh antagonists (Dettbarn, 1984). It has been further suggested that early administration of AChE reactivators reduce the severity of the IS

(Nutley and Cocker, 1993), but there are no reported clinical studies demonstrating this (Wood, 1999).

2.1.5.3 Organophosphate-induced delayed polyneuropathy

Historically, incidence of organophosphate-induced delayed polyneuropathy (OPIDP) have been documented for over a century with the earliest recorded case attributed to the use of tri-o-cresyl phosphate (TOCP)-containing creosote oil for treatment of pulmonary tuberculosis in France in 1899 (Smith *et al.*, 1930; Abou-Donia, 1981, Abou-Donia and Lapadula, 1990). TOCP was also discovered to be the cause of 50,000 cases of OPIDP in the southern and Mid Western region of the USA (Smith *et al.*, 1930; Abou-Donia, 1981; Abou-Donia and Lapadula, 1990). OPIDP is a neurodegenerative disorder characterized by a delayed onset of prolonged ataxia and upper motor neurone spasticity as a result of a single or repeated exposure to OP esters (Smith *et al.*, 1930, Johnson, 1975; Abou-Donia, 1981, Abou-Donia and Lapadula, 1990). It is a persistent symmetric neurological impairment that may ensue a few weeks after the recovery from acute poisoning (Ray and Richards, 2001; Salvi *et al.*, 2003; Miranda *et al.*, 2004). It is a sensory and motor neuropathy that results from axonal degeneration and secondary demyelination of long and large diameter axons in peripheral nerves, the spinal cord, anterior horn cells and the medulla oblongata (Ahmed and Glees, 1971; Lotti, 1992; Lotti *et al.*, 1986; Moretto and Lotti, 1993).

The distal portion of the lower limbs is primarily affected, but the upper limbs are also involved in most severe poisoning (Lotti, 1992). It is a dying-back axonopathy characterized by sensory and motor symptoms that develop days to weeks after poisoning by certain OPs (WHO, 1990). Although OPIDP has both motor and sensory components, clinical reports have

shown that motor impairment seems to be a more dominant feature (Miranda *et al.*, 2004). The neuropathological lesion is a central-peripheral distal axonopathy caused by a chemical transaction of the axon (known as Wallerian-type degeneration), followed by myelin degeneration of the distal portion of the long and large-diameter tracts of the CNS and peripheral nervous system (Cavanagh and Patangia, 1995). Schwannn cell proliferation and macrophage accumulation (Cavanagh, 1973; Krinke *et al.*, 1997). The mechanisms causing axonopathy involve alterations of the transport mechanisms in the axon membrane, loss of ionic balance across the membrane, abnormal water uptakes, axonal swelling and subsequent degeneration of the axon (Miranda *et al.*, 2004). The extent of delayed polyneuropathy is determined by the differences in the structural activity of different OP insecticides (Ecobichon, 1996).

Organophosphate-induced delayed polyneuropathy has been divided into three classes, Type I-III (Abou-Donia, 2003): Type I is caused by the pentavalent phosphates and phosphonates as well as their sulphur analogues (Abou-Donia, 2003). Type II is produced by trivalent phosphates (Abou-Donia, 1992), while Type III is induced by phosphines (Abou-Donia *et al.*, 1996; Abdel-Rahaman *et al.*, 1997). All the three OPIDP types are produced by OP compounds (Abou-Donia, 2003). Type II differs from type I in terms of susceptibility of rodents and the presence of neuropathological lesions in neuronal cell bodies (Abou-Donia, 1992).

Clinical manifestations include parethesis in the legs and arms, leg cramping and calf pain, and weakness which appears initially in the distal leg muscles causing foot drops, followed by small muscles of the hands. This may later progress to involve truncal muscles. Gait is

disproportionate and in severe cases there is paralysis of limb muscles (Keifer and Mahurin, 1997; Singh and Sharma, 2000). The prognosis of patients with mild neuropathy is usually good, but persistent deficits such as weakness, claw hand, foot drop, loss of reflexes, persistent atrophy, sensory impairments, spasticity and ataxia are usually observed in severe neuropathy (Davies, 1990; Singh and Sharma, 2000). A non-classical form of OPIDP has been reported in young mice (Lush *et al.*, 1998).

2.1.5.3.1 Mechanisms of organophosphate-induced delayed polyneuropathy

The proposed mechanism for the pathogenesis of OPIDP is independent of AChE inhibition, but it involves phosphorylation and subsequent ageing of the nervous system enzyme, neuropathy target esterase (NTE) (Johnson, 1969; Du toit *et al.*, 1981; Lotti *et al.*, 1986; Lotti and Moretto, 1999). Neurotoxic OPs bind irreversibly by means of phosphorylation to the neuropathy target esterase (NTE). This process starts with hydrolysis of an ester or amide bond, leaving ionized acidic groups on the phosphorous atom (Stuart and Oehme, 1982; Meerdink 1989). It is time-dependent (ageing), which explains the prolonged time interval between exposure and development of toxicity (Stuart and Oehme, 1982; Meerdink, 1989). Studies have shown that OPIDP is initiated when the OP has inhibited NTE activity by more than 70%, generally within 48 hours after the poisoning episode (Stuart and Oehme, 1982; Lotti, 1992). It has been suggested that "ageing" process in NTE promoted by certain neuropathic OP is essential for the development of OPIDP (Lotti, 1992). Ageing involves the loss of an alkyl group from the phosphoryl residue attached to NTE leaving a negatively charged phosphorylated NTE (Miranda *et al.*, 2004). It has been shown that the development of OPIDP may occur without ageing (Lotti *et al.*, 1986). However, some other studies showed that ageing is essential for OPIDP to occur. This is because as OP binds covalently to NTE,

and thus precludes the possibility of ageing, which does not result in OPIDP. Besides, their binding to NTE prevents certain OPs from producing OPIDP (Wood, 1999). Although the exact function of NTE is not fully known, it has been suggested to be important for interactions in the developing nervous system (Glynn, 1999; Moser *et al.*, 2000).

However, the role of NTE in the mechanism of pathogenesis of OPIDP has been trailed with some controversy. This is based on the fact that Type III OPIDP is not accompanied by inhibition of NTE (Abou-Donia *et al.*, 1996; Abdel-Rahaman *et al.*, 1997), and most importantly it has been found that NTE knock-out mice are sensitive to the development of OPIDP (Bus *et al.*, 2003; O'Callahan, 2003; Winrow *et al.*, 2003), indicating that this enzyme is not involved in the mechanisms of OPIDP (Abou-Donia, 2003). Therefore, an alternative mechanism has been proposed to explain the delayed neurotoxicty observed with some OP compounds. Some OP compounds have been shown to cause accumulation of tubulo-vesicular profiles within axons prior to degeneration (Abou-Donia and Lapadula. 1990), a pathology that is consistent with stagnation of membrane traffic (Chretien *et al.*, 1981; Souyri *et al.*, 1981). Therefore, the mechanism of OPIDP has been related to the inhibition of axon transport (Terry *et al.*, 2003). It has been suggested that the development of OPIDP involves protein kinase-mediated phosphorylation of cytoskeletal proteins (Abou-Donia, 2003). The increased aberrant protein kinase-mediated phosphorylation of cytoskeletal proteins could result in the destabilization of microtubules and neurofilaments, leading to their aggregation and deregulation in the axon (Abou-Donia, 1995).

2.1.5.4 Organophosphorous ester-induced chronic neurotoxicity

This is a chronic neurotoxicity that persists for years after exposure to a single large dose or small subclinical doses of OP (Jamal, 1997). It is a nervous system disorder induced by OP compounds which involves neuronal degeneration and subsequent neurological, neurobehavioural and neuropsychological consequences (Abou-Donia, 2003). This disorder has been variously named by different authors as "chronic organophosphate-induced neuropsychiatric disorder" (Jamal, 1997); "psychiatric sequelae of chronic exposure" (Gershon and Shaw, 1961); "CNS effect of chronic exposure" (Dille and Smith, 1964); "chronic neurological sequelae" (Callender *et al.*, 1994; Steenland *et al.*, 1994) among others. All these terms have now been harmonized and named organophosphate ester-induced chronic neurotoxicity (OPICN) (Abou-Donia, 2003). Clinical signs which continue from weeks to years after OP exposure consists of neurological and neurobehavioural abnormalities (Abou-Donia, 2003). Damage is observed in both the central and the peripheral nervous systems. In the brain, neuronal cell death resulting from early necrosis or delayed apoptosis is observed in various regions, including the cortex, hippocampal formation and cerebellum (Abou-Donia, 2003).

Organophosphorous ester-induced chronic neurotoxicity has been divided into two based on the extent and duration of exposure to OP. The first class is observed following exposure to large toxic dose of OP, which involves patients who have experienced severe acute poisoning and have eventually recovered (Abou-Donia, 2003). Clinical signs observed in such individuals include decreased verbal attention, visual memory, motoricity and affectivity (Maroni *et al.*, 1986), increased incidence of depression, irritability, confusion and social isolation (Savage *et al.*, 1988) and other forms of deficits in neurobehavioural,

neuropsychological and psychomotor functions (Rosenstock *et al.*, 1991; McConnel *et al.*, 1994; Yokoyama *et al.*, 1998a and b). Some of these symptoms have been attributed to sequelae of convulsions, anoxia, respiratory failure and cardiac arrhythmias that the patients might have suffered during the initial acute cholinergic syndrome (Singh and Sharma, 2000).

The other type of OPICN occurs following sub-clinical exposure to low-doses of OP, without any previous acute poisoning episode. Although health effects of this type of OPICN have been largely inconsistent, many studies have demonstrated anxiety, depression, chronic fatigue, autonomic dysfunction, and extrapyramidal symptoms such as dystonia, resting tremor, bradykynesia, postural instability and rigidity of face muscles; non-responsiveness to levodopa treatment; impairments in neurobehavioural and neuropsychological performance, persistent long-term cognitive dysfunction and defects in concentration and memory (Levin *et al.*, 1976; Kaplan *et al.*, 1993; Stokes *et al.*, 1995; Beach *et al.*, 1996; London *et al.*, 1997; Salvi *et al.*, 2003). Signs associated with peripheral nervous system involvement include lethargy, tremor, peripheral neuropathy, slowing of reaction times, hearing reaction times, and reduced grip and hand strength (Tabershaw and Cooper, 1996; Kilburn, 1999; Miranda *et al.*, 2004). In monkeys, an increase in electrocorticogram was observed up to one year after a single symptomatic exposure or a series of subclinical exposures to OP sarin (Burchfield *et al.*, 1976), which might be attributed to persistence of the acute OPs resulting in elevation of cholinergic activity (Duffy *et al.*, 1979).

2.1.5.4.1 Mechanisms of organophosphorous ester-induced chronic neurotoxicity

Large toxic doses of OP have been shown to cause early convulsive seizures and subsequent encephalopathy, leading to necrotic death of brain neuronal cells, whereas small doses produce

delayed apoptotic death (Abou-Donia, 2003). The large toxic doses of OP, which induce early seizures activate the glutamatergic system and involve Ca^{2+}-related excitotoxic process, possibly mediated by N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Solberg and Belkin, 1997; Raveh *et al.*, 1999). De Groot *et al.* (2001) hypothesized that accumulated acetylcholine, resulting from acute inhibition of AChE by OP compounds leads to activation of glutamatergic neurones and the release of the excitatory L-glutamate amino acid neurotransmitter. This in turn produces increased depolarization and subsequent activation of the NMDA subtype of glutamate receptors and the opening of NMDA ion channels resulting in massive Ca^{2+} fluxes into the post-synaptic cell thereby causing neuronal degeneration. Thus, glutamate-induced neuronal degeneration during seizures may occur as a result of lowering of the threshold for glutamate excitation at NMDA receptor sites. Activation of nitric oxide synthase, following stimulation of NMDA receptor sites, increases the level of nitric oxide, which functions as a signaling or cytotoxic molecule responsible for neuronal cell death (Dawson *et al.*, 1992). As a retrograde messenger, nitric oxide induces the release of several neurotransmitters, including excitatory amino acid, L-glutamate (Montague *et al.*, 1994), which alters neurotransmitter balance and affect neuronal excitability. The production of nitric oxide is enhanced in AChE-inhibitor–induced seizures (Bagetta *et al.*, 1993; Kim *et al.*, 1997). Infact, the involvement of nitric oxide in organophosphate-induced seizures and the effectiveness of nitric oxide synthesis inhibitors in preventing such seizures has been demonstrated (Kim *et al.*, 1997).

The small subclinical doses of OPs cause delayed neuronal death due to FR generation. OPs cause mitochondrial damage or dysfunction, thereby resulting in ATP depletion and increased generation of reactive oxygen species (ROS) leading to oxidative stress (Tsujimoto, 1997;

Murphy *et al*., 1999). Increased ROS production induced by OP causes fatal depletion of mitochondrial energy (ATP), induction of proteolytic enzymes and DNA fragmentation leading to apoptotic death (Tsujimoto, 1997; Floyd, 1999; Gupta *et al*., 2001).

Increased expression of AChE gene observed in OP poisoning has been suggested to play a role in the pathogenesis of OPICN, similar to that reported for Alzheimer disease (Calderon *et al*., 1998). AChE has been shown to be toxic both *in vivo* and *in vitro* (Yang *et al*., 2002), and brain AChE has been shown to be toxic to neuronal (Neuro 2a) and glial-like (B12) cells (Calderon *et al*., 1998). Therefore, the aggregation of AChE further initiates apoptotic neuronal cell death (Abou-Donia, 2003). In addition, chronic OP poisoning has been found to result in gradual loss of brain stem cholinergic muscarinic and nicotinic (Dawson and Jarrot, 1981; Schwab *et al*., 1981; Costa and Murphy, 1983; McDonald *et al*., 1988) and serotonergic (Prioux-Guyonneau *et al*., 1982; Fernando *et al*., 1984) receptors. Neurotoxic OP compounds increase the permeability of the brain-blood barriers (Dambska *et al*., 1984), which also contribute to neurological syndromes observed in OPICN. There is also evidence that in the brain, low-level doses of organophosphate pesticides that do not inhibit AChE may alter cholinergic neurotransmission via direct effects on muscarinic and nicotinic receptor function (Abdallah *et al*., 1992; Katz and Marquis, 1992; Bomser and Casida, 2001; Jones *et al*., 2003). It has been postulated that disruption of kinesin-dependent intracellular transport could account for some of the long-term effects of organophosphates on the CNS and the peripheral nervous system (Gearharta *et al*., 2007). The occurrence and severity of OPICN is influenced by factors such as environmental exposure to other chemicals or individual genetic differences (Abou-Donia, 2003). Stress as observed in oxidative stress which decrease the threshold level required to produce neuronal damage also exacerbates OPICN (Abou-Donia, 2003).

2.1.5.5. Organophosphate-Induced myopathy

This is a well-characterized syndrome in experimental animals, although relatively little is said about it in human subjects (de Bleecker *et al.*, 1992, 1994). The syndrome appears to result from overstimulation of the muscles by excess ACh, resulting from inhibition induced by OPs. The prolonged weakness which is observed beyond acute OP intoxication has been attributed to damage to voluntary muscles resulting from over-exposure to OPs (Whorton and Ohrinsky, 1983). A connection has been made between intermediate syndrome and OP-induced myopathy (Balali-Mood and Balali-Mood, 2008). Myopathy has been observed in study with tabun, soman and sarin in experimental animals (Furlong *et al.*, 2000). Oxidative stress and increased lipid peroxidation have been implicated as the main mechanism in OP-induced myopathy (Yang and Dettbarn, 1998).

2.2 Chlorpyrifos

Chlorpyrifos (0, 0-diethyl 0-3,5,6-trichloro-2-pyridylphosphorothioate) is a moderately toxic broad spectrum chlorinated OP that is widely used for pest control, including arthropod pests in agriculture and turf, throughout the world (Giesy *et al.*, 1999; Smegal, 2002; Taylor and Di Marco, 2003). It was first produced by Dow Chemical Company, Midland, Michigan, USA in 1962, and was introduced into the American market in 1965. Chlorpyrifos is used to control many types of insect pests in a wide range of crops and ornamentals. It is also used to control mosquitoes, flies, and household pests, including termites (Richardson, 1995, Sultato *et al.*, 1982). The compound is applied in emulsifiable concentrates, dust, flowable pellet, spray and granular wettable powder (US National Library of Medicine, 1999). It is applied to the soil surrounding or beneath buildings as protection against termites (Barron and Woodburn, 1995) and seventy percent of the urban CPF is estimated to be used for termite control (Smegal,

2002). In the past, it has been used via direct application to water bodies as a larvicide and pest control on cattle and sheep through spray, dip or pour-on applications (Racke, 1993).

2.2.1 Incidence of poisoning and sources of exposure

Chlorpyrifos was the most widely used insecticides in the USA, both in crop production and non-agricultural applications (Aspelin, 1994). Its non-agricultural use in the USA was estimated at between 4.1 and 5.4 million kilograms (9 and 12 million pounds) per year (Aspelin, 1994). However, estimates for agricultural use vary. The United States Environmental Protection Agency (US EPA) estimated that between 4.5 and 15 million kg (10 and 15 millions pounds) are used annually (Aspelin, 1994), while the US Department of Agriculture (USDA) estimated agricultural uses at 9.5 million kilograms (21 million pounds) per year (USDA, 1994). In American homes, lawns and gardens over two hundred million CPF applications are made annually and over 17% of US households use CPF (Cox, 1994). About 800 registered products on the market contain CPF and these products are used for a number of purposes, including pest control on a variety of crops, turfs and ornamental plants, green houses, and sod; indoor pest control, structural pest control and pet collars (Smegal, 2002). These alarming figures were true until year 2000, when US EPA revised the risk assessment of this and other OP pesticides and phased out or eliminated some of their residential uses (US EPA, 2002). Despite this restriction, CPF remains one of the most widely used insecticides. Data from population-based National Health and Nutrition Examination Survey III showed that 3,5,6-trichloro-2-pyridinol, the primary urinary metabolite of CPF was detected in most of the subjects examined (Centre for Disease Control and Prevention, 2002). For example, the metabolite of CPF was detected in 93% of the 102 children in the Minnesota children exposure study in 2000 (Adgate *et al.*, 2001).

In the homes, following residential application, CPF can cling to toys and other adsorbent surfaces such as window and upholstered furniture creating a reservoir of contamination (Gurunathan *et al.*, 1998). Interior use of CPF can also result in lingering indoor air contamination, and CPF may be detected years after application (Davis and Ahmed, 1998). It is one of the pesticides most frequently found to contribute to unacceptable levels of OP exposure to children from certain foods (Wiles, 1998). Persistent outdoor application can allow for concentration in household dust level of CPF and other OP to be higher in farm workers homes, especially those close to where the application is being used (Simcox *et al.*, 1995). This pervasiveness combined with its neurotoxic properties has led to alarming cases of poisoning that finally resulted in the restriction of some its domestic and residential uses.

Chlorpyrifos also causes adverse effects on the health of animals. The domestic animals most sensitive to CPF exposure appear to be cats, newborn calves, adult bulls, and newborn pigs. Cats have been poisoned or killed following treatments of their owners' homes with CPF (Jaggy and Oliver, 1990). Newborn calves are about 30 times more sensitive to CPF than older calves (Palmer *et al.*, 1980). Bulls have been killed by CPF exposure and found to exhibit poisoning symptoms and a decline in sperm production (Everett, 1982. Lein *et al.*, 1982, Haas *et al.*, 1983; Putnam and Edwards, 1984; Scaratt and Blodget, 1986). Pigs have been poisoned and killed after a chlorpyrifos-containing aerosol spray was used to treat wounds formed where the umbilical cord and the tail were removed (Long *et al.*, 1986, Scheidt *et al.*, 1987). Sources of exposure to man and animals may also be from meat and dairy products. For example, residues were found in mutton following a pour-on treatment to kill flies (Ivey and Palmer, 1981), in beef following dipping to kill ticks (Ivey *et al.*, 1972), and in pork following

a pour-on treatment to kill lice (Ivey and Palmer, 1979). In all the cases fat appeared to have higher CPF concentration than other tissues. It was also found in milk and cream obtained from cows experimentally fed CPF (McKellar *et al.*, 1976). Under certain conditions, CPF residues on food can be very persistent. Treatment of rice storage sacks with CPF resulted in the rice grains absorbing the CPF; residues were measured for six months following treatment (Tejada *et al.*, 1990). CPF residues have been detected in eggs (Narahari and Amutha, 2007). Another source of exposure to CPF is through workplace exposures. Over 1,300 occupational exposures to CPF were reported to poison control centres between 1985 and 1992 (US EPA Office of Prevention, Pesticides and Toxic Substances, 1994).

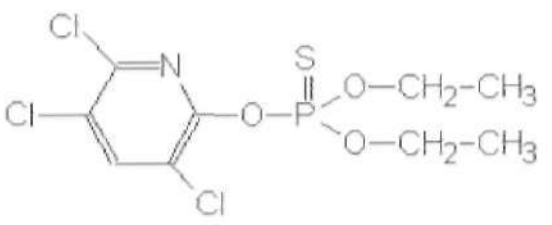


Figure 2.5 Chemical structure of chlorpyrifos

2.2.2 Toxicokinetics and biotransformation of chlorpyrifos

Chlorpyrifos is known to be absorbed through the respiratory tract in humans and animals (ATSDR, 1997). About 72% of the inhaled vapour is absorbed. Based on the LD_{50} inhalation of formulated CPF is more toxic to mice and rats than is oral ingestion (Berteau and Deen, 1978). CPF is readily absorbed from the GIT. Based on the percent of administered dose excreted in the urine, absorption of CPF in humans was about 70% in a single dose oral study (Nolan *et al.*, 1984) and in rats ranged from 84-90% in single gavage studies (US EPA, 2000b). The half-life of absorption from the GIT into the body is 78 minutes in rats (Ahdaya

and Guthrie, 1982). In humans, about 1.3% of the CPF applied through the dermal route is absorbed within 48 hours (Nolan *et al.*, 1984). However, dermal absorption in animals is much higher, but results were confounded by dermal irritation and even blistering, which compromised the integrity of the skin (EXTOXNET, 1993). Therefore, dermal uptake varies considerably within and between species. Dermal absorption may be as low as 1% for man, 60% for rats and 90% for target (and non-target) insect pests (Racke, 1993; DOW Agroscience LLC, 2001). Greater permeability of CPF through the skin of younger animals has been demonstrated in piglets up to 36 hours (Long *et al.*, 1986) and in 33 day-old rats in comparison with 82-day-old (Shah *et al.*, 1981).

Studies in rats, rabbits, chickens, goats, pigs, cows and humans showed that CPF which entered the general circulation is rapidly distributed throughout the body (NRA, 2000). Its metabolism is fast and extensive. The main feature of CPF metabolism has been discussed by various authors (Ma and Chambers, 1994; ATSDR, 1997; Atterberry *et al.*, 1997). Once CPF enters the general circulation, it undergoes the following metabolic processes:

(a) It is activated to CPF oxon by the action of mixed function oxidases cytochrome P450-dependent monooxygenases in target tissue. This involves oxidative desulphuration of the P=S moiety to P=O, in humans cytochrome P450 isozymes that activate CPF are CYP1A2 and CYP2B6 at low CPF concentration (environmentally relevant) (Burrati *et al.*, 2003), and CYP3A4 and also CYP3B6 at higher concentration (Tang *et al.*, 2001; Burratti *et al.*, 2003). Metabolic activation occurs predominantly in the liver and plasma (Richardson, 1995). Oxidative desulphuration is believed to proceed via an electrophilic phosphooxathiran intermediate (Kamataki and Neil, 1976) (Figure 2.6). Another CPF oxon-binding protein, a serine hydrolase enzyme has been identified in the brain of mice and has been described as the

principal enzyme for metabolizing low level of CPF in the brain (Nomura *et al.*, 2005). Large doses of CPF may result in unmetabolized CPF in body fluids (Drevenkar *et al.*, 1993).

(b) Detoxification of CPF by cytochrome P450 dearylation resulting in 3,5,6-trichloro-2-pyridinol (TCP) and diethylthiophosphate (DETP). Isozymes involved in dearylation of CPF are CYP2C19 and CYP3a4 (Tang *et al.*, 2001). A-esterases such as paraoxonase (PON I) and CPF oxonase also aid in the detoxification of CPF and CPF oxon by hydrolyzing their phosphate ester bonds. This results in the formation of TCP and DETP for CPF, and diethylphosphate for CPF oxon. Genetic polymorphism has been shown to cause variation in the level of activity of PON I (Davies *et al.*, 1996), hence resulting in differences in the capacity of individual to metabolize CPF oxon. This difference has also been observed in animals. For example, rabbit paraoxonase hydrolyzes CPF oxon with a much higher turnover number than does rat paraoxonase (Li *et al.*, 1993; Costa *et al.*, 1999). CPF oxonase on the other hand is a calcium-dependent A-esterase that hydrolyses CPF oxon (Furlong *et al.*, 1989), and it has been found to have lower activity in younger animals than in adults (Mortensen *et al.*, 1996).

(c) The last stage in the detoxification involves the formation of glucoronide and sulphate conjugate of TCP, predominantly in the liver but also plasma.

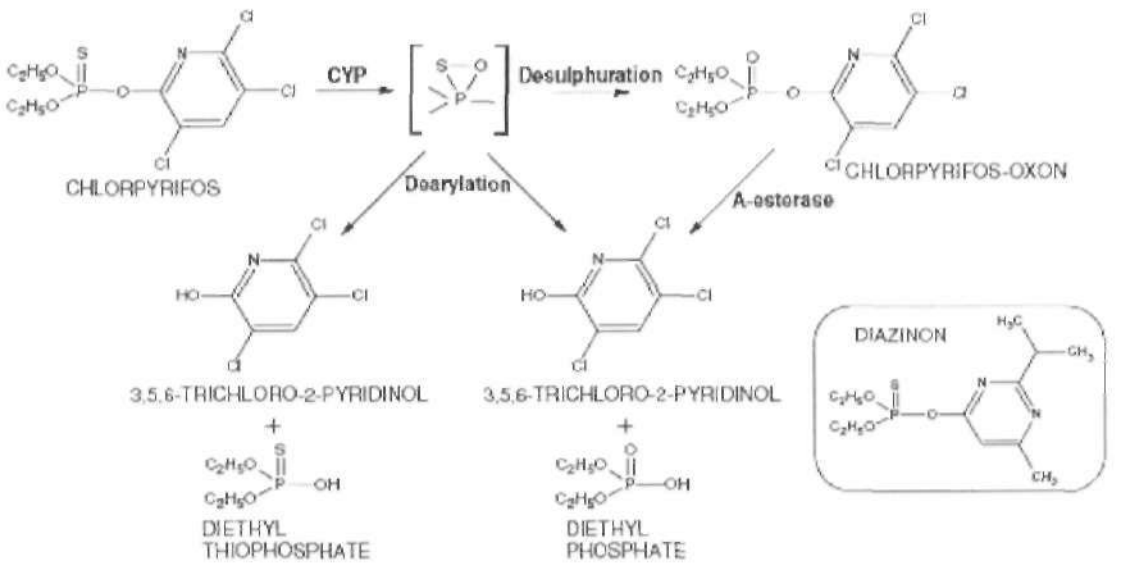


Figure 2.6 Pathway for biotransformation of chlorpyrifos (Inset is another organophosphate *compound, diazinon*) (adapted from Sams *et al.*, 2004).

Chlorpyrifos and its oxon are not detected or are found only in trace amounts in blood or urine, except following very high exposures. The metabolite TCP is the principal form found in circulation (ATSDR, 1997; Meeker *et al.*, 2006)

Chlorpyrifos and CPF oxon and their metabolites have been shown to be rapidly eliminated in humans via the kidneys (US National Library of Medicine, 1999), therefore urine is the primary means of elimination (ATSDR, 1997; FAO/WHO, 1999). In rats oral administration of CPF resulted in 75% elimination in urine, 7% in exhaled air, 8% in faeces and 4% in organs (Neskovic *et al.*, 1992). Oral feeding of cows with CPF found unchanged pesticides in the faeces, but not in urine or milk. However, it was detected in the milk of cows for 4 days following spray dipping (Gallo and Lawryk, 1991). Human volunteer studies indicated that approximately 3% of dermally applied CPF and up to 93% of orally administered CPF was detected as urinary metabolites (Nolan *et al.*, 1984; Griffin *et al.*, 1999). The rate of

metabolism of urinary dialkyl phosphates is rapid, the elimination half-life of an oral dose is 15.5 hours, and dermal dose was 30 hours (Griffin *et al.*, 1999) and a half-life of 27 hours for 3,5,6-trichloro-2-pyridinol (Nolan *et al.*, 1984). Studies in rats (National Academy of Sciences, 1993) and other mammalian species (Gallo and Lawryk, 1991) indicate that CPF is redistributed in adipose tissue, forming a depot for slow release. Thus, OP may be retained for longer period than is evident using plasma measurement (Terry *et al.*, 2003). Despite this, studies in animals have shown that only low concentration of residues remain in the tissue such as fats, lungs, liver and brain several days after administration of CPF (NRA, 2000).

2.2.3 Mechanisms of toxicity of chlorpyrifos

Like any other OP insecticide, the main mechanism of acute toxicity is due to inhibition of AChE, the enzyme responsible for binding and termination of the biological activity of the neurotransmitter ACh (Carr and Chamber, 1996). Therefore, the mechanism is similar to what has been described under OPs (see 2.1.4). Although CPF is directly toxic to the nervous system through its interaction with AChE, CPF oxon, the activated product of CPF, is about 3,000 times as potent against the nervous system as CPF itself (Chambers *et al.*, 1989). The inhibition of AChE activity caused by CPF oxon is more persistent than that caused by other OPs, with measurable inhibition two weeks after exposure in one study (Chambers and Carr, 1993), one month after exposure in another study (Sakai, 1990) and six to seven weeks in other studies (Pope *et al.*, 1992; Bushnell *et al.*, 1994). It is believed that this is because CPF is lipophilic (attached to the non-water soluble, fatty parts of body tissues). This means that CPF is stored in the fatty tissue but then released and transformed to CPF oxon, so that effects occur over a long interval (Chambers and Carr, 1993).

Although CPF is a well-known AChE inhibitor, recent evidence has shown that some of its neurotoxicity is independent of AChE inhibition (Auman *et al.*, 2000; Crumpton *et al.*, 2000b). For example, CPF has been shown to bind directly to muscarinic receptors in the *corpus striatum* thereby resulting in covalent modification of the receptor. The receptor modification is responsible for the inhibition of adenyl cyclase activity. The post-receptor signal transduction by CPF oxon may interfere with normal cellular signaling, thereby resulting in impairment of neurological function, constituting an alternative pathway in the neurotoxicity of CPF (Huff *et al.*, 1994).

Chlorpyrifos has also been shown to impede internal respiration in the hepatic cells of laboratory animals. This results from the mitochondrial damage, which eventually affects the activity of ATPase, an enzyme important in cellular respiration (Sakai, 1990). This eventually leads to the induction of oxidative stress and finally apoptotic death (Tsujimoto, 1997). Furthermore, CPF oxon inhibits the enzyme cholesterol ester hydrolase; inhibition of this enzyme eliminates one of the normal reactions to stress in rats (Civen *et al.*, 1977).

Chlorpyrifos has also been shown to acutely stimulate cytokine synthesis, molecules that relay the inflammation and immune message to the brain (Gordon and Rowsey, 1999; Rowsey and Gordon, 1999). Cytokines are involved in anxiety-like responses and psychiatric illness syndrome (Kronfol and Remick, 2000; Dantzer, 2001; Anisman and Merali, 2003), similar to the psychological symptoms observed in OP-exposed soldiers in the Gulf war (Ferguson and Cassaday, 1999).

Acute CPF exposure has also been suggested to have a relationship in the aetiopathogenesis of Parkinson diseases. CPF through its persistent inhibition of cortical and striatal cholinesterase and a reduction of total muscarinic binding sites (Pope *et al.*, 1992) has the potential to disrupt cholinergic neurotransmission in the striatum mediated by the cholinergic interneurones (Bowman and Rand, 1980). Given the existence of ACh receptors on the terminals of nigro-striatal dopaminergic neurones, ACh inhibition could disrupt the normal integrity of this dopaminergic striatal input. Li *et al.* (2000) have shown that CPF can affect dopamine turnover in the striatum, as indicated by significant increases in the dopamine metabolite 3, 4-dihydroxyphenylacetic acid. Therefore, it is conceivable that inhibition of AChE at the synaptic terminals of cholinergic interneurones, from CPF exposure, could produce Parkinson disease-related alterations of normal motor behaviour. In addition, CPF has been shown to be capable of inducing an increase in cell stress, as measured by decreased mitochondrial integrity (Karen *et al.*, 2001), which may cause nigrostriatal cell degeneration, a factor implicated in the pathogenesis of Parkinson disease. Indeed, acute and reversible Parkinsonism has been reported following exposure to OP (Bhatt *et al.*, 1999).

2.2.4 Health effects of chlorpyrifos

Like any other OP insecticide, the health effects of CPF have been traditionally classified as acute cholinergic syndrome, intermediate syndrome, delayed neuropathy and chronic syndrome related to neurotoxicity (OPICN) (Abou-Donia, 2003), developmental neurotoxicity, reproductive and genotoxicity, and immunotoxicity. Nand *et al.* (2007) described a patient who experienced the three phases of acute cholinergic syndrome, intermediate syndrome and OPIDN following exposure to CPF.

2.2.4.1 Acute cholinergic syndrome

This is the most commonly described and encountered syndrome (Pease *et al*., 1993; Robbinson *et al*., 1993). It may arise from accidental or suicidal intent (Aiuto *et al*, 1993). The syndrome is related mainly to large single exposure to CPF. As observed in other OP insecticides, the syndrome is mainly due to AChE inhibition, resulting in accumulation of ACh in the cholinergic synapses and leading to signs associated with autonomic and CNS symptoms. Signs of cholinergic hyperstimulation include excessive salivation, rapid and laboured breathing, coughing, wheezing, copious secretion from mouth and nose, twitching and rapid contractions of muscles, coarse and generalised body tremors, lacrimation, urination, miosis, increased frequency of defaecation, depression, prostration, epileptoid tremors, convulsions, paralysis, respiratory failure due to respiratory muscle paralysis and death (Richardson, 1995; Selden and Curry, 1987; Taylor and Di Marco, 2003). Dermal exposure to CPF also results in skin irritation, blistering and lesions causing increasing permeation (Sha *et al*., 1981).

2.2.4.2 *Intermediate syndrome*

The syndrome is characterized by delayed onset of muscular weakness, respiratory arrest, hypotonia and flaccid paralysis (Guadarramma-Naveda *et al*., 2001; Mattingly *et al*., 2003; Lee and Lin, 2006). The syndrome has been described in a 16-month-old baby (Mattingly *et al*., 2003) and in a 42-year-old woman following suicide attempt (Lee and Lin, 2006). In these cases, symptoms persisted for some time despite adequate pradiloxime therapy. The persistence of the symptoms may be due to lipid solubility of CPF and its longer half-life, causing delayed inhibition of AChE and subsequent exhaustion of the neuro-muscular junction from acetylcholine overstimulation (Guadarramma-Naveda *et al*., 2001).

2.2.4.3 Delayed polyneuropathy

Chlorpyrifos oxon has been shown to cause delayed neuropathy due to its ability to inhibit NTE. However, unlike other OPs, a high dose is required to induce delayed neuropathy in chicken (Capodicasa *et al.*, 1991; Richardson *et al.*, 1993a and b) and cats (Fikes 1992). Delayed neuropathy can also be caused by low repeated exposure to CPF as observed in hens that develop quadriplegia after 145 days of dosing (Francis *et al.*, 1985). Studies in animals revealed that the delayed neuropathy caused by CPF is 'atypical' (Francis *et al.*, 1985; Fikes, 1992). In cats, the symptoms were less severe, but more persistent than is typical (Fikes, 1992); while in hens, the delay before the onset of symptoms was longer than expected, and some symptoms were reversible (Francis *et al.*, 1985). Delayed neuropathy due to CPF exposure has been observed in humans (Aiuto *et al.*, 1993; Kaplan *et al.*, 1993; Steeland *et al.*, 1994). Clinical symptoms involve distal degeneration of nerves together with ataxia or lower limb paralysis. In severe cases, the upper limb may also be affected, and typically develop 2-4 weeks after a single exposure, but this interval is not predictable following chronic exposures (Lotti, 1986; Richardson, 1995, Meggs, 2003). A case of atrophy and permanent paralysis of hands and feet has been reported following acute dermal exposure to CPF (Meggs, 2003). Vocal cord paralysis and loss of normal reflexes have been observed following single exposure to OP (Aiuto *et al.*, 1993).

Although inhibition of NTE has been traditionally linked to the development of OP, an increasing body of evidence suggests that one mechanism underlying the delayed neurotoxicity associated with some OPs is the inhibition of axonal transport (AXT). OPs that produce delayed neurotoxicity (at high doses like CPF) cause accumulations of tubulo-vesicular profiles within axons prior to degeneration (Abou-Donia and Lapadula, 1990), a

condition that is consistent with the stagnation of membrane traffic (Chretien *et al.*, 1981; Souyri *et al.*, 1981).

2.2.4.4 Chronic neurotoxicity

It has been established that long-term repeated or chronic exposure to CPF results in cognitive and behavioural impairments (Upchurch and Wehner, 1987; Bushnell *et al.*, 1991, 1994; Pope *et al.*, 1992; Cohn and MacPhail, 1997; Prendergast *et al.*, 1997; 1998; Stone *et al.*, 2000; Cañadas *et al.*, 2005, Moser *et al.*, 2005; Samsam *et al.*, 2005). It also induces neuropsychiatric disorders, including generalized, panic and social anxiety disorders (Salvi *et al.*, 2003). In addition, chronic CPF exposure results in alteration in motor function, including reduced locomotor activity (Pope *et al.*, 1992; Mattsson *et al.*, 1996; Moser, 1995), and reduced response rates (Bushnell *et al.*, 1993, 1994; Cohn and MacPhail, 1997; Maurissen *et al.*, 2000). Some of these neurobehavioural sequelae of CPF poisoning are independent of AChE inhibition or muscarinic binding, and persisted long after the CPF exposure (Pope *et al.*, 1992; Cañadas *et al.*, 2005). Chronic CPF poisoning at levels that causes appreciable inhibition of B-esterases produce adverse effects on peripheral nerve electrophysiologic parameters suggestive of subclinical neuropathy (Albers *et al.*, 2007).

Although chronic neurotoxicity may result from repeated exposure to CPF, studies and anecdotal evidences have revealed that long-term neurobehavioural alterations such as neurocognitive deficits may accompany acute exposure. For example, studies have shown that acute OP poisoning resulted in long-term functional neurotoxicity such as peripheral and central neuropathy, and neurocognitive deficits, including impairment in memory and information processing (Bushnell *et al.*, 1993, 2001; Kaplan *et al.*, 1993; Sanchez-Amate *et*

al., 2001; Terry *et al.*, 2007). Apoptosis and reduction of mitochondrial function of the cortical neurones have been implicated in CPF-induced neurotoxicity (Caughlan *et al.*, 2004). CPF oxon has been found to be slightly more potent in inducing apoptosis than CPF (Caughlan *et al.*, 2004). According to Gultekin *et al.* (2007), increased glutaminergic activity as a result of increased expression of NR2A and NR2B subunits of NMDA receptor in the hippocampus observed in CPF poisoning may cause neuronal damage, resulting in impairment of learning and memory.

2.2.4.5 Chlorpyrifos- A developmental neurotoxicant

There has been increasing concern about the propensity of the widely used OP insecticides, especially CPF to elicit developmental neurotoxicity at undetected exposures due to the absence of systemic signs of intoxication (Landrigan, 2001; Slotkin, 2005; Colborn, 2006; Costa, 2006). In addition to heightened vulnerability to CPF because of their developing nervous systems, children are likely to be exposed to higher levels of CPF than adults for several reasons. Children eat, breathe, and drink more per kg of body weight than adults, so the effects of any CPF-contaminated food, water or air is magnified relative to that experienced by adults. They also play on the floor and in the grass where pesticide residues collect and exhibit hand-to-mouth behaviours that increase their potential for exposure (Furlong *et al.*, 2006). Several studies conducted with rats indicate that CPF is a developmental neurotoxicant directly affecting developing cells (Whitney *et al.*, 1995; Song *et al.*, 1997; Crumpton *et al.*, 2000a, Jett and Navoa 2000). Although, TCP, the metabolite of CPF and CPF oxon selectively accumulates in the foetal brain (Hunter *et* al., 1999), it does not contribute to neurotoxicity (Caughlan *et al.*, 2004). Mechanisms of developmental neurotoxicity induced by CPF have been subject of much research, since the role of AChE

inhibition in this instance has been discountenanced. This is because the foetus and neonates recover from cholinesterase inhibition faster than adults (Chakraborti *et al.* 1993; Lassiter *et al.* 1998), yet display greater overall toxicity and damage to the CNS.

Furthermore, doses of CPF below those that will inhibit AChE still cause developmental neurotoxicity (Slotkin *et al.*, 2007). Therefore, other mechanisms independent of AChE inhibition are involved in this type of neurotoxicity. The neurochemical and physiological bases are still being investigated, but hypotheses put forward include: the non-cholinergic targeting of the expression and function of major nuclear transcription factors involved in brain cell replication and differentiation (Crumpton *et al.*, 2000a), induced oxidative stress in the developing brains of rats (Jett and Navoa 2000), the inhibition of DNA and protein synthesis in the brain (Whitney *et al.* 1995), and action on adenyl cyclase that functions in the coordination of cell development (Song *et al.* 1997). As a developmental neurotoxicant, CPF has also been shown to affect the processes of neuronal and glial cell replication and differentiation, axonogenesis, synaptogenesis, synaptic function and apoptosis and, therefore, the potential to evoke behavioural teratogenesis at subtoxic doses (Pope, 1999; Slotkin, 1999, 2004; Barone *et al.*, 2000; Crumpton *et al.*, 2000a; Casida and Quistad, 2004; Jameson *et al.*, 2006). CPF interferes with the expression or causes a deficit in fibroblast growth factor, a superfamily of neurotrophic factors that plays critical roles in neural cell development, brain assembly, and recovery from neuronal injury (Slotkin *et al.*, 2007). *In utero* exposure to CPF has been shown to produce neurobehavioural deficits that extend to adulthood as a result of decrease in the number of surviving neurones in the meninges of rats (Abou-Donia *et al.*, 2005).

2.3 Chlorpyrifos and oxidative stress

Organophosphate insecticides have been shown to induce oxidative stress in experimental studies (Kourakis *et al.*, 1996; Abdollahi *et al.*, 2004b) as well as acute poisoning cases in humans (Banerjee *et al.*, 1999; Seth *et al.*, 2001) and in workers occupationally exposed to OPs (Ranjbar *et al.*, 2002).

The basis of organophosphate toxicity in the production of oxygen free radical (OFR) may be due to;

a) Their "redox-cycling" activity- They readily accept an electron to form free radicals and then transfer them to oxygen to generate superoxide anions and, hence, hydrogen peroxide through dismutation reaction (Ryrfeldt *et al.*, 1992).

b) Generation of free radicals probably because of the alteration in the normal homeostasis of the body resulting in oxidative stress, if the requirement of continuous antioxidants is not maintained (Vidyasagar *et al.*, 2004).

Several clinical and experimental evidences have expressed that CPF produces part of its neurotoxicity through oxidative mechanisms (Bagchi *et al.*, 1995; Crumpton *et al.*, 2000b; Jett and Navoa 2000; Gupta 2004; Qiao *et al.*, 2005; Slotkin *et al.*, 2005, 2007; Verma *et al.*, 2007). This oxidative-stress induced neuronal cytotoxicity may involve disturbances in intracellular calcium homeostasis (Giordano *et al.*, 2007). CPF may also exert more generalized cytotoxicity from oxidative stress that affects the developing brain because of its lower reserve of antioxidants (Bagchi *et al.*, 1995, 1996; Crumpton *et al.*, 2000a). Apart from the nervous system, an overwhelming body of evidence has shown that CPF induce oxidative stress and depletion of the antioxidant defense system such as zinc, catalase, superoxide

dismutase, glutathione, glutathione-s-transferase in other organs such as the liver and kidneys (Gultekin *et al.*, 2001, 2006; Oncu *et al.*, 2002a and b; Bebe and Penamangalore, 2003; Goel *et al.*, 2005; Slotkin *et al.*, 2007; Verma *et al.*, 2007; Zama *et al.*, 2007). In addition, several studies have demonstrated the protective effect of some antioxidant molecules including vitamins C and E on toxicity produced by exposure to CPF (Gultekin *et al.*, 2001; Oncu *et al.*, 2002a; Goel *et al.*, 2005; Ambali *et al.*, 2007; Verma *et al.*, 2007; Zama *et al.*, 2007,; Tuzmen *et al.*, 2008).

In conclusion, the available information reviewed in the current literature shows clearly a strong preponderance in FR or ROS-induced deleterious actions of CPF on body cells, tissues, organs and systems, especially the nervous system. It is, therefore, conceivable that the target of a FR approach to prophylactic and therapeutic interventions may be of immense value in reducing or eliminating the adverse effects of CPF on the health of animals and man.

2.4 Oxidative stress

Oxidative stress coined by Sies (1985) is an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage (Sies, 1991). Oxidative stress occurs when the oxidative stress–related molecules, generated in the extracellular environment or within the cells exceed the intracellular antioxidant defences (Vrba and Modriansky, 2002). The term has been used to describe a number of chemical reactions involved in the production of free radicals (FRs) and other reactive molecules that are able to induce cellular injury (Kennedy *et al.*, 2005). It results in loss of normal balance between FR species production and the antioxidant system (Himmelfarb and Hakim, 2003) or a disruption of the equilibrium between pro- and antioxidant systems (Lazarova *et al.*, 2004).

2.4.1 Free radicals

Free radicals are chemical species that have an unpaired delocalized electron in an outer orbital (Castagne *et al.*, 1999; Campanella *et al.*, 2007), hence they are molecules with an "open" or half bond (McCord, 1985) and are capable of independent existence no matter how brief (Halliwell and Gutteridge, 1999). This unstable configuration causes these chemical species to be very aggressive and to have a short life span (Halliwell, 1987). The unpaired electron confers on these molecules a strong propensity to react with target molecules, by withdrawing one electron from target molecules to complete their own orbital (Younes, 1999; Halliwell and Gutteridge, 1999), and thereby stabilizing themselves. Thus, FR causes the molecule whose electron had been withdrawn to become non-functional and even become a FR itself (Halliwell and Gutteridge, 1999). The resulting FR can in turn react with another molecule until the chain of reaction is terminated either by random collision of two or three FRs to form a molecule with stable bond or by one of the cellular defence mechanisms (Freeman and Crapo, 1982; McCord, 1985; Southorn and Powis, 1988; Goldfarb, 1999). In view of their high reactivity and instability, FRs can react with several chemical compounds such as proteins, lipids, nucleic acids, carbohydrates present in cellular compartments and other classes of molecules like extracellular matrix glycosaminoglycans (hyaluronic acid) and damaging them (Halliwell and Gutteridge, 1985; Schimdley, 1990; Campanella *et al.*, 2007).

Free radicals are often classified according to the functional group in their molecules (Halliwell, 1987; Halliwell and Gutteridge, 1992; Kehrer, 1993; Morrisey and O'Brien, 1998). They are most often oxygen-, carbon-, or nitrogen-based molecules (Kalyanaraman, 1982; Kehrer, 1993; Halliwell, 1996b). Oxygen molecules with unpaired electron in their outer orbits are called reactive oxygen species (ROS) and they include hydroxyl ($OH^{\cdot}$), superoxide

($O_2^{\cdot}$) and peroxy ($ROO^{\cdot}$) radicals (Bahorun *et al.*, 2006). FRs which have nitrogen molecules with unpaired electron in their outer orbits are called reactive nitrogen species (RNS) and they include nitric oxide ($NO^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$) (Bahorun *et al.*, 2006). Carbon-centred radicals can arise following hydrogen extraction from unsaturated bonds in fatty acids during lipid peroxidation or metabolism of certain xenobiotics (Kehrer, 1993). Reactive oxygen/nitrogen species such as peroxynitrite ($OONO^{-}$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3), nitrous acid (HNO_2) and dinitrogen trioxide (N_2O_3) are not FRs but can easily lead to FR reactions in living organisms. Depending on the pathway of generation, or the major ROS formed, the phenomenon of oxidative stress can be sub-specified as metabolic oxidative stress, environmental oxidative stress, photooxidative stress (Sies and Stahl, 2004), drug-dependent oxidative stress, or nitrosative stress (Sies, 2000).

2.4.1.1 Sources of free radicals

Free radicals are produced in small amounts by normal cellular processes (Schmidley, 1990). In living organisms, they have endogenous and exogenous sources (Chihuailaf *et al.*, 2002). The group generated endogenously includes those produced intracellularly that act inside and outside the cell (Machlin and Bendich, 1987). The generation of these FRs takes place in four well-defined sources:

(a) Normal metabolic reactions: During the normal metabolic reaction, the mitochondrial electron transport system is designed to add four electrons to O_2, consequently reducing it to water and avoiding the reactive species produced by single electron reduction of oxygen. However, "leaks" in mitochondrion electron transport allow a small fraction of O_2 to accept a single electron, and are metabolized via a univalent reaction that inevitably results in

intermediate products such as superoxide radicals, hydrogen peroxide and hydroxyl radical (Schmidley, 1990; Pal, 1994, Morrisey and O'Brien, 1998).

(b) FRs are also produced by peroxisomes containing Acyl CoA oxidase, dopamine β-hydroxylase (involved in cathecolamine metabolism, urate oxidase and other reactions catalyzed by prostaglandin hydroperoxidase), and are also produced by products of the normal or pathological functions of several other enzymes (Freeman and Crapo., 1982; Morrisey and O'Brien, 1998).

(c) Xenobiotic metabolizing enzymes in the endoplasmic reticulum or nuclear membrane such as the enzymatic microsomal cytochrome p-450 reductase system, cytochrome b_5 and NADPH-dependent cytochrome reductases are important source of endogenous FR production (Chihuailaf *et al.*, 2002). These pathways constitute a primary defense against several xenobiotics and endogenous substances, but they activate molecular oxygen or allow superoxide to "leak" out.

(d) Free radicals are also produced endogenously during the process of phagocytosis. Superoxide and other FR anions are produced when phagocytes destroy cells infected by bacteria or viruses through an oxidizing discharge, composed basically of hypochlorite, hydrogen peroxides, nitric acid and superoxides (Stoian *et al.*, 1996; Morrisey and O' Brien, 1998; Younes, 1999; Chihuailaf *et al.*, 2002) or during the handling of other xenobiotics in a process known as respiratory burst (Ji, 1995). Increased level of FRs can also be observed from other sources such as ischaemia or reperfusion injury following surgery or artery blockage, and following inhibition/reduction in the level of antioxidants, which affects the ability of the body to handle increased level of FR (Reed, 1995).

Exogenous sources, which outstandingly increase the ROS production, include a high availability of some transition metals in the diet such as copper (Cu) and iron (Fe) that promote FR generation from peroxides (Halliwell and Gutteridge, 1984, Morrisey and O'Brien, 1998; Chihuailaf *et al.*, 2002). The ability of these ions to gain and lose electrons is the basis for the creation and propagation of several toxic reactions (Halliwell, 1987; Halliwell and Chirico, 1993). Other exogenous sources include radiation, cigarette smoke, certain organic solvents and pesticides (Machlin and Bendich, 1987; Lunec, 1996), products obtained from food lipid peroxidation (Kubow, 1993), sun radiation, mycotoxins (Miller *et al.*, 1993), trauma, hyperoxia and over-exercising (Halliwell *et al.*, 1992; Chihuaialaf *et al.*, 2002).

2.4.1.2 Formation of free radicals

Oxygen is an indispensable element essential for maintenance of life, but can be life threatening in higher concentration due to its ability to generate FR (Bartnikowska, 1994). Approximately 85-90% of the inhaled O_2 is used by several oxidase and deoxidase enzymes as well as by direct oxidation chemical reactions (Halliwell and Gutteridge, 1999; Bahorun *et al.*, 2006). At the terminal part of the electron transport chain, cytochrome oxidase removes one electron from each one of the four cytochrome reduced molecules, oxidizing them and add the four electrons to the O_2 in order to form water (around 95-98% from the 85-90% mentioned above) (Equation 1). The remaining 2-5% is univalently reduced into metabolites called ROS. Tetravalent oxygen reduction is shown in Equation 1 below:

$$O_2 + 4c^- + 4H^+ \rightarrow 2H_2O + \text{energy} \qquad (1)$$

Due to the electronic configuration, oxygen has strong tendency to accept one electron at a time. The univalent conversion of oxygen to water is presented below:

(a) The addition of one electron to one oxygen molecule in its fundamental state generates the formation of the superoxide radical ($O_2^{\circ-}$) (Equation 2)

$$O_2 + e^- \rightarrow O_2^{\circ-} \quad (2)$$

(b) The superoxide that receives more than one electron and two hydrogen ions forms hydrogen peroxide (H_2O_2) in a dismutation reaction process (Equation 3). This reaction is catalyzed by superoxide dismutase (SOD) found in high amounts in mammalian cells.

$$O_2^{\circ-} + 2H^+ \xrightarrow{SOD} H_2O_2 \quad (3)$$

(c) When H_2O_2 receives more than one electron and one hydrogen ion, the most toxic intermediates hydroxyl radical (OH°) is formed. The OH° is the most destructive of the ROS. It may also be formed when H_2O_2 reacts with Fe^{2+} or Cu^{2+} in a reaction known as Fenton reaction (Equation 4)

$$Fe^{2+}/Cu^{2+} + H_2O_2 \rightarrow OH^{\circ} + OH^- + Fe^{3+}/Cu^{2+} \quad (4)$$

The ions of the transition metal may as well catalyze the reaction between H_2O_2 and superoxide, leading to production of OH° radical in a reaction known as Haber-Weiss reaction (Equation 5)

$$H_2O_2 + O_2^{\circ-} \xrightarrow{Fe/Cu} OH^{\circ} + OH^- + O_2 \quad (5)$$

Besides, the superoxide radical may react directly with the nitric oxide (NO) to generate peroxynitrite ($ONOO^-$), which can eventually result in the generation of hydroxyl radicals (Equation 6)

$$O_2^{\circ-} + NO \rightarrow ONOO^- \rightarrow ONOO^- + H^+ \rightarrow OH^{\circ}$$

Peroxynitrite itself is a non-radical species that is very reactive and can induce a chain reaction of events such as direct biological damage by excessively oxidizing –SH groups and also leading to the production of nitric dioxide, which is a powerful initiator of lipid peroxidation (Schmidt and Walter, 1994; Halliwell, 1996b; Muijsers *et al.*, 1997). Peroxynitrite can also react with carbon dioxide in tissues and fluids, leading to protein damage via the formation of nitrotyrosine and lipid oxidation (Klein and Ackerman, 2003).

Free radical mediated chain oxidation proceeds by a chain reaction consisting of three steps: chain initiation, chain propagation and chain termination (Noguchi and Niki, 1999). The chain initiation involves the formation of a FR and its attack on a lipid. The FR attack on the lipid results in lipid radicals. Lipid radicals then react with oxygen molecules, becoming peroxyl radicals, which then attack more lipid radicals, and react with more oxygen and propagate the chain reaction. The chain propagating radical is a lipid peroxyl radical, regardless of the initiating FR species. Chain termination occurs when a lipid radical or a lipid peroxyl radical is scavenged by an antioxidant such as vitamins C and E or when two lipid peroxy radicals react to produce a non-radical ketone and alcohols.

2.4.1.3 Cellular targets and consequence of free radical damage

Free radicals can react with and damage proteins, enzymes, nucleic acids, carbohydrates, lipids and other classes of molecules such as the extracellular matrix glycosaminoglycans in cells and tissues (Schmidley, 1990; Singh *et al.*, 2004). The sulphur-containing amino acids, polyunsaturated fatty acids and DNA molecules are vulnerable to damage by FRs (Halliwell and Gutteridge, 1986; Schmidley, 1990). Singh *et al.* (2004) have shown that FRs induce undesirable oxidation, causing membrane damage, protein modification, DNA damage and

cell death induced by DNA modification and lipid peroxidation. The amino acid residues of proteins are highly sensitive to oxidative actions. Studies have shown that modification of a protein starts from the interaction of $OH^{\cdot}$ with the main molecular chains, with the lateral ones and with the functional groups of the amino residues (Campanella *et al.*, 2007). Hydroxy radicals targets all the amino residues but the preferred ones are tyrosine and histidine (aromatic amino acids), methionine and cysteine (sulphur-containing amino acids) (Stadtman, 1986, 1992, 1993; Wolff *et al.*, 1986; Ames *et al.*, 1993; Barlett and Stadtman, 1997; De Zwart *et al.*, 1999; Stadtman and Levine, 2003). The oxidative modifications enhance the degradation of the essential enzymes involved in the preservation of the integrity of the cell and thus extend the damage to the cell resulting in its total destruction (Campanella *et al.*, 2007).

The oxidative reactions cause the breakage of the polynucleotide chains, which may result in the formation of bridge bonds that are able to produce processes leading to mutagenesis, carcinogenesis or cellular deaths in the DNA structure (Campanella *et al.*, 2007). ROS also cause inactivation of the thiol-containing Ca-ATPase enzyme, essential for maintaining calcium (Ca^{2+}) ion homeostasis (Kehrer, 1993). The consequence of this is the alteration in the transportation of Ca^{2+}, thereby increasing its intracellular concentration. This results in the activation of proteases, which attack cytoskeleton and nucleases, which fragment DNA (Halliwell *et al.*, 1992). Oxidative injuries on proteins produce peroxides and carbonyls (Cheeseman and Slater, 1992).

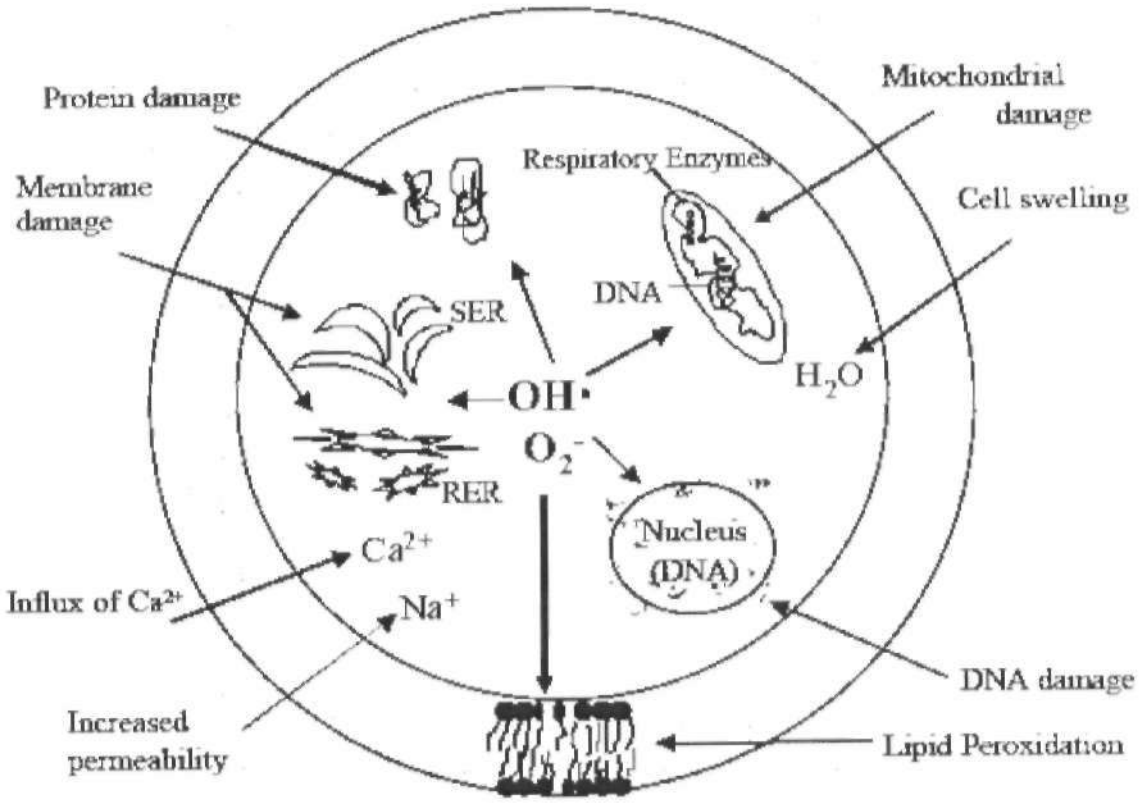


Figure 2.7: Cell injury by reactive oxygen species (ROS) or free radical-induced oxidative stress (adapted from Marks *et al.* 1996).

Fatty acids are most susceptible to FR attack at the alpha-methylene carbons, and the adjacent carbon-carbon double bonds. Therefore, polyunsaturated fatty acids (PUFA) with several double bonds per molecule are particularly vulnerable to FR damage (Halliwell and Gutteridge, 1985). The double bond on the carbon weakens the carbon-hydrogen bond allowing for easy dissociation of the hydrogen by a FR, creating the acetylated fatty FR (Chihuailaf *et al.*, 2002). Oxygen is quickly added to this molecule and transformed to a peroxide fatty acid FR that acts as a transporter in the chain reaction, attacking more PUFA

(Chihuailaf *et al.*, 2002). The resulting lipid hydroperoxides are not completely stable *in vivo* and in the presence of metals or metal complexes (such as Fe) can further decompose to reactive radicals, which will propagate the chain reactions started by the initial FR attack (Halliwell and Gutteridge, 1985). Lipid hydroperoxides also fragment to produce aldehydes, which can in turn cross-link proteins, rendering them incapable of serving as receptors or enzymes (Schmidley, 1990). Potential consequences of damage to membrane lipids include change in fluidity and permeability and in the orientation of proteins embedded in the bilayer of the plasma membrane and other cellular endomembranes. The shape and function of many intrinsic membrane proteins depend on specific interactions between their hydrophobic domains and membrane phospholipids (Demopoulos *et al.*, 1979; Halliwell and Gutteridge, 1985). These phospholipid-dependent proteins are particularly susceptible to FR damage of the fatty acid component of the membrane phospholipids (Demopoulos *et al.*, 1979). The consequence of lipid peroxidation is the alteration of the receptor function (Chan, 1988), which affects cellular function that leads to lethality (Schmidley, 1990).

Carbohydrates, such as glucose and other related monosacharides are peroxidized under certain conditions to form dicarbonylic compounds and hydrogen peroxide (Kubow, 1993). This is important because once activated, these biomolecules can interact with one another and form new molecular associations (Chihuailaf *et al.*, 2002).

The DNA is another important target for FR damage, especially the mitochondrial DNA (Machlin and Bendich, 1987; Bandyopadhyay *et al.*, 1999). Due to its location, the mitochondrial DNA is constantly exposed to a high flow of ROS from the respiratory chain. This DNA is also very susceptible to ROS assault because of its lack of histone, and because

its repair mechanisms are less efficient (Halliwell, 1997; Chaudiere and Ferrari-Iliou, 1999). DNA damage involves direct attacks of oxidants on individual nucleotides in DNA (Halliwell and Gutteridge, 1999). Guanine is the DNA base most susceptible to oxidative attack and damage. The nucleotide oxidation product is 8-deoxyguanine and the nucleoside derivative is 8-hydroxy-2-guanosine (Halliwell and Gutteridge, 1999), which is the main product of oxidative injury (Chiuailaf *et al*., 2002). DNA can also be damaged by RNS, some of which can be derived from nitrosamines (Traber and Kamal-Eldin, 2007). The alterations experienced by this DNA include deoxyribose oxidation, chain intererossing and modification of nitrogen bases, which will be significant if they are intense enough and are incapable of affording the repair mechanisms before replication occurs (Cheeseman and Slater, 1992). ROS and RNS can cause DNA strand breaks and point mutations (Traber and Kamal-Eldin, 2007).

In summary, cell injury results as a consequence of damage to the biomolecules. This injury results from conformational changes in the cell structure and alteration in metabolic functions (Chihuailaf *et al*., 2002). This can result from loss of catalytic activity, changes in protein and enzyme polarity, modification of ionic channels, alteration in hormone response receptors and neurotransmitters, or in the covalent union between FR and receptors or biomolecules that modify the cellular antigenic structure (Slater, 1984; Machlin and Bendich, 1987). Lipid peroxidation due to oxidative stress is a common cause of inflammation and tissue damage (Geetha *et al*., 2001). Oxidative damage has been suggested to constitute early, and possibly signaling, events in apoptotic cell death (Kane *et al*., 1993; Sato *et al*., 1995). Indeed, cell death via apoptosis or necrosis is the end-point of FR damage in cells (Kannan and Jain, 2000).

Due to their highly biologically reactive properties, excessive production of FRs has beeen implicated in the pathogenesis of various disease states, including ageing, ischaemia reperfusion injury, dementia, atherosclerosis (Schisterman *et al.*, 2001; Surekha *et al.*, 2007), various forms of cancers (Ames, 1983; Goldstein and Witz, 1990, Guyton and Kensler, 1993; Gonzales, 1999) and diabetes mellitus (Ceriello *et al.*, 1998). They have also been implicated in neurodegenerative processes (Southorn *et al.*, 1988; Halliwell, 1994; Klein and Ackerman, 2003) and neuropsychiatry diseases (Lohr, 1991).

Free radicals also play an important role in the mechanisms of myocardial infarction (Simpson and Lucchesi, 1987; Drewa *et al.*, 1997), gastritis and duodenitis in children (Bala *et al.*, 1996), peptic ulcer disease (Demir *et al.*, 2003), respiratory tract diseases (Bartnikowska, 1994; Drewa *et al.*, 1995) and CNS diseases (Swincow *et al.*, 1994; Dalkara *et al.*, 1994; Drewa *et al.*, 1995). They are involved in the toxic and therapeutic effects of antineoplastic drugs and radiation, and the deleterious consequence of environmental pollution (Cross *et al.*, 1987; Southorn and Powis, 1988). Oxidative stress has been shown to play a role in the pathogenesis of pre-eclampsia (Madazli *et al.*, 2002), male and female infertility (Agarwal *et al.*, 2005) and even endometriosis (Jackson *et al.*, 2005). It is also believed to be involved in the pathophysiology of autoimmune diseases such as rheumatoid arthritis, immune complex-mediated vasculitis, inflammatory bowel diseases, cataract and cerebral ischaemia (Schmidley, 1990).

2.4.2 Oxidative stress and the nervous system

Free radicals play an important role in the functioning of the brain, and in physiologic and pathologic conditions (Drewa *et al.*, 1998). Neurotransmitters function by means of membrane

lipid utilization. Lipid peroxidation might disturb brain functioning inspite of normal neurotransmitter concentration (Drewa *et al.*, 1998). The brain due to its biochemical and physiological properties is especially sensitive to FRs, which destroy its functions and structure (Drewa *et al.*, 1998). Under normal conditions, the brain physiological processes utilize about 20% of the organism's oxygen, which favour FR production (Drewa *et al.*, 1998). ROS such as $O_2^{-\bullet}$, $OH^{\bullet}$, $NO^{\bullet}$, nitrite, nitrate and H_2O_2 are normally produced in the brain (Walz *et al.*, 2000). The neuronal membranes are rich in PUFAs, which are particularly susceptible to FR attack (Schmidley, 1990). The brain is very susceptible to oxidative stress because in addition to harbouring large amount of oxygen in a relatively small mass, it contains a significant quantity of metals (Fe), and has fewer antioxidant mechanisms than other tissues (Halliwell and Gutteridge, 1999; Naffa-Mazzacoratt *et al.*, 2001). For example, the CNS is relatively poorly endowed with superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase and is also relatively lacking in vitamin E (Halliwell and Gutteridge, 1985).

The sources of ROS in the brain include inefficiency of the electron-carrying components, the mitochondrial, nitric oxide synthase, arachidonic acid metabolism, xanthine oxidase, monoamine oxidase and cytochrome P450 enzymes (Schulz *et al.*, 2000). Oxidants can be produced essentially in all cells in the brain. For example the NADH oxidase, the superoxide generating enzymes in the phagocytes is expressed by microglial, astrocytes and the neurones (Tammariello *et al.*, 2000). Although superoxide can lead to generation of $OH^{\bullet}$ through slow process of Fenton and Haber-Weiss reactions, respectively (Beckman, 1991), the toxicity of superoxide can be increased by its interaction with nitric oxide to produce peroxynitrite ($ONOO^{-}$), that modifies proteins to form nitrotyrosine essential for the induction of neuronal

injury (Brennan *et al.*, 2002). The role of Fe^{2+} in the oxidative damage in the brain cannot be overemphasized as it serves as catalyst in the formation of FR. For Fe^{2+} to perform its catalytic role, it must not be bound to protein. Since, unlike most extracellular fluids, the cerebrospinal fluid (CSF) has low concentration of Fe^{2+}-binding proteins, Fe^{2+} released from brain cells is more readily available to catalyze the formation of $OH^{\cdot}$. Fe^{2+} can also donate electron to H_2O_2 (a non-radical, non-polar compound that can easily cross the membrane to form $OH^{\cdot}$ (Schmidley, 1990).

The brain regions including *substantia nigra* that are rich in cathecolamines, adrenaline, noradrenaline, and dopamine are exceptionally vulnerable to FR generation (Singh *et al.*, 2004). This is because cathecolamines can spontaneously break down (auto-oxidise) to chinone and semichinone, which are metabolized to H_2O_2 by endogenous enzymes such as monoamine oxidase (Singh *et al.*, 2004). The *substantia nigra*, in addition contains a greater amount of Fe^{2+} than other brain structures (Drewa *et al.*, 1998).

Perry *et al.* (2002) has established a connection between antioxidant depletion and brain degeneration. Mutation of α-tocopherol transfer protein gene resulted in retinal degeneration and gait abnormalities in homozygous mice at one year of age, which correlated with degeneration of the posterior column of the spinal cord (Yokota *et al.*, 2001). In addition, mice devoid of SOD develop gait abnormalities at 12 days of age, associated with vacuolisation in the brain stem and cortical regions (Melov *et al.*, 1998). These indicate that the loss of antioxidant genes can lead to neuronal degeneration.

Oxygen free radicals through the destructive effects on biological membranes cause disturbances in physiological and biochemical processes such as mitochondrial oxidative phosphorylation, ion and membrane transport and maintainance of electrical potential difference. These disturbances may cause cell and neuronal depolarization (Southorn and Powis, 1988). Excess of calcium ions results in functional protein activation, endonuclease and cell death (apoptosis), which in neurones may cause neurodegenerative diseases (Paschen and Mengensdorf, 2005). The phenomenon of excitotoxicity has been linked to over-production of FRs (Pazdernik *et al.*, 2001; Freitas *et al.*, 2003). Lipid peroxidation is an index of irreversible neuronal damage of cell membrane phospholipids (Freitas *et al.*, 2005).

2.4.3 Antioxidant defence system

Biological systems have evolved endogenous defence mechanisms to help protect against FR-induced cell damage (Singh *et al.*, 2004). These are called antioxidants. Antioxidants was originally defined as any substance that when present in low concentration compared with that of the oxidizable substrate, significantly prevents the prooxidant-initiated oxidation of that substrate (Halliwell and Gutteridge, 1985; Black, 2004). However, because of the imperfection in the definition in relation to the concentration of antioxidants (Halliwell, 2007), Halliwell and Gutteridge (2007) has redefined antioxidants as any substance that delays, prevents or removes oxidative damage to a target molecule. Higher organisms have developed a remarkably efficient antioxidant defense system over the course of evolution. The antioxidants neutralize FR by donating an electron to stabilize the FR (Chihuailaf *et al.*, 2002). The antioxidant molecules "internalize" the loss of an electron through resonance between carbon bonds. The antioxidant systems can prevent oxidative damage by (Jenkins, 1993; Sen, 1995):

proton to yield a chemically reduced non-radical product and ascorbyl radical. The ascorbyl radical dismutates to ascorbate and dehydroascorbic acid (DHA) (Carr *et al.*, 2000; Halliwell, 2001).

Vitamin E is the major lipid soluble antioxidant present in all cellular membranes where it protects the membranes against lipid peroxidation (Machlin, 1980). Vitamin E can directly react with a variety of oxy radicals, including the peroxyl radical ($ROO^{\cdot}$), carbon trichloride (CCl_3^{-}), $OH^{\cdot}$ as well as with $O_2^{\cdot -}$ (Fukuzawa and Gebicki, 1983; Ozawa *et al.*, 1983) and singlet oxygen (Fahrenholtz *et al.*, 1974; Littarru *et al*, 1984).

Beta-carotene, a plant pigment, is the major carotenoid precursor of vitamin A (Machlin and Bendich, 1987). ß-carotene and vitamin A protect the cell membrane against lipid peroxidation, especially those induced by xanthine oxidase system and they eliminate superoxide and peroxyl radical (Chihuailaf *et al.*, 2002). β-carotene is the most efficient quencher of singlet oxygen known in nature (Burton and Ingold, 1989). It is found in cellular membranes including those of the lysosomes (Mayne and Parker, 1986).

Glutathione is a tripeptide molecule composed of non-essential amino acid (Machlin and Bendich, 1987). It is a low molecular weight compound and is the most abundant thiolic compound in mammalian cells (Powers and Lennon, 1999; Chihuailaf *et al.*, 2002). Due to its chemical properties, glutathione reacts with several oxidants such as hydrogen peroxide, superoxide, hydroxyl and reactive carbon species (Halliwell and Gutteridge, 1984; Yu, 1994). It reduces dehydroascorbate and ascorbyl and tocopheroxyl FR to their active forms (Powers and Lennon, 1999), thereby effectively recycling the pool of bioavailable vitamins C and E

(Stocker and Keaney Jr, 2004). Glutathione also plays a central role in the activity of glutathione peroxidase and aids in the detoxification of xenobiotics via the action on glutathione transferases (Hayes and Strange, 1995; Strange *et al.*, 2001).

The antioxidant property of ceruloplasmin is through its oxidase activity, which is directed towards aromatic amines, phenols (Attansiu *et al.*, 1998; Verma *et al.*, 2005) and ferrous ions (ferroxidase activity) (Holmberg and Laurell, 1951). It also inhibits ferrous ion stimulated by lipid peroxidation and decomposes lipid peroxides (Osaki *et al.*, 1966).

2.4.3.1.2 The antioxidant enzymes

The antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase (CAT) (Drewa *et al.*, 1998; Schneider and de Oliveira, 2004), glutathione reductase and transferases, thiol disulfide oxidoreductases and peroxiredoxins (Halliwell and Gutteridge, 1990; Stocker and Frei, 1991; Stocker and Keaney, 2004) are primary enzymes involved in the direct elimination of toxic oxygen species, while other enzymes including glucose-6-phosphate dehydrogenase and cytosolic glutathione sulphur-transferase (GST) are secondary enzymes, which help in the detoxification of ROS by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione or NADPH necessary for optimum function of the antioxidant enzymes (Vendemiale *et al.*, 1999; Singh *et al.*, 2003). The antioxidant enzymes are primarily intracellular and, thus, extracellular FRs either produced extracellularly or from the environment must be inactivated by circulating antioxidants such as vitamins and ceruloplasmins (Machlin and Bendich, 1987). Their effectiveness varies with the stage of development and other physiological aspect of the organism (Halliwell and Gutteridge, 1999; Livingstone *et al.*, 2001). The antioxidant enzymes

require micronutrients such as selenium, zinc, iron, copper and manganese as cofactors for optimum catalytic activity and effective antioxidant defence mechanisms (Halliwell and Gutteridge, 1992; Halliwell, 2001).

Superoxide dismutase has three forms in the mammalian systems: the copper-zinc SOD (Cu-Zn SOD), Manganese SOD (MnSOD) and the extracellular SOD (EC-SOD) (Stocker and Keaney, 2004). The ubiquitous form is the Cu-Zn SOD, found in virtually all cells, where most of it is located in the cytosol, with some activity in lysosomes, peroxisomes, nucleus, and the space between inner and outer mitochondrial membrane (Stocker and Keaney, 2004). Mn-SOD is largely located in the mitochondrial matrix (Chaudiere and Ferrari-Iliou, 1999), while EC SOD is an extracellular form of the enzyme. It also contains copper-zinc but the significant amounts of it are present in the normal arterial wall outside cells (Strålin *et al.*, 1995). SOD catalyzes the univalent reduction and oxidation of $O_2^{\cdot-}$ to H_2O_2 and molecular oxygen (Equation 7).

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (7)$$

Catalase (CAT) is a haeme protein containing four groups, with a wide intracellular distribution. It is concentrated mainly in the peroxisomes and mitochondria (Murray *et al.*, 1994; Powers and Lennon, 1999), where it catalyzes hydrogen peroxide (from superoxide dismutation) into water and oxygen (Chihuailaf *et al.*, 2002) (Equation 8).

$$H_2O_2 + H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O \qquad (8)$$

Glutathione peroxidase (GSH-Px) is a selenoprotein located in the mitochondrial matrix and cytosol of animal cells (Chihuailaf *et al.*, 2002). The mechanism of protection against

oxidative stress is by converting the reduced glutathione (GSH) into oxidized glutathione (GSSG), removing H_2O_2 and forming water (Ferrari *et al.*, 1985). GSH-Px also acts on other hydroperoxides other than hydrogen peroxides (Murray *et al.*, 2004).

$$2GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG + 2\,H_2O \qquad (9)$$

From the foregoing, the three primary antioxidant enzymes (SOD, CAT, GSH-PX) prevent the accumulation of superoxide radical and hydrogen peroxide, which are substrate for the formation of the highly active hydroxyl radical, that lacks defensive enzymatic detoxification system (Pal, 1994). The perfect balance between the primary antioxidant enzymes is of great importance for the maintenance of cellular integrity (Schneider and de Oliveira, 2004).

Glutathione reductase catalyzes the reduction of low molecular weight disulphides (such as GSSG) using NADH as an electron source (Yu, 1994; Marks *et al.*, 1996). Some glutathione transferases show peroxidase-like activity including that against phospholipid hydroperoxides, suggesting its contribution to cellular antioxidant defence (Stocker and Keaney Jr, 2004).

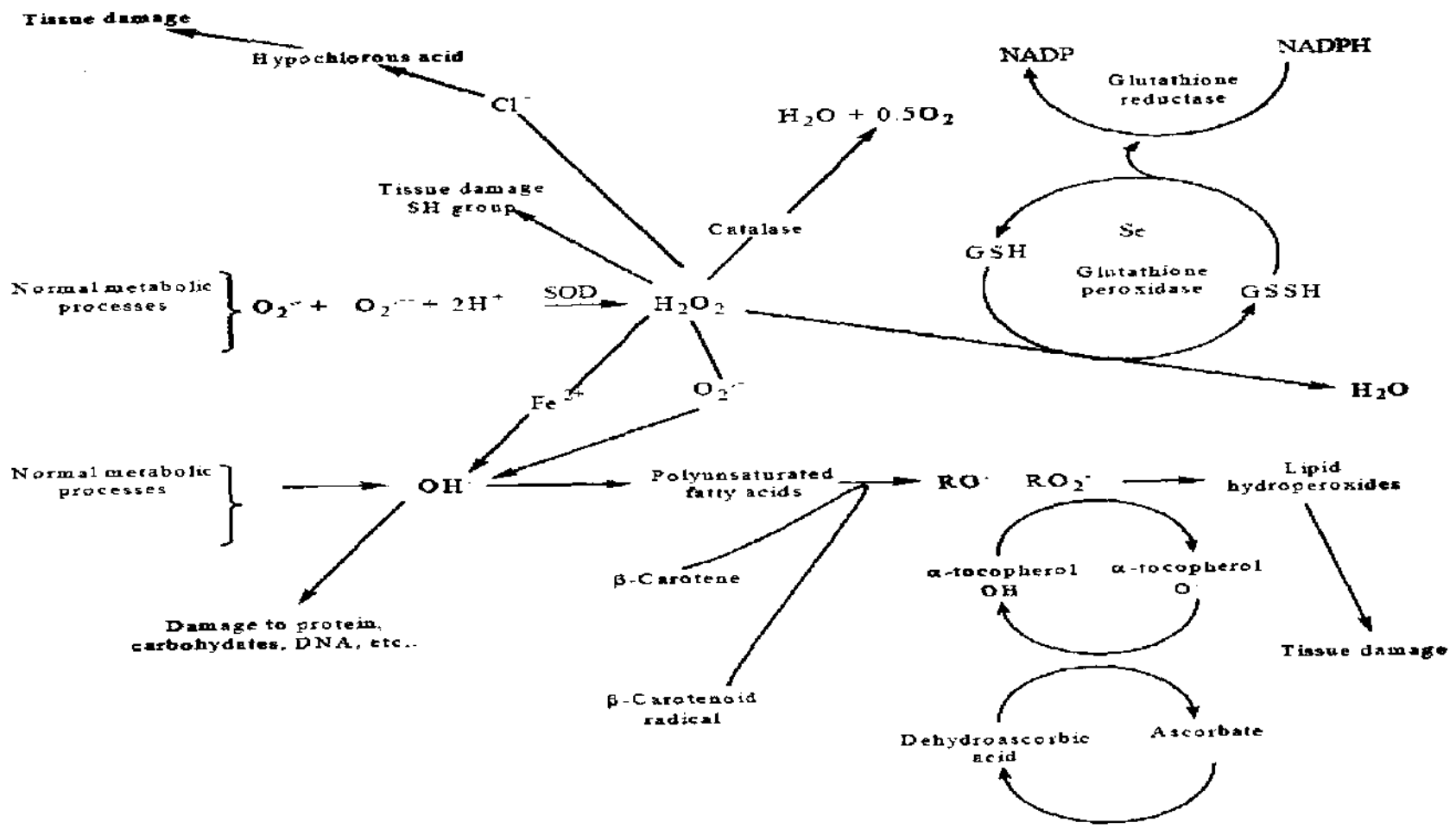


Figure 2.8: Inter-relationship between reactive oxygen species and antioxidants (Adapted from Bahorun *et al.* (2006)

2.5 Vitamin C

Vitamin C (Ascorbic acid, AA) is an essential water-soluble vitamin present in plant and animal cells. The chemical name is 2-oxo-l-threo-hexono-1,4-lactone-2,3-enediol (Naidu, 2003). Its essentiality as a micronutrient has been known since the late 1900s, when the British Navy supplemented the diet of their sailors with citrus fruits to prevent scurvy. It was first isolated in 1928 by the Hungarian biochemist and Nobel laureate winner SczentGyorGyi (Iqbal *et al.*, 2004). AA is widely distributed in fresh fruits and vegetables. It is a six-carbon lactone synthesized by most plants and animals from D-glucose or D-galactose (Naidu, 2003; Padayatty *et al.*, 2003). The site for the synthesis of AA for many animals is the liver (Naidu, 2003; Padayatty *et al.*, 2003), while kidney is the major site of synthesis in birds and other non-human primates (FAO, 2000). However, guinea pigs, fruit-eating bats, reptiles, primates including man cannot synthesize AA *de novo* due to substantial mutation in the gene responsible for the synthesis of L-gulonolactone oxidase, the final enzyme in its biosynthetic pathway from glucose (Burns, 1959; Nishikimi *et al.*, 1994; Nishikimi and Yagi, 1996). Therefore, humans and all the animals that cannot synthesize AA must have their diet supplemented with AA. The pathway for the synthesis of AA from glucose or galactose is highlighted in Figure 2.9.

Due to its asymmetric ring structure, AA may exist in four stereoisomers. L-ascorbic acid and dehydroascorbic acid (DHAA) are the major dietary forms of AA, and are the most biologically active form (Moser and Bendich, 1990; Trout, 1991). The pH influences the stability of AA in food as it exhibits maximal stability between pH of 4 and 6 (Moser and Bendich, 1990). It is a

labile molecule, and thus may be lost from food during cooking or processing (Naidu, 2003).

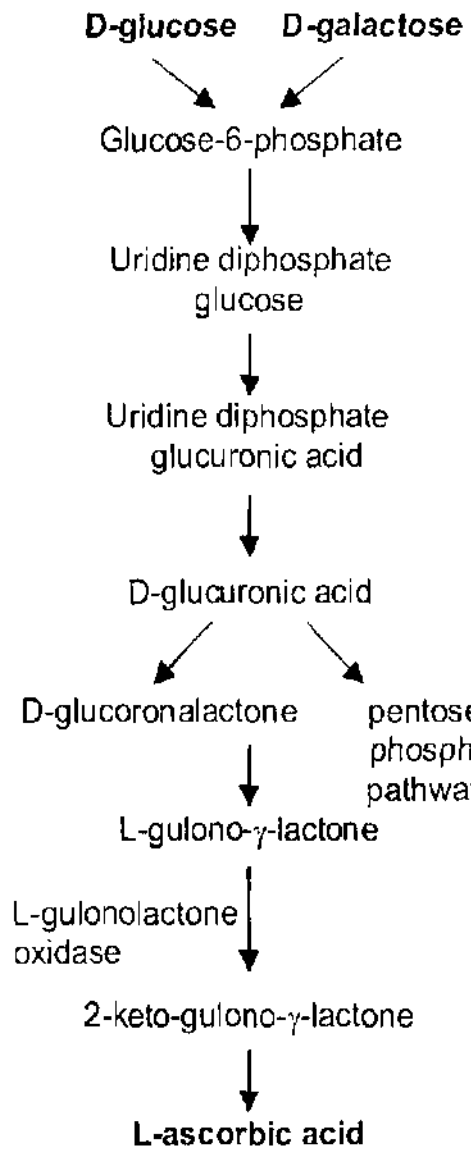


Figure 2.9 Biosynthesis of L-Ascorbic acid in animals (Source: Naidu, 2003)

Therefore, most plants containing AA are best consumed raw to avoid any loss in the content of the vitamin (Anon, 2007). AA is equally sensitive to air, light, heat and easily destroyed by prolonged storage and over-processing of food (Naidu, 2003).

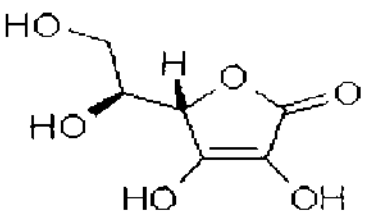


Figure 2.10 – Chemical structure of ascorbic acid

2.5.1 Absorption, metabolism and excretion

In humans and guinea pigs, the absorption of AA occurs in the buccal mucosa, stomach and the small intestine (Iqbal *et al.*, 2004). The buccal absorption of AA is mediated by passive diffusion through the membrane of the buccal cavity (Iqbal *et al.*, 2004). However, absorption from the gastrointestinal tract (GIT) is by an active, sodium-dependent energy requiring carrier-mediated transport mechanism (Stevenson, 1974; Gaby and Singh, 1991; McCormick and Zhang, 1993). The major intestinal AA transporter is sodium-dependent vitamin C transporter 1 (SVCT 1) (Anon, 2007). Some AA may be oxidized to DHAA and transported into the enterocytes via glucose transporters. Dietary DHAA is absorbed from the lumen of the small intestine precisely in the jejunum (Groff *et al.*, 1995) into the enterocytes (Anon, 2007). The process of intestinal absorption is saturable because as the intake increases, the tissue becomes progressively more saturated, and the degree of intestinal absorption decreases (Groff *et al.*, 1995). Eighty to ninety five percent of the AA found in food is absorbed (Groff *et al.*, 1995). However, the bioavailability of the synthetic and the "natural" form of the vitamin differs very little (Groff *et al.*, 1995; Jacob, 1999). A number of factors impair the absorption of AA. They include the administration of a single large dose, which saturates the enzyme kinetics for AA, leading to excess AA in the intestinal lumen. High iron concentration in the GIT may cause oxidative

destruction, which in turn impair uptake (Groff *et al.*, 1995). Pectin and zinc also impair the absorption of AA by an unknown mechanism (Groff *et al.*, 1995).

All DHAA within the enterocytes is reduced to AA via reduced glutathione and dehydrascorbate reductase, and AA leaves the enterocytes to enter first the portal and subsequently the systemic circulation (Anon, 2007). AA is distributed to various tissues of the body (Groff *et al.*, 1995; Anon, 2007). Some tissues have greater concentration of AA than others. The highest concentration is found in the adrenal medulla, the pituitary gland, fibroblasts, chondrocytes and osteoblasts (Groff *et al.*, 1995). Large amounts of AA are also found in the neutrophils, monocytes, macrophages, the eyes and the nervous tissue (Stipanuk, 2006). Erythrocytes are the only cells that do not take up AA (Stipanuk, 2006). Tissue concentration exceeds those found in the plasma by three to ten times. This is because of the effect of energy-driven transport pumps (Groff *et al.*, 1995). There are two known mechanisms for transporting AA (Liang *et al.*, 2001). A universal system present in all cells is responsible for transportation of AA as DHAA into the tissue, via facilitative glucose transporters (GLUT-1, GLUT -3 and GLUT-4) (Vera *et al.*, 1993; Stipanuk, 2006, Anon, 2007). The second transport system is functional in specialized cells, where AA is directly transported into cells via sodium-dependent vitamin C transporters ($SVCT_1$ and $SVCT_2$) (Tsukaguchi *et al.*, 1999). $SVCT_1$ is responsible for transportation of AA into the intestinal, liver and kidney cells (Anon, 2007). The transporter $SVCT_2$ aids in the transportation of AA into the aqueous humour of the eye (Anon, 2007). The GLUT-1 transport system is responsible for the transportation of AA to the brain and neutrophils after its oxidation to DHAA. In the brain DHAA is transported across the blood-brain barrier by facilitative diffusion via GLUT-1. The DHAA is next transported through GLUT-1 at the surface of the blood-brain

barrier endothelial cells. DHAA is transported out of the endothelial cells through GLUT-1. DHA in the brain is then reduced to AA, which is essentially trapped in the brain since it cannot be transported through GLUT-1 (Anon, 2007).

Ascorbic acid is either oxidized back to DHAA, which can either be reduced back or dismutate to AA or otherwise hydrolyse irreversibly to 2,3-diketoglutaric acid (Padayatty *et al.*, 2003), formed by irreversible rupture of the lactone ring structure that is part of AA, scorbyl radical and DHAA. 2,3-diketoglutaric acid is further metabolized into xylose, xylonite, lyxonate, threonate and oxalate (Lewin, 1976; Halliwell, 1996a; Padayatty *et al.*, 2003; Anon, 2007) (Figure 2.11). The formation of oxalate is of clinical significance because hyperoxaluria (overexpression of oxalate) can result in oxalate kidney stone in some individuals (Padayatty *et al.*, 2003).

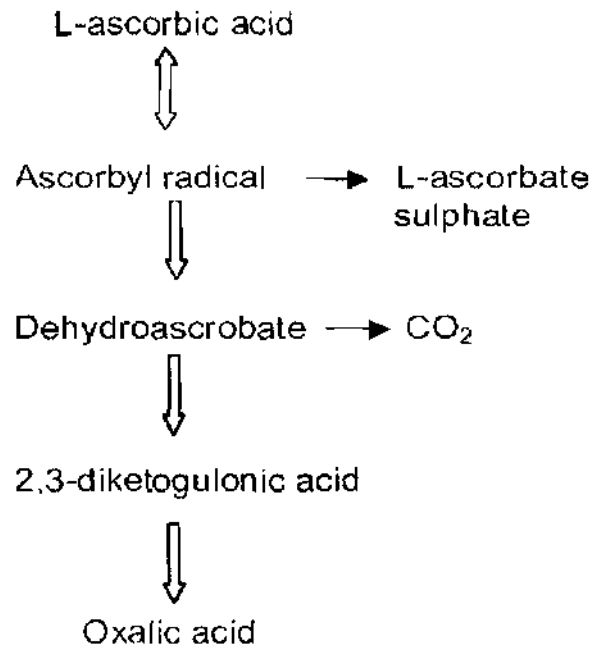


Figure 2.11 Metabolic Pathway for Ascorbic Acid (Adopted from Naidu, 2003)

As a water-soluble vitamin, AA and its metabolites are excreted via the kidneys (Groff *et al.*, 1995; Naidu, 2003). The average half-life of AA is believed to be between 16-20 days (Jacob, 1999), and this is inversely related to the intake (Groff *et al.*, 1995). The physiologically efficient renal tubular reabsorption mechanism retains AA in the tissues up to a whole body content of ascorbate of about 20 mg/kg body weight (Kallner *et al.*, 1979). The renal reabsorption process has a threshold (0.8-1.4 mg/dl of plasma ascorbate) and when this value exceeded the threshold, then AA will be excreted rather than reabsorbed by the kidneys (Groff *et al.*, 1995; Levine *et al.*, 1996; Graumlich *et al.*, 1997).

2.5.2. Physiological role of ascorbic acid

The physiological functions of AA are largely dependent on its oxido-reduction properties (Naidu, 2003). AA acts as an electron donor for 11 enzymes (Englard and Seifter, 1986; Levine, 1986). Three of those enzymes are found in fungi, but not in humans and other mammals (Wondrack *et al.*, 1978; Stubbe, 1985), and are involved in the reutilization pathways for pyrimidines and deoxyribose moiety of deoxynucleosides. Among the eight remaining enzymes found in humans and other mammals, three participate in collagen hydroxylation (Kivirikko and Myllyla, 1985; Peterkofsky, 1991; Prockop and Kivirikko, 1995) and two in carnitine biosynthesis (Dunn *et al.*, 1984; Rebouche, 1991). Of the three enzymes which participate in collagen hydroxylation, one is necessary for the biosynthesis of norepinephrine from dopamine (Kaufman, 1974; Levine *et al.*, 1992), one is necessary for amidation of peptide hormones (Eipper *et al.*, 1992, 1993, Levine *et al.*, 2000) and one is involved in tyrosine metabolism (Lindblad *et al.*, 1970; England and Seifter, 1986).

One of the most important functions of AA is its role in hydroxylation reaction that is essential for the formation of collagen. This is possible because it allows for a tight cross-linking of the triple helix, thereby resulting in the stabilization of peptides (Groff *et al.*, 1995). It is also believed that AA may be involved in collagen gene expression through mechanism that is well known (Anon, 2007).

Ascorbic acid serves as a reducing agent in the synthesis of carnitine (Hulse *et al.*, 1978; Groff *et al.*, 1995). Carnitine facilitates the beta-oxidation of fat, through its role in transporting long chain fatty acids from the cytoplasm into the mitochondrial matrix of cardiac and skeletal muscle for ATP generation (Levine *et al.*, 2000). AA is also involved directly in the enzyme activity of two copper-dependent mono-oxygenases, which are important in the formation of norepinephrine and serotonin (Groff *et al.*, 1995; Levine *et al.*, 1995; 2000; Jacob, 1999).

In the CNS, AA regulates the activity of some neurones within the brain, such as neurotransmitter membrane receptor synthesis, and neurotransmitter dynamics. Animal studies have also shown that it is an important factor in the development of the nervous system, specifically in the maturation of glial cells and myelin (Groff *et al.*, 1995).

Ascorbic acid is necessary for the transformation of cholesterol to bile acids as it modulates the microsomal 7α-hydroxylation, the rate limiting step in cholesterol catabolism in the liver (Naidu, 2003). In AA deficiency, this process slows down, thereby resulting in an accumulation of cholesterol in the liver, hypercholesterolaemia and formation of cholesterol gall stones (Ginter *et al.*, 1982; Naidu, 2003).

Ascorbic acid also enhances the availability and absorption of iron from soluble non-haeme iron sources (Hallberg *et al.*, 1987), probably as a result of reduction of iron by AA or possibly by chelation (Hallberg *et al.*, 1982; 1987; Bendich and Cohen, 1990). AA may also have vasodilatory and anticlotting effect within the body by stimulating nitric oxide release (Anon, 2007). AA is protective against the development of atherosclerosis by inhibiting oxidative modification of low-density lipoprotein (LDL) (Frei, 1991), primarily by scavenging FRs and other ROS in the aqueous milieu (Frei *et al.*, 1989).

Ascorbic acid plays an indirect regulatory role throughout the entire body due to its involvement in the synthesis of hormones, hormone-releasing factors and neurotransmitters (Groff *et al.*, 1995). AA intake at moderate doses is important and safe for bone maintenance and, therefore, a factor in mitigating or delaying osteoporosis (Gaby and Singh, 1991).

On the immune system, AA modulates the functions of phagocytes such as chemotaxis (Vohra *et al.*, 1990; Maderazo *et al.*, 1991; Johnston *et al.*, 1992; Levy *et al.*, 1996), as well as activity of natural killer cells and the functions and proliferation of lymphocytes (Smit and Anderson, 1990; Heuser and Vojdani, 1997; Hemila, 1997). It also affects the production of immune proteins such as cytokines and antibodies as well as complement components (Haskell and Johnston, 1991; Tanaka *et al.*, 1994; Hemila, 1997). Delayed hypersensitivity response is also modulated by the antioxidant micronutrient, AA (Kelley and Bendich, 1996; Bendich, 1997).

High doses of AA have been advocated in the treatment and prevention of recurring cancer (Cameron and Pauling, 1973; Douglas *et al.*, 2000; Drisko *et al.*, 2003a and b; Zou *et al.*, 2006).

The benefits of AA include increased sense of well-being or much improved quality of life, prolongation of survival times in terminal patients and complete regression in some cases (Cameron and Pauling, 1976; 1978; Murata *et al.*, 1982). Although the molecular mechanisms of anti-carcinogenic effect of AA have not been completely elucidated, studies have shown that it may inhibit cell proliferation by interfering with cell cycle (Naidu *et al.*, 2003). Considerable epidemiological evidence has shown that high intake of AA reduces the incidence of cancer. Biochemical and physiological evidences suggest that AA functions as a free radical scavenger and inhibits the formation of potentially carcinogenic N-nitroso compounds from nitrates, nitrites in stomach and, thus, offers protection against stomach cancer (Schorah *et al.*, 1991, Sobala *et al.*, 1991; Drake *et al.*, 1996).

2.5.3 Vitamin C as an antioxidant

Ascorbic acid is an important water-soluble antioxidant in biological fluids (Frei *et al.*, 1989, 1990). It readily scavenges physiologically relevant ROS and RNS such as superoxide, hydroxyl and aqueous peroxyl radicals, as well as non-radical species such as singlet oxygen, ozone, peroxynitrite, nitrosating species (N_2O_3/N_2O_4), nitroxide radicals, nitrogen dioxide and hypochlorous acid (Nishikimi, 1975; Bodannes and Chan, 1979; Halliwell, 1996b; Carr and Frei, 1999); thus, it effectively protects the body's substrate from oxidative damage. In addition to direct quenching of FRs, AA interacts with tocopheroxyl radical and generates the reduced tocopherol (Bendich *et al.*, 1984, 1986). Therefore, administration of high concentration of AA leads to higher concentration of vitamin E. AA has also been shown to regenerate urate, glutathione and β-carotene from their respective one-electron oxidation products; that is, urate, glutathyl and β-carotene radical cations (Halliwell, 1996a and b; Edge and Truscott, 1997).

As an antioxidant, the primary role of AA is to neutralize FRs. Since AA is water soluble, it can work both inside and outside the cells to combat FR damages (Iqbal *el al.,* 2004). This antioxidant effect is observed in virtually all the body systems. AA protects the DNA of the cells from the damage caused by free radicals and mutagens. It prevents harmful genetic alterations within cells and protects lymphocytes from mutations to the chromosomes (Gaby and Singh, 1991). AA prevents free radical damage in the lungs and may even help to protect the CNS from such damage (Kronhausen *el at.,* 1989). AA protects sperm from oxidative damage (Fraga *el al.*, 1991), improves sperm quality in smokers (Dawson *el al.,* 1992) and is effective in treating sperm agglutination, a condition which causes sperm to stick together (Dawson *el al.,* 1983). AA fights off widespread environmental pollutants, including CO, hydrocarbons, pesticides and heavy metals by stimulating enzymes in the liver that detoxify the agents (Iqbal *el al.,* 2004).

2.5.3.1 Mechanisms of antioxidant effects of vitamin C

Two major properties of AA make it an ideal antioxidant. First, the low one-electron reduction potentials of ascorbatc (282 mV) and its one-electron oxidation product, the ascorbyl radicals (2174 mV), which enables ascorbatc and the ascorbyl radicals to react with and reduce basically all physiologically relevant radicals and oxidants (Halliwcll, 1996a). For this reason, AA has been said to be "at the bottom of the pecking order" and to act as the terminal water-soluble small molecule antioxidant in biological system (Buettner, 1993). The second property is the stability and low reactivity of the ascorbyl radical formed when ascorbate scavenges ROS and RNS(Ilalliwell, 1996a).

Ascorbic acid owes its biochemical and physiological actions to ability to donate electron (Padayatty *et al.*, 2003). Therefore, as an electron donor, AA is a reducing agent. It serves as an antioxidant because by donating its electron, it prevents other compounds from being oxidized. By the nature of the reaction leading to this process, AA is also oxidized (Padayatty *et al.*, 2003). The species formed after the loss of one electron is a FR, semidehydroascorbic acid or an ascorbyl radical, which is fairly unreactive relatively stable compound, with a half-life of 10^{-15} seconds (Padayatty *et al.*, 2003). Therefore, the interaction of AA with a possibly harmful reactive FR results in the reduction of the latter, while the resultant ascorbyl radical formed is less harmful and less reactive. This process is known as FR scavenger or quenching (Padayatty *et al.*, 2003). Hence, AA is a good FR scavenger due to its chemical properties (Bielski *et al.*, 1975; Buettner and Mosley, 1993). Ascorbyl radical, with its unpaired electron, is not a long-lived compound, as it forms DHAA upon the loss of a second electron (Padayatty *et al.*, 2003). The stability of DHAA depends on factors such as temperature and pH, but it is often in minutes (Washko *et al.*, 1993). Once formed, ascorbyl radical and DHAA can be reduced back to AA by at least three enzyme pathways, and by reducing compounds in biological systems such as glutathione (Padayatty *et al.*, 2003). The ascorbyl radical readily dismutates to form ascorbate by an NADH-dependent semihydroascorbate reductase (Tsao *et al.*, 1987; Packer and Fusch, 1997; Wells and Jung, 1997) (Figure 2.12). The 2-electron oxidation product of ascorbate, DHAA, can itself be reduced back to ascorbate by glutathione, the glutathione-dependent enzyme glutathione:dehydroascorbate oxidoreductase (glutathione dehydrogenase (ascorbate) or glutaredoxin) or the NADPH-dependent selenoenzyme thioredoxin reductase (Tsao *et al.*, 1987; Packer and Fusch, 1997; Wells and Jung, 1997). If DHAA is not reduced back to AA, it is hydrolysed rapidly and metabolized into xylose, xylonite, lyxonate and oxalate (Lewin, 1976;

Halliwell, 1996b). In humans, there is only partial reduction of DHAA back to AA, therefore not all AA that is oxidized is recovered, as some is lost via hydrolysis, otherwise humans would not get scurvy (Padayatty *et al.*, 2003).

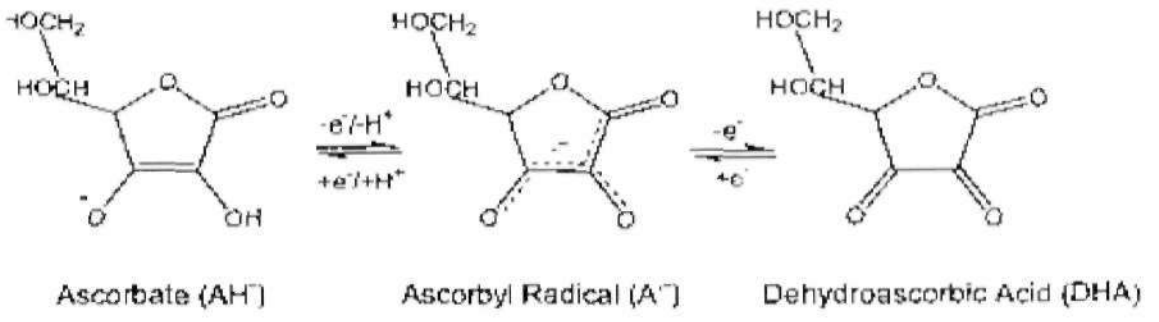


Figure 2.12: Oxidation of ascorbate (AH^-) by two successive one electron oxidation steps to give the ascorbyl radical (A^-) and a dehydroascorbic acid (DHAA), respectively (Adopted from Carr *et al.*, 1999)

2.6 VITAMIN E

Vitamin E is the collective name for the eight molecules synthesized by plants that have a chromanol head group and a phytyl tail (tocopherols) or an isoprenoid tail (tocotrienols) and exhibit the antioxidant activity of α-tocopherol (Sheppard *et al.*, 1993; Traber and Kamal-Eldin, 2007). These eight molecules are α, β, γ and δ -tocopherols, and α-, β-, γ- and δ-tocotrienols. Vitamin E is an essential micronutrient that was discovered in 1922 by Herbert M. Evans, while conducting research in nutrition and fertility (Evans and Bishop, 1922). He observed that foetal resorptions occurred in vitamin E-deficient diet, and was therefore described as a plant extract essential for maintaining fertility, from where the term tocopherol was coined. Tocopherol came from the word *Tocos*, the Greek word for 'birth', *Ferein*, Greek word for 'bringing', while the ending 'ol' represents an 'alcohol' (Papas, 1999a). The four tocopherols were isolated during the late 1930s (Evans *et al.*, 1936) and the four tocotrienols were isolated many years later (Pennock *et al.*, 1964). Vitamin E is an essential micronutrient and, therefore, must be obtained from the

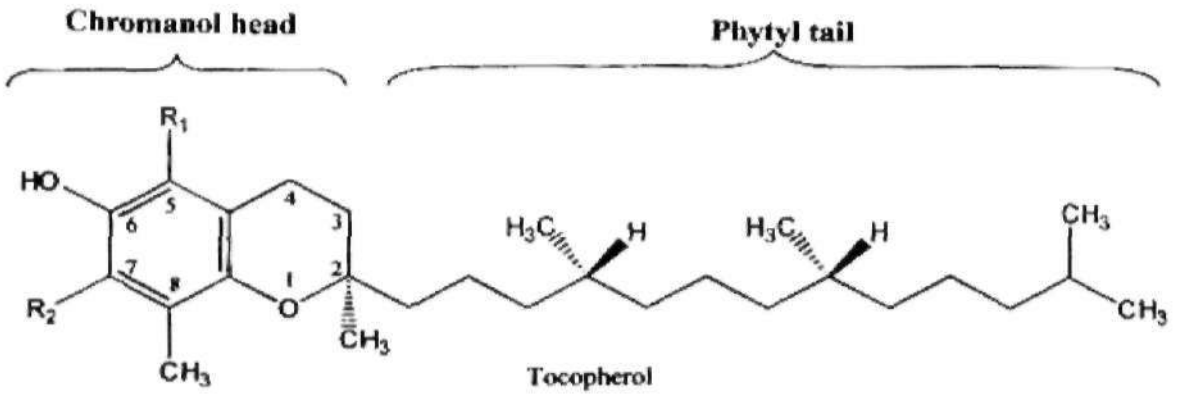
diet. The most biologically active form of vitamin E is RRR-α-tocopherol (formerly D-α-tocopherol), which accounts for approximately 90% of the vitamin E found in animal tissues (Bjorneboe *et al.*, 1990; Traber *et al.*, 1992; Bramley *et al.*, 2000; Debier and Larondelle, 2005).

2.6.1 Absorption, distribution, metabolism and excretion of vitamin E

Vitamin E is a fat soluble vitamin and therefore is absorbed the same way as other lipids. The absorption of vitamin E from the intestinal lumen is dependent upon processes necessary for fat digestion and uptake into the enterocytes (Traber and Kamal-Eldin, 2007). Absorption of vitamin E occurs in the jejunum via passive diffusion (Erdman *et al.*, 1988; Sokol, 1988). This process is facilitated by pancreatic esterases and bile acids, which are required for the release of free fatty acids from dietary triglycerides and the formation of micelles for absorption (Sokol *et al.*, 1983).

Absorption of vitamin E is also dependent on the fat content of food, with little absorption from vitamin E supplement in the absence of fats (Bender, 1992; Borel *et al.*, 2001; Hayes *et al.*, 2001; Bruno *et al.*, 2006). A study has shown that a mixture of carotenoids, ß-carotene, lycopene and lutein reduce the absorption of vitamin E, while the γ-tocopherol reduces the aborption of the α-form (Reboul *et al.*, 2007).

After absorption by the intestinal wall, the micelles containing the tocopherols forms chylomicrons and these are secreted into the lymph. In the lymph, the chylomicrons are catabolized by lipoprotein lipase, and some tocopherols are transferred from the chylomicron remnants to lipoproteins or tissues (Bruno *et* al., 2006; Traber and Kamal-Eldin, 2007). Most chylomicron remnants become bound by apolipoprotein E and travels to the liver, which has specific apolipoprotein E receptors.



R_1	R_2	Compound
CH_3	CH_3	α
CH_3	H	β
H	CH_3	γ
H	H	δ

Figure 2.13 - Structures of various isomers of vitamin E

Once chylomicron remnants reach the liver, the dietary fats are repackaged and secreted into the plasma in very low density lipoproteins (VLDL) (Havel, 1994). The α-tocopherol transfer protein (α-TTP) present in the liver preferentially binds and transfer α-tocopherol to the endoplasmic reticulum for its incorporation into nascent VLDL and their subsequent release into

the circulation (Kayden and Traber, 1993). Plasma α-tocopherol concentrations are dependent upon the hepatic α-TTP (Hosomi *et al.*, 1997; Terasawa *et al.*, 2000; Yokota *et al.*, 2001; Leonard *et al.*, 2002); and in humans, a genetic defect in α-TTP results in severe vitamin E deficiency (Cavalier *et al.*, 1998). A loss of α-TTP also impairs the ability to discriminate between RRR- and SRR- α -tocopherols (Leonard *et al.*, 2002; Traber *et al.*, 1993). In the plasma, VLDLs are hydrolyzed by lipoprotein lipases to low density lipoproteins (LDLs), which contain the highest concentration of plasma tocopherols and LDL readily exchange tocopherols with high density lipoproteins (HDL) (Herrera and Barbas, 2001; Traber and Kamal-Eldin, 2007). Since HDL readily transfers vitamin E to other lipoproteins, vitamin E is distributed to all circulating proteins (Traber *et al.*, 1990). It has been demonstrated that phospholipids transfer proteins catalyze vitamin E exchanges between lipoproteins (Kostner *et al.*, 1995). Tocopherols in HDL may be transferred back to chylomicron remnants in the plasma and return to the liver (Papas, 1999b). The process of tissue uptake of tocopherol is not completely understood. As VLDLs are hydrolyzed by lipoprotein lipase, tocopherols may enter tissues, or the tocopherols incorporated in LDL may be transferred to tissues via the LDL receptors located on tissue surfaces. Other tocopherols may be taken up by membrane binding sites recognizing apolipoprotein A-I and A-II present on HDL (Traber, 1996) or by direct transfer of tocopherol across the tissue cell membranes down a concentration gradient (Traber and Kayden, 1984; Groff *et al.*, 1995; Papas, 1999b). Tocopherols may be transported via HDL lipid uptake by the scavenger receptor-B1(SR-B1) [this latter route is important for vitamin E delivery to the lungs (Guthman *et al.*, 1997; Kolleck *et al.*, 2000; 2002), brain (Goti *et al.*, 2000; 2001) and liver (Witt *et al.*, 2003); as well as mediated by various transporters such as ABCA1 (Oram *et al.*, 2001)].

Once inside the cell, tocopherol binds to tocopherol binding proteins for transport to cells, mitochondrial and microsomal membranes (Farrell and Roberts, 1994).

Vitamin E is distributed throughout the body tissues. A large percentage is stored in the adipose tissue with smaller amounts in the liver, lungs, heart, muscles, adrenal glands and the brain (Burton *et al.*, 1998). The low vitamin E storage in the liver may explain its low toxicity (Kappus and Diplock, 1992). The amount of vitamin E in the adipose tissue increases as the intake increases. However, the levels in other tissues remain constant or increase at a very slow rate. When the intake of vitamin E is low, there is fairly rapid withdrawal of vitamin E from the liver and plasma, but that from the adipose tissue occurs slowly (Diplock, 1985). The release of α-tocopherol from adipose tissue seems to play an important role in keeping plasma levels under deficiency conditions. In contrast to what happens during lipolysis, tocopherol does not decrease in adipose tissue when food intake is reduced in obese subjects (Schaefer *et al.*, 1983) or under fasting conditions in subjects with normal weight (Brouwer *et al.*, 1998a). These indicate that there is another mechanism for the release of α-tocopherol from the adipose tissue different from the one normally invoved in the release of triglycerides. In summary, the mechanisms for the release of α-tocopherol from tissues are unknown and no organ is known to function as a storage organ for α-tocopherol, releasing it on demand (Traber and Kamal-Eldin, 2007). However, the contents of α-tocopherol in peripheral nerves has been correlated to its concentration in adipose tissue (Kayden *et al.*, 1983), while its content in adipose tissue of vitamin E-deficient animals has been shown to be lower than normal (Kayden *et al.*, 1983), suggesting the capacity of adipose tissue to release α-tocopherol.

Studies have shown that α–tocopherol in a group of tissue is in rapid equilibrium with the plasma α-tocopherol pool (Ingold *et al.*, 1987; Burton *et al.*, 1990; 1998; Leonard *et al.*, 2002). Erythrocytes, liver and spleen have a high turn-over times where they quickly replace 'old' with 'new' α-tocopherol, while other tissues such as heart or muscle have a slower α-tocopherol turn-over times (Traber and Kamal-Eldin, 2007). The α-tocopherol content of the CNS is spared during α-tocopherol depletion (Vattassery, 1978; Meydani *et al.*, 1986; Bourre and Clement, 1991). However, the peripheral nerves (Sokol, 1993), especially the sensory neurones are the most susceptible to vitamin E deficiency (Traber *et al.*, 1987).

Within the tissues, the highest concentration of tocopherols is found in the subcellular fraction tissues within membraneous organelles such as microsomes and mitochondria, which are involved in highly oxidative-reductive reactions (Taylor *et al.*, 1976).

The vitamin E metabolites, α-CEHC (2,5,7,8-tetramethyl-2-(2-carboxyethyl)-6-hydroxychroman) and γ-CEHC (2,7,8-trimethyl-2-(2carbo-xyethyl)-6-hydroxychroman) are derived from α-tocopherol and α-tocotrienol, and from γ-tocopherol and γ-tocotrienol, respectively (Birringer *et al.*, 2002; Lodge *et al.*, 2001). These forms of vitamin E appear to be metabolized similarly to xenobiotics in that they are ω-oxidized by cytochrome P_{450s} (CYPs), then conjugated and excreted in urine (Brigelius-Flohe and Traber, 1999) or bile (Kiyose *et al.*, 2001). Hepatic CYP 4F2 catalyzes the ω-oxidation of α- and γ-tocopherols (Sontag and Packer, 2002), but CYP 3A may also be involved (Parker *et al.*, 2000; Birringer *et al.*, 2001, 2002; Ikeda *et al.*, 2002). Similar to other xenobiotics, CEHCs are sulphated or glucuronidated (Stahl *et al.*, 1999; Swanson *et al.*, 1999; Pope *et al.*, 2002). Alpha-tocopherol is excreted into bile via MDR2

(p-glycoprotein) (Mustacich *et al.*, 1998), an ATP-binding cassette phospholipid transporter that facilitates the excretion of biliary phospholipids (Traber and Kamal-Eldin, 2007). The summary metabolic pathways for vitamin E is shown in Figure 2.14

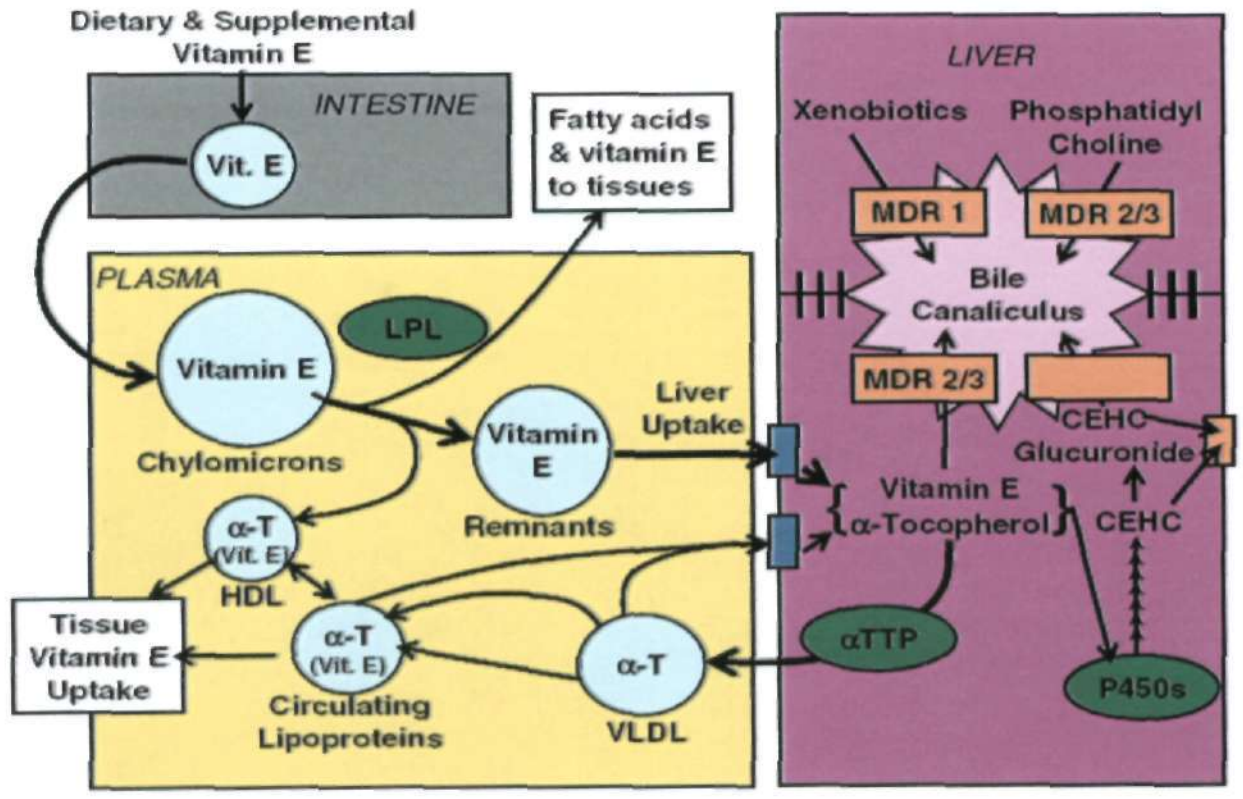


Figure 2.14 Metabolic pathways for vitamin E (adapted from Traber, 2007)
All forms of vitamin E are absorbed in the intestine (*gray*) and secreted into the circulation in chylomicrons. Lipoprotein lipase (lpl) hydrolyzes the chylomicron triglyceride and transfers fatty acids, as well as vitamin E, to tissues. During the formation of chylomicron remnants in the plasma compartment (*yellow*), some of the vitamin E is transferred to high-density lipoproteins (HDLs) and subsequently to other lipoproteins. The chylomicron remnants are taken up by the liver, where the α-tocopherol transfer protein (α-TTP) salvages α-tocopherol (α-T) from the lysosomal degradation pathway and returns it to the circulating lipoproteins, principally very-low-density lipoproteins (VLDLs). During lipoprotein catabolism in the circulation, α-T is redistributed among the various lipoproteins. Lipoproteins are taken up by the liver (and peripheral tissues) by various receptors (*blue rectangles*), and thus tocopherols are delivered to tissues by this process. In the liver, excess α-T and other vitamin E forms can be excreted into bile via the multidrug-resistance gene products (MDR 2/3), e.g., p-glycoprotein [ATPbindingcassette (ABC) and other transporters; *orange rectangles*]. Excess vitamin E is also metabolized by a cytochrome P450 (CYP)-mediated process to carboxy ethyl hydroxy chromans (CEHCs) that can be glucuronidated (or sulfated) and excreted in bile or urine. High α-T concentrations in the liver upregulate various xenobiotic pathways, including CYP3A and MDR1.

2.6.2 Vitamin E as an Antioxidant

Vitamin E is a major lipid soluble antioxidant present in all cellular membranes protecting against lipid peroxidation (Machlin, 1980). Thus, it assists in stabilizing the cell membranes and maintenance of their permeability (Bjørneboe *et al.*, 1990, Navarro *et al.*, 1999). It functions as chain-breaking antioxidant by preventing chain initiation and propagation of FR reactions and lipid peroxidation in cell membranes (Ingold, 1961; Tappel, 1962; Burton and Ingold, 1986; Kamal-Eldin and Appelqvist, 1996). Vitamin E acts directly on a variety of oxyradicals, including peroxy radical, (d_3 and $OH^{\cdot}$) (Burton *et al.*, 1985; McCay, 1985), superoxide radical ($O_2^{\cdot-}$) (Fukuzawa and Gebicki., 1983; Ozawa *et al.*, 1983) and singlet oxygen (Fahrenholtz *et al.*, 1974; Littarru *et al.*, 1984). Alpha-tocopherol is both an intracellular and intercellular antioxidant. Despite the fact that the concentration of α-tocopherol in the cell membrane is low (less than 1 molecule of α-tocopherol per 1000 lipid molecules), it is considered the main lipid soluble antioxidant in the body (Herrera and Barbas, 2001). Vitamin E may also affect oxidative changes which occur in other cell organelles (Ibrahim *et al.*, 2000). Moreover, it is known that antioxidants such as Vitamin E, coenzyme Q, AA, glutathione (GSH) and selenium may act synergistically, preventing lipid peroxidation and cell destruction (Beyer 1994; Chen and Tappel, 1995; Navarro *et al.*, 1999; Lass and Sohal, 2000). In addition to its antioxidant function, vitamin E influences the cellular response to oxidative stress through modulation of signal-transduction pathways (Azzi *et al.*, 1992).

2.6. 2.1 Mechanisms of antioxidant action of vitamin E

The phytyl tail of the tocopherol molecule allows the positioning of the molecule within the membrane bilayer so that the active chroman ring lies close to the surface of the membrane

(Kagan *et al.*, 1990). As a phenolic antioxidant, vitamin E readily donates hydrogen from the hydroxyl (-OH) group on the ring structure to the FRs, making them unreactive. On donating the hydrogen, vitamin E becomes relatively unreactive FR because the unpaired electron on the oxygen atom is usually delocalized into the aromatic ring structure thereby increasing its stability (Scott, 1997). Specifically, the pathway for achieving the antioxidant effect of α-tocopherol on lipid peroxidation is highlighted in Figure 2.15 below. Briefly the FR in lipid membrane is initiated when one PUFA in the cell membrane phospholipid loses one hydrogen by reacting with FR ($R^{\cdot}$) to become FR of PUFA ($PUFA^{\cdot}$). $PUFA^{\cdot}$ then react with oxygen to produce peroxyl FR of PUFA ($PUFA\text{-}OO^{\cdot}$), which may also affect other PUFA in a chain propagation step. Tocopherol (TocH) then react with $PUFA\text{-}OO^{\cdot}$ resulting in the production of hydroperoxide of PUFA (PUFA-OOH), which is no longer a FR, and α-tocopheroxyl radical ($Toc^{\cdot}$). This process is possible because the phenolic hydroxyl group of the chromanol ring reacts with an organic peroxyl radical to form the corresponding or $PUFA\text{-}OO^{\cdot}$ and the $Toc^{\cdot}$ (Burton *et al.*, 1985). $Toc^{\cdot}$ may also combine with PUFA-OOH and again produce $PUFA\text{-}OO^{\cdot}$, which may be considered part of the prooxidant effect of α-tocopherol (Nagaoka *et al.*, 2000).

However when lipid hydroperoxides are oxidized to peroxyl radicals ($PUFA\text{-}OO^{\cdot}$), they react 1000-times faster with α-tocopherol than with PUFA (Traber and Kamal-Eldin, 2007). Meanwhile, the prooxidant action of α-tocopherol is suppressed when TocH is regenerated by the reaction of AA, a hydrogen donor, together with reduced form of glutathione (GSH) (Miki *et al.*, 1987) at the interphase of cell membrane and aqueous phase (cytosol) or with ubiquinone (UQH2) within the cell membrane. All these reactions are essentially proton-hydrogen transfer reactions (Herrera and Barbas, 2001). In this TocH regeneration reaction, the AA is oxidized to

ascorbyl radical, while Toc° returns to its reduced state (Buettner, 1993). The ascorbyl radical is subsequently reduced back to its reduced form through the action of glutathione. By the action of phospholipase A_2 on PUFA-OOH in membrane phospholipids, free PUFA-OOH are released into the cytosol (Herrera and Barbas, 2001). Through the action of antioxidant enzymatic systems such as superoxide dismutase and catalase, PUFA-OOH are converted to hydroxyl polyunsaturated fatty acid (PUFA-OH).

Although α-tocopherol has been considered as the major antioxidant molecule present in vitamin E, many other studies have demonstrated the superiority of tocotrienols over α-tocopherol (Serbinova *et al.*, 1991; Serbinova and Packer, 1994). This may be due to the fact that tocotrienols have better tissue penetration and better distribution in the fatty layers of the cell membrane because of its unsaturated side chain (Suzuki *et al.*, 1993). Tocotrienols have been shown to possess powerful neuroprotectant antioxidant, anti-cancer and cholesterol lowering properties (Pearce *et al.*, 1992; 1994; Sen *et al.*, 2006).

2.6.3 Non-antioxidant function of vitamin E

Although most of the action of vitamin E is due to its antioxidant effect, not all its properties and actions are as a result of this action (Azzi and Stocker, 2000; Azzi *et al.*, 2000). Among the functions and mechanisms of action of vitamin E that are unrelated to its antioxidant molecular mechanisms include:

(i) Prevention of spermatogenesis loss in males and foetal resorption in the rat (Evans and Bishops, 1922; Wu *et al.*, 1973).

(ii) Inhibition of α-tropomyosin expression (Aratri *et al.*, 1999).

(iii) Inhibition of scavenger receptor class A (SR-A) (Teupser *et al.*, 1999).

(iv) Inhibitory action on protein kinase C (Azzi *et al.*, 1992; Özer *et al.*, 1998), which plays a key role on the effect of vitamin E inhibition of cell proliferation in vascular smooth muscles.

(v) Up-regulation of cytosolic phospholipase A_2 and cyclooxygenase activities (Chan *et al.*, 1998).

(vi) Vitamin E effects on bone occur by increasing bone trabecular formation (Xu *et al.*, 1995) and preventing bone calcium loss due to an oxidising agent, ferric nitrilotriacetate,(Yee and Ima-Nirwana, 1998) and prevention of bone calcium loss in ovariectomised rats (Norazlina *et al.*, 2000).

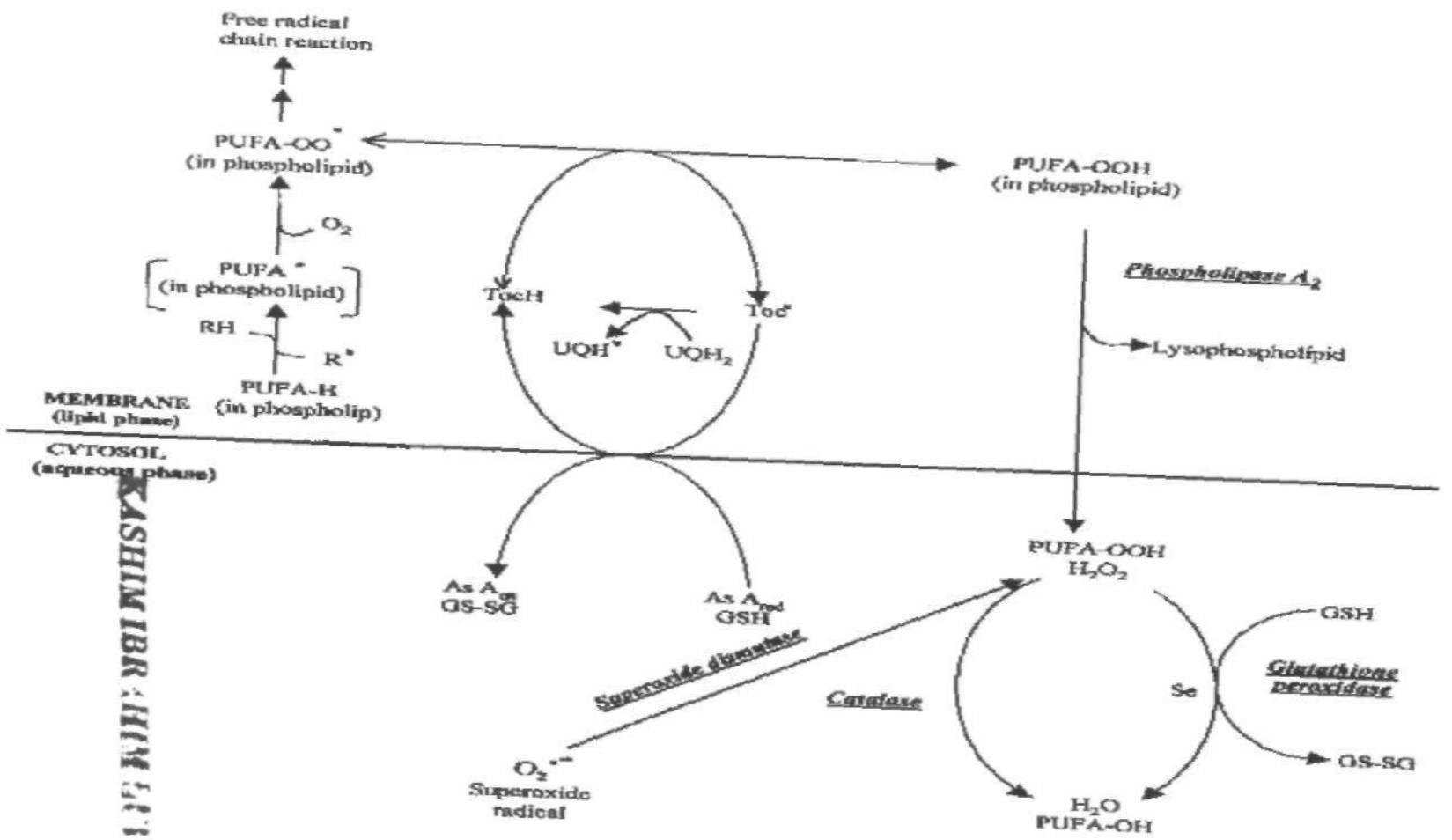


Figure 2.15 Scheme of the interaction and synergism between antioxidant systems operating in membrane (lipid phase) and cytosol (aqueous phase) of the cell, with special attention to part of the antioxidant, pro-oxidant and regeneration reactions of tocopherol (TocH) (Adapted from Herrera and Barbas, 2001)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental animals

Sixty two thirteen-week old Wistar rats of both sexes weighing between 95 and 110g used for this study were obtained from the Department of Veterinary Surgery and Medicine, Ahmadu Bello University, Zaria. The animals were kept in the Teaching Laboratory of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria. They were allowed to acclimatize for at least two weeks in the laboratory before they were used for the experiment. They were fed on pellets made from grower mash, maize bran and groundnut cake at a ratio of 4:2:1. The rats were provided with water *ad libitum*.

3.2 Chemical acquisition and preparations

Chlorpyrifos (CPF) marketed as Termicot® (Sabero organics, Gujarat limited, India), was purchased from a reputable Agrochemical Company in Zaria. Termicot® is a 20% emulsifiable concentrate of CPF. It was prepared by reconstituting in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria) to make 10% stock solution.

Ascorbic acid tablets (100 mg/tablet) with brand name Med Vit C® (Dol-Med Laboratories Limited, Lagos, Nigeria) was obtained from a Pharmaceutical Store in Zaria. Each tablet was dissolved in 1ml of distilled water to obtain 100 mg/ml suspension, just prior to its daily administration.

Vitamin E (100 mg/capsule) from Pharco Pharmaceuticals, Egypt, was obtained from a reputable Pharmaceutical Store in Zaria. Prior to daily use, each capsule was aspirated into a syringe (0.15 ml oil-based content/capsule) and then reconstituted with soya oil to 100% v/v.

3.3 Determination of median lethal dose (LD_{50})

Median lethal dose (LD_{50}) was evaluated using the method of Lorke (1983). Briefly, the evaluation was divided into two stages. In the first stage, 9 rats were divided into 3 groups of three animals each. Rats in group 1 were dosed with CPF at 100 mg/kg orally, while those in groups 2 and 3 were dosed with CPF at 300 mg/kg and 500 mg/kg orally, respectively. The animals were observed for death over a period of 48 hours. Based on the result obtained, the second stage of the test was conducted as follows: 3 rats, individually caged and numbered I, II, and III were dosed with reconstituted CPF at a dose of 100, 90 and 80 mg/kg, respectively. LD_{50} was then determined based on the dose-response (death) as described by Lorke (1983).

3.4 Chronic toxicological study

Fifty adult Wistar rats of both sexes (25 male, 25 females) weighing 95 to 110g were used for this phase of the study. The rats were weighed and then divided into 5 groups of 10 rats each, comprising 5 males and 5 females per group. Rats in group I served as the passive control group (S/oil group) termed control group and were given only soya oil at the dose of 2ml/kg. Rats in group II (CPF group) served as the active control and they were administered with CPF only at a dose equivalent to $1/8^{th}$ of the LD_{50}. Rats in group III (VC+CPF) were pretreated with 100 mg/kg of vitamin C, then dosed with CPF (dose equivalent to $1/8^{th}$ LD_{50}) 30 minutes later. Rats in group IV (VE+CPF) were pretreated with vitamin E at a dose of 75 mg/kg and then dosed with CPF

only at a dose equivalent to 1/8th of its LD_{50}, while those in group 5 (VC+VE+CPF) were pretreated with both vitamins C and E at a dose of 100 mg/kg and 75 mg/kg, respectively, and 30 minutes later administered with CPF only at a dose equivalent to 1/8th of its LD_{50}. The different treatment regimens were administered daily for a period of 17 weeks. During this period, signs of toxicity and death were recorded. In addition, the effect of the different regimens on body weight changes in rats was evaluated by weighing the animals on weekly basis using digital weighing balance. In addition, periodic evaluations of some neurobehavioural changes were conducted on the rats from each group.

3.4.1 Evaluation of the effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on neurobehavioural changes

3.4.1.1 Evaluation of the effect of treatments on open-field activities

The open-field apparatus as described by Zhu *et al.* (2001) was used to evaluate open-field activities of rats exposed repeatedly to all the treatment regimens. The open-field activities were examined on each animal in all the groups prior to the commencement of the study (day 0) and subsequently on day 2, 5 and 7, and thereafter on weekly basis for the rest of the test period. The open-field maze was constructed using cardboard box (50 x 50 x 46 cm high) with clear plexiglass on the inner surface. The floor of the box was divided into 25 equal squares. The parameters analysed in the open-field maze included motor activity and anxiety, which was evaluated by the frequency of rearing, grooming, defaecation, urination and stretch-attends posture. All the open-field parameters were assessed at 8.00-11.00 h every week.

In general, motor (locomotor) activity was assessed by placing an animal in the box and then allowing it to walk freely for 3 minutes to familiarize itself with the environment. Then the

number of squares crossed with all the paws during the next 2 minutes was recorded. Rearing was measured by the frequency at which the rat stood on its hind-limbs with either the fore-paws lifted up freely or leaning against the wall of the maze during the next 2 minutes, after an initial period of habituation that lasted 3 minutes. Frequency of grooming was measured by the number of times the rats licked the fore-paws during the next 2 minutes, after the initial 3 minutes of habituation. The frequency of stretched-attend postures refers to the number of times the animals stood still with the body elongated; usually with its rear end raised, while the head was lowered to the platform in the next 2 minutes after the initial 3 minutes of habituation. The frequency of urination and defaecation was the number of puddles or urine streaks and faecal boli produced, respectively, during the 5 minutes duration in the open-field maze. Soapy water followed by 90% alcohol solution was used to clean the inner surface of the box between trials to remove the interfering odours left by the previous animal.

3.4.1.2 Assessment of neuromuscular coordination

The effect of CPF and the ameliorating effect of vitamins C and/or E on neuro-muscular co-ordination of each rat were evaluated based on their ability to walk through obstacles using inclined plane and ladder walk tests as described by Petrich (2006) with slight modifications. In addition, the ability of rats to right its reflex was performed using the surface righting reflex method as described by Laviola *et al.* (2006) as modified. Briefly the activity of the rats on an inclined plane was evaluated by placing each rat on an apparatus made with an angled rough wooden plank with thick foam pad at its bottom end. The plank was first raised to an inclination of 35°, and thereafter gradually increased stepwise by 5° until the subject could no longer stay and be situated horizontally on the plank for 3s, without sliding down. Angles were measured

and marked on the apparatus beforehand, and were obtained by propping the plank on a vertical bar with several notches. The test was performed with the head of the rat first facing left and then right handside of the experimenter. The highest angle at which each rat stayed and stood horizontally, and facing each direction was recorded. Two trials were performed for each testing period (Petrich, 2006). This procedure was carried out on each animal from all the groups on Day 0, week 7 and 14 of the study.

For the assessment on ladder walk, every rat from each group was encouraged to walk across a black wooden ladder (106 cm x17 cm, with 0.8-cm diameter rungs, and with 2.5-cm spaces between them. The number of times each rat missed a rung was counted by one rater on each side (Petrich, 2006). Performance on ladder walk was evaluated on Day 0, weeks 7 and 14.

The surface righting reflex was assessed by holding a rat at the base of its tail on a levelled flat surface. With a rapid clockwise motion of the hand, the rat was turned from a prone to a supine position (Laviola *et al.*, 2006). The time needed to return to the normal position was noted and graded on an ordinal scale of 0, 1 and 2, thus:

Grade 0: Animal with a normal reflex that had ability to right its reflex immediately (within 2 seconds)

Grade 1- Animal with a slightly impaired motor coordination reflex that righted itself within 3-5 seconds.

Grade 2- Animal with markedly impaired surface righting reflex, which was able to right itself in more than 5 seconds.

The surface righting reflex was evaluated on day 0, week 4, 8, 12 and 16 of the study.

3.4.1.3 Assessment of coordinated gait

The assessment of coordinated gait was performed using the beam walk performance task as described by Petrich (2006). Briefly, the beam walk performance was evaluated by allowing individual rat from each group to walk across a wooden black beam of 106-cm length, beginning at 17.2-cm width and ending at 1.0-cm width. Periodic widths were marked on the side of the apparatus. On each side of the narrowing beam, there was a 1.8-cm step-down to a 3.0-cm area where subjects may step if necessary. As the subject walked across, the width of the beam at which they stepped down was recorded by one rater on each side, and this was repeated twice during each trial session.

3.4.1.4 Assessment of sensorimotor reflex

A battery of standardized tests such as postural reflex, forelimb-placing (visual, tactile and proprioceptive forelimb-placing responses) and orienting to vibrissae touch as described by Abou-Donia *et al.* (2001) with slight modification were used to assess the sensorimotor reflex of each animal in the various treatment groups and the control.

3.4.1.4.1 Assessment of postural reflex

For the evaluation of postural reflex, each rat was held gently by the tail, one meter above the floor and observed for forelimb extension. A normal rat extended both forelimbs. Consistent flexion of the forelimb is an abnormal response. Any rat with consistent flexion of the forelimbs was further assessed by placing it on a wooden dowel that can be gripped with both forepaws. With the tail held, gentle pressure was then applied behind the shoulder of the rat until the forelimb slid several millimetres. This manoeuver was repeated five times in each direction.

Normal rat resisted lateral pressure by gripping the wooden dowel. The response of each rat was graded as follows (Abou-Donia *et al.* 2001):

Grade 0- Rats without consistent limb flexion when held above the floor

Grade 1- Rats with consistent forelimb flexion

Grade 2- Otherwise grade 1 rats that did not resist lateral pressure on at least three of five trials in either direction.

The assessment of this neurobehavioural parameter was done on day 0, week 7 and 14.

3.4.1.4.2 Evaluation of the effects of treatments on forelimb-placing responses

Forelimb-placing neurobehavioural assessment consisted of visual placing, tactile placing and proprioceptive placing. For visual placing, each rat was held by tail in the hands of the experimenter 10 cm above the tabletop, with forelimbs hanging free. The rat was then slowly tilted towards the table with both forepaws. The evaluation of tactile placing performance was conducted by allowing the dorsal and then the lateral portions of the forepaws to touch the table edge. Intact rats immediately placed the paw on the surface of the table. Proprioceptive placing was tested by pushing the forepaw onto the table edge, with care taken to avoid the vibrissae from touching the table. The response of each rat was scored by an independent trained assessor oblivious of the different treatment regimens to avoid bias, as follows (Abou-Donia *et al.*, 2001):

Grade 0- Placing response was immediate

Grade 1- The placing response was slowed or delayed

Grade 2- The placing response did not occur within 2 seconds

These neurobehavioural parameters were conducted on day 0, week 4, 8, 12 and 16.

3.4.1.4.3 Effects of treatments on orientation to vibrissae touch

To assess the response of the animals to this neurobehavioural test, each rat was placed on top of an inverted polycarbonate cage and allowed one minute for habituation. Its vibrissae were then touched slightly with a cotton swab. The response was then assessed thus:

Grade 0- Rat oriented to the side of the probe at the two of the three trials from each side

Grade 1- Rat failed to orient on at least two of three trials on either side (Abou-Donia *et al.*, 2001).

This parameter was evaluated on day 0, week 4, 8, 12 and 16.

3.4.1.4.4 Assessment of the level of excitability scores

This neurobehavioural parameter was evaluated using the excitability scores as described by Kanaan *et al.* (2003), Maria *et al.* (2004) and Ayo *et al.* (2006) with slight modifications. Briefly, each rat was held by the tail upside down and held in that position for 30 seconds. Response of each rat was then scored using an ordinal scale of 0-5 as follows:

Grade 0- Rat did not show any form of wriggling at all.

Grade 1- Rat's wriggling was low with feeble forepaw movement.

Grade 2- Rat responded through a stronger wriggling and forepaw movement.

Grade 3- Rat vigorously wriggled and a strong fore- and hind-limbs movement

Grade 4- In addition to observation in grade 3 above, rat made unsuccessful attempt to climb on its tail.

Grade 5- In addition to observations in grade 3 above, rat sucessfully climbed the tip of its tail.

The excitability scores were assessed on day 0, week 4, 8, 12 and 16 of the study

3.4.1.5 Effect of treatments on motor strength

The forepaw grip time was used to evaluate the motor strength of the rats, as described by Abou-Donia *et al*. (2001). This was conducted by having rats hung down from a 5 mm diameter wood dowel gripped with both forepaws. The time spent by each rat before releasing their grips was recorded in seconds. This parameter was evaluated on day 0, week 4, 8, 12 and 16.

3.4.2 Evaluation of the effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on cognition

The effect of various groups on cognitive ability of each rat was assessed via learning and short-term memory test.

3.4.2.1 Assessment of the effect of treatments on learning

The effect of CPF on learning task in rats and the possible ameliorative effect of antioxidant vitamins were assessed 48 hours to the termination of the test using the step-down inhibitory avoidance learning task as described by Zhu *et al.* (2001). The apparatus used for the learning test was an acrylic chamber 40 x 25 x 25 cm consisting of a floor made of parallel 2-mm-caliber stainless steel bars spaced 1 cm apart. An electric shock was delivered through the floor bars. A 2.5-cm-high, 8 x 25 cm wooden platform was placed on the left extreme of the chamber. Each animal was gently placed on the platform. Upon stepping down, the rat immediately received a single 80-volt foot shock. If the animal did not return to the platform, the foot shock was repeated every 5s. A rat was considered to have learned the avoidance task if it remained on the platform for more than 2 min. The number of foot shocks was recorded as an index of learning acquisition.

3.4.2.2 Assessment of the effect of treatments on short-term memory

Short-term memory was assessed in individual rat from each group using the step-down avoidance inhibitory task as described by Zhu *et al.* (2001) twenty four hours after the assessment of learning. The apparatus used for the memory test was the same used earlier for the assessment of learning. In this test, the rat was again placed gently on the platform 24 hours after performing the learning task. The time an animal remained on the platform was recorded as an index of memory retention. Staying on the platform for 2 min was counted as maximum memory retention (ceiling response).

3.4.3 Evaluation of the effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on haematological parameters

At the end of the test period, rats from each group were sacrificed by severing the jugular vein after light ether anaesthesia. Blood was then collected into sample bottles containing sodium citrate. The haematological parameters analysed included packed cell volume (PCV), erythrocyte count, haemoglobin concentration and absolute and differential leucocyte counts.

3.4.3.1 Determination of the effect on packed cell volume

Packed cell volume was determined using the method described by Dacie and Lewis (1991). Heparinized capillary tubes (John Poulten Limited, England) were inserted into the blood-containing sample bottles, leaving about 15 mm of the tubes unfilled. The open end of each of the tubes was then carefully sealed in a fine flame. The tubes were then loaded onto a microhaematocrit centrifuge (Hawksley, England) and then centrifuged at 1000g for 10 minutes. The PCV value for each of the tube was read using the haematocrit reader (Hawksley, England)

3.4.3.2 Determination of the effect of treatments on total erythrocyte count

The total erythrocyte count was determined using the method described by Dacie and Lewis (1991). Briefly, blood was sucked into RBC diluting pipette to the 0.5 mark and then diluted with 100 ml of RBC diluting fluid to the 101 mark (1:200). The counting was done in an improved Neubaeur counting chamber under a light microscope at X 40 magnification.

3.4.3.3. Evaluation of the effect of treatments on haemoglobin concentration

The cyanmethaemoglobin method described by Baker and Silverton (1985) was used to determine the haemoglobin concentration. Briefly, 20 µl of blood was pipetted into a tube and then diluted with 5 ml of modified Drabkin's fluid and allowed to stand for at least 3 minutes. The absorbance of the mixture was read using a spectrophotometer (Beckman Coulter, Model BU520, Austria) at a wavelength of 540 nm against reagent blank. The haemoglobin concentration was determined using the following formula:

$$Hb = \frac{T \times C \times D}{A \times 1000} \quad (g/dL)$$

where T is the test absorbance, A is the standard absorbance, C is the concentration of cyanmethaemoglobin standard (mg/dL), while D is the dilution factor (1: 250); 1000 converts the Hb concentration from mg/dL to g/dL.

3.4.3.4 Evaluation of the effect of treatments on absolute and differential leucocyte counts

Absolute and differential leukocyte counts were determined using the method described by Dacie and Lewis (1991). Briefly, a drop of blood was placed on one end of a glass slide (UNISCOPE, England) and using a spreader, a blood film was made and allowed to air-dry. Leishman stain was then poured to cover the film for 2 minutes. The film was then rinsed in buffered distilled

water and allowed to stand for 10 minutes. The film was then later viewed under a microscope in an oil immersion at x 1000 magnification. The total number of leukocytes was counted in absolute values, while the differential leukocyte counts were evaluated as both absolute and % values of each of the different types of leukocyte in relation to the total leukocytes.

3.4.3.5 Evaluation of the effect of treatments on *in vitro* erythrocyte osmotic fragility

Evaluation of the *in vitro* erythrocyte osmotic fragility was done using the method described by Faulkner and King (1970) as modified using different concentrations of physiological buffer saline (PBS, pH 7.4) from 0.0, 0.1, 0.3, 0.5, 0.7, 0.9 g/L. Freshly obtained whole blood (0.02 ml in sodium citrate anticoagulant) from each rat was pipetted into the test tubes containing varying concentrations of PBS and then followed by careful mixing and incubation for 30 minutes at room temperature, 26-28°C. The test tubes were then centrifuged at 2,000g for 10 minutes using a centrifuge model IEC HN-SII (Damon/IEC Division, Germany). The supernatant was transferred into a glass cuvette and the absorbance of the supernatant measured colorimetrically with Spectronic 20 (Bausch and Lomb, USA) at wavelength of 540 nm. The percent haemolysis for each sample was then calculated using the formula of Faulkner and King (1970).

$$\% \text{ haemolysis} = \frac{\text{Optical density of test solution}}{\text{Optical density of standard solution}}$$

3.4.4 Evaluation of the effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on serum biochemical parameters

Blood samples were collected into test tubes and allowed to clot. They were then centrifuged at 1000g for 10 minutes. Thereafter, the straw coloured serum was collected from each test tube into a clean sample tube, which was subsequently used for the evaluation of serum biochemical

parameters. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Gamma-glutamyl transferase (GGT) and creatine kinase (CK) were analysed using an autoanalyzer (Bayer Clinical Chemistry Analyzer, Germany).

Total serum proteins was analysed using biuret reaction as described by Armstrong and Carr (1964) while serum albumin concentration was measured using the method described by Henry (1974). Serum globulin concentration was obtained by subtracting serum albumin concentration from total serum protein concentration. Serum glucose concentration was measured using the glucose oxidase method described by Ware and Marbach (1968). Serum urea concentration was determined according to the method of Wybenga *et al.* (1971). Concentration of serum uric acid was determined using the method of Pileggi *et al.* (1972) while the serum concentration of creatinine was measured using the modified method of Miller and Miller (1951).

Serum lipid profiles such as triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-c) and high density lipoprotein cholesterol (HDL-c) were also analysed. Serum TG concentration was determined by the enzymatic colorimetric method of Wahlefeld (1974). TC was analysed using the enzymatic colorimetry method of Flegg (1973), while HDL-c was assessed using the colorimetry method of Finley *et al.* (1978). LDL-c was calculated using the formula of Friedewald *et al.* (1972) thus:

$$\text{LDL cholesterol (mg/dL)} = \text{Total cholesterol} - \text{HDL cholesterol} - (0.20 \times \text{triglycerides})$$

The concentration of electrolytes such as Na^+, K^+, Cl^- and HCO_3^- were also evaluated. The serum Na^+ and K^+ were measured by flame photometry, while Cl^- was analysed using the method of

Schales and Schales (1941). Serum HCO_3^- was analysed using the titrimetric method of Segal (1955) as modified by Ibbott *et al.* (1974).

The thyroid function was also evaluated by determining the serum concentration of thyroid stimulating hormone (TSH), triiodothyronine (T_3) and thyroxine (T_4) using enzyme-linked immunosorbent assay (ELISA) kits (microwell TSH, T_3, T_4 kits) from Synthron Bioresearch, Inc, USA. The sensitivity of the kits for TSH was 0.5 µlU/ml, while that of T_3 was 20 ng/L.

3.4.5 The effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on lipid peroxidation

The level of thiobarbituric acid reactive substance (TBARS), malonaldehyde (MDA) as an index of lipid peroxidation was evaluated in the serum, brain and liver samples using the double heating method of Draper and Hadley (1990). The principle of the method was based on spectrophotometric measurement of the colour developed during reaction of thiobarbituric acid (TBA) with malonadehyde (MDA). Briefly, the liver and brain samples from each animal in all the groups were weighed and then homogenized in a known volume of ice-cold phosphate buffer to obtain a 10% homogenate, which was then centrifuged at 6,000 g for 10 minutes to obtain the supernatant. The supernatant was then used to assess the level of protein and MDA in the samples. For the assessment of lipid peroxidation in the liver, the Draper and Hadley method as modified by Yavuz *et al.* (2004) was used. Briefly, the assessment of MDA concentration in the supernatant of liver homogenates was evaluated thus: 2.5 ml of 100 g/L trichloroacetic acid (TCA) solution was added to 0.5 ml liver homogenate in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling under tap water for 5 minutes, the mixtures were then centrifuged at 1000 g for 10 min. 2 ml of the supernatant was added to 1 ml of 6.7 g/L TBA

solution in a test tube and placed in a boiling water (100°C) bath for 15 min. The solution was then cooled under tap water and the absorbance measured using a UV spectrophotometer (Jenway, 645, Japan) at 532 nm.

For the determination of lipid peroxidation in the brain, the method of Draper and Hadley (1990) as modified by Freitas *et al*. (2005) was used. Briefly, 0.5 ml of the brain homogenate of each animal was mixed with 1 ml 10% TCA and 1 ml 0.67% thiobarbituric acid. The mixture was then heated in a boiling water bath for 15 min, and butan-2-ol (2:1, v/v) was added to the solution. After centrifugation (800 x g, 5 min), TBARS, MDA was determined from the absorbance at 532 nm.

The serum MDA level was also analysed for each of the sample using the method of Draper and Hadley (1990) modified according to the protocol of Kanter *et al*. (2005). For this purpose, 2.5 ml of 100 g/L trichloroacetic acid solution was added to 0.5 ml serum in each centrifuge tube and placed in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at 1000 g for 10 min, and 2 ml of the supernatant was added to 1 ml of 6.7 g/L TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a UV spectrophotometer (Jenway, 645, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5 /cm, and expressed in µmol/ml.

The concentration of MDA in both the liver, brain and serum samples was calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5/cm/M and expressed in nmol for the

serum or nmol/mg of protein for the brain and liver. The concentration of protein in the liver and brain homogenates was evaluated using the biuret reaction as described by Armstrong and Carr (1964).

3.4.6 Gross and histopathological lesions of rats chronically exposed orally to soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E

Gross pathological examinations were conducted and the observed lesions were recorded. Samples of the brain, lung, liver, heart, spleen, intestine, stomach, pancreas and kidneys were obtained and prepared for histopathological processing using the method described by Luna (1960). Briefly, the tissue samples were fixed in 10% formalin, embedded in paraffin and cut to 5µm. Sections of the tissue were stained with haematoxylin-eosin and examined under light microscope (x 400) and lesions observed were recorded.

3.4.7 Statistical analysis

Values were obtained as mean ± SEM. One-way analysis of variance (ANOVA) with Dunnett's multiple comparison post-hoc test was performed using GraphPad Prism version 4.0 for windows from GraphPad Software, San Diego, California, USA (www.graphpad.com) to compare the level of significance between the test groups and those in the control, treated with soya oil only. Tukey multiple comparison post-hoc test was used to evaluate the sex difference among groups. Values of $P < 0.05$ were considered to be the least significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Determination of median lethal dose (LD_{50}) for chlorpyrifos

All the three rats from each of the three groups dosed at the phase I with CPF at 100 mg/kg, 300 mg/kg and 500 mg/kg died within 24 hours post-administration. Clinical signs observed prior to death included depression, huddling, piloerection, tremor, intermittent convulsion, respiratory depression, ataxia and death. Post-mortem gross findings observed included haemorrhagic gastroenteritis, stomach filled with gas bubbles, congestion and petechial haemorrhages on the liver, congestion of the lungs, heart, kidney, testes and brain vessels, and flabby thigh muscle.

The result obtained in phase II from the three groups of one animal each, is presented in Table 4.1

Therefore, based on the method of Lorke (1983), the result of the $LD_{50} = \frac{90+80}{2} = 85$ mg/kg

4.2 Chronic toxicological study

4.2.1 Clinical signs

There were no apparent clinical signs observed in the soya-treated control animals and those pretreated with vitamins C and/or E. On the contrary, rats exposed to CPF only showed serous ocular discharges, conjunctivitis and congested ocular mucous membranes.

4.2.2 Effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on body weight changes in adult wistar rats

The pattern of body weight changes in rats exposed to the different treatments throughout the study period are shown in Figure 4.1. There was a general progressive increase in body weight

gain in animals in all the experimental groups. However, rats in the CPF group did not show considerable rapid progression in their body weight compared to those observed in other groups. Specifically, there was no difference ($P > 0.05$) between various test groups compared to the soya oil-treated control at day 0, week 1, 4, 8, 12 and 17. However, a significant increase in weight gain was observed in rats from the VC+CPF and VE+CPF ($P < 0.01$), and VC+VE+CPF ($P < 0.05$) groups compared to the control group by week 2 of the study. On the whole, there was a significant increase ($P < 0.01$) in body weight of animals in each of the groups when the initial weight on day 0 was compared to the final weight at termination of the study. However, the final mean weight of rats exposed to CPF only at termination was lower than the other groups (Figure 4.2). There was 54.6% increase in weight gain between commencement and termination of the study in the control, compared to 49.4% increase observed in the CPF group. The percentage increase in weight gain observed in the VC+CPF, VE+CPF and VC+VE+CPF groups were 53.4%, 51.3% and 55.5%, respectively. There was no significant difference in the body weight of male and female controls before week 12 of the experiment. However, an increase in the mean body weight was observed in the males compared to the females by week 12-17, week 13-17 and week 15-17 in the control, CPF, VE+CPF and VC+VE+CPF groups, respectively. However, no significant body weight changes between males and females in the VC+CPF group were observed.

4.2.3 Evaluation of neurobehavioural changes following chronic exposure to soya oil, chlorpyrifos only and chlorpyrifos and vitamin C and/or E in adult Wistar rats

4.2.3.1 Effect of treatments on open-field activities

The open-field parameters examined included motor activity and anxiety (as measured by frequency of defaecation, urination, rearing, grooming and stretch-attends posture).

Table 4.1: Summary of results from Phase II median lethal dose (LD_{50}) evaluation

Group	Dose (mg/kg)	No dead / No dosed
1	100	1/1
2	90	1/1
3	80	0/1

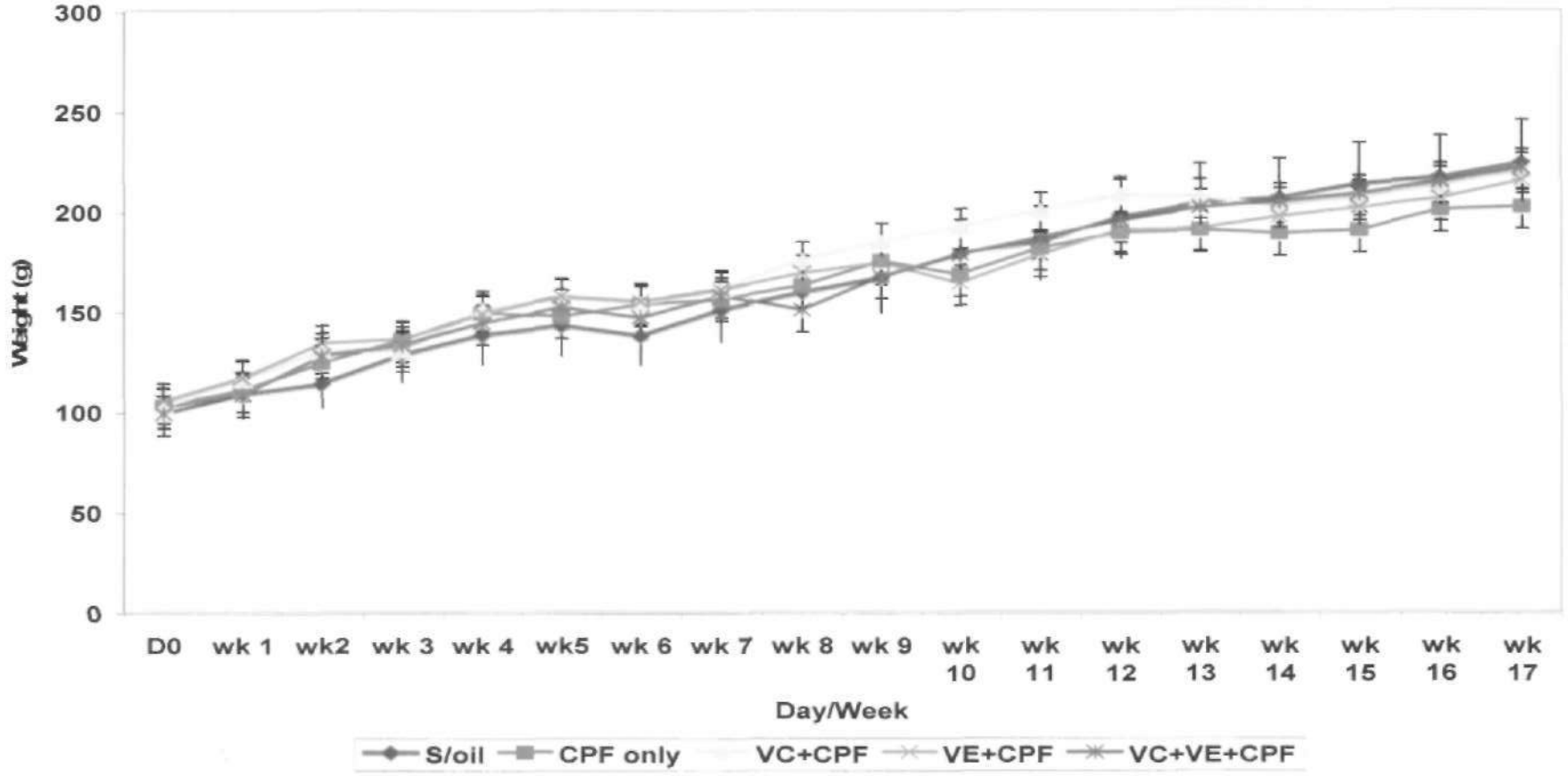


Figure 4.1: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on the dynamics of weekly body weight changes in adult Wistar rats (n=10).

D0= Day 0
Wk= Week

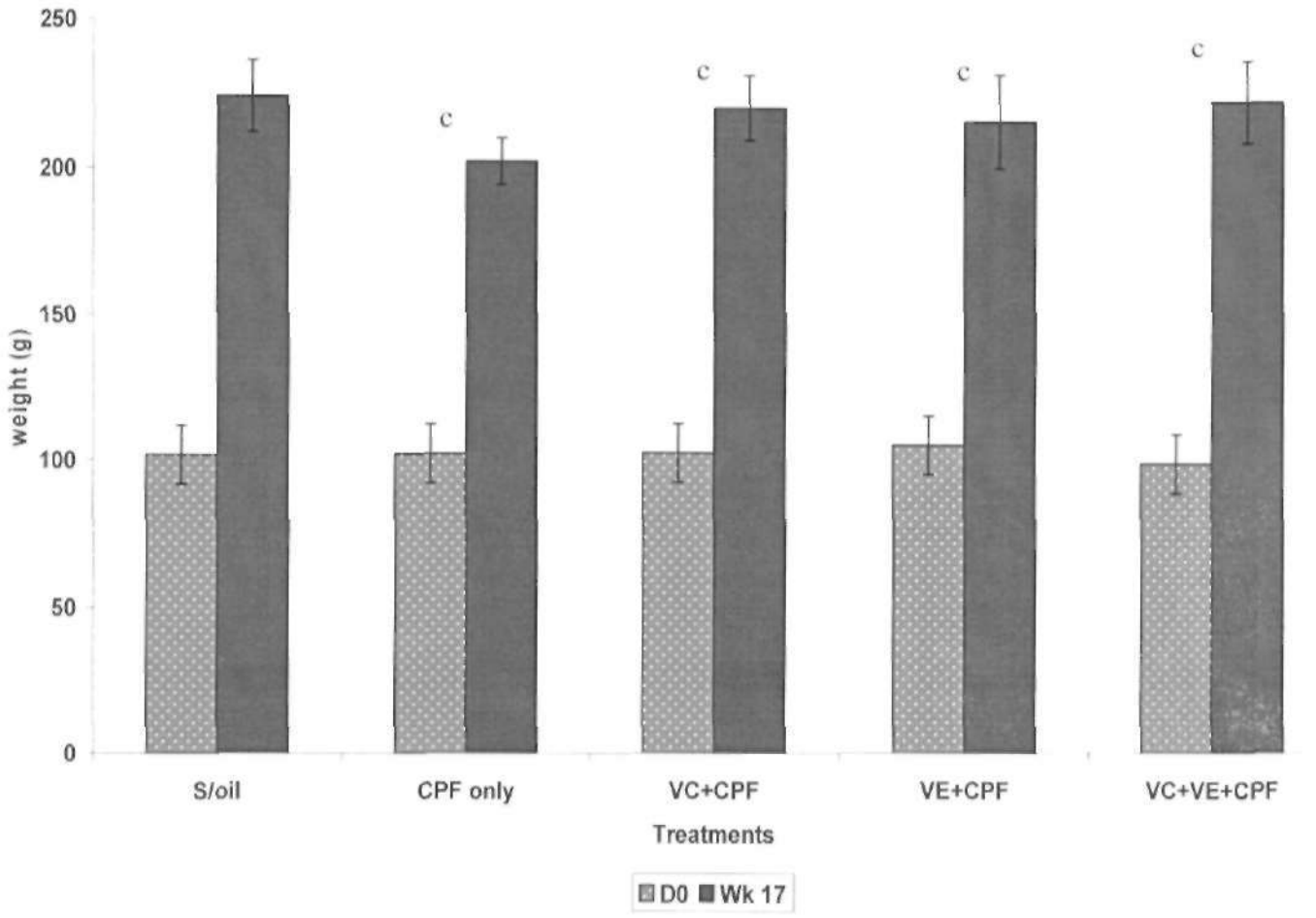


Figure 4.2: Body weight changes of Wistar rats after chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) at day 0 (D0) and weeek 17 (Wk 17) of the study

c= significant difference ($P < 0.01$) in weight gain between initial weight and those obtained at termination

4.2.3.1.1 Effect on motor (locomotor) activities

The general pattern of locomotor activity in rats from all the groups during the entire period of the study is shown in Figure 4.3. There was a rapid decline in the general pattern of motor activity in the CPF group throughout the study period. Pretreatment with vitamins C and/or E resulted in general improvement in motor activity. A non-significant change ($P>0.05$) in the locomotor activity was observed in the CPF and vitamins-pretreated groups compared to the soya oil-treated control on day 0, week 1, 8, 12 and 17. However, a significant decrease in motor activity ($P<0.05$) was observed in the CPF group (20.6 ± 1.7) compared to the control (25.4 ± 2.8) by day 2. A significant decrease ($P<0.01$) in motor activity was observed in CPF (13.6 ± 2.8) and VC+CPF (15 ± 1.9) groups compared to the control (27.2 ± 1.6) by week 2. Furthermore, a significant increase ($P<0.05$) in motor activity was observed in rats in the VC+VE+CPF group (25.2 ± 2.4) compared to the control (15.6 ± 2.5) by week 4. On the overall, the cummulative locomotor activity throughout the study period was significantly lower ($P<0.05$) in the CPF group compared to the control (Figure 4.4). There was no significant change in cummulative locomotor activity in the vitamins pretreated groups compared to the control. In addition, there was no significant difference in locomotor activity between male and female rats from each of the groups throughout the period under study, except in the VE+CPF group where motor activity in the female was more than the male ($P<0.001$) by day 5, and week 3 and 6.

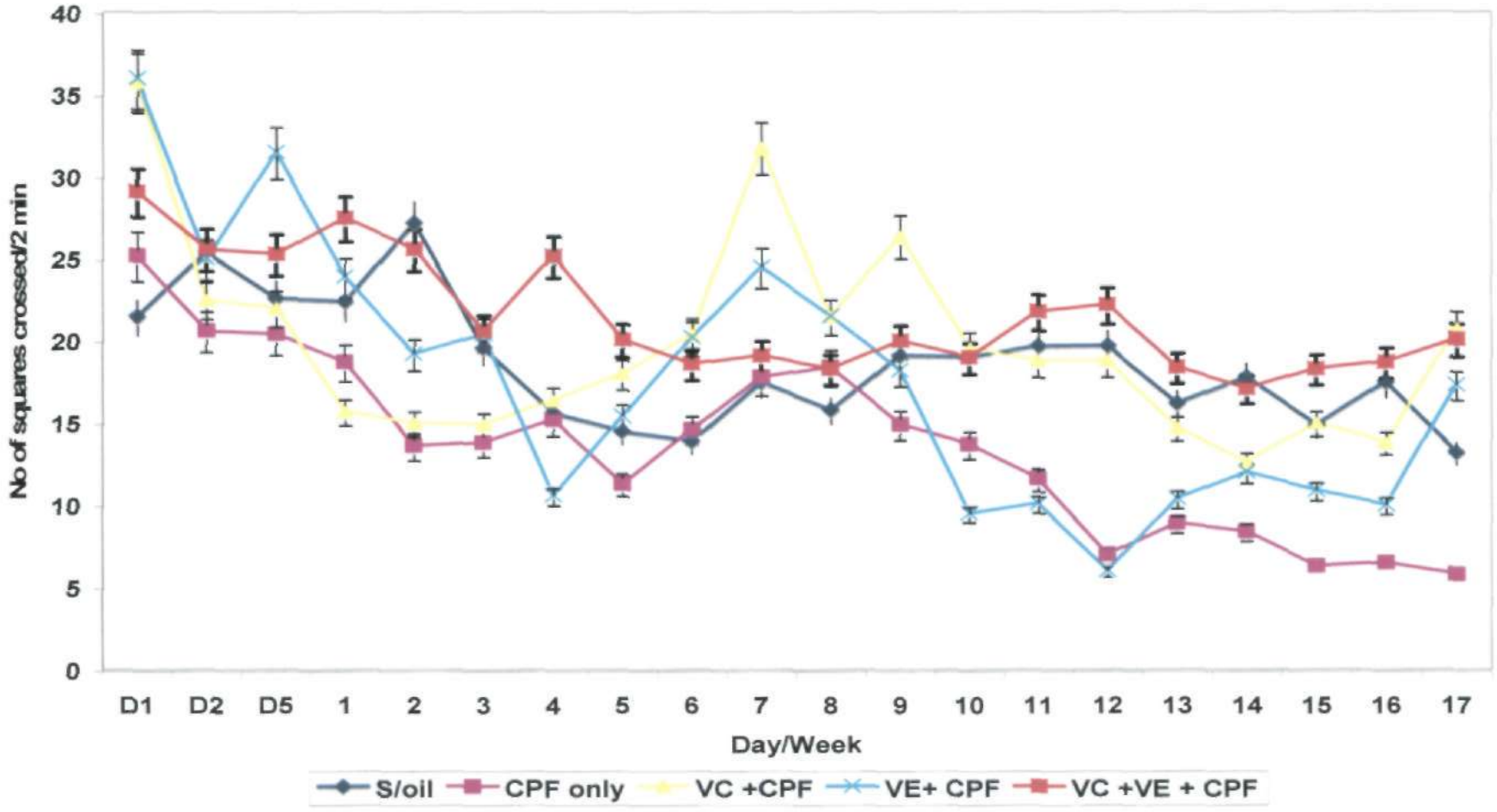


Figure 4.3: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on the time-course of locomotor activity pattern in adult Wistar rats (n=10)

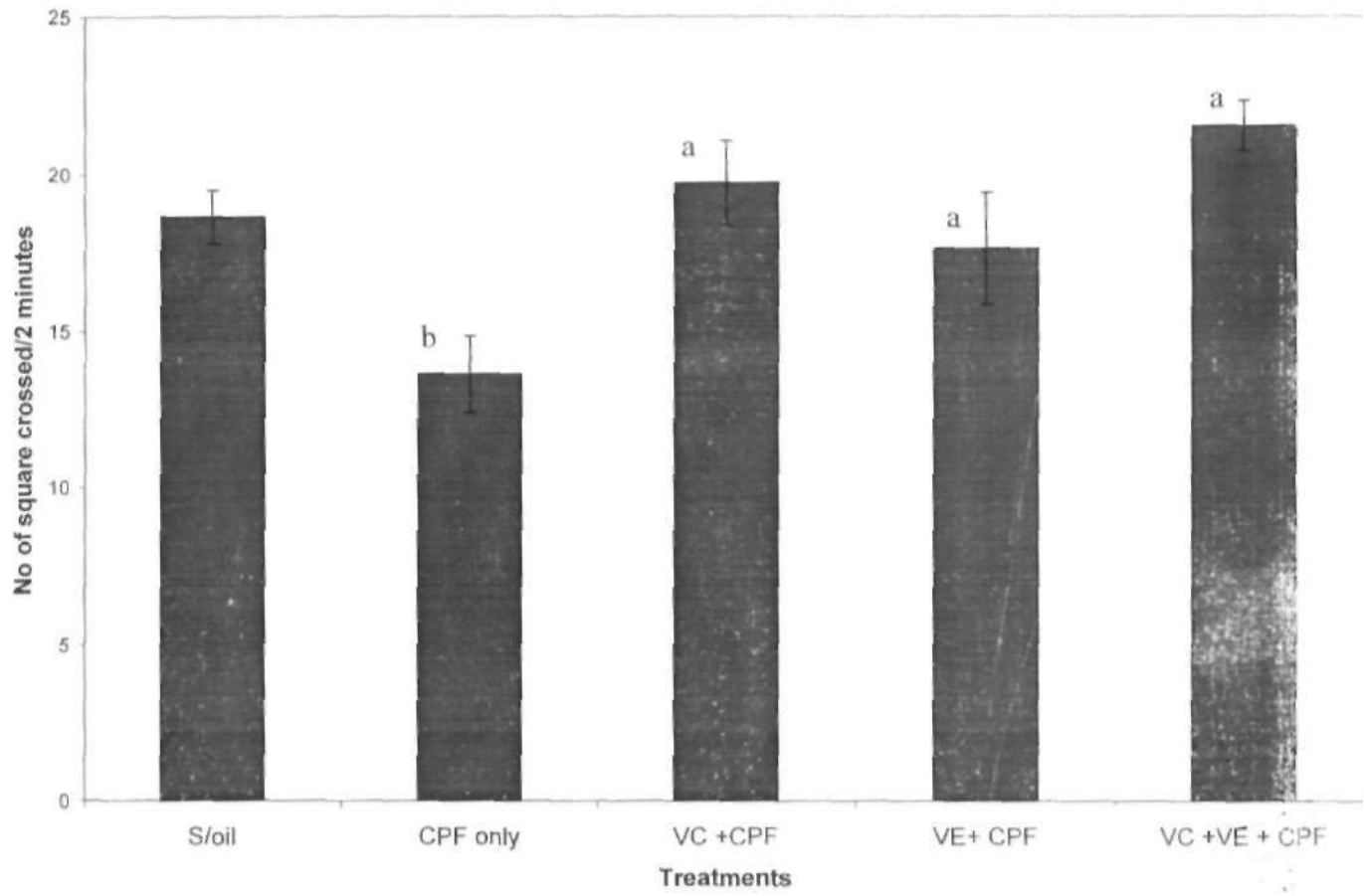


Figure 4.4: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative mean locomotor activity adult Wistar rats (n=10)

4.2.3.1.2 Effect on frequency of rearing

The pattern in the frequency of rearing was that of a general decline with increasing duration of exposure of rats to CPF only, compared to the other experimental groups (Figure 4.5). On the contrary, rats in the control and vitamins-pretreated groups showed a general rise in frequency of rearing with increasing duration of exposure to various regimens. A decrease ($P < 0.05$) in the frequency of rearing was observed in the CPF group compared to the control by week 17. A significant decrease in frequency of rearing was observed in the VE+CPF and VC+VE+CPF groups in week 4 ($P < 0.05$) and in VC+VE+CPF group by week 8 ($P < 0.01$) compared to the soya oil-treated control. No significant change in frequency of rearing was observed in the other treatment groups compared to the control during the period under study. On the whole, a decrease ($P < 0.01$) in the cummulative mean frequency of rearing was recorded in CPF group compared to the control. No significant change in the cummulative frequency of rearing was recorded in the VC+CPF and VE+CPF groups compared to the control. The rats in VC+VE+CPF groups showed an increase ($P < 0.01$) in the cummulative frequency of rearing compared to the control (Figure 4.6). No sex-specific difference in the frequency of rearing was observed in all the treatment groups.

4.2.3.1.3 Effect on frequency of grooming

The pattern in the frequency of grooming was that of a general decrease in that of the control and the vitamins-pretreated groups compared to those in the CPF group, which did not show considerable change throughout the study (Figure 4.7). In addition, the frequency of grooming was generally higher in the CPF group compared to the values obtained in the other groups.

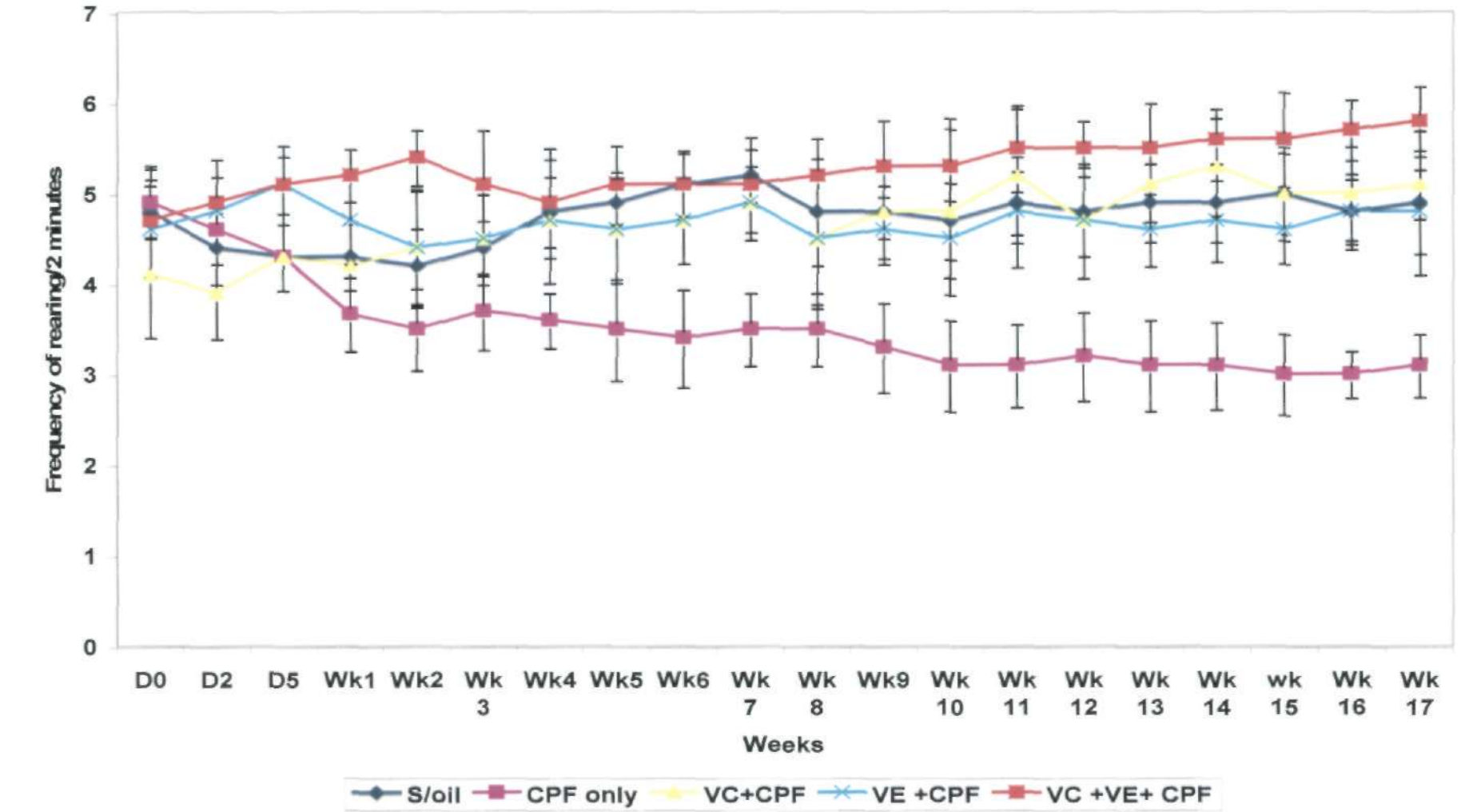


Figure 4.5: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on the pattern of frequency of rearing in adult Wistar rats (n=10).

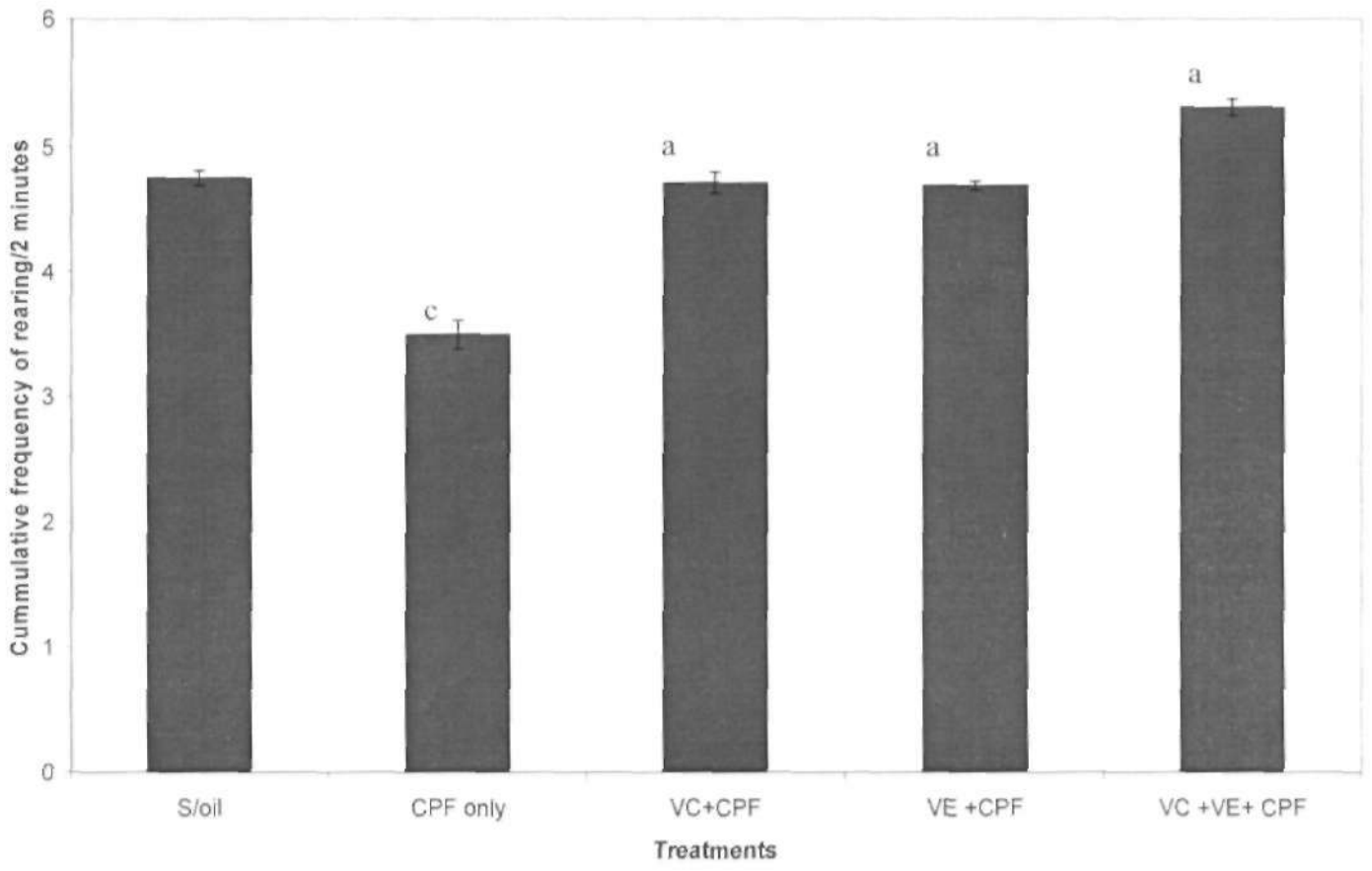


Figure 4.6: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative mean frequency of rearing in adult Wistar rats (n=10).

a =No significant difference (P >0.05) compared to the control
c = Significant difference (P< 0.01) compared to the control

Thus, the frequency of grooming decreased ($P<0.01$) in the VE+CPF (0.1 ± 0.1) and VC+VE+CPF (0.8 ± 0.4) groups, compared to the control (2.8 ± 0.4) by week 2 of the study. There was an increase ($P<0.01$) in the frequency of grooming in the CPF group by week 8, 12 and 17 compared to the control. No significant change in the frequency of grooming was observed between the various treatment groups compared to the control by day 2, week 1, 2, 4 and 16 of the study (Figure 4.7). On the overall, there was a marginal but non-significant increase in the cummulative frequency of grooming in rats in the CPF group compared to the control. Rats in groups pretreated with vitamins C and/or E showed a non-significant decrease in cummulative frequency of grooming compared to the control (Figure 4.8). There was no sex difference in the frequency of grooming in both the various treatment groups and the control.

4.2.3.1.4 Effect on stretch-attends posture

There was an initial decrease by day 2 and then a progressive rise until week 1, then followed by a gradual decrease in stretch-attends posture in the CPF group rats. On the other hand, rats from both the control and vitamins pretreated groups showed a consistent decrease in the frequency of stretch-attends posture throughout the study period. Generally, the frequency of stretch-attends posture was generally higher in the CPF group compared to the rest groups (Figures 4.9 and 4.10). A significant increase ($P < 0.05$) in the frequency of stretch-attends posture occurred in the CPF group in week 1 (2.1 ± 0.4) and 17 (1.1 ± 0.4), while a decrease ($P < 0.05$) in the frequency of stretch-attends posture was observed in week 4 in VC+CPF group (0.2 ± 0.1) compared to the soya oil-treated control (Figure 4.9). On the whole, the cummulative frequency of stretch-attends posture in the CPF group was insignificantly higher compared to the control. The cumulative frequency of stretch-attends posture in the VC+CPF and VC+VE+CPF groups were insignicantly lower than the control. The rats in the VE+ CPF group had a lower ($P <0.05$) cummulative

frequency of stretch-attends posture compared to the control (Figure 4.10). In addition, no significant difference in the frequency of stretch-attends posture was obtained between male and female rats from any of the groups.

4.2.3.1.5 Effect on frequency of defaecation

The effect of the various treatments on the pattern of frequency of defaecation is shown in Figure 4.11. There was a general progressive decrease in the frequency of defaecation throughout the study period. Rats dosed with CPF only showed a higher frequency of defaecation than the rest groups. Rats in the CPF group showed a decrease in frequency of defaecation by day 2, with subsequent increase till week 7, and thereafter the number of faecal mass recorded decreased. There was a progressive decrease in the frequency of defaecation in the vitamins-pretreated groups, especially in the VC+VE+CPF group. There was no significant change in the pattern of defaecation between the various treatment groups compared to the control by day 0, weeks 2, 4 and 8. However, there was a significant increase in the frequency of defaecation in the CPF group compared to the control by week 1 ($P < 0.01$), 12 and 17 ($P < 0.05$). There was a significant increase ($P< 0.01$) in the cummulative mean number of faecal boli produced by the rats in the CPF group throughout the period of study compared to the control. There was no significant change in the cummulative mean frequency of defaecation in the vitamins-pretreated group throughout the period of the experiment compared to the control (4.12). The difference in the frequency of defaecation between male and female rats in each of the groups was insignificant.

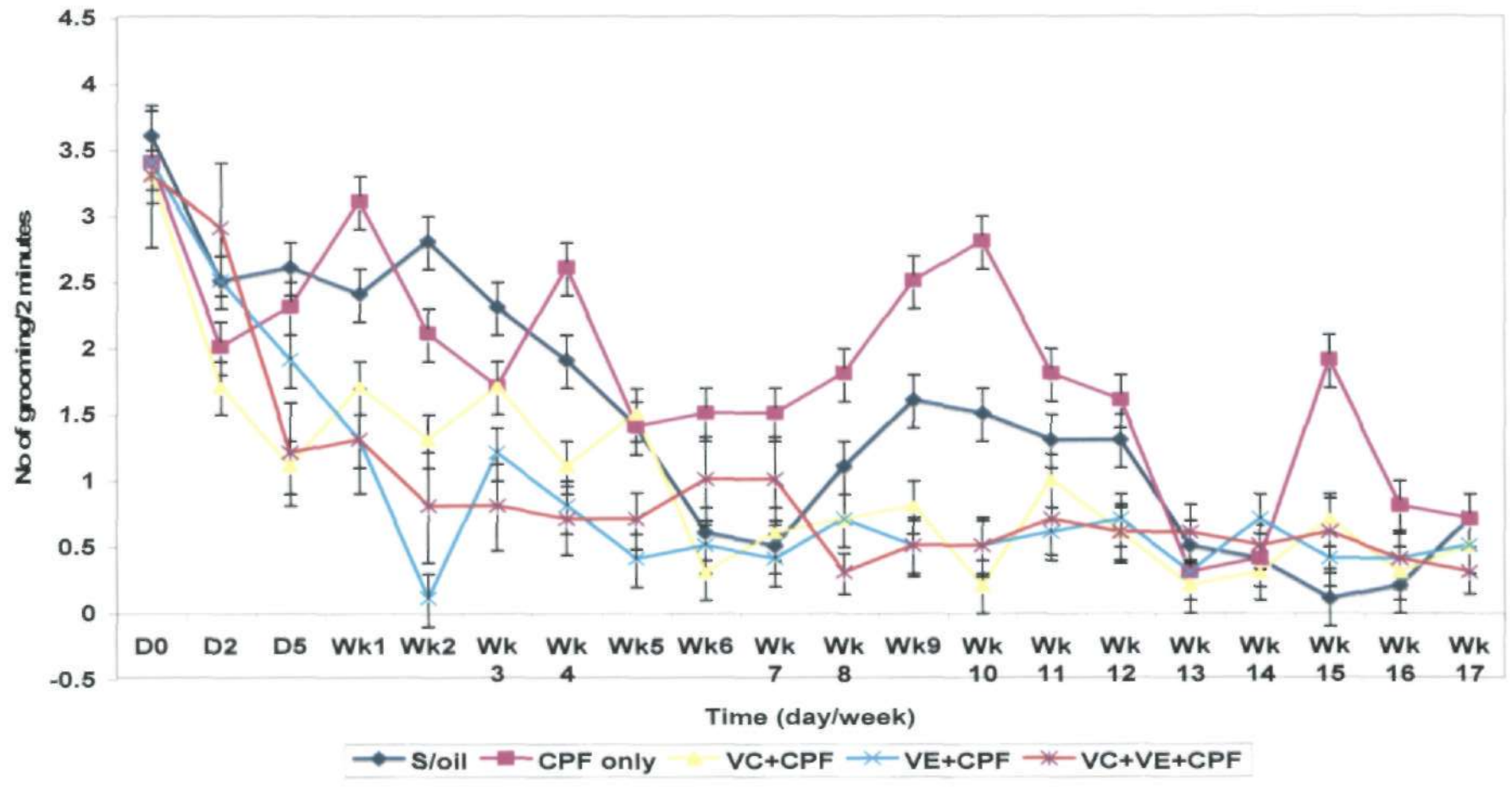


Figure 4.7: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on the pattern of frequency of grooming of adult Wistar rats (n=10).

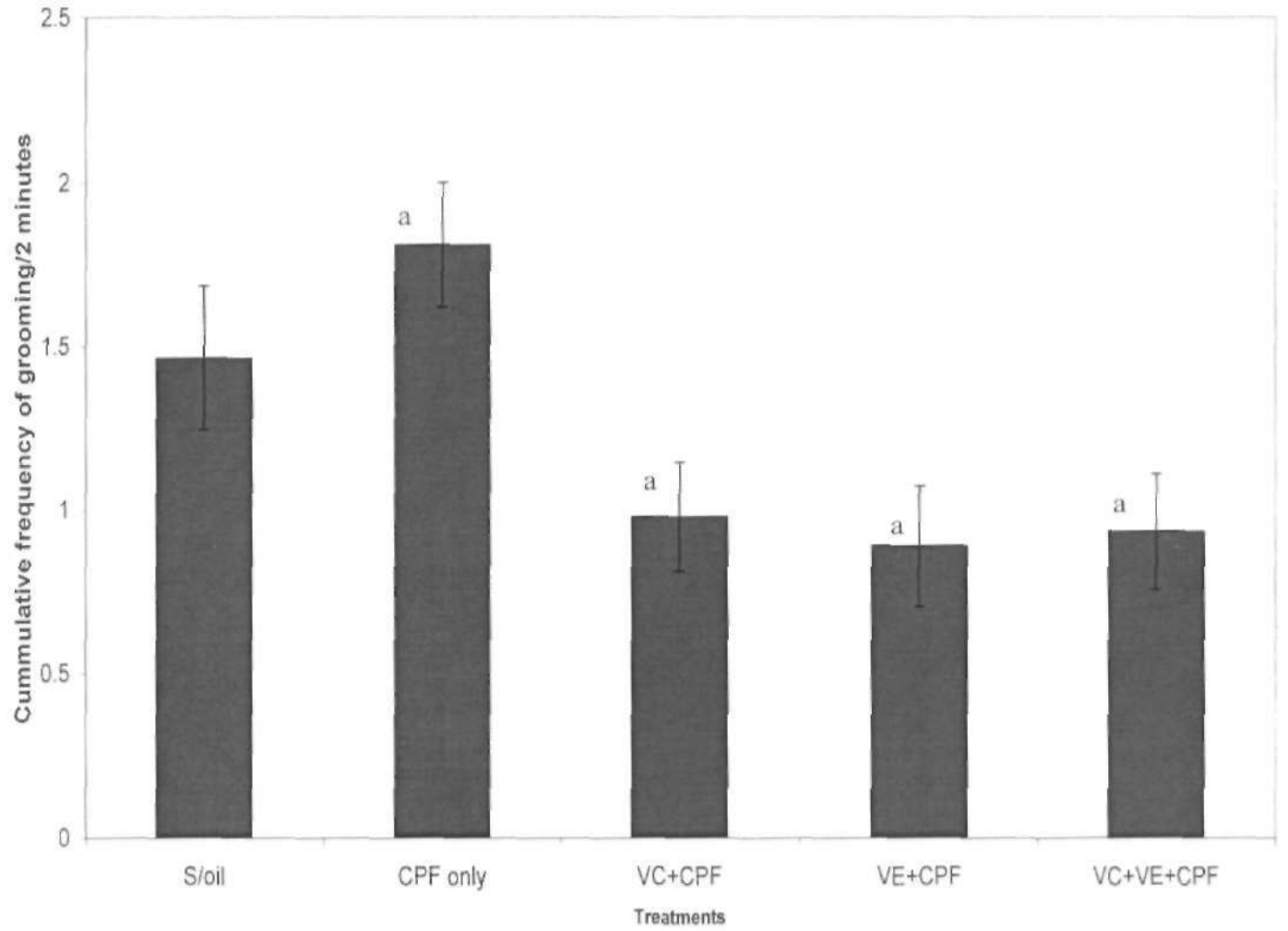


Figure 4.8: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative mean frequency of grooming in adult Wistar rats (n=10).

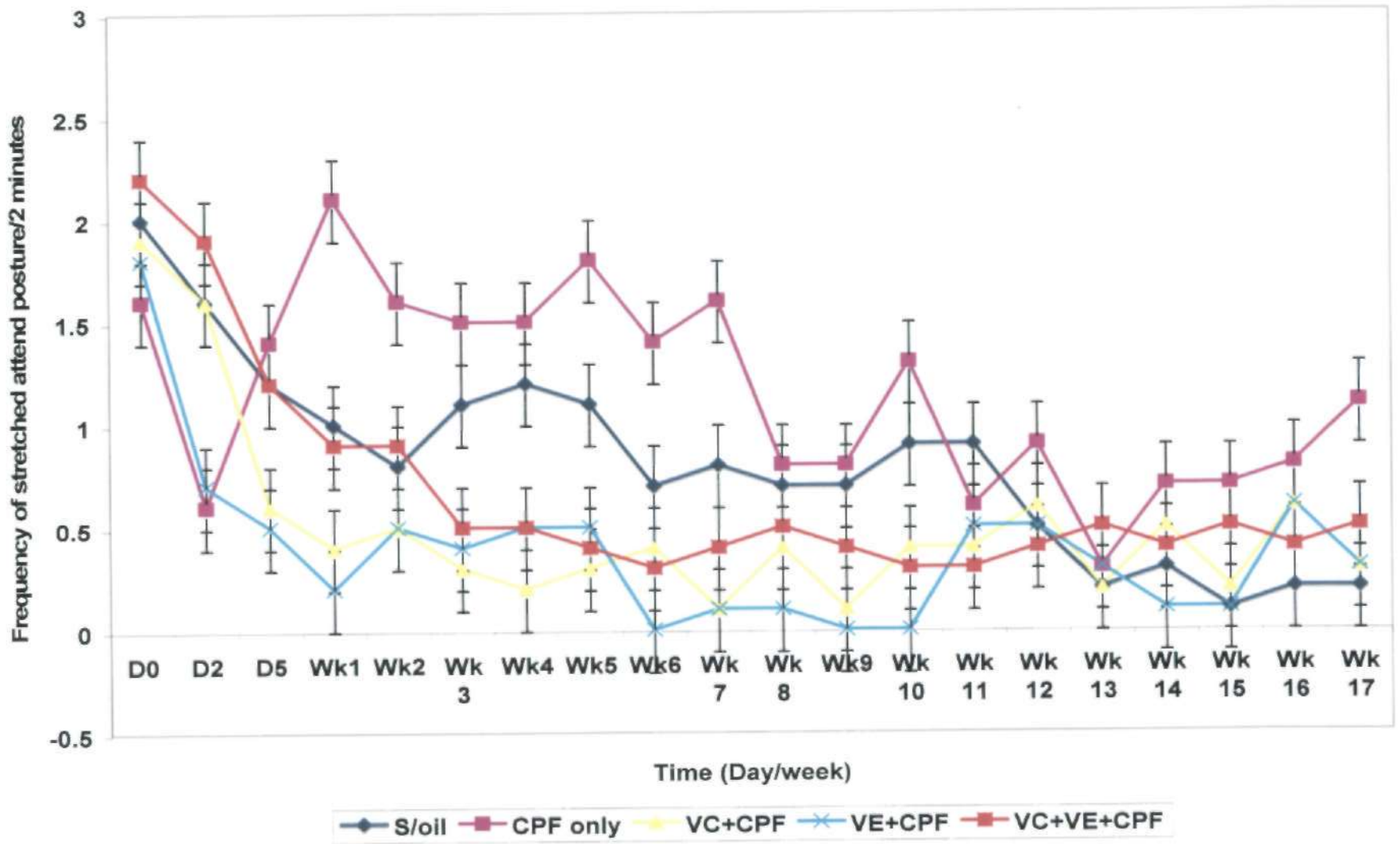


Figure 4.9: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on the pattern of frequency of stretch-attends posture in adult wistar rats (n=10)

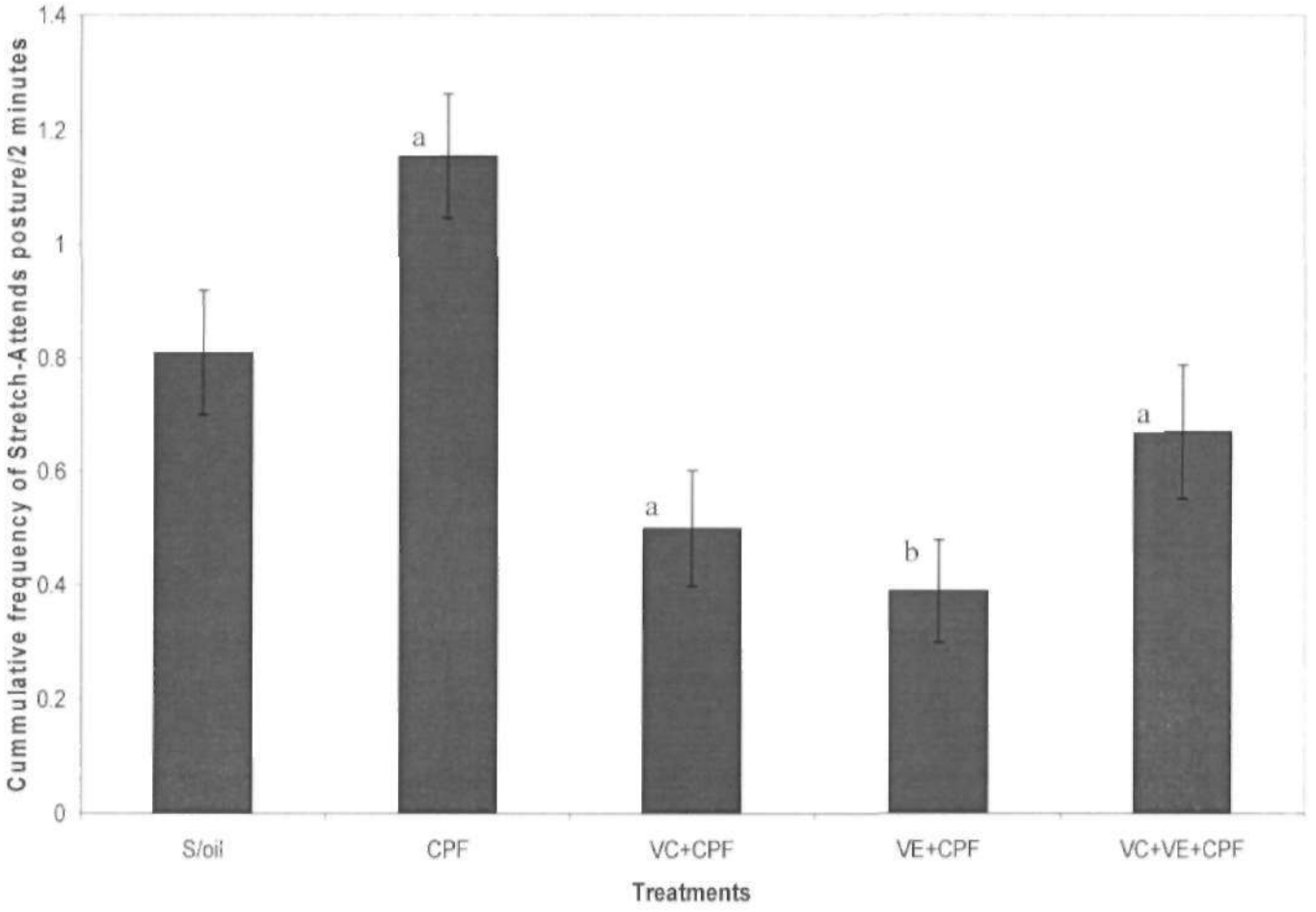


Figure 4.10: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative mean frequency of stretch-attends posture in adult Wistar rats (n=10).

a =No significant difference (P >0.05) compared to the control
b = Significant difference (P < 0.05) compared to the control

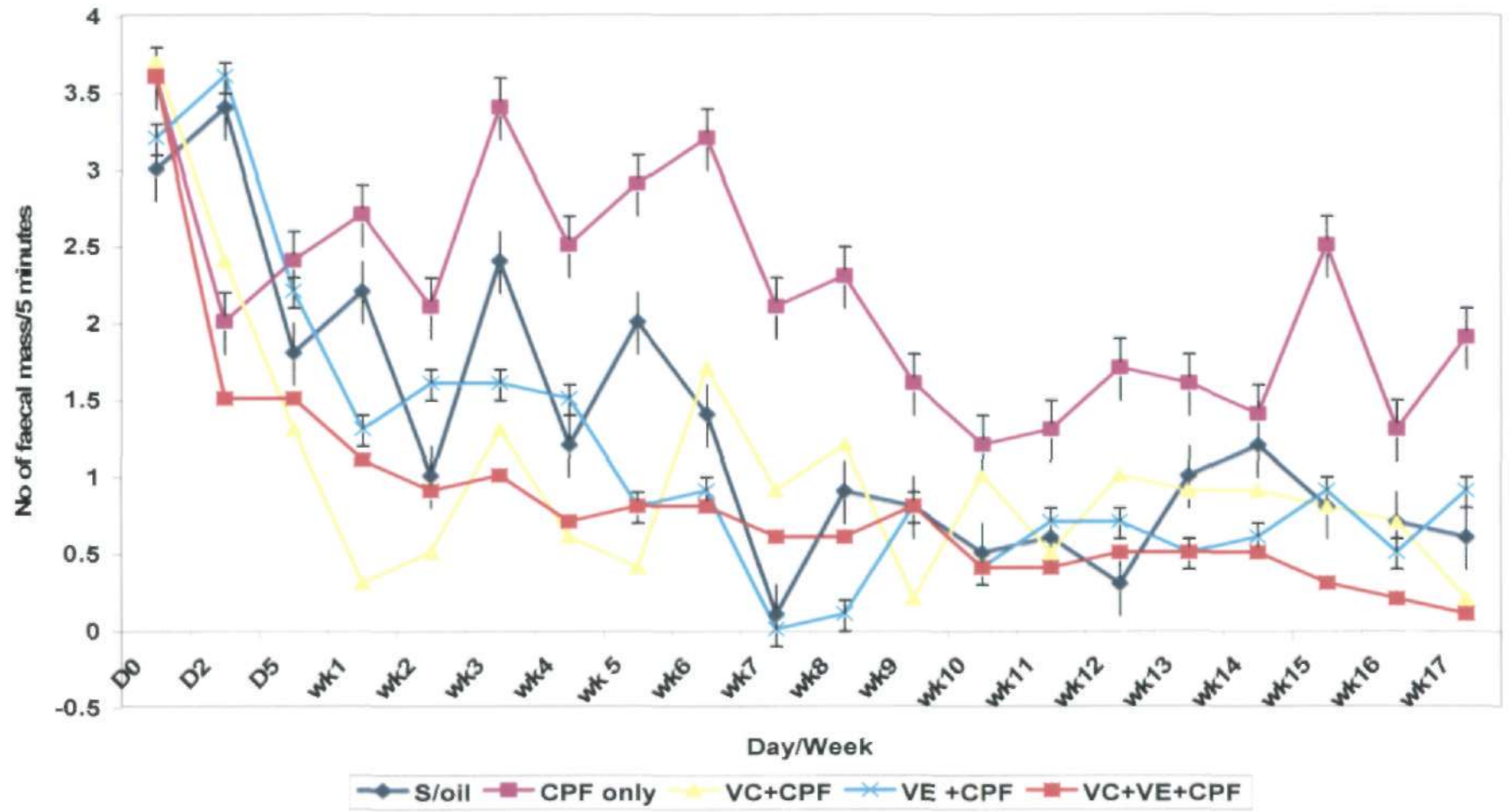


Figure 4.11: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on the time-course pattern in the frequency of defaecation in adult Wistar rats (n=10)

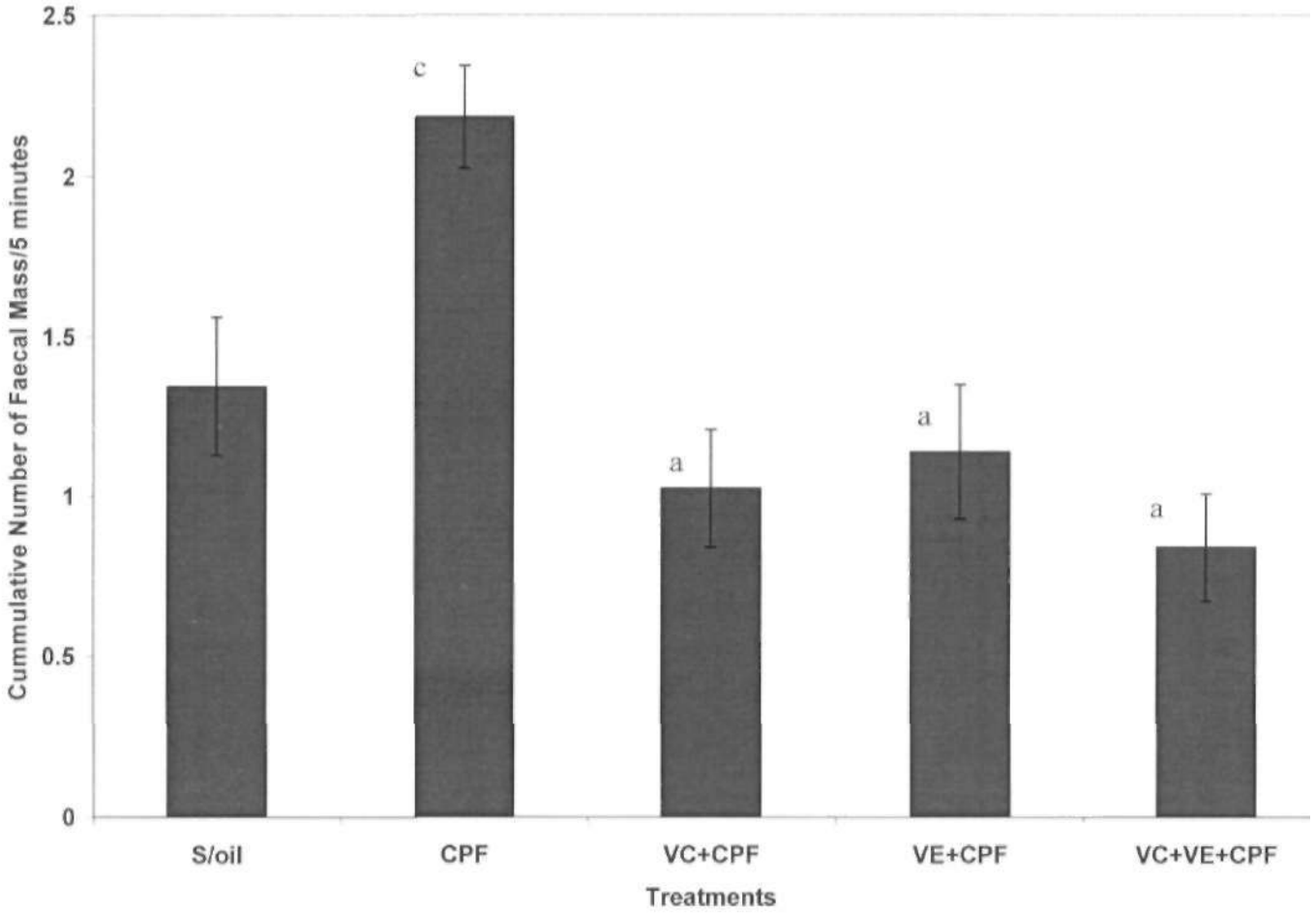


Figure 4.12: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative mean number of faecal mass in adult Wistar rats (n=10).

a =No significant difference ($P > 0.05$) compared to the control

c = Significant difference ($P < 0.01$) compared to the control

4.2.3.1.6 Effect on frequency of urination

Generally, there was a progressive decrease in the frequency of urination in all the experimental animals throughout the study period. Rats in the CPF group had a higher frequency of urination than both the control and the vitamins-pretreated groups. Rats in the CPF group showed a relatively consistent decrease in the frequency of urination until week 12 to week 16, when a consistent increase was observed. An increase in the frequency of urination was observed in CPF group by day 5 ($P < 0.05$) and week 12 and 17 ($P < 0.01$) compared to the control. On the other hand, a significant decrease in the frequency of urination occurred in the CPF group (0.2 ± 0.1) ($P < 0.05$) compared to the control (0.6 ± 0.2) by week 8. Similarly, a decrease in the frequency of urination in VC+VE+CPF ($P < 0.05$), VC+CPF and VE+CPF ($P < 0.01$) compared to the control was observed by week 8. No significant change in the frequency of urination was recorded in the other treatment groups compared to the control by week 8 (Figure 4.13). On the overall, the cummulative frequency of urination in the CPF group was significantly higher ($P < 0.05$) compared to the control. There was no significant change in the frequency of urination in vitamins-pretreated groups compared to the control (Figure 4.14). In addition, the difference in the frequency of urination in male rats compared to the females from each of the groups was insignificant throughout the study period.

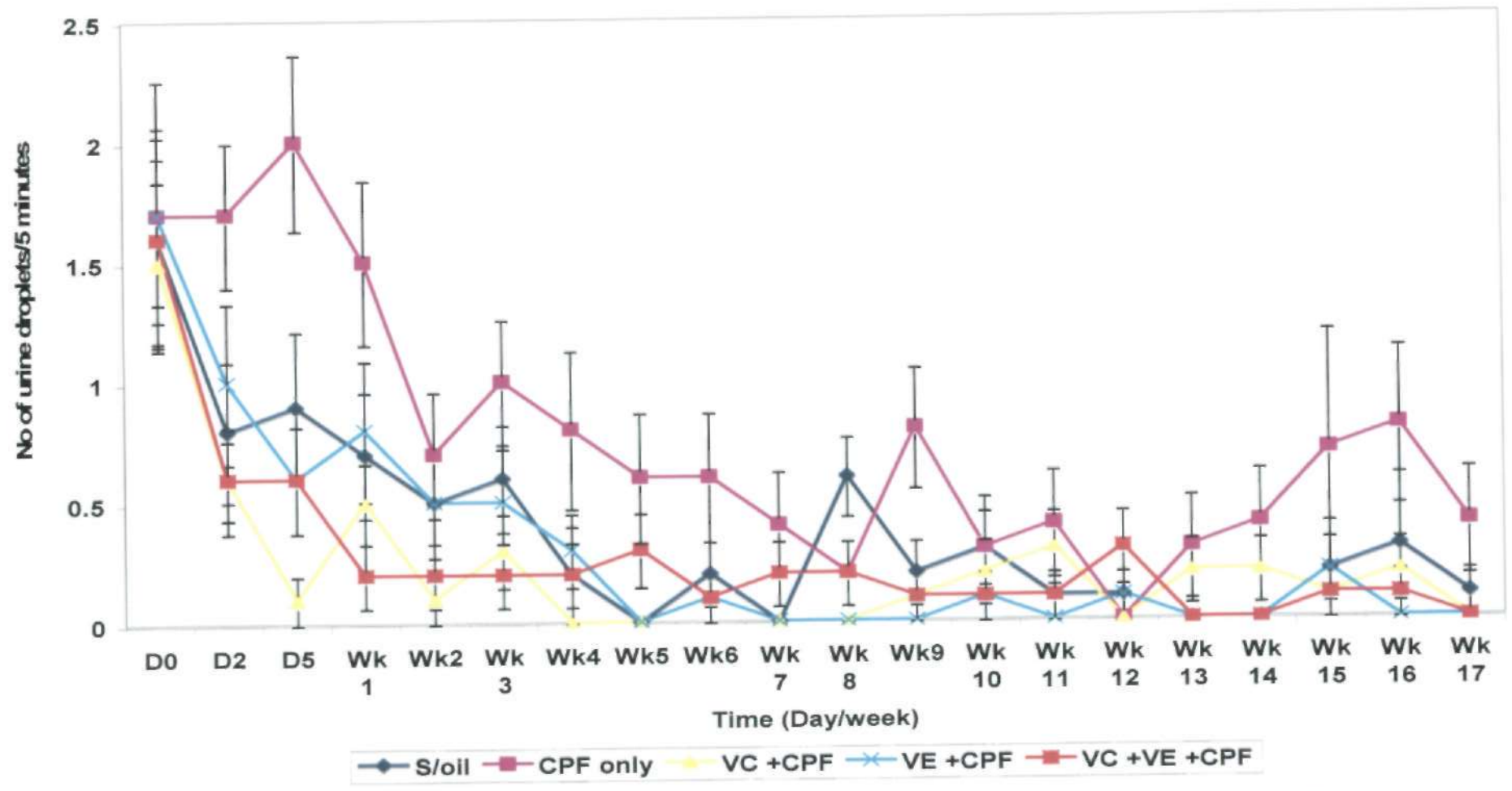


Figure 4.13: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on the pattern of frequency of urination in adult Wistar rats (n=10)

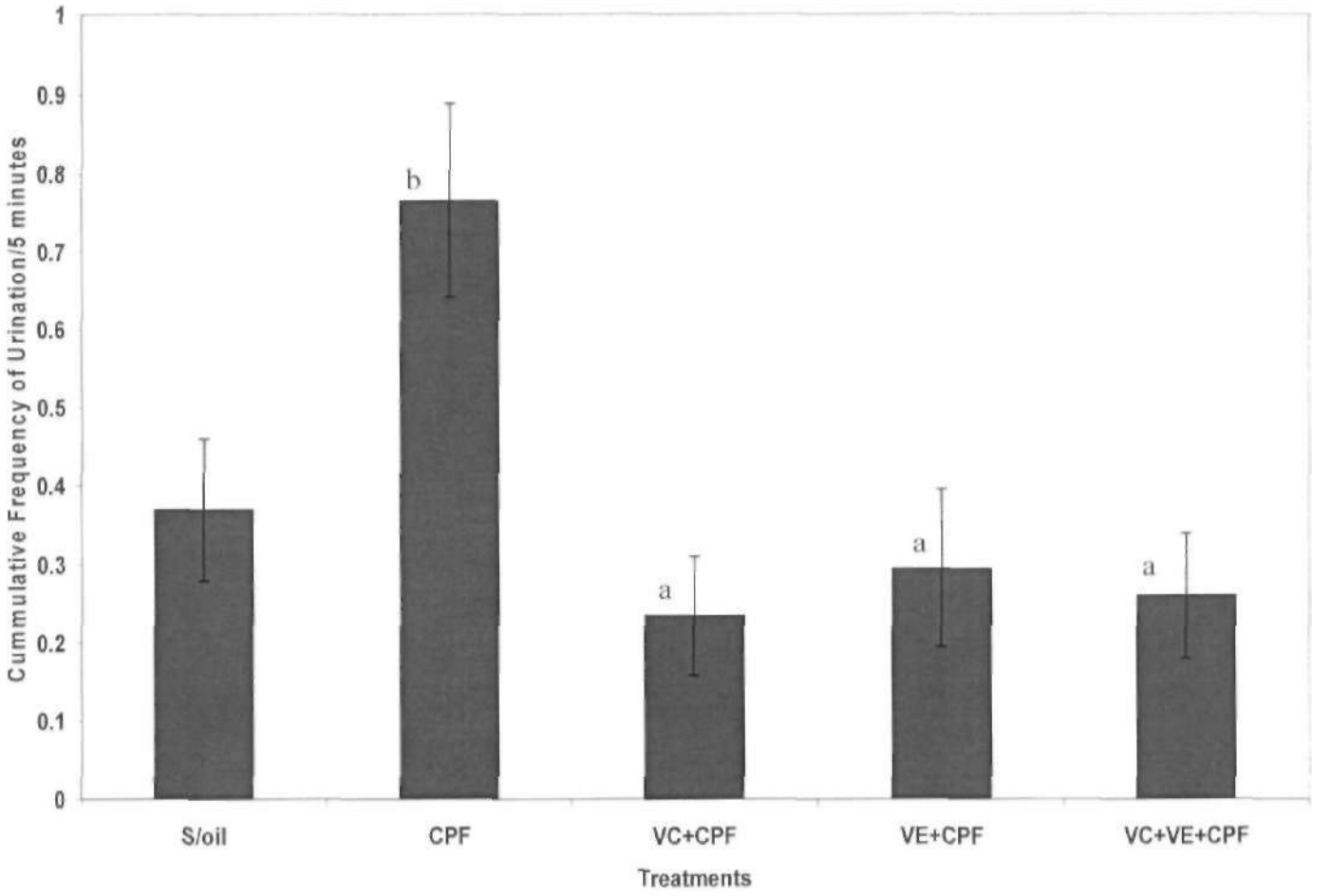


Figure 4.14: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative mean frequency of urination throughout the study period in adult Wistar rats (n=10).

a =No significant difference ($P > 0.05$) compared to the control
b = Significant difference ($P < 0.05$) compared to the control

4.2.3.2 Effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on neuromuscular coordination

4.2.3.2. 1 Effect of treatments on inclined plane performance

The effect of various treatments on inclined plane performance is shown in Figure 4.15. There was a significant ($P < 0.01$) decrease in the performance of rats in the CPF group on inclined plane at week 7 and 14 compared to the soya oil-treated control. On the other hand, no significant difference in the inclined plane performance was observed in the vitamins-pretreated groups compared to the control in both weeks 7 and 14 of the study. Male rats in the CPF group showed a better inclined plane performance than their female counterpart rats in week 7 only (Table 4.2).

4.2.3.2.2 Effect of treatments on ladder-walk performance

There was no significant change in the number of missed rungs in all the treatment groups by day 0, and in all the vitamins-pretreated groups by weeks 7 and 14, compared to the soya oil-treated control. On the contrary, a significant low ladder-walk performance characterised by a decrease in the number of missed rungs was observed in the CPF group by week 7 ($P < 0.01$) and 14 ($P < 0.05$), compared to the soya oil-treated control (Figure 4.16). There was no significant difference in the ladder-walk performance of male rats compared to the females in all the groups except in the soya-oil treated control ($P < 0.05$), where female had better ladder-walk performance than the male at week 7 only.

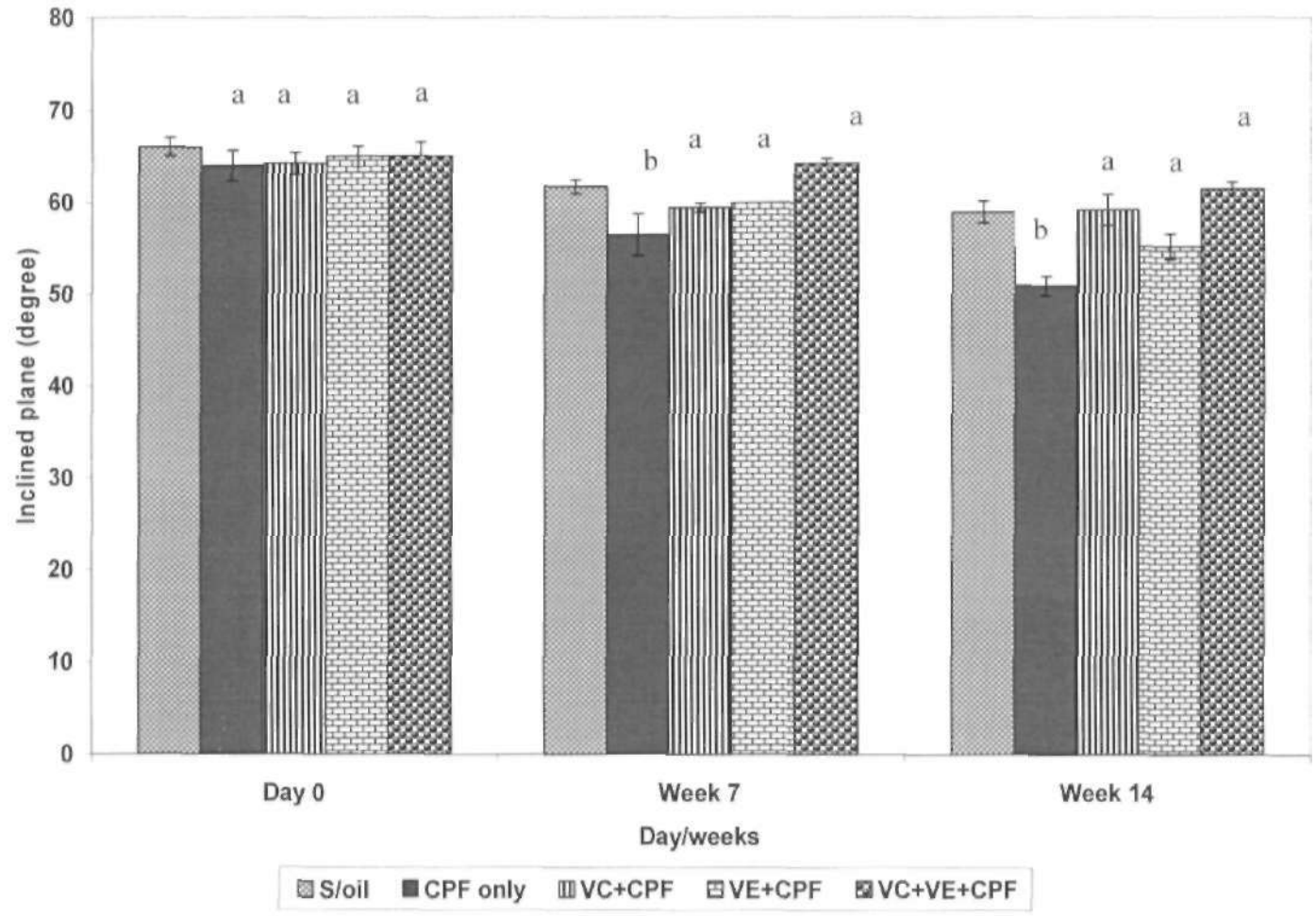


Figure 4.15: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on inclined plane performance in adult Wistar rats (n=10).

a = No significant difference (P>0.05) compared to the control
b= Significant difference (P < 0.05) compared to the control

Table 4.2: Sex differences on inclined plane performance following chronic administration of soya oil, chlorpyrifos and chlorpyrifos and/or vitamins C and/or E to rats

Time	Sex	Soya oil (degree)	Chlorpyrifos (CPF) only (degree)	Vitamin C+CPF (degree)	Vitamin E +CPF (degree)	Vitamin C+Vitamin E+CPF (degree)
Day 0	Female	66±1.9	65±2.2	64±1.9	65.5±1.7	67.5± 1.6
	Male	66±1.0	63±2.6	64.5±1.7	64.5 ±1.7	62.5±2.2
Week 7	Female	60.5±0.5	51.5±2.9*	59±1	60±0.0	63.5±2.2
	Male	63±1.2	61.5±1.5*	60±0.	60±0.0	65±0.0
Week 14	Female	60±1.4	51.5 ±1.5	60±2.7	55±2.7	61±1.0
	Male	58±2.0	50.5±1.7	58.5±2.2	55±0.9	62±1.2

* Significant difference in inclined plane performance ($P < 0.05$) between the male and female rats

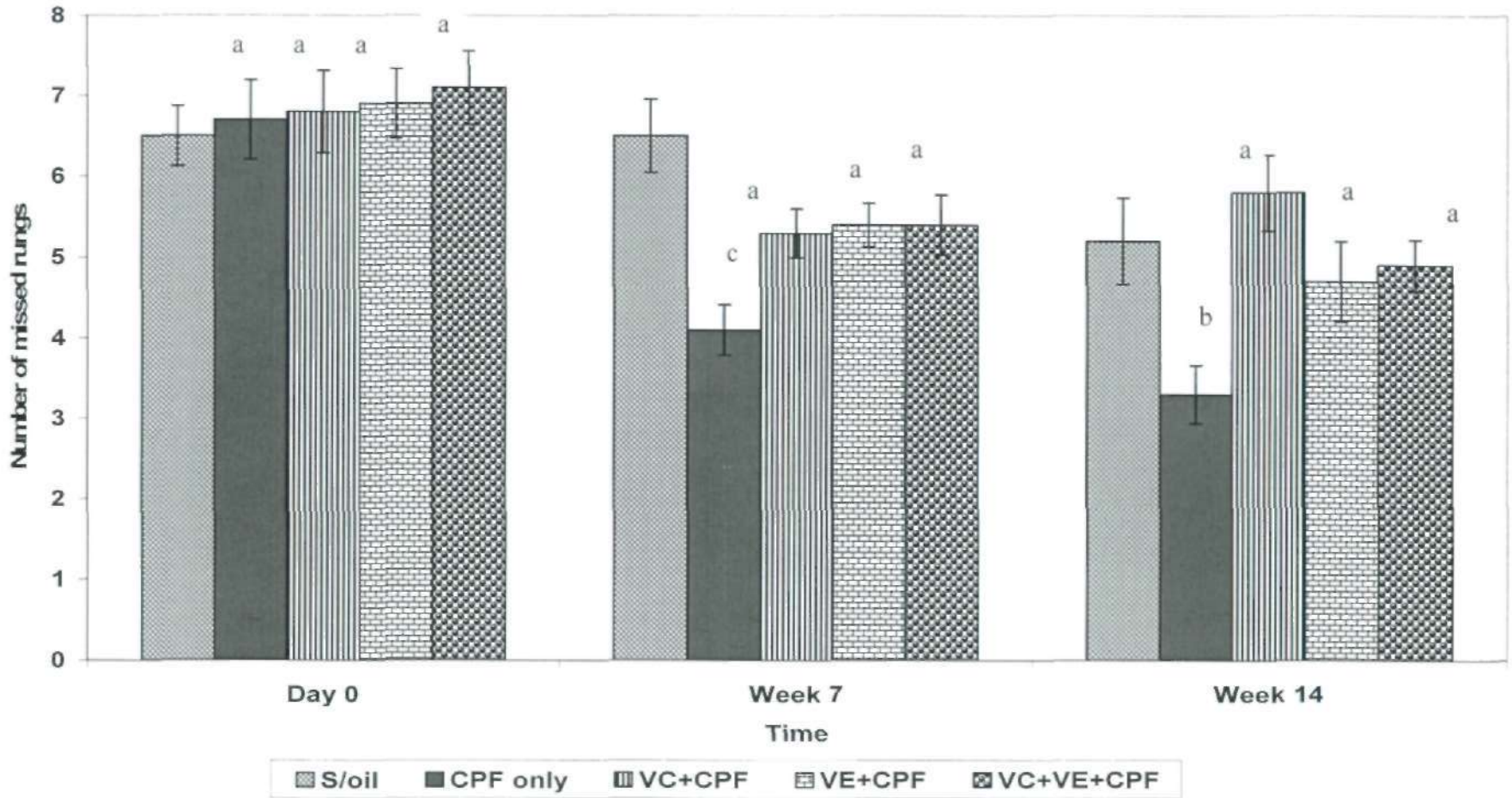


Figure 4.16: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on ladder-walk of adult Wistar rats (n=10)

a =No significant difference (P >0.05) compared to the control
b = Significant difference (P < 0.05) compared to the control
c = Significant difference (P< 0.01) compared to the control

4.2.3.2.3 Effect of treatments on surface righting reflex

The effect of various treatment groups on surface righting reflex is shown in Figure 4.17. All the rats from the control group had 100% 0 rating throughout the study period. However, all rats in the CPF group had 0 rating [100%(0)] by day 0; 30% (0), 70% (1) by week 4; 10% (0), 60 % (1) and 30% (2) by week 8; 10% (0), 40% (1); 50% (2) by week 12 ; while by week 16, 20% (0); 60% (1) and 20% (2). The peak of impairment of reflex righting in CPF group occurred in week 12, when 50% of the rats in the group had a poor rating of 2. By week 16, there was a slight drop in the number of animals with a rating of 2 and an increase in the number of animals with a rating of 1. Groups pretreated with vitamins C and/or E significantly had lower surface reflex righting time in a large percentage of animals than those in the group dosed with CPF only (Figure 4.17). On the overall, the number of rats that had rating of 0 were lower ($P < 0.01$) in the CPF group than the control. The cumulative percentage mean of rats in the CPF groups with rating of 1 and 2 was significantly higher ($P < 0.05$) in the CPF group than those in the control. There was no significant difference in the cumulative percentage mean of animals with a respective rating of 0, 1 and 2 in the vitamins pretreated groups compared to the corresponding rating in the control (Figure 4.18). The difference in the response of all the treatment groups to reflex righting time in female and male rats was insignificant ($P > 0.05$), although the female rats generally had a slightly lowered surface reflex righting time than the males.

4.2.3.4 Effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on coordinated gait

4.2.3.4.1 Effect of treatments on beam-walk score

The beam walk lengths between various treatment groups were not different from that of the control prior to the commencement of the treatment (day 0). However, there was a significant

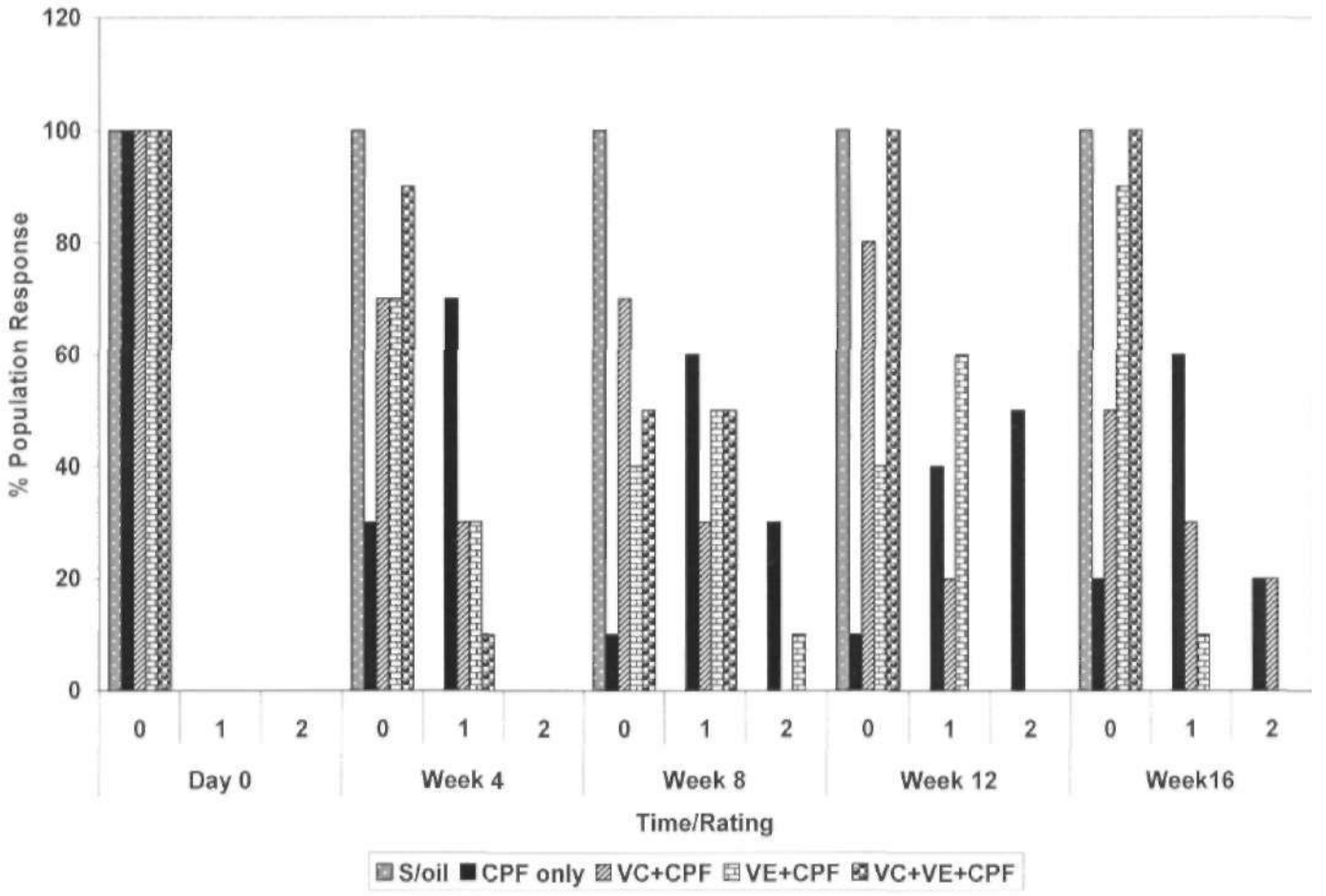


Figure 4.17: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on surface righting reflex of adult Wistar rats (n=10)

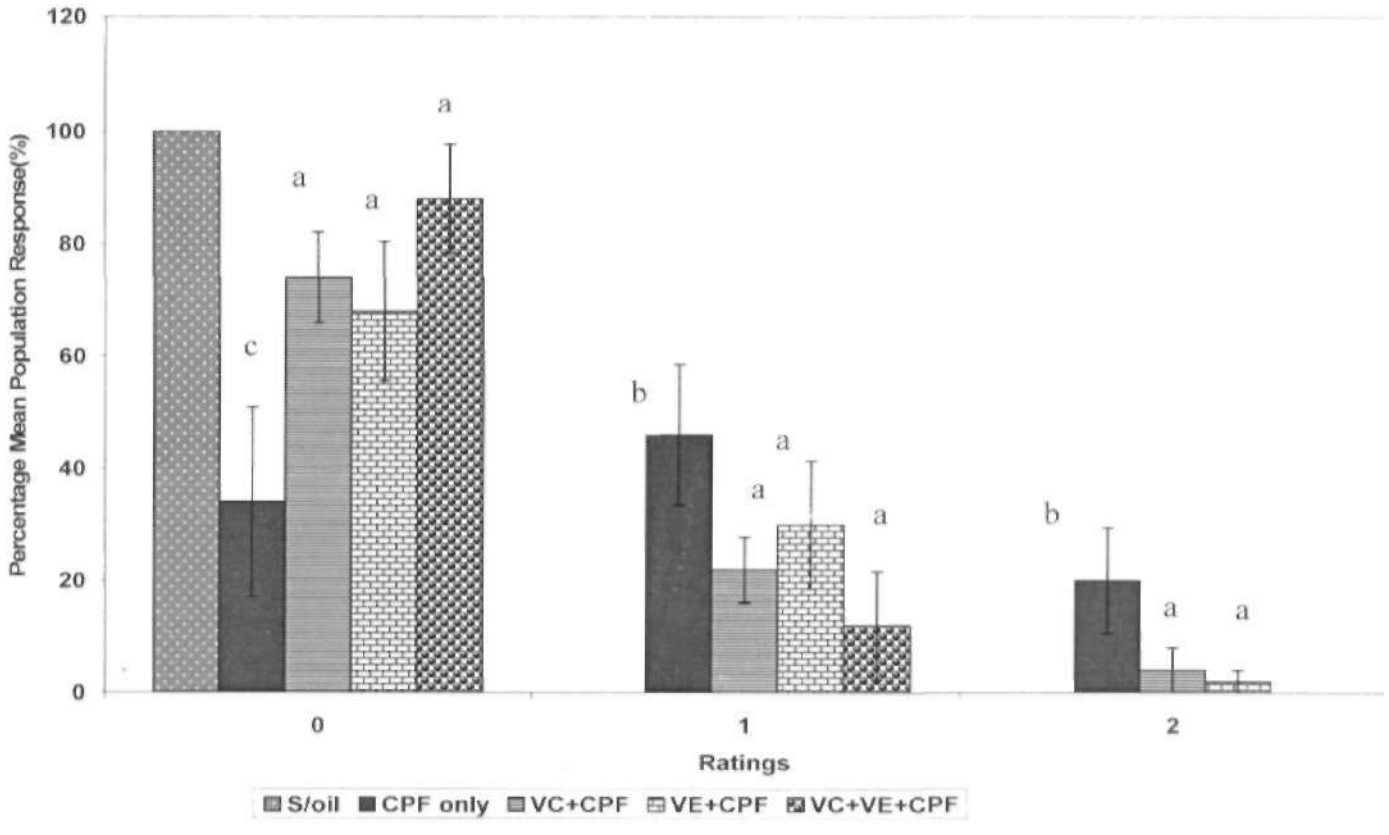


Figure 4.18: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative mean surface righting reflex of adult Wistar rats (n=10)

decrease ($P<0.01$) in the beam walk length, characterized by increase in the width by which the rat's limbs slipped off the beam apparatus, in CPF group compared to the control in weeks 7 and 14. On the contrary, there was a significant decrease ($P < 0.05$) in the width by which animals in the VC+VE+CPF group slipped off the beam by week 7 than even the control (Figure 4.19). On the whole, there was a significant increase in beam walk score in the vitamins-pretreated groups compared to the CPF group, with group pretreated with the combination of vitamins C and E showing the best beam walk score (Figure 4.19). There was no significant difference in the beam walk score between male and female rats in the different experimental groups.

4.2.3.4 Effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on sensorimotor reflexes

4.2.3.4.1 Evaluation of postural reflex

The effect of the various treatment groups on postural reflex is shown in Figure 4.20. Rats from all experimental groups demonstrated inconsistent flexion of their fore-limbs (score 0) by day 0. However, 10% and 90% of rats in the CPF group had a rating of 1 and 2, respectively, at week 7, while 10% and 90% of the rats in the CPF group also had a rating of 0 and 2, respectively, at week 14. A large percentage of the animals in the vitamins-pretreated group had a lower number of animals with 1 or 2 score rating compared to the CPF group (Figure 4.20). For example, 60% of the rats in the VC+CPF group had 0 rating at week 14, compared to 10% observed in the CPF group. On the overall, the percentage of rats that showed a rating of 0 was significantly lower ($P < 0.01$) in all the test groups compared to the control, with rats in the CPF group having the least percentage. The overall percentage of rats that had a rating of 1 in both CPF and VE+CPF groups were not significantly different from the control. The overall percentage of rats in VC+CPF and

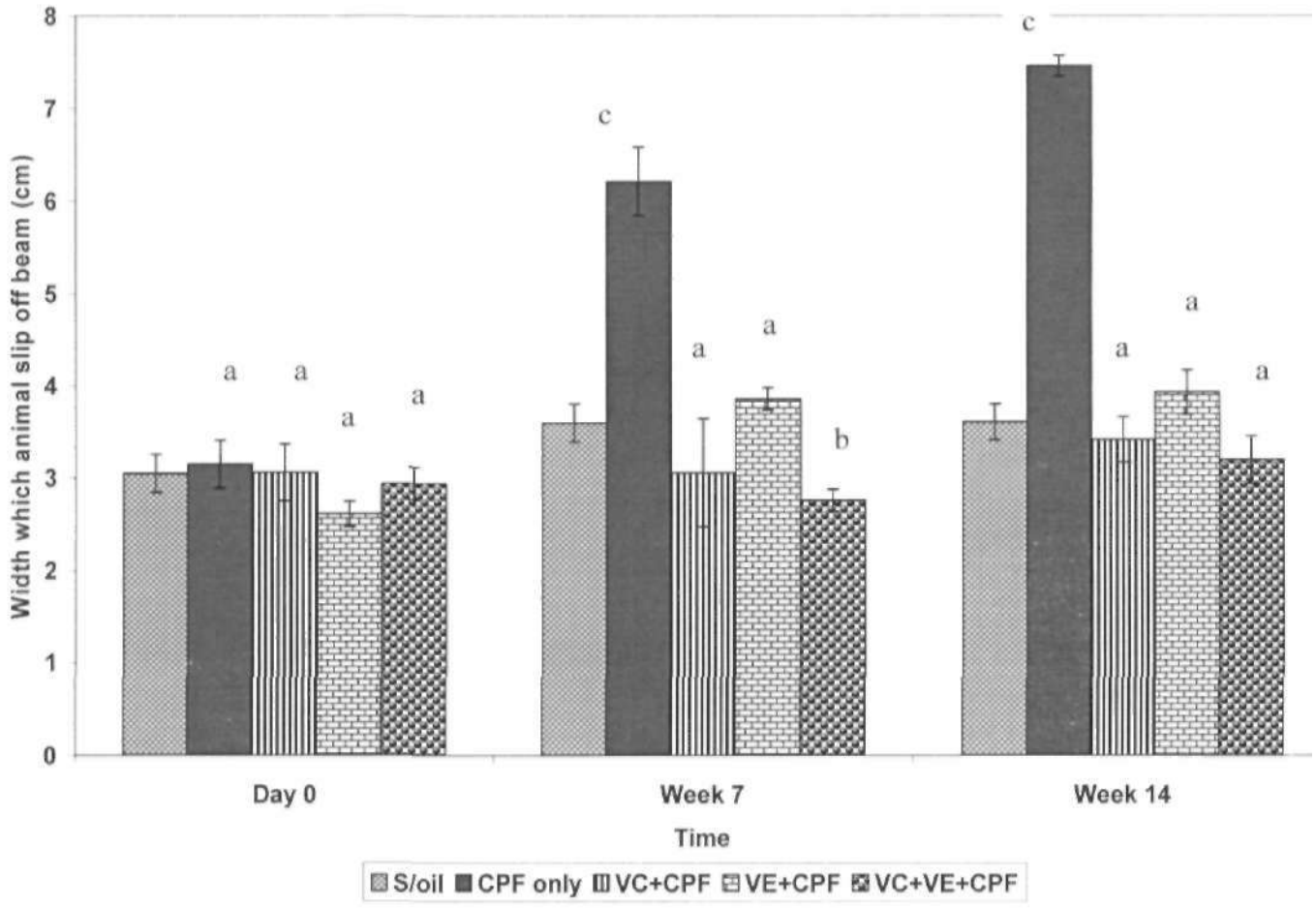


Figure 4.19: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on beam walk score of adult Wistar rats (n=10)

a = Non significant difference ($P > 0.05$) compared to the control
b = Significant difference ($P < 0.05$) compared to the control
c = Significant difference ($P < 0.01$) compared to the control

VC+VE+CPF groups that had a rating of 1 were higher ($P < 0.01$) than that in the control group,. The cummulative percentage of animals with rating of 2 was higher ($P < 0.01$) in the CPF group compared to the control. There was no significant difference in the cumulative percentage of rats in the vitamins pretreated groups that had a rating of 2 when compared to the control (Figure 4.21). There was no difference in the response of male and female rats in each group to postural reflex test.

4.2.3.4.2 Assessment of forelimb-placing response

4.2.3.4.2.1 Evaluation of visual-placing response

All the animals in the experimental group had a good visual-placing response by day 0 (100% with 0 rating). Animals in the soya oil-treated control had rating of 0 throughout the period of study. However at week 4, all rats in the CPF group experienced a poor visual-placing response with rating of 1; and by the 8th week of the study, 20% of the animals in the CPF group had rating of 0, while 80% had a rating of 1. By week 12, 10% and 90% of rats in the CPF group had rating of 0 and 1, respectively. At week 16, 30% and 70% of the rats in the CPF group had a rating of 0 and 1, respectively. On the other hand, 100% of the rats in VE+CPF and VC+VE+CPF groups, and 50% of the rats in VC+CPF group had a rating of 0 by week 12 (Table 4.3). On the whole, the overall percentage mean of rats that had rating of 0 was significantly lower ($P<0.01$) in both the CPF and VC+CPF groups compared to the control. The cumulative percentage mean of rats in VE+CPF and VC+VE+CPF groups with rating of 0 and 1 was not significantly different from those in the control (Figure 4.22). There was no significant difference in the visual-placing response of male rats compared to the females in all the experimental groups.

Table 4.3: Effect of chronic administration of soya oil, chlorpyrifos and chlorpyrifos and vitamins C and/or E on visual-placing response of adult Wistar rats (n=10)

Group	Percentage Population Response														
	Day 0			Week 4			Week 8			Week 12			Week 16		
	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
Soya oil	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
Chlorpyrifos (CPF) only	100	0	0	0	100	0	20	80	0	10	90	0	30	70	0
Vitamin C +CPF	100	0	0	80	20	0	60	40	0	50	50	0	50	50	0
Vitamin E + CPF	100	0	0	100	0	0	60	40	0	100	0	0	70	30	0
Vitamins C+E+CPF	100	0	0	80	20	0	60	40	0	100	0	0	90	10	0

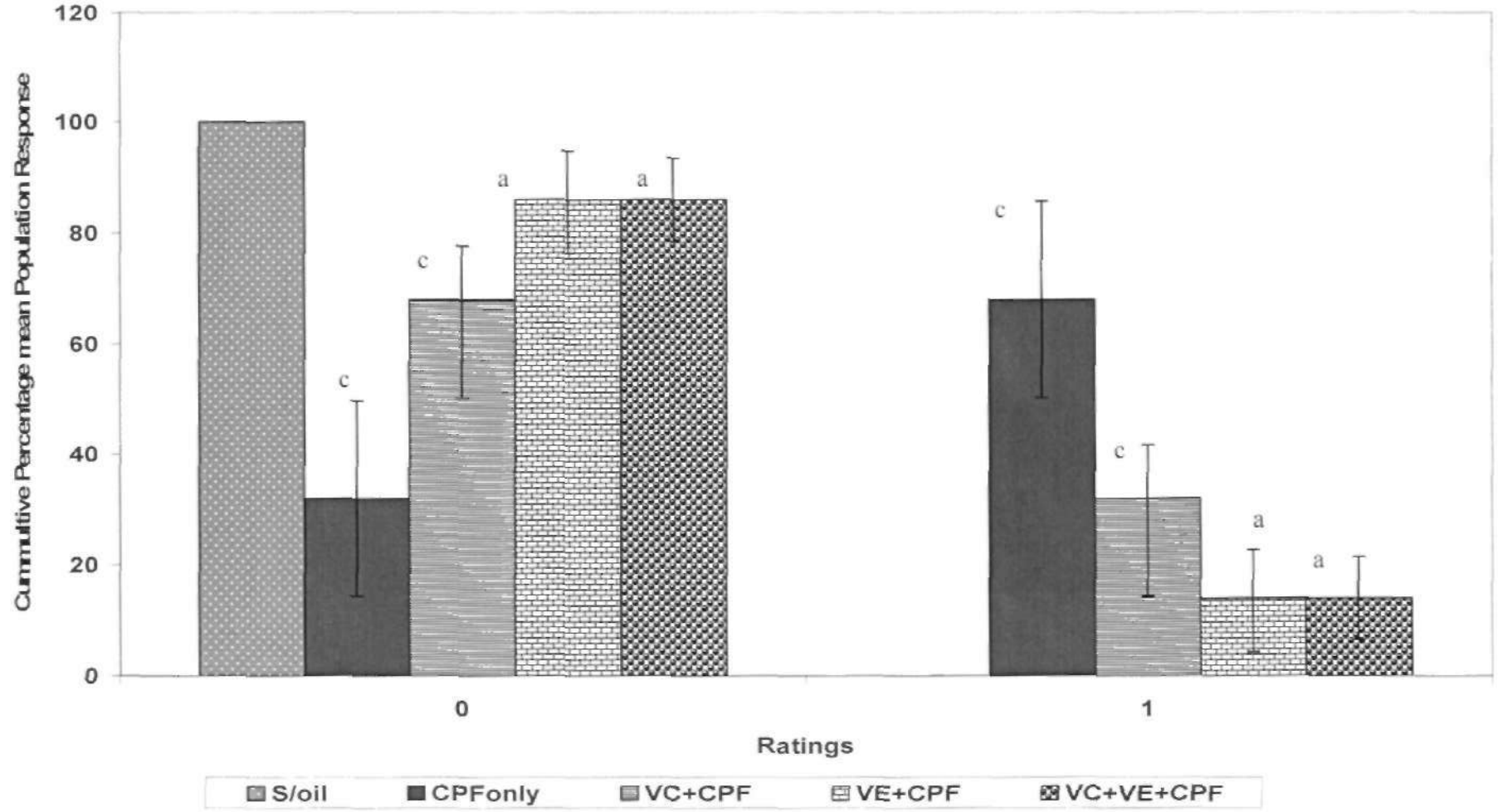


Figure 4.22: Effect of chronic administration of soya oil, chlorpyrifos and chlorpyrifos and vitamins C and/or E on visual-placing response of adult Wistar rats (n=10)

a = Non significant difference ($P > 0.05$) compared to the control

c = Significant difference ($P < 0.01$) compared to the control

4.2.3.4.2.2 Evaluation of tactile-placing response

There was no significant difference in tactile-placing response on day 0 in rats from all the experimental groups. However, by week 4, only 10% of the rats in the CPF group had intact tactile-placing response rating of 0 diminished to 10% in the CPF group. A significant increase occurred by week 8, 12 and 16 with 50% of the rats had rating of 0. Ten percent (10%) of the animals in the CPF group had a very poor rating of 2 by week 16. On the other hand, 70%, 90% and 100% of the rats, respectively in the VC+CPF, VE+CPF and VC+VE+CPF groups had 0 rating by week 12, compared to 50% in rats exposed to CPF only (Table 4.4). There was a significant decrease in the cumulative percentage mean of rats with rating of 0 in the CPF group ($P<0.01$) and those in VC+CPF ($P<0.05$) and VE+CPF ($P<0.05$) groups compared to the control. There was no significant change in the cumulative percentage mean of rats that showed a rating of 0 in the VC+VE+CPF group compared to the control (Figure 4.23). There was no difference between male and female rats from each group in response to tactile placing throughout the period of the study.

4.2.3.4.2.3 Evaluation of proprioceptive-placing response

Rats from all the experimental groups had a proprioceptive rating of 0 by day 0. All the rats in the soya oil-treated control group demonstrated very good proprioceptive placing response (0) throughout the period of study. However, 10% and 90% of the rats in the CPF group showed a rating of 0 and 1, respectively by week 4. 20%, 40% and 80% of the rats had a rating of 0 by week 8, 12, and 16, respectively. 70%, 60%, 80% and 50% of the rats in the VC+CPF group, respectively had a rating of 0 by week 4, 8, 12 and 16, respectively. 80%, 50%, 100% and 70%

Table 4.4: Effect of chronic administration of soya oil, chlorpyrifos (CPF) only and chlorpyrifos and vitamins C and/or E on tactile-placing response of adult Wistar rats (n=10)

	Percentage Population Response														
	Day 0			Week 4			Week 8			Week 12			Week 16		
	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
Soya oil	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
Chlorpyrifos (CPF) only	100	0	0	10	90	0	50	50	0	50	50	0	50	40	10
Vitamin C +CPF	100	0	0	80	20	0	70	30	0	70	30	0	70	30	0
Vitamin E + CPF	100	0	0	80	20	0	60	40	0	90	10	0	80	20	0
Vitamins C+E+CPF	100	0	0	90	10	0	80	0	0	100	0	0	80	20	0

of the rats in VE+CPF group had a rating of 0 by week 4, 8, 12 and 16, respectively. 90%, 70%, 100% and 70% of rats in the VC+VE+CPF group had rating of 0 by week 4, 8, 12 and 16, respectively (Table 4.5). There was a significant decrease in the cumulative percentage mean of rats that had rating of 0 throughout the period of study in the CPF ($P<0.01$) and VC+CPF ($P < 0.05$) groups compared to the control. No significant change in the cumulative percentage mean of rats with rating of 0 was observed in VE+CPF and VC+VE+CPF groups compared to the control (Figure 4.24). There was no significant difference in the response of the male animals compared to the females in all the experimental groups to this neurobehavioural parameter.

4.2.3.4.3 Evaluation of treatments on orientation to vibrissae touch

A sharp response to vibrissae touch was observed in all the experimental rats on day 0. In addition, all animals in the soya oil-treated control showed a rating of 0 throughout the period of the study. On the contrary, animals in the CPF group showed a poor orientation to vibrissae touch, and 20%, 10%, 40% and 50% of the animals had rating of 0 by week 4, 8, 12 and 16, respectively. However, 90%, 80%, 90%, 50% of rats pretreated with vitamin C, had rating of 0 by week 4, 8, 12, 16, respectively. 70%, 80%, 90% and 60% of rats pretreated with vitamin E, respectively had a rating of 0, by week 4, 12, 16, respectively (Figure 4.25). A rating of 0 was obtained in 70%, 100%, 100% and 90% of rats in VC+VE+CPF group by week 4, 8, 12 and 16, respectively. On the whole, there was a significant decrease ($P<0.01$) in the cumulative percentage of rats in the CPF group with a rating of 0 compared to the control. The cumulative percentage of rats with a rating of 1 was higher ($P<0.05$) in the CPF group compared to the control. The cumulative percentage of rats in the vitamins-pretreated groups with rating of 0 and 1 were not significantly different from the control (Figure 4.26). The response to vibrissae touch

observed in female rats was not different from that of the male rats in all groups throughout the study period.

4.2.3.4.4 *Evaluation of the effect of various treatments on excitability scores*

The effect of various treatments on excitability score is shown in Figure 4.28. At day 0, prior to dosing, rats in all the experimental groups showed a high excitability score of 5. Soya oil-treated control rats maintained the score of 5 throughout the period of the study. On the contrary, by the 4^{th} week of dosing, the percent number of animals in the CPF group with excitability scores of 5 and 4 decreased to 30% and 70%, respectively. The score progressively decreased by week 8, with 10%, 20%, 50% and 20% of the animals having a score of 2, 3, 4 and 5, respectively. By week 12, the percent numbers of rats with excitability score of 2, 3, 4 and 5 were 10%, 10%, 70% and 10%, respectively. At week 16, 20%, 10%, 30% and 40% of the rats had excitability scores of 1, 2, 3 and 4, respectively. Rats in the vitamins-pretreated groups showed a significant improvement in their excitability score. Thus, by week 16, 70% of the animals in VC+VE+CPF group had an excitability score of 5, compared to 0% in the CPF group. On the overall, the cumulative percentage mean of rats with excitability scores of 1, 2 and 3 were not significantly different in all test groups compared to the control, although many rats in the CPF group had lower excitability scores than the other groups (Figure 4.29). The cummulative percentage mean of rats with excitability score of 4 and 5 were significantly lower in the CPF ($P < 0.01$), VC+CPF ($P < 0.01$), VE+CPF ($P < 0.05$) and VC+VE+CPF ($P < 0.05$) groups compared to the control. There was no significant difference in the excitability scores between male and female rats in each experimental group.

4.2.3.5 Effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on motor strength

4.2.3.5.1 Evaluation of the effect of various treatments on forelimb grip time

There was no significant difference in the grip time values between the various treatment groups and the control on day 0 (Figure 4.29). By week 4, a significant decrease ($P<0.01$) in grip time was recorded in the CPF (59.4 ± 8.0) and VE+CPF (58.1 ± 6.9) groups compared to the soya oil-treated control (97±4.1). However, there was no significant change in the values of grip time obtained in the VC+CPF and VC+VE+CPF groups compared to the control. By week 8, the grip time reduced significantly in the CPF and VE+CPF ($P < 0.01$), and VC+VE+CPF ($P < 0.05$) groups compared to the control. There was no significant change in the grip time in the VC+CPF group compared to the control by week 8. However by week 12 and 16, a significant decrease ($P < 0.01$) in the grip time was observed in all the test groups compared to the control (Figure 4.29). The values of grip time recorded in the male and female rats from any of the experimental groups did not differ ($P > 0.05$).

Table 4.5: Effect of chronic administration of soya oil, chlorpyrifos and chlorpyrifos only and vitamins C and/or E on proprioceptive-placing response of adult Wistar rats (n=10)

	Percentage Population Response														
	Day 0			Week 4			Week 8			Week 12			Week 16		
	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
Soya oil	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
Chlorpyrifos (CPF) only	100	0	0	10	90	0	20	80	0	40	60	0	50	50	0
Vitamin C +CPF	100	0	0	70	30	0	60	40	0	90	10	0	50	50	0
Vitamin E + CPF	100	0	0	80	20	0	50	50	0	100	0	0	70	30	0
Vitamins C+E+CPF	100	0	0	90	10	0	70	30	0	100	0	0	70	30	0

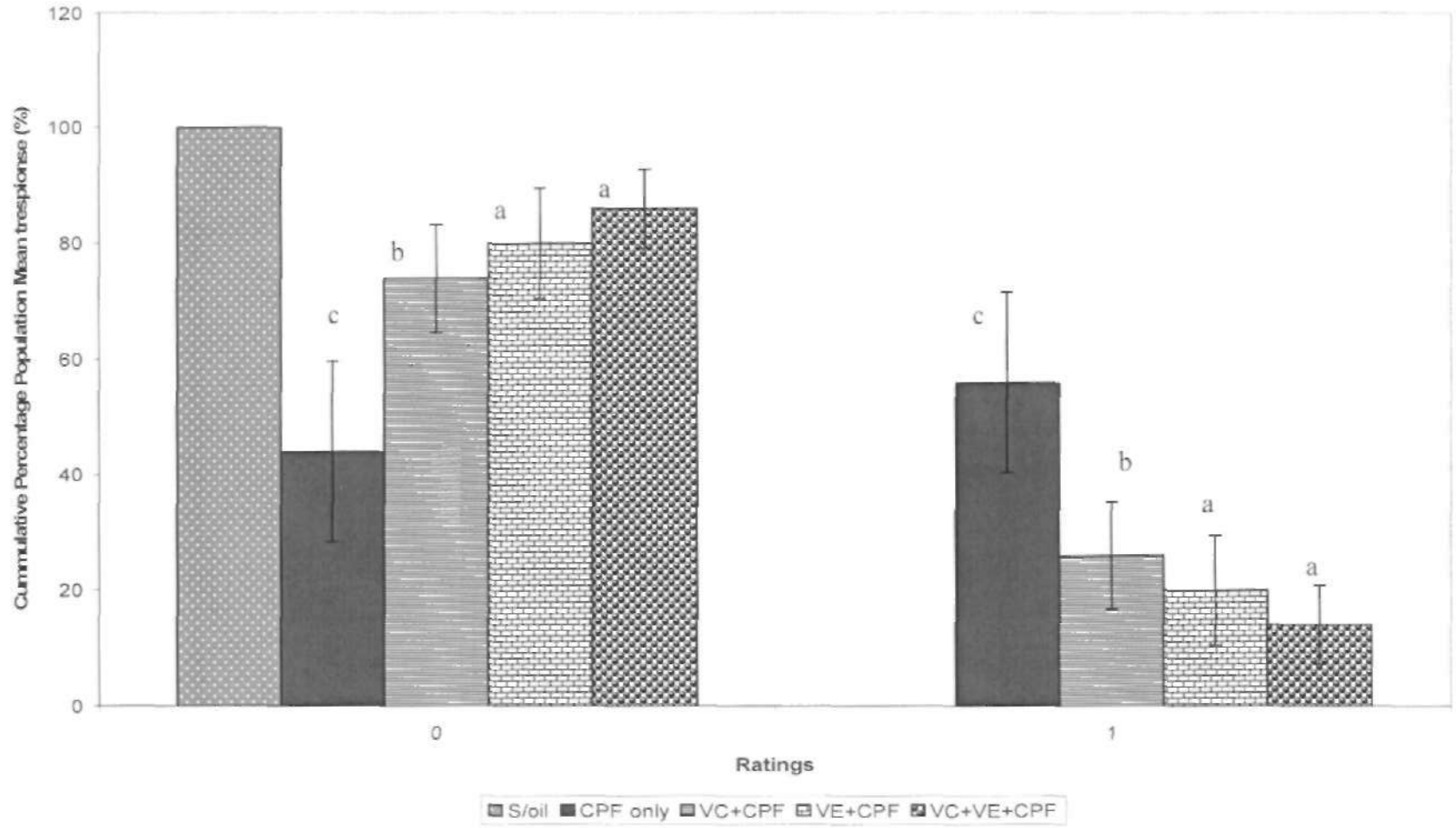


Figure 4.24: Effect of chronic administration of soya oil, chlorpyrifos and chlorpyrifos only and vitamins C and/or E on cumulative mean proprioceptive-placing response of adult Wistar rats (n=10).

a = Non-significant difference ($P > 0.05$) compared to the control
b = Significant difference ($P < 0.05$) compared to the control
c = Significant difference ($P < 0.01$) compared to the control

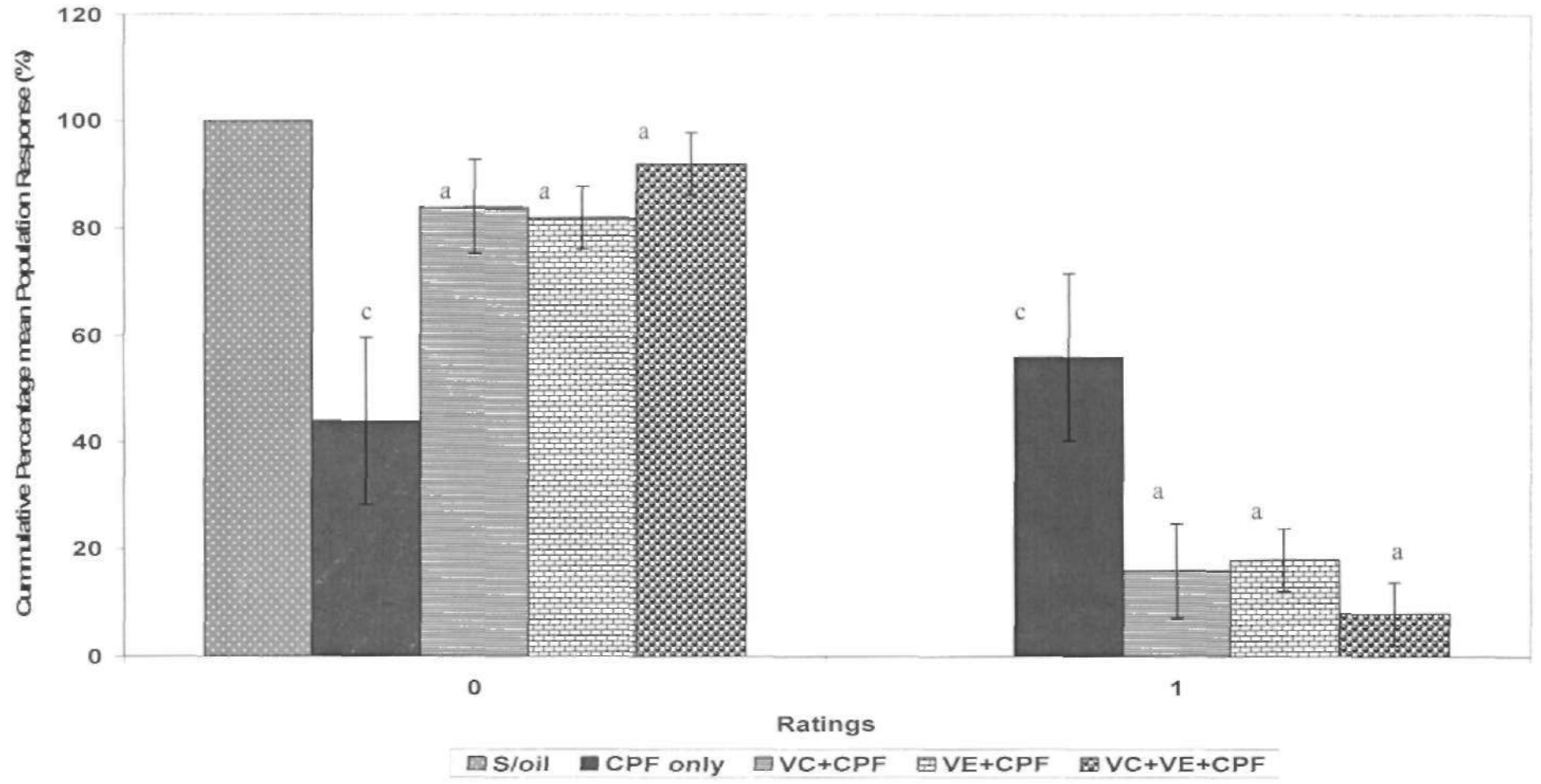


Figure 4.26: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative vibrissae touch in adult Wistar rats (n=10).

a = Non-significant difference ($P > 0.05$) compared to the control
b = Significant difference ($P < 0.05$) compared to the control
c = Significant difference ($P < 0.01$) compared to the control

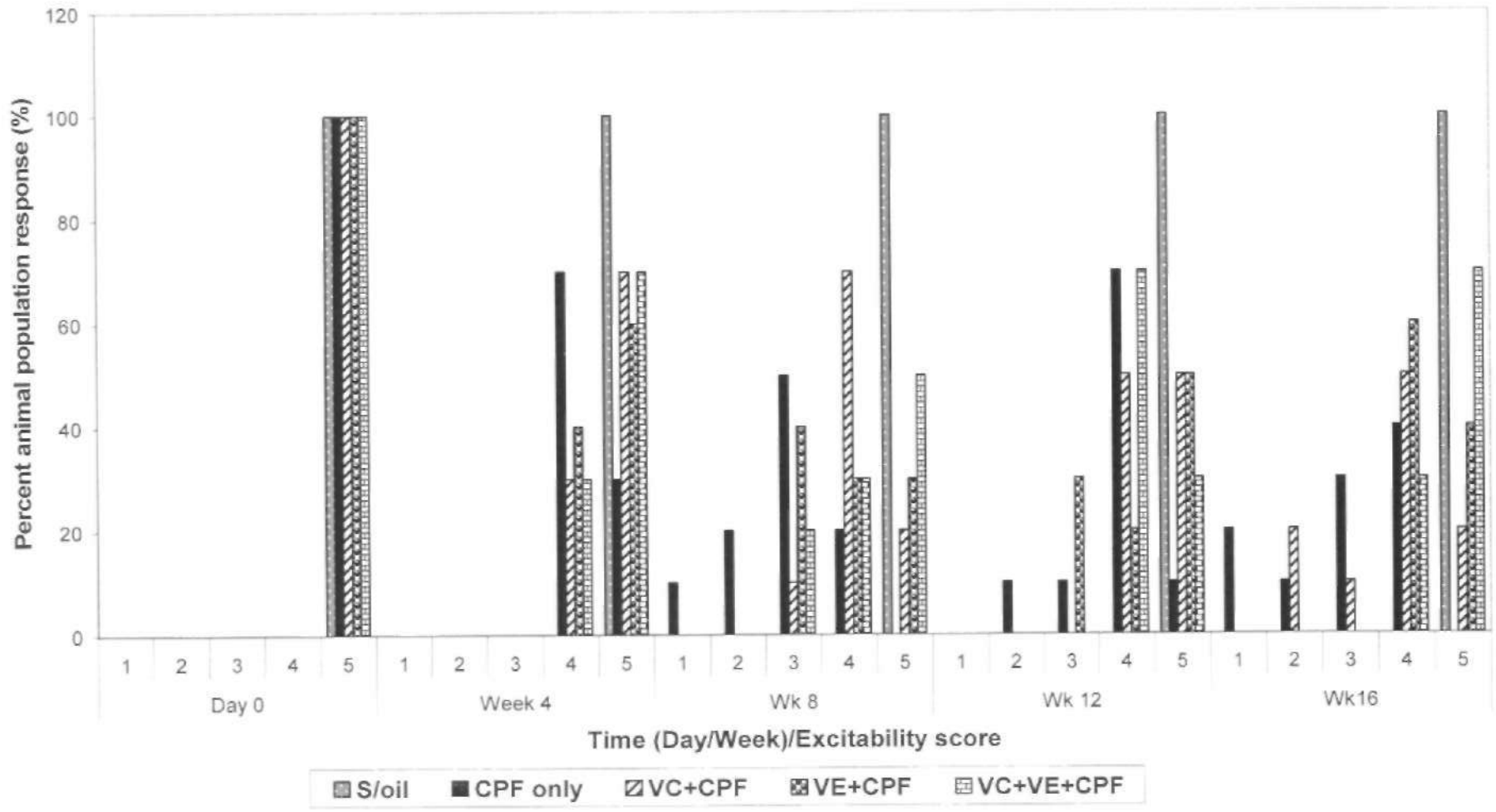


Table 4.27: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on excitability score in adult Wistar rats (n=10).

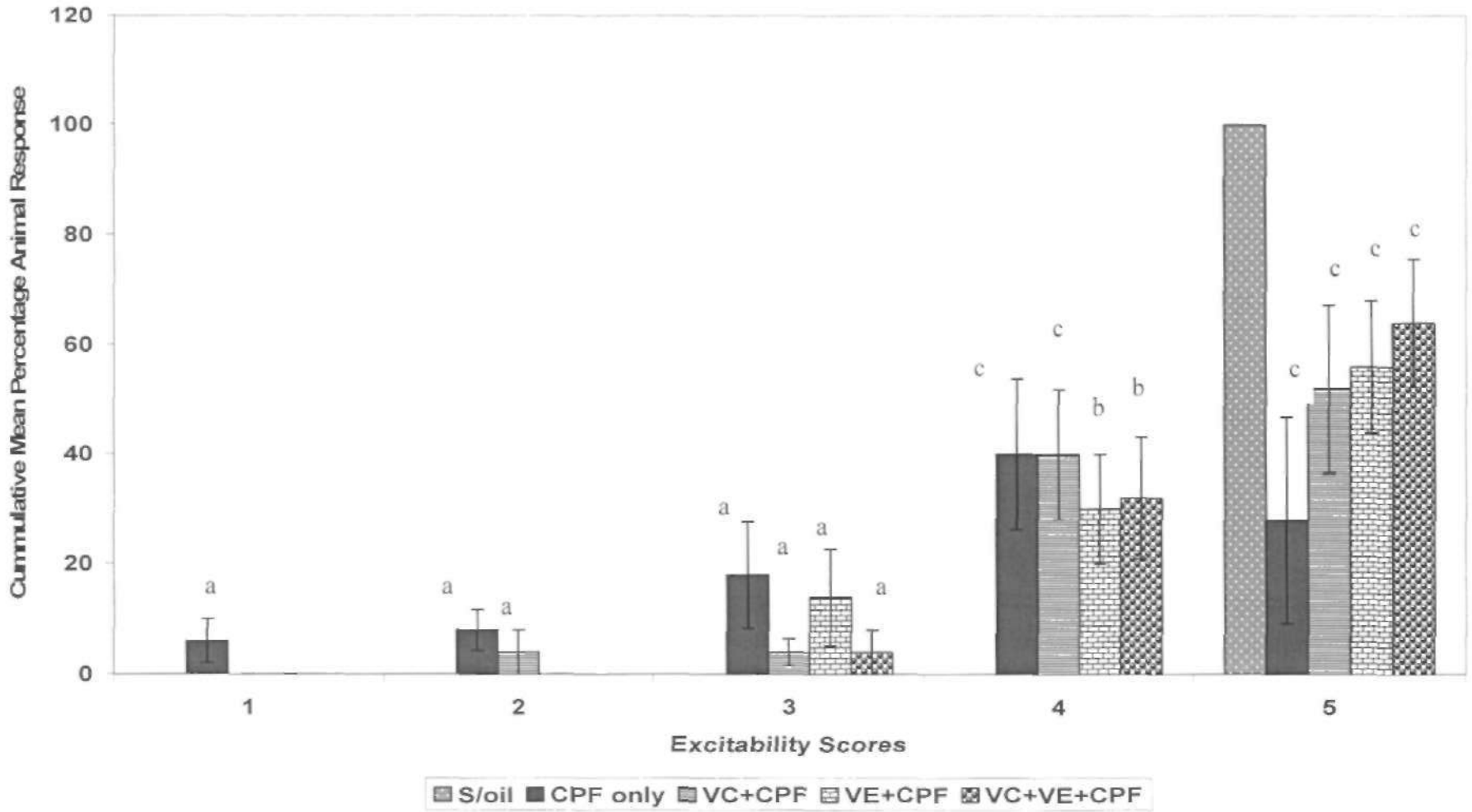


Figure 4.28: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on cummulative excitability score of adult Wistar rats (n=10)

a = Not Significantly different ($P > 0.05$) from the control

b = Significantly different ($P < 0.05$) from the control

c = Significantly different from the ($P < 0.01$) control

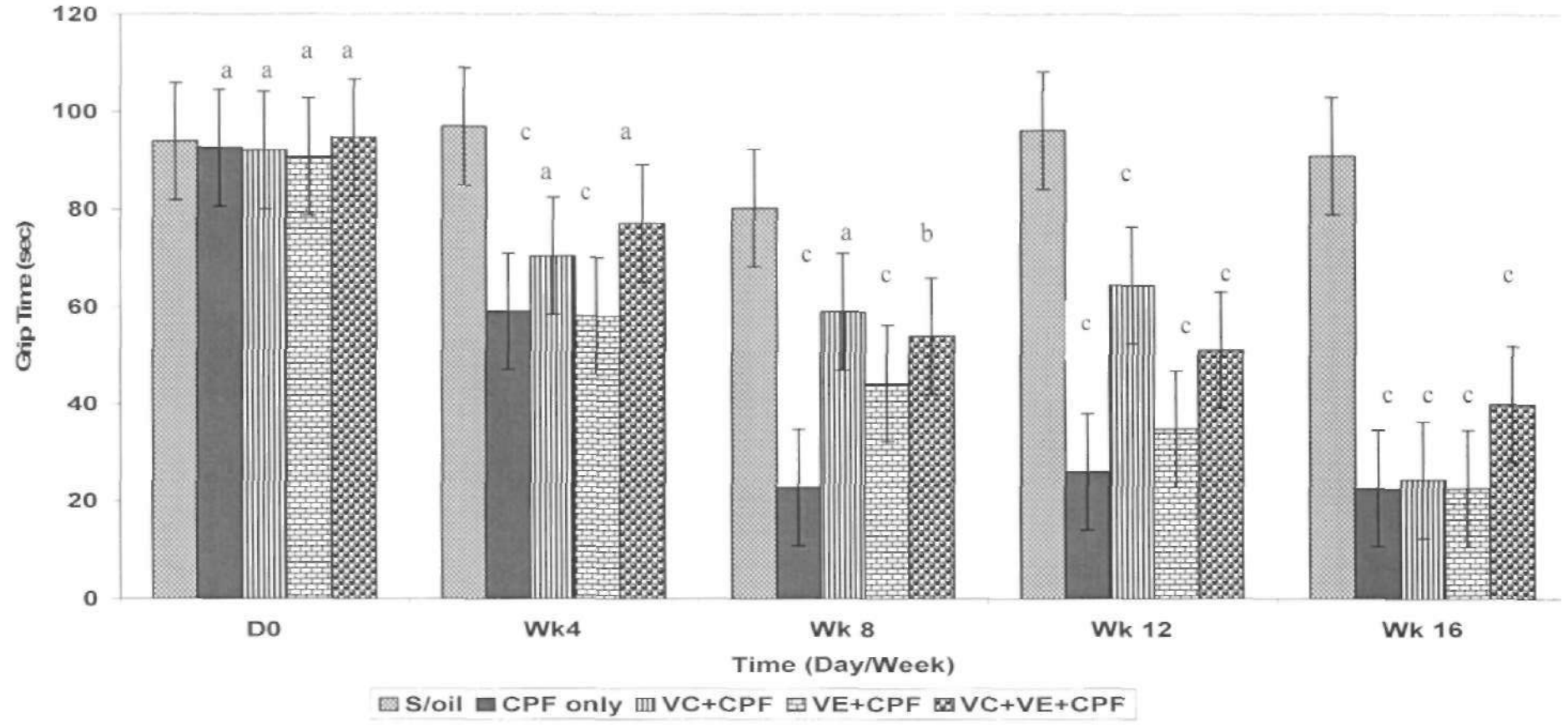


Figure 4.29: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on grip time in adult Wistar rats (n=10).

a = Not Significantly different ($P > 0.05$) from the control
b = Significantly different ($P < 0.05$) from the control
c = Significantly different from the ($P < 0.01$) control

4.2.9 Effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on cognition

4.2.9.1 Effect on learning

The effect of various treatments on learning is shown in Figure 4.30. There was a significant increase ($P < 0.05$) in the number of footshocks applied to rats in the CPF group (3.9 ± 0.6) compared to the control (2.4 ± 0.4), reflecting poor learning ability in rats from the CPF group compared to the soya oil-treated control. Although there was insignificant reduction in the number of footshocks applied in rats from the vitamins-pretreated groups compared to the control. This shows that rats in the vitamins-pretreated groups had a slight increase in the performance of learning task than those in the control. In addition, there was a significant reduction in the number of footshocks applied in the vitamins-pretreated groups compared to those in the CPF group, indicating significant increase in the performance of learning task in groups pretreated with vitamins over those exposed to CPF only. There was a significant reduction ($P < 0.05$) in the number of footshocks applied to male rats in the CPF group compared to the females indicating difference in the sex response to learning task in the CPF group, with the male showing a better learning ability than the female. No significant difference in the sex response in the number of footshocks hence learning task was observed in the rest groups.

4.2.9.2 Effect on short-term memory

The duration of staying on the platform by rats in the CPF group was comparatively shorter ($P<0.05$) than that recorded in the soya oil-treated control group. No significant difference was observed in the vitamins-pretreated group compared to the control group. In general, rats pretreated with vitamins C and/or E stayed longer on the platform than those exposed to CPF

only. In addition, rats in the VC+VE+CPF group had a longer stay on the platform (114.9 ± 4.0 secs) than the soya oil-treated control group (94.6 ± 12.03 secs), although the difference was not significant (Figure 4.31). There was no significant difference in the response of the male and female rats in the duration of their stay on the platform in all the experimental groups.

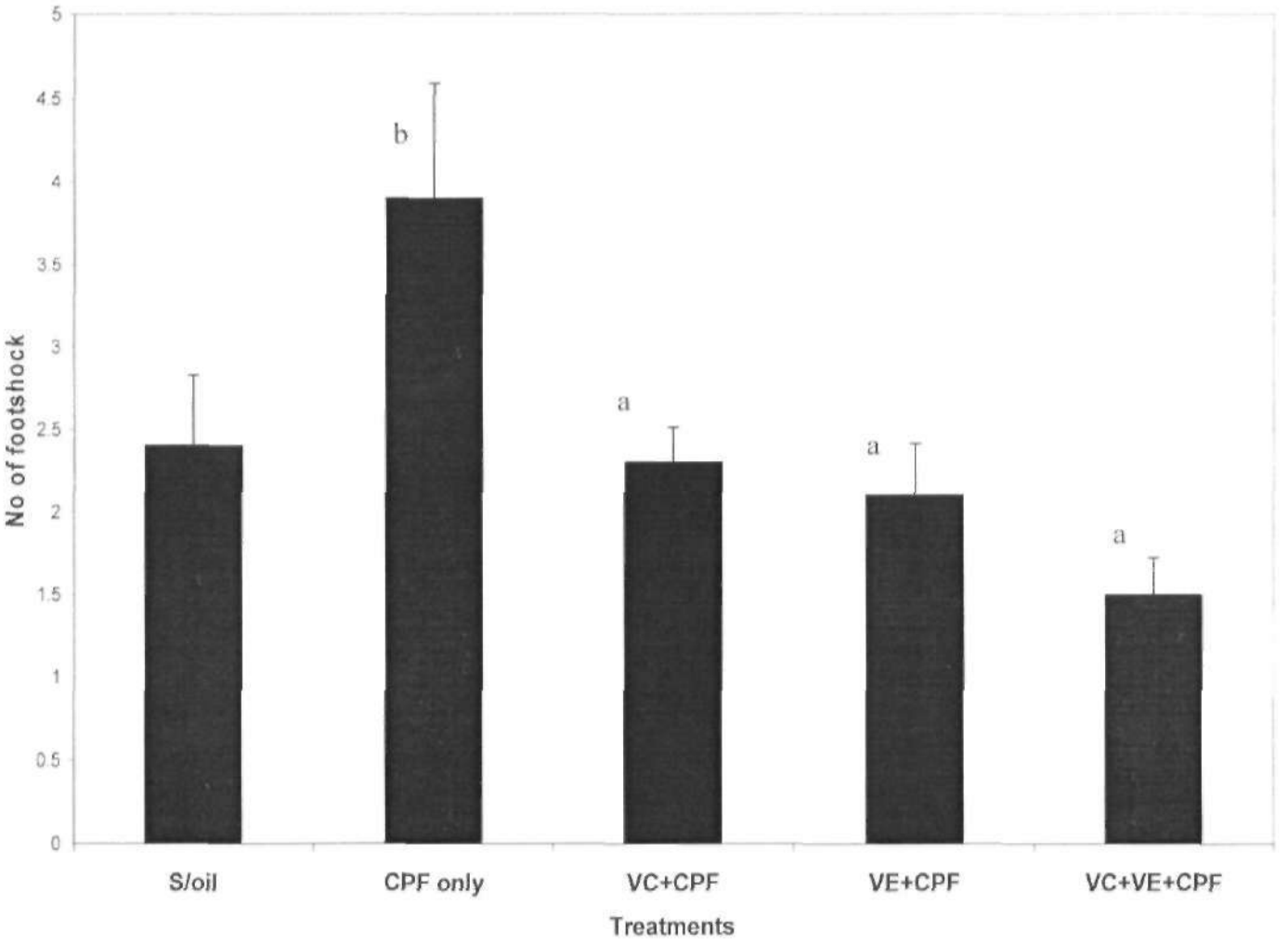


Figure 4.30: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on learning in adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control
b = Significantly different ($P < 0.05$) from the control

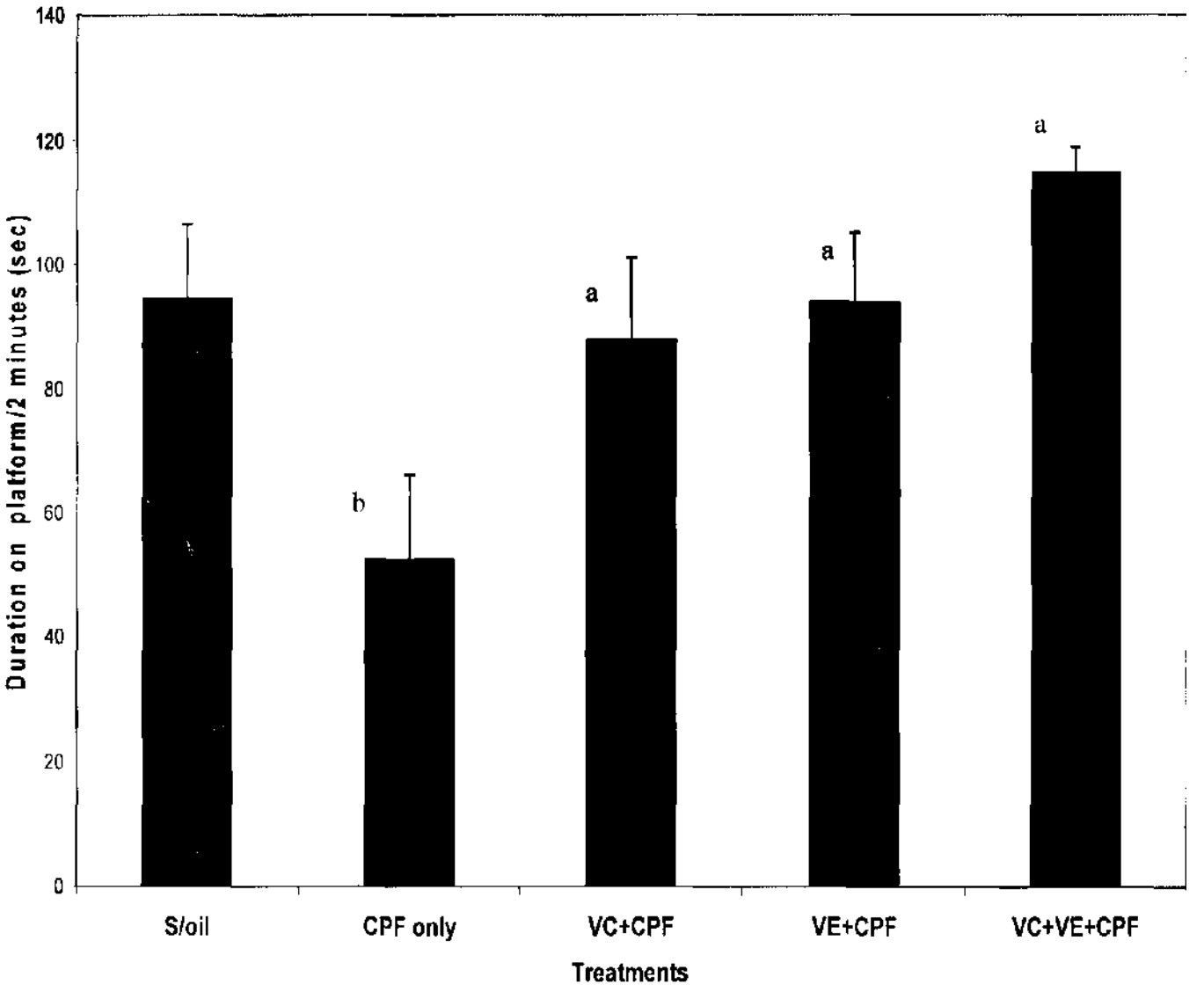


Figure 4.31: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on short-term memory in adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control
b = Significantly different ($P < 0.05$) from the control

4.2.4 Effect of chronic repeated exposure to soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on haematological parameters in adult Wistar rats

4.2.4.1 Effect on packed cell volume

There was a significant reduction ($P < 0.05$) in the PCV of rats exposed to CPF only (35.5 ± 1.1) compared to the control (42.2 ± 1.6). The PCV in rats pretreated with vitamins C and/or E did not change significantly from that of the control rats (Figure 4.32). There was a significant increase in the PCV of male rats in the control ($P < 0.05$) and VC+VE+CPF ($P < 0.01$), compared to the female rats in both groups. The PCV in the male and female rats were not significantly different in the CPF, VC+CPF, and VE+CPF groups.

4.2.4.2 Effect on total red blood cell count

There was a significant decrease ($P < 0.01$) in the total RBC count in rats repeatedly exposed to CPF only (3.7 ± 0.1 x 10^{12}/L) compared to the control (5.8 ± 0.5 x 10^{12}/L). On the other hand, there was no significant change in total RBC count in vitamin C and/or E pretreated groups compared to the control (Figure 4.33). The total RBC count recorded in the male rats did not differ from those obtained in the females in any of the groups.

4.2.4.3 Effect on haemoglobin concentration

A significant reduction ($P < 0.05$) was observed in the Hb concentration of rats repeatedly exposed to CPF only (11.8 ± 0.4 g/dL) compared to the control (14.1 ± 0.5 g/dL). Rats pretreated with vitamins C and/or E showed no significant change in Hb concentration compared to the control (Figure 4.34). There was a significant increase in Hb concentration of male rats compared to the female rats in the control ($P < 0.05$) and VC+VE+CPF ($P < 0.01$) groups.

However, no significant change was recorded in the Hb concentration of male rats in the CPF, VC+CPF and VE+CPF groups compared to the females.

4.2.4.4 Effect on white blood cell counts

The effect of various treatments on absolute WBC count is shown in Figure 4.35. A significant decrease ($P < 0.01$) in absolute WBC counts was observed in the rats exposed to only CPF ($5.6 \pm 0.3 \times 10^6$ g/dL) compared to the control ($9.8 \pm 0.7 \times 10^6$ g/dL). The leucopaenia observed in the CPF group was due to neutropaenia (Table 4.6). There was no significant change in the absolute WBC count in rats from the vitamins pretreated groups compared to the control. There was no significant difference in the absolute WBC count of male rats compared to the female in all the groups, except in VC+VE+CPF where male rats had a significant increase ($P < 0.01$) in WBC values compared to the females.

4.2.4.5 Effect on *in vitro* erythrocyte osmotic fragility

The effect of the various treatments on haemolysis is illustrated in Figure 4.36. There was complete haemolysis (100%) in the control solvent (distilled water). Generally, there was no significant difference in the percent haemolysis in the test groups when compared respectively to the control at each selected sodium chloride ion concentration. Although there were some differences between the control and the CPF groups when compared respectively to the vitamin pretreated groups, especially at 0.3% and 0.5% NaCl concentration, the differences were not significant. However, percent haemolysis was shown to be highest in the CPF group, especially

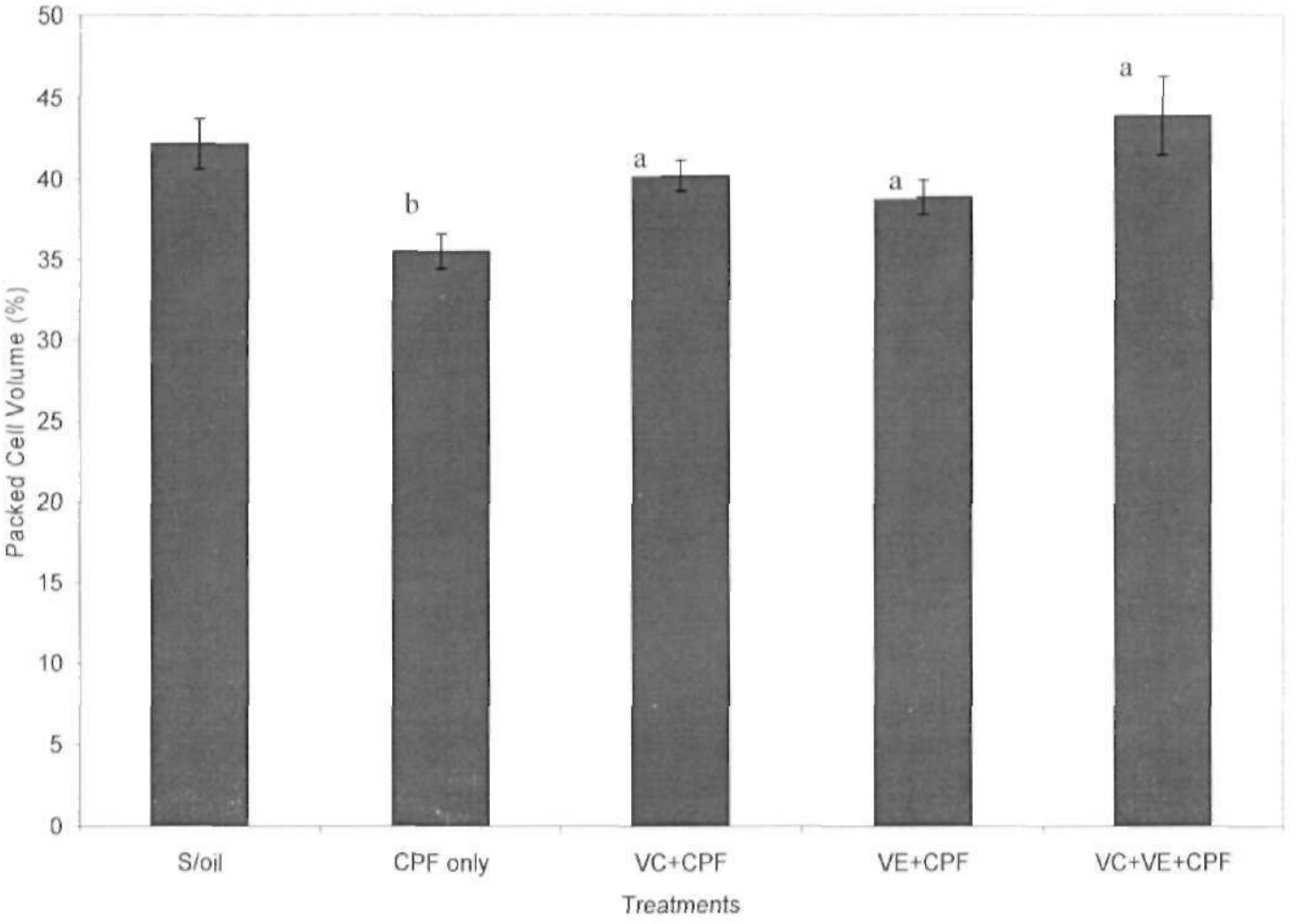


Figure 4.32: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on packed cell volume in adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control
b = Significantly different ($P < 0.05$) from the control

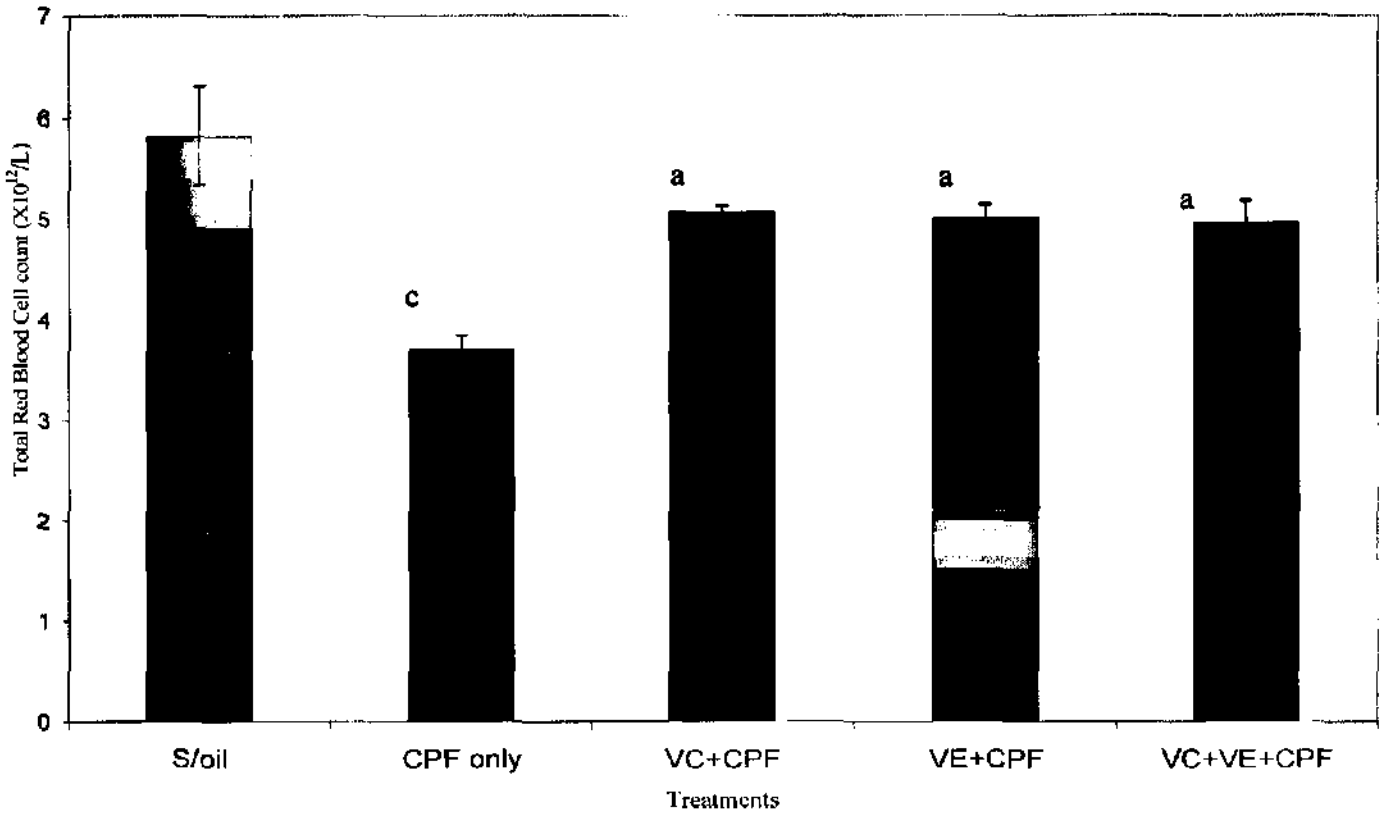


Figure 4.33: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on total red blood cell count in adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control

c = Significantly different ($P < 0.01$) from the control

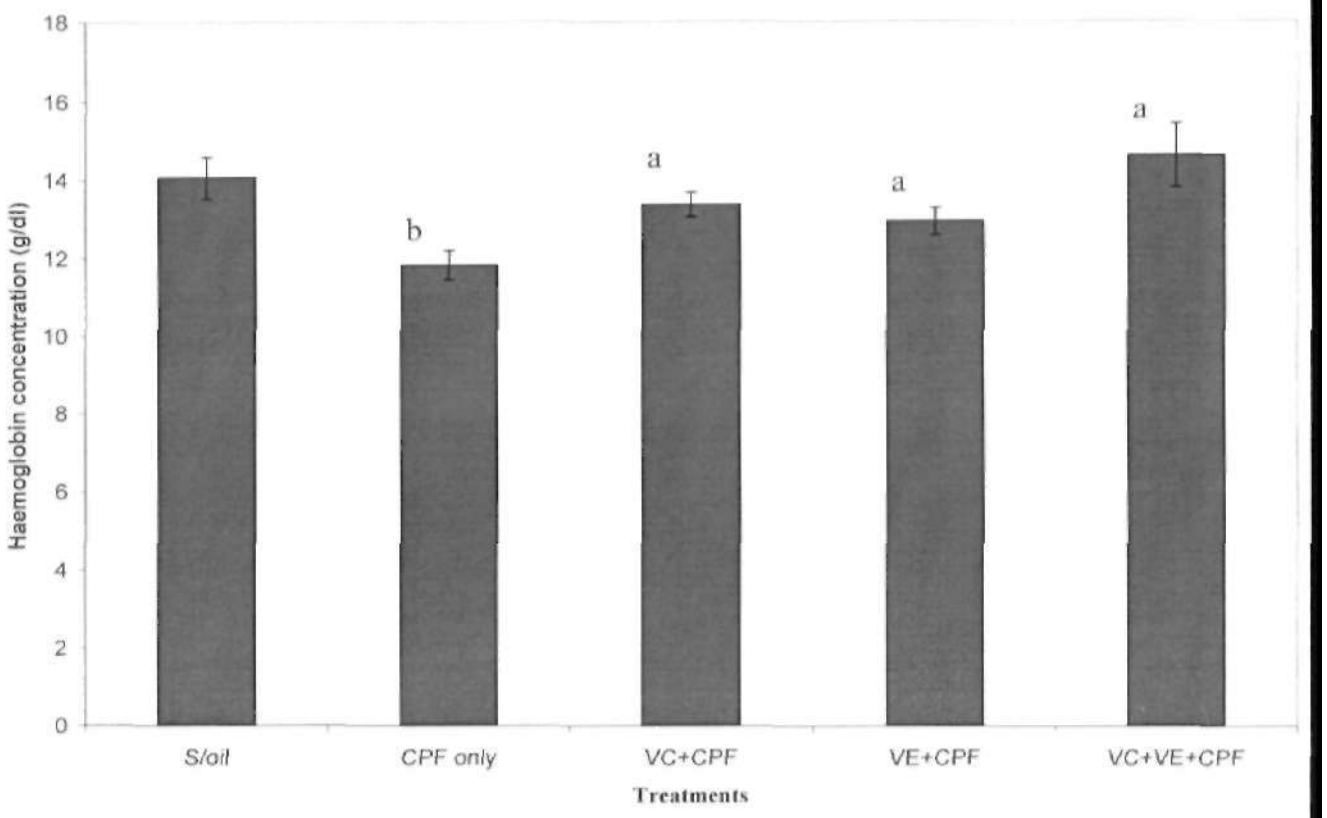


Figure 4.34: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC+CPF) and/or E (VE+CPF; VC+VE+CPF) on *haemoglobin concentration in adult Wistar rats (n=10).*

a = Not significantly different ($P > 0.05$) from the control
b = Significantly different ($P < 0.05$) from the control

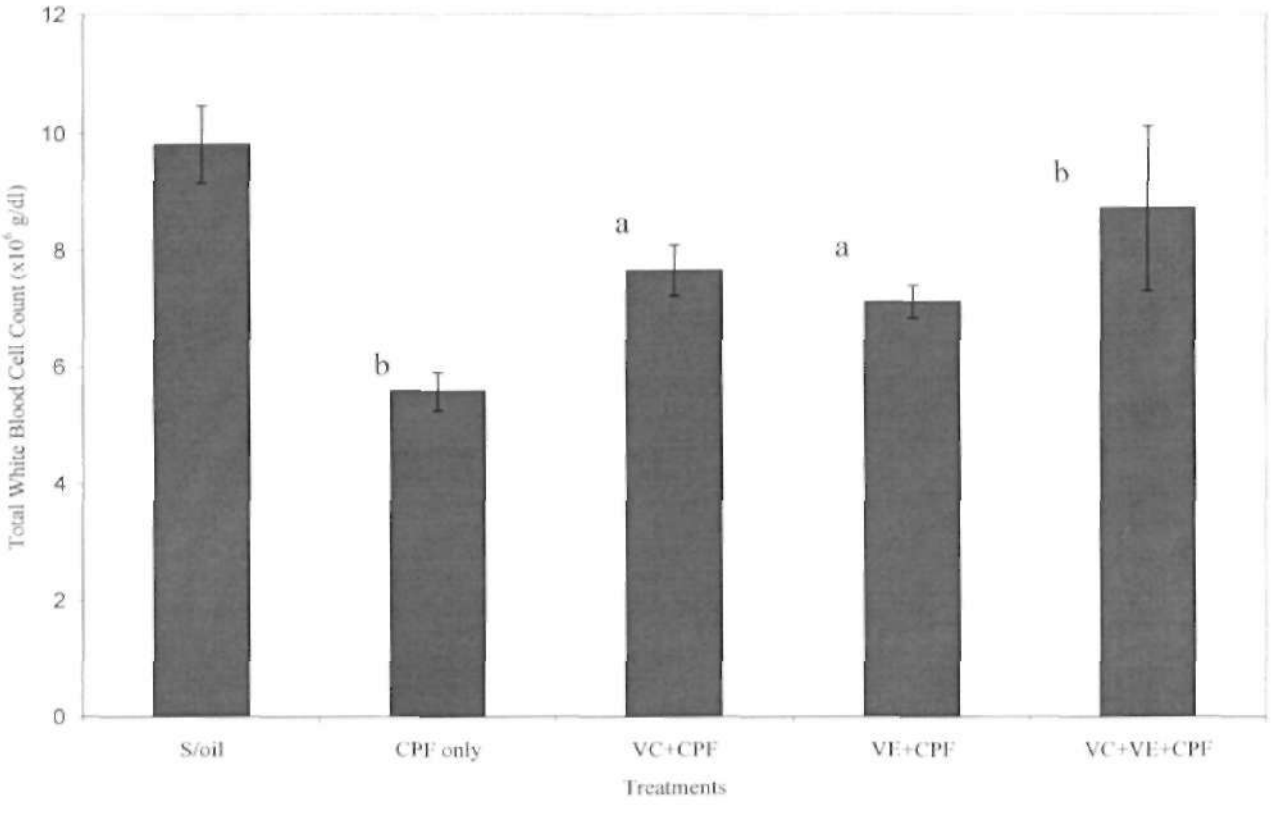


Figure 4.35: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on total leucocyte count in adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control
b = Significantly different ($P < 0.05$) from the control

Table 4.6: Effect of chronic administration of soya oil, chlorpyrifos and chlorpyrifos only and vitamins C and/or E on differential leukocyte count of adult Wistar rats

Groups	Absolute ($x10^6$ g/dL)	Neutrophil (%)	Lymphocytes (%)	Monocytes (%)	Band (%)
S/oil	9.8 ± 0.7	4.5 ± 0.07 (45.7 ± 1.0)	5.3 ± 0.008 (53.7 ± 1.0)	0.07 ± 0.002 (0.7 ± 0.3)	-
CPF only	5.6 ± 0.3	1.6 ± 0.006 (28.7 ± 2.1)	3.9 ± 0.1 (71.3 ± 2.1)	-	-
VC+CPF	7.7 ± 0.4	3.3 ± 0.006 (43.3 ± 1.4)	4.4 ± 0.07 (57.7 ± 1.7)	0.03 ± 0.0008 (0.5 ± 0.2)	0.01 ± 0.0007 (0.17 ±0.17)
VE +CPF	7.1 ± 0.3	2.85 ± 0.0003 (40.2 ± 1.1)	4.2 ± 0.008 (59.8 ± 2.6)	-	-
VC+VE+CPF	8.7 ± 1.4	3.8 ± 0.02 (44 ± 1.7)	4.8 ± 0.02 (55.5 ± 1.8)	0.03 ± 0.03 (0.3 ± 0.2)	-

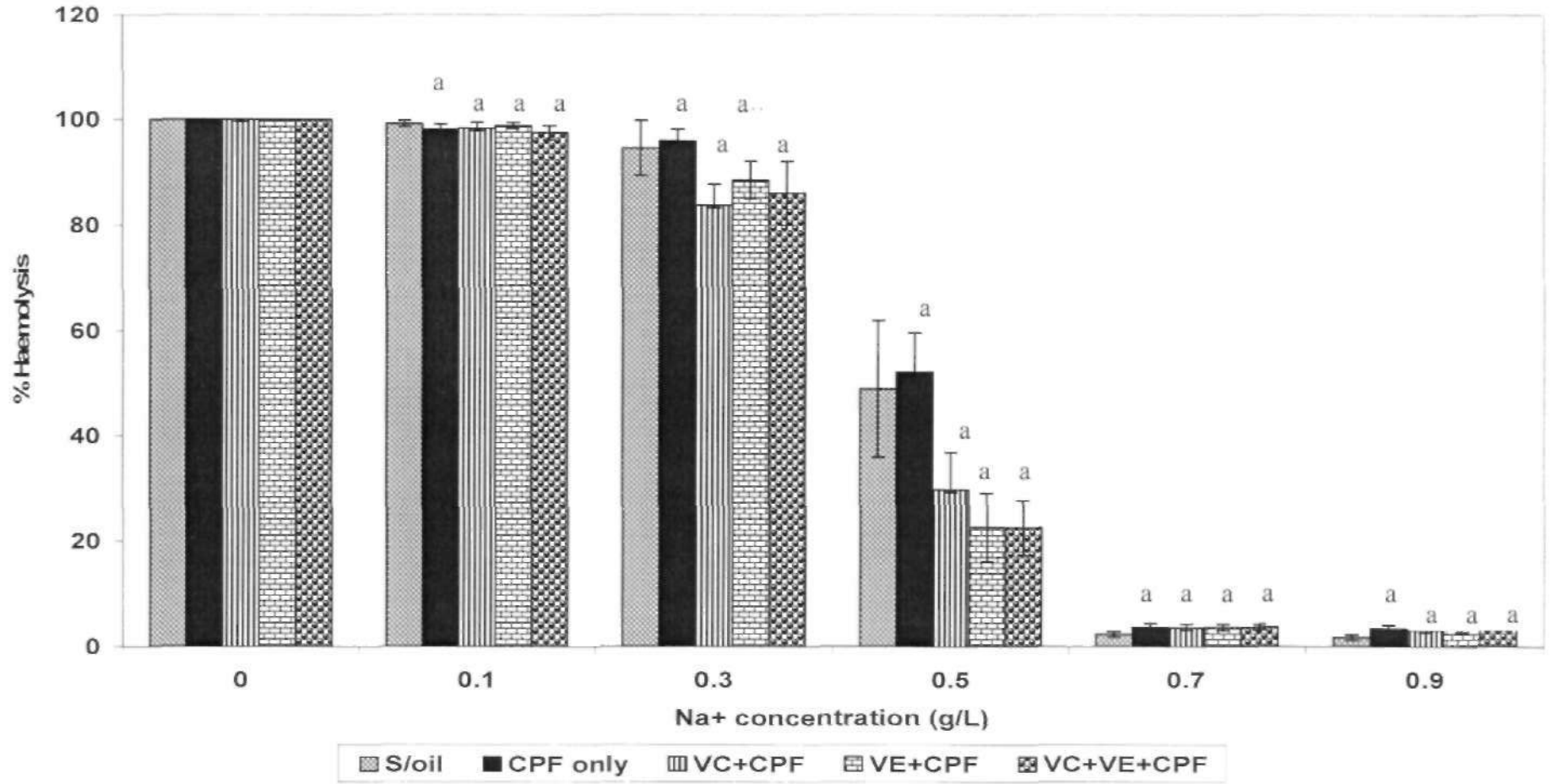


Figure 4.36: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on *in vitro* erythrocyte osmotic fragility of adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control

4.2.5 Effect of chronic administration of soya oil, chlorpyrifos and chlorpyrifos and vitamins C and/or E on Serum biochemical parameters

4.2.5.1 Effect of treatments on serum glucose concentrations

There was a significant increase ($P < 0.05$) in the serum glucose of rats exposed to CPF only (5.2 ± 0.4 dL) compared to the soya oil-treated control (4.2 ± 0.1 g/dL) Rats pretreated with vitamins C and/or E did not show any significant change in the serum glucose concentration compared to the control (Figure 4.37).There was no significant difference in the glucose values in male and female rats in each treatment group.

4.2.5.2 Effect of treatments on serum electrolytes concentration

The effect of various treatments on serum electrolytes concentration is shown in Figure 4.38. There was no significant difference in the concentration of serum Na^+, K^+, Cl^- and HCO_3^- in all the test groups compared to the soya oil-treated control. Furthermore, no significant difference in the concentration of electrolytes was recorded between male and female rats in each treatment group.

4.2.5.3 Effect of treatments on serum proteins

The effect of the various treatments on serum proteins in all the experimental groups is shown in Figure 4.39. There was no significant difference in the serum concentration of total proteins in all treatment groups compared to the control, although rats from the CPF group exhibited a lower total serum protein concentration than all the other experimental groups. Rats in the vitamin pretreated groups had higher but insignificant serum protein concentration than control group. There were no significant differences in total serum proteins in the male and female rats in all the experimental groups.

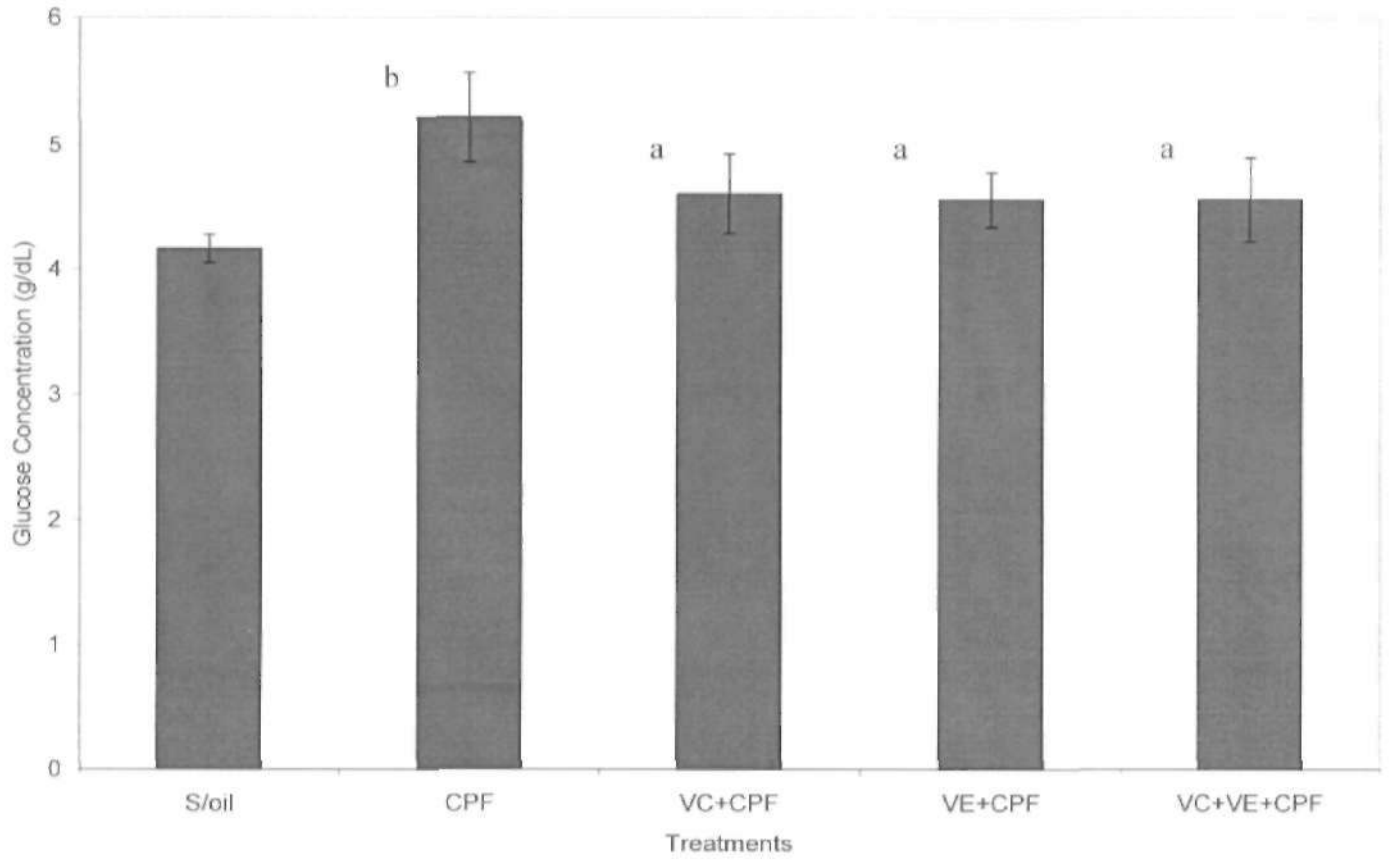


Figure 4.37: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on serum glucose concentration in adult wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control
b = Significantly different ($P < 0.05$) from the control

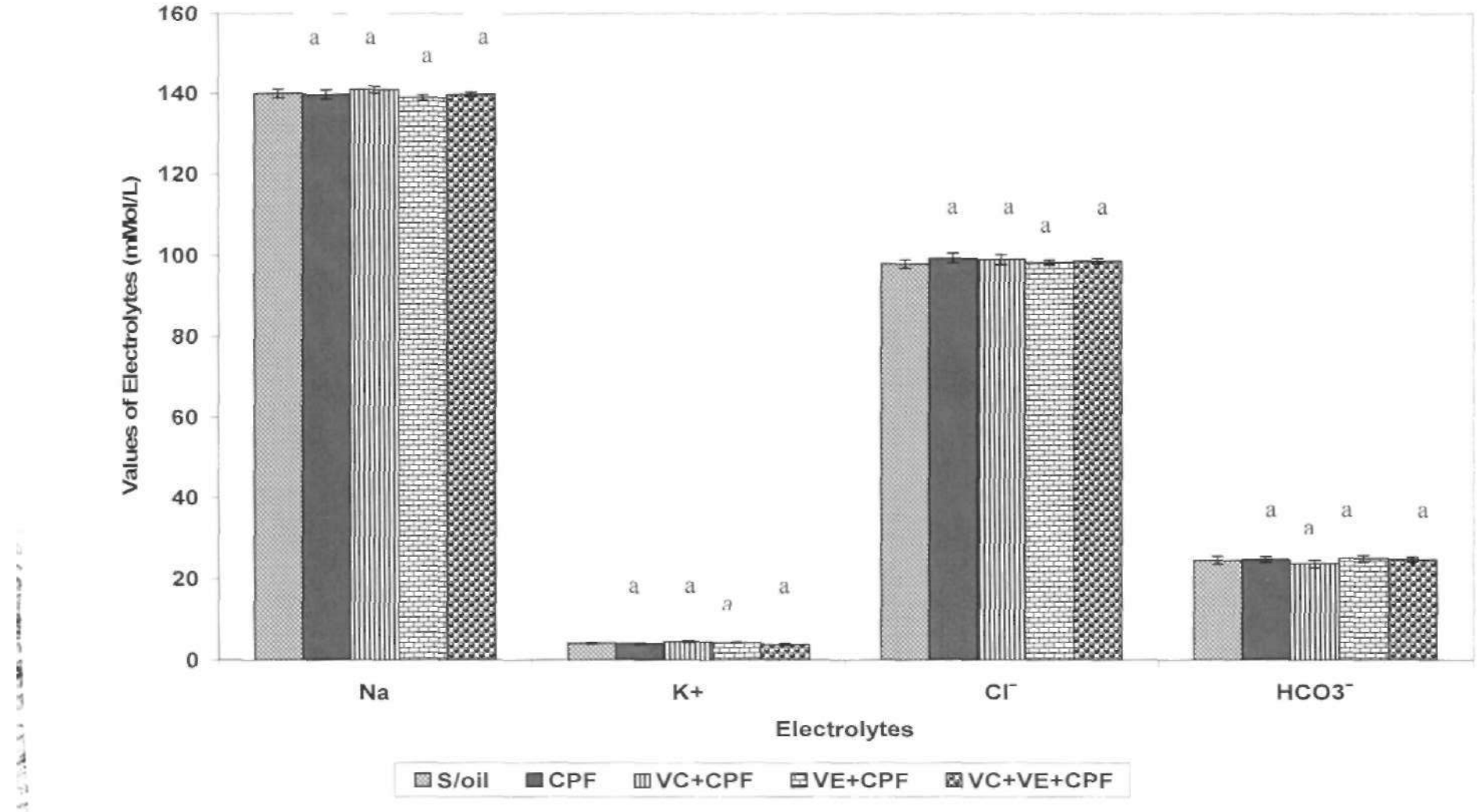


Figure 4.38: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on serum electrolytes in adult Wistar rats (n=10).

a= Not significantly different (P > 0.05) from the control

There was no significant difference in the concentration of serum albumin in all the test groups when compared respectively to the soya oil-treated control. However, the concentration of serum albumin was comparatively lower in the CPF group than in any other experimental group (Figure 4.39). The concentration of serum albumin in the male rats did not significantly differ from that of the female rats in all the experimental groups.

The difference in the concentration of serum globulin was higher ($P < 0.05$) in the VE+CPF group than the control. The rats from the soya oil-treated control group had a comparatively lower serum globulin than the test groups (Figure 4.39). Alteration in serum globulin values in the male rats did not differ from that of the females in each of the experimental group.

4.2.5.4 Effect of treatments on serum uric acid

The serum concentration of uric acid in all the test groups did not differ significantly ($P > 0.05$) from the control. However, the concentration of serum uric acid in the CPF group was the lowest compared to any other experimental group. The serum uric acid concentration in the VC+CPF group was slightly higher than what was obtained in any other experimental group (Table 4.6). The concentrations of serum uric acid in the male and female rats did not differ in any of the experimental groups.

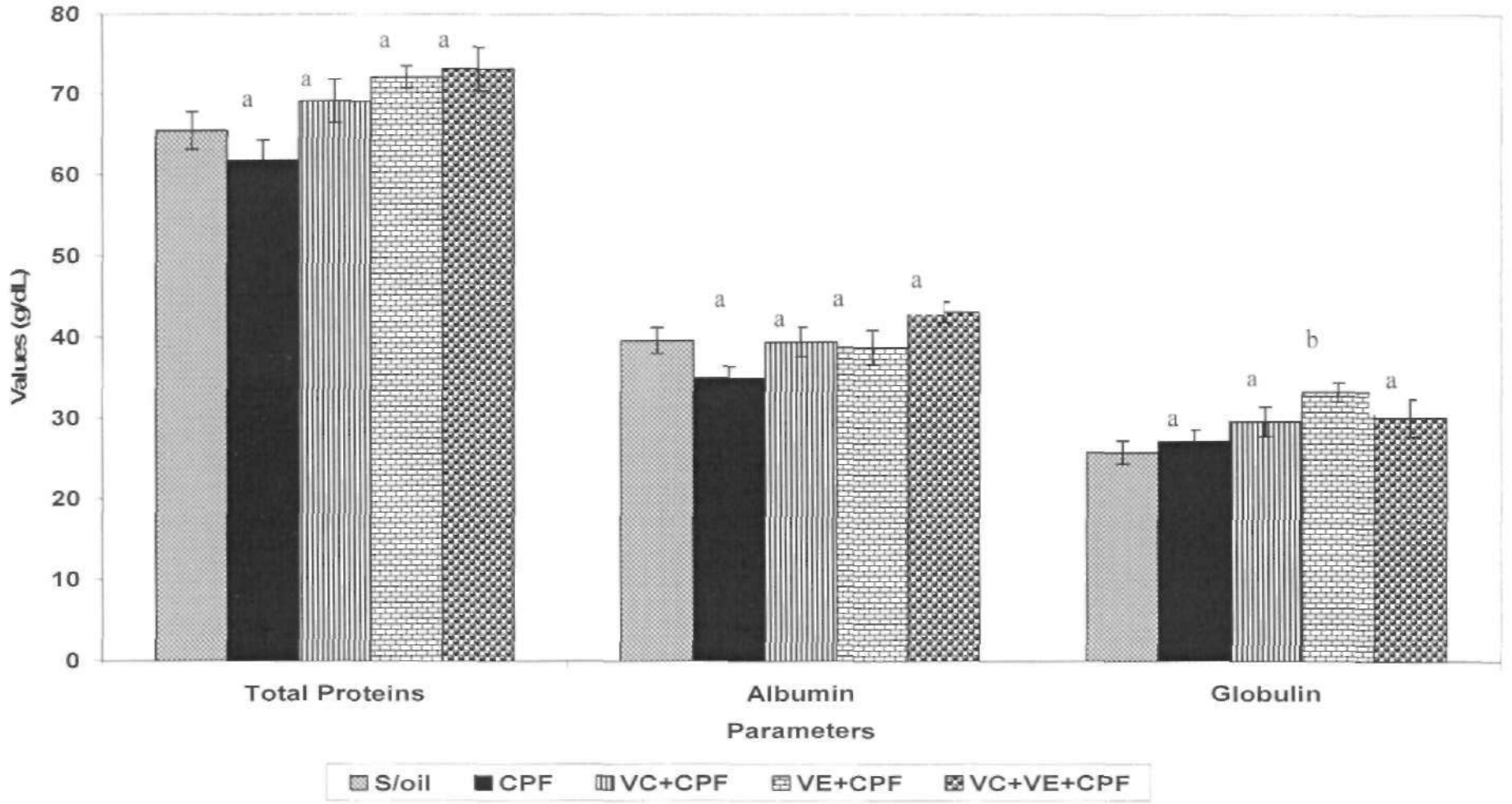


Figure 4.39: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on serum proteins of adult Wistar rats (n=10)

a = Not significantly different ($P > 0.05$) from the control

b = Significantly different ($P < 0.05$) from the control

4.2.5.5 Effect of treatments on serum urea and creatinine

There was no significant change in the concentration of urea in all the test groups compared to the soya oil-treated control. However, the urea level was comparatively higher in the CPF group compared to the other experimental groups. Animals in the VC+CPF and VC+VE+CPF groups have lower urea level compared to the other groups. The values of urea in the female and male rats were not significantly different in all the experimental groups (Table 4.6).

There was a significant increase ($P < 0.05$) in the concentration of serum creatinine in animals from the CPF group compared to the control. The concentration f urea in the vitamin pretreated groups was slightly lower than the control and CPF groups (Table 4.6). The concentration of serum creatinine in the female and male rats did not differ significantly in all the experimental groups.

4.2.5.6 Effect of treatments on serum liver enzymes

There was a significant increase ($P < 0.05$) in the activity level of AST in the CPF group (24 ± 1.4 IU/L) compared to the control (17.7 ± 2.1 IU/L), but no significant change in the activity level of AST was obtained in the vitamins pretreated groups when compared respectively to the control. Animals in the VC+VE+CPF group showed the lowest activity of AST compared to the other vitamin pretreated groups and even the control (Figure 4.40). In addition, the activity levels of AST between male and female rats were not significantly different in all the experimental groups.

Table 4.7: Effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on serum uric acid, urea and creatinine of adult Wistar rats

Parameters	Soya oil control	Chlorpyrifos only	Vitamin C +Chlorpyrifos	Vitamin E +Chlorpyrifos	Vitamin C+Vitamin E+Chlorpyrifos
Uric acid (mMol/L)	205± 9.6	198.8 ± 11.7*	227 ± 11.8*	201.8 ± 8.8*	199 ± 11.2*
Urea (mMol/L)	4.4 ± 0.2	4.6 ± 0.2*	4.3 ± 0.1*	4.4 ± 0.1*	4.3 ± 0.2*
Creatinine (mMol/L)	81.5± 3.6	97..3 ± 4.3**	77.7 ± 3.8*	76 ± 4.6*	80.7 ± 2.9*

* = Not different from the control
**= significant ($P < 0.05$) increase compared to the control

The activity level of serum ALT was significantly higher ($P < 0.05$) in the CPF group (44.5 ± 1.4 IU/L) than that of the control (35.2 ± 2.4 IU/L). Serum ALT activity was lower ($P<0.05$) in the VC+CPF group compared to the control. A slight decrease in the activity level of ALT was observed in the VE+CPF and VC+VE +CPF groups compared to the control (Figure 4.40). No significant difference was observed in the activity level of ALT in male and female rats in each of the experimental groups.

There was no significant change in the activity level of ALP between the test groups and the control. The activity level in group exposed to CPF is slightly higher than those in the control. The activity level of ALP in VE+CPF and VC+VE+CPF groups was comparatively lower than that obtained in other experimental groups (Figure 4.40). No significant difference was observed in the activity level of ALP between male and female rats in each of the experimental groups.

There was no significant change in the activity level of GGT in rats from each treatment group when compared to the control. However, the activity level of GGT in rats in the CPF group was slightly higher compared to that of the control. The activity of GGT in vitamins pretreated groups was insignificantly lower than that obtained in rats in the soya oil-treated control group (Figure 4.40). The activity levels of GGT in female and male animals in all the experimental groups were not significantly different.

4.2.5.7 Effect of treatments on serum creatine kinase

No significant change was observed in the activity level of CK in the test groups when compared, respectively to the control group. However, the activity level of CK was slightly lower in the CPF groups compared to the rest experimental groups (Figure 4.41). The activity level of CK in the male and female rats was not significantly different in all the experimental groups.

4.2.5.8 Effect of treatments on thyroid hormones

There was no significant change in the concentration of serum TSH, T_3 and T_4 in the test groups when compared respectively to the control group. The concentration of TSH was slightly lower in the CPF group than the control, but the difference was insignificant. In addition, there was an insignificant elevation in the concentration of TSH in rats pretreated with the combination of vitamins C and E than either of the two vitamins. Similarly, the concentration of T_3 in rats exposed to CPF was insignificantly lower than in any of the other experimental groups. The concentration of T_3 in VE+CPF and VC+VE+CPF was also slightly but insignificantly elevated compared to the CPF only and VC+CPF groups. Similarly, the concentration of T_4 in the group exposed to CPF was insignificantly lower than the control. However, groups pretreated with antioxidant vitamins had higher concentration of serum T_4 compared to group given CPF only and even the control, with those pretreated with vitamin C showing the highest T_4 level (Figure 4.42). The responses of the male and female animals in all experimental groups to TSH, T_3 and T_4 were not significantly different.

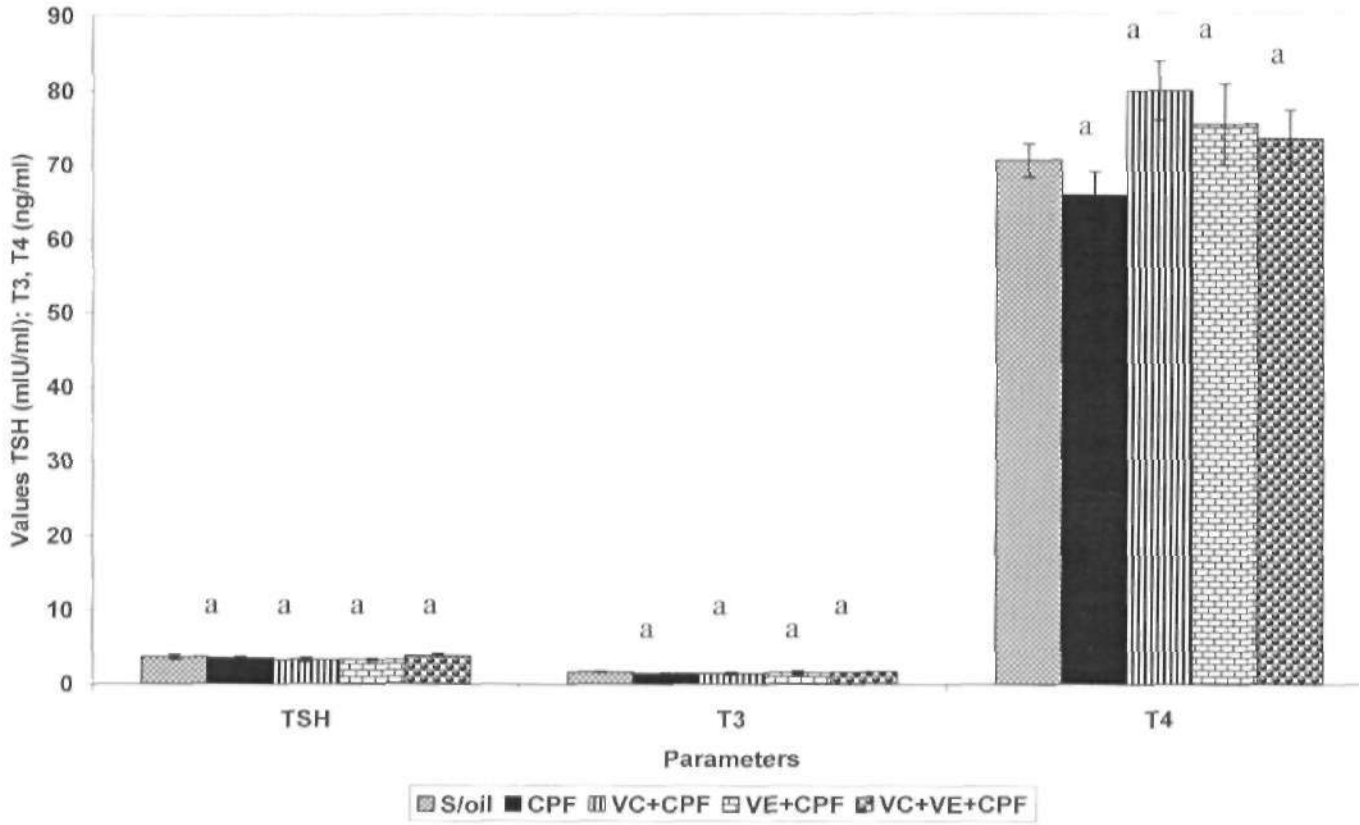


Figure 4.42: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on thyroid stimulating hormone (TSH) and thyroid hormones (T_3 and T_4) in adult Wistar rats (n=10)

a = Not significantly different ($P > 0.05$) from the control

4.2.5.9 Effect of treatments on lipid profiles

The serum concentrations of TC, TG, HDL-c and LDL-c in the test groups did not significantly differ ($P>0.05$) compared to the control. However, the concentration of TC was comparatively lower in the CPF group than in the other experimental groups. The rats from the vitamin pretreated groups, especially those in the VC+CPF and VE+CPF groups, had higher TC level than those in the control and CPF-treated groups, but the difference in the values was insignificant (Figure 4.43). There were insignificant changes in the serum concentration of TC among male and female rats in all the experimental groups. The TG concentrations in the treatment groups were insignificantly lower compared to those of the control group. Rats in the VC+VE+CPF group had the lowest TG concentration (Figure 4.43). There were no differences in the level of TG between male and female rats in the different experimental groups. The serum concentrations of HDL-c in rats in the treatment groups were insignificantly lower than those of the control. Rats pretreated with combination of vitamins C and E showed a relatively higher HDL-c than those in groups pretreated with either of the vitamins and those administered CPF only. No significant difference in the level of HDL-c was observed between male and female rats in each of the experimental groups. The concentration of LDL-c in CPF group was higher than the value obtained in the control. Rats in groups pretreated with either vitamins C or E had an insignificantly higher concentration of LDL-c compared to the CPF and the control groups. However, the concentration of LDL-c in group pretreated with a combination of vitamins C and E was slightly lower than those obtained in the CPF group. The concentration of LDL-c in male and female rats did not significantly differ in all the experimental groups.

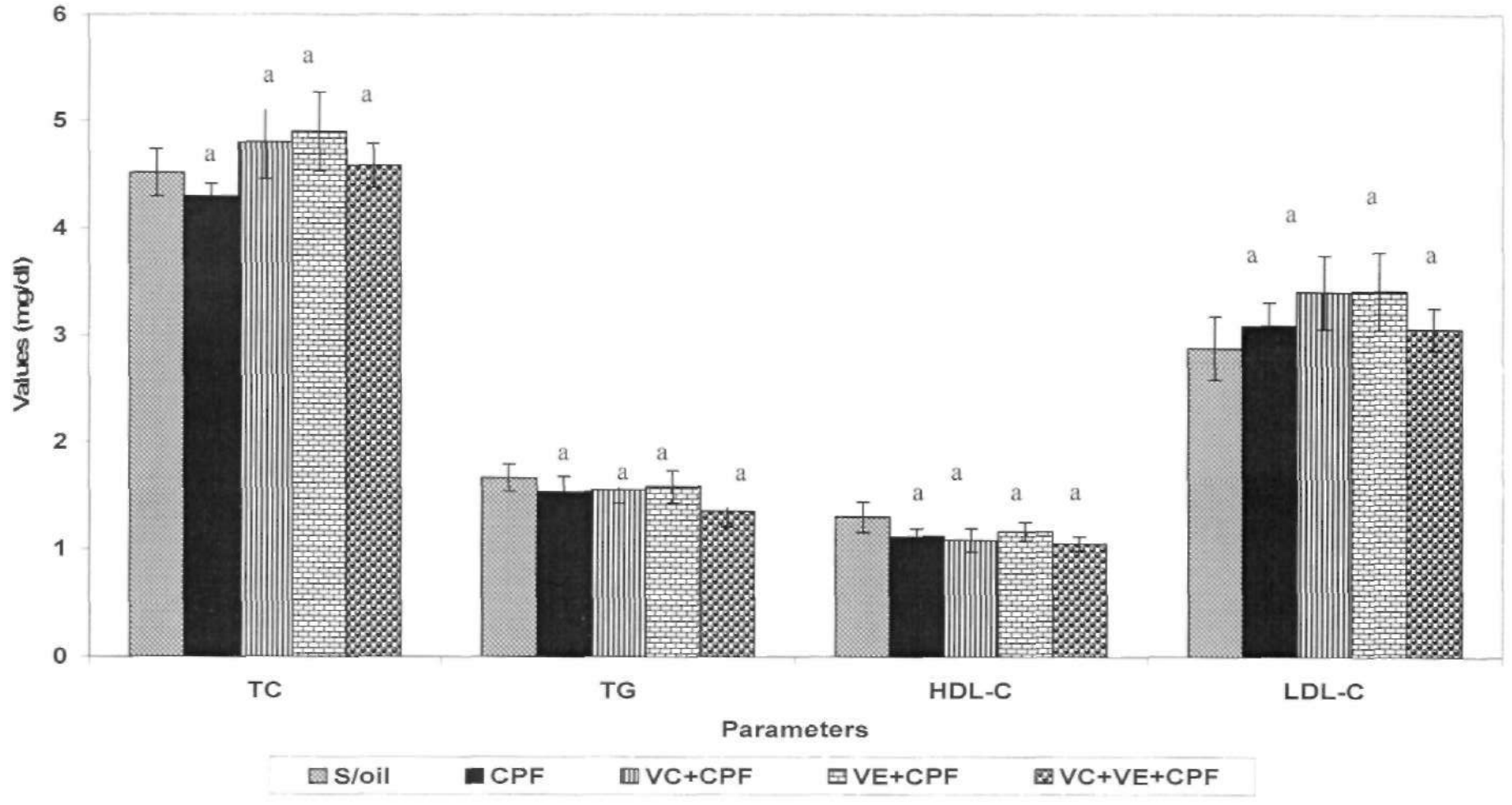


Figure 4.43: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on serum lipid profiles of adult Wistar rats (n=10)
a = Not significantly different (P > 0.05) from the control

4.2.5.10 Effect of chronic administration of soya oil, chlorpyrifos only and chlorpyrifos and vitamins C and/or E on lipid peroxidation

4.2.5.10.1 Effect on malonaldehyde concentration in the liver

The effect of various treatments on lipid peroxidation in the liver is shown in Figure 4.44. There was an increase ($P<0.01$) in the concentration of MDA in the CPF group (0.38 ± 0.02 nmol/mg tissue protein) compared to the control (0.23 ± 0.03 nmol/mg tissue protein). However, there was no significant change in the concentration of MDA in the vitamins-pretreated groups compared to the control, with the group pretreated with combination of vitamins C and E having the least MDA concentration. No significant difference in the concentration of MDA was observed between male and female rats in each of the experimental groups.

4.2.5.10.2 Effect on malonaldehyde concentration in the brain

There was a rise ($P<0.01$) in the concentration of MDA in rats exposed to CPF only (0.46 ± 0.02 nmol/mg tissue protein) compared to the control (0.26 ± 0.02 nmol/mg tissue protein). The concentration of MDA in rats belonging to VC+CPF, VE+CPF and VC+VE+CPF groups did not differ significantly ($P>0.05$) compared to the control. Rats from VC+VE+CPF group had the least brain MDA concentration and the value of 0.24 ± 0.02 nmol/mg tissue protein obtained in the group was insignificantly lower than even that of control (0.26 ± 0.02 nmol/mg tissue protein) (Figure 4.45). The brain MDA concentrations in male and female rats from each of the experimental groups were not significantly different.

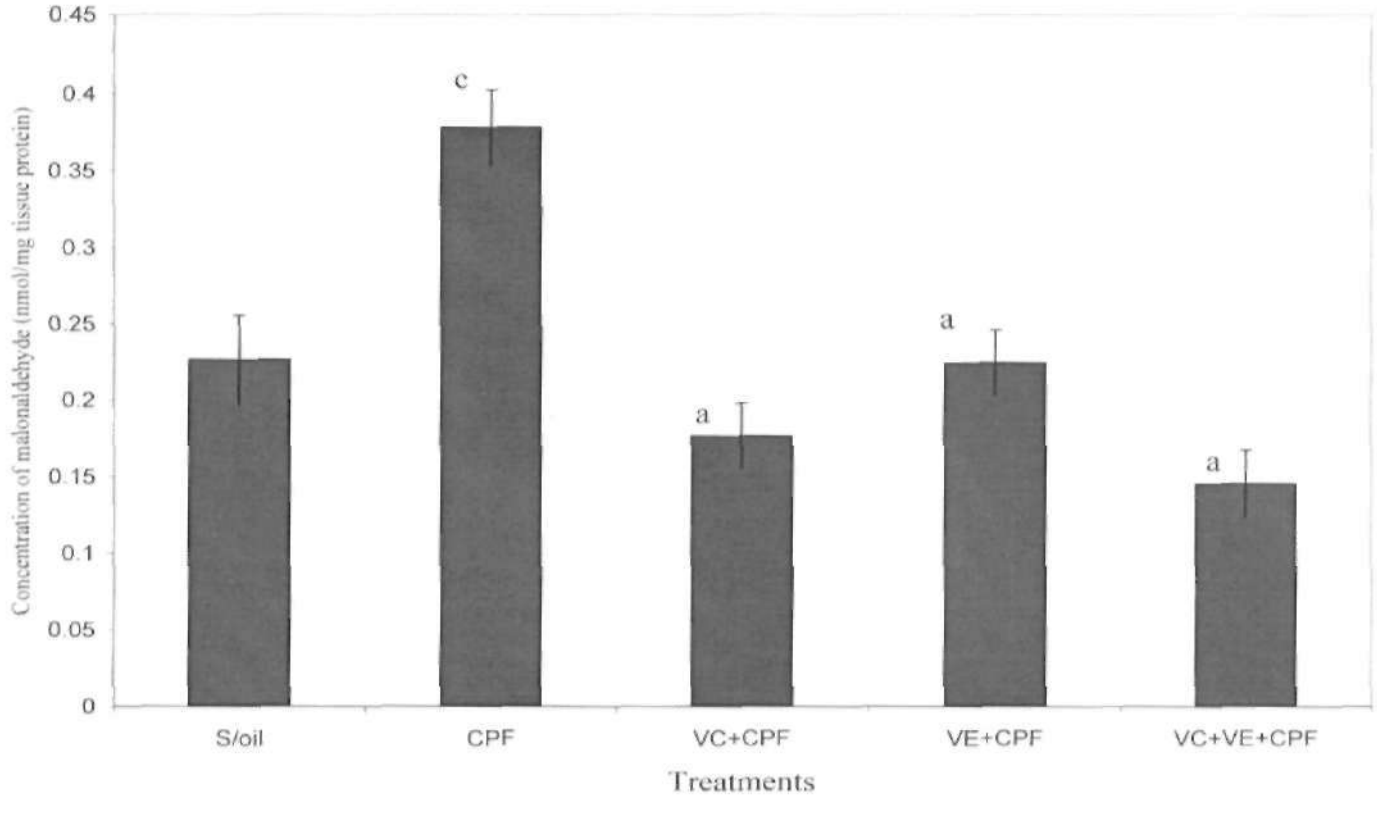


Figure 4.44: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on the concentration of malonaldehyde in the liver of adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control
c = Significantly different ($P < 0.01$) from the control

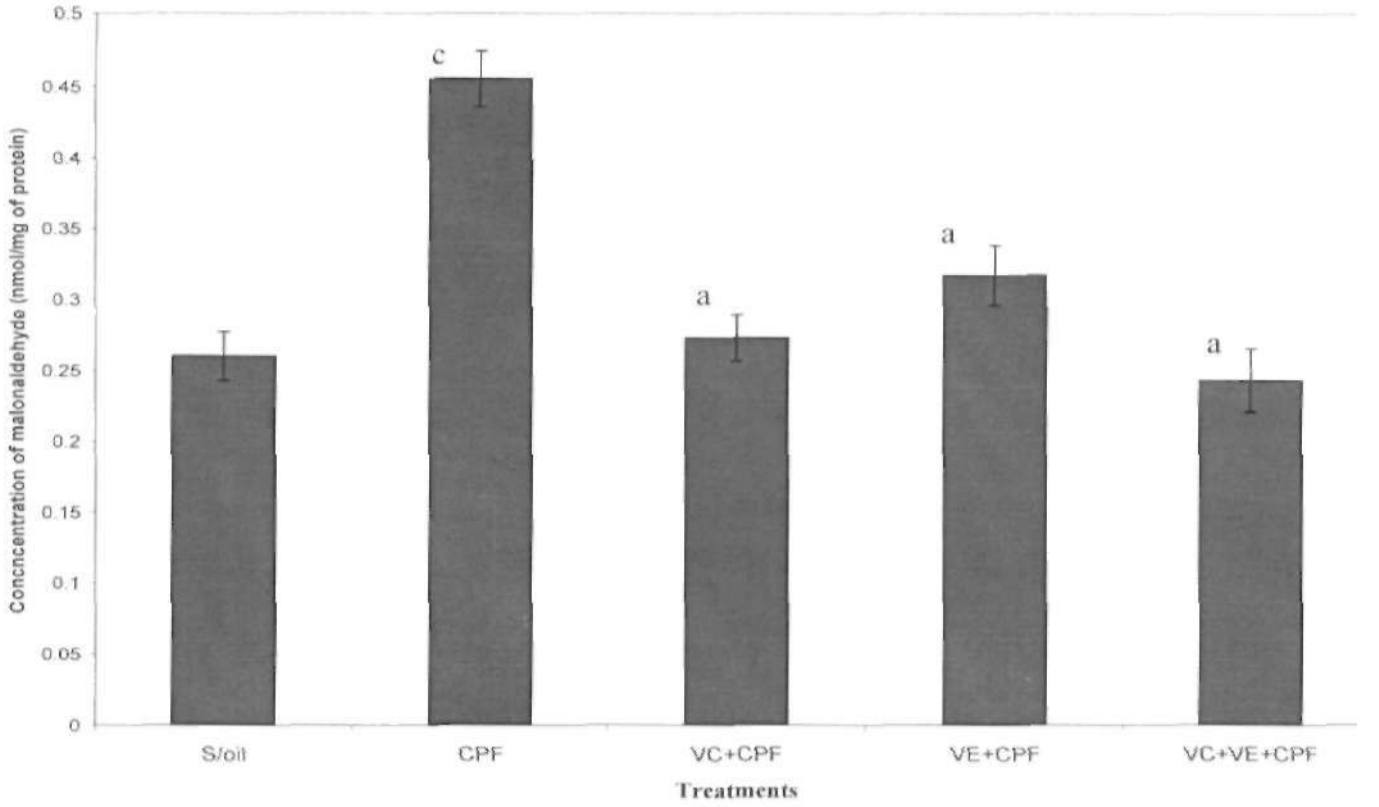


Figure 4.45: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on concentration of malonaldehyde in the brain of adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control
c = Significantly different ($P < 0.01$) from the control

4.2.5.10.3 Effect on serum malonaldehyde concentration

An increase in MDA concentration ($P<0.05$) was observed in CPF group (0.3 ± 0.02 nmol) compared to the control (0.21 ± 0.03 nmol). Rats pretreated with VC+CPF, VE+CPF and VC+VE+CPF had lower serum MDA values than the control but the differences in the values were not significant (Figure 4.46). No significant changes were obtained in the serum concentrations of MDA in the male and female rats in all the experimental groups.

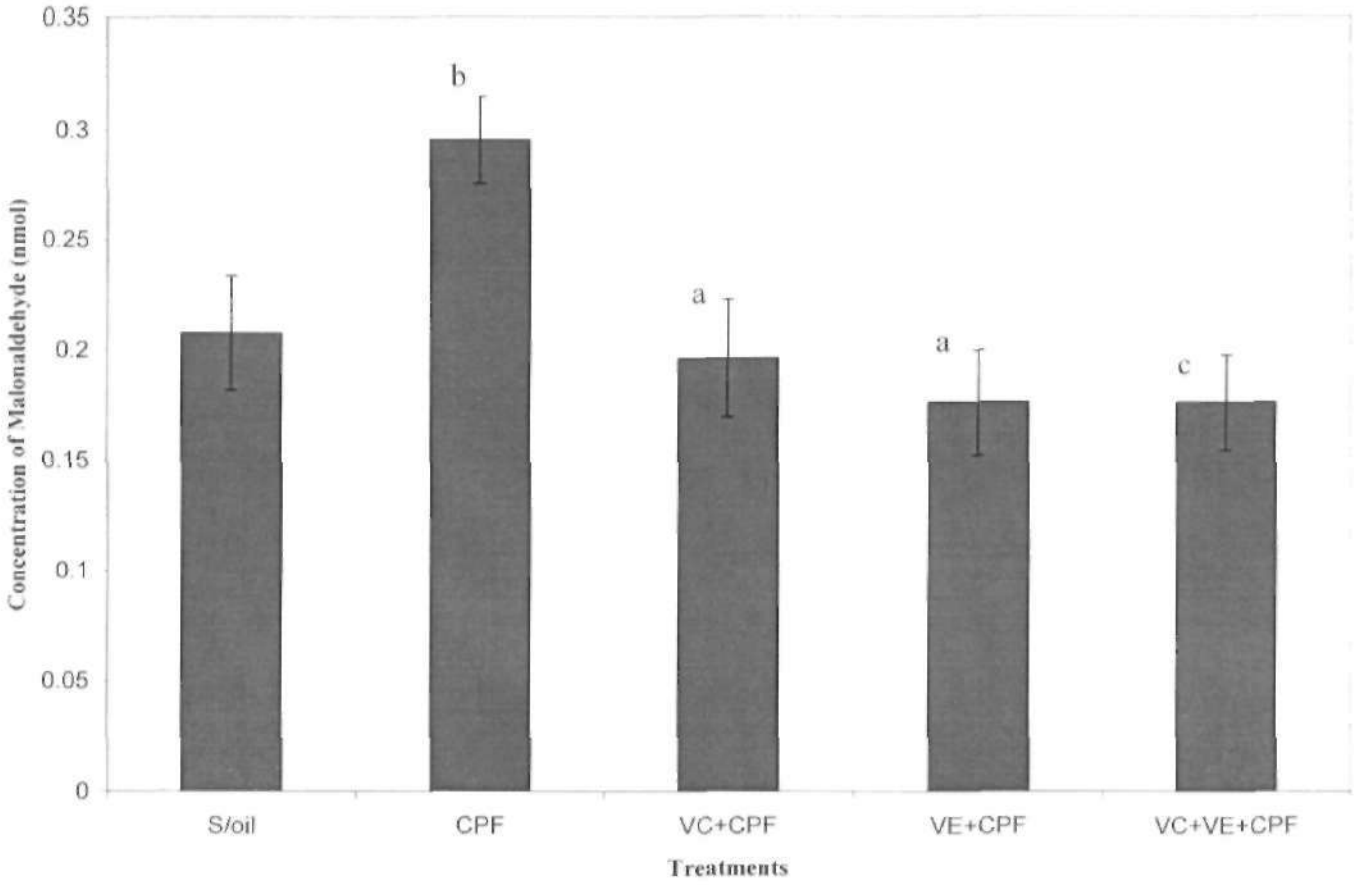


Figure 4.46: Effect of chronic administration of soya oil (S/oil), chlorpyrifos (CPF) and chlorpyrifos and vitamins C (VC) and/or E (VE) on concentration of malonaldehyde in the serum of adult Wistar rats (n=10).

a = Not significantly different ($P > 0.05$) from the control
b = Significantly different ($P < 0.05$) from the control

and *tunica muscularis* in rats from the CPF and VC+CPF groups. There were areas of necrosis of the intestinal villi involving the *lamina propria,* submucosa and *tunica muscularis* with leucocytic infiltration in the necrotised areas in VE +CPF and VC+VE+CPF groups.

Spleen- No observable microscopic lesion occurred in rats in the control group. Rats in the CPF group showed degenerated lymphoid cells in both red and white pulps. There was no observable microscopic lesion in the spleen of rats in VC+CPF and VC+VE+CPF groups. Rats in VE+CPF group had degenerated lymphoid tissues in both red and white pulps, haemosiderosis and haemosiderin-laddened macrophages.

Liver- Fewer areas of degenerated hepatic cells were observed in rats in the control group (Plate VII). Massive areas of degenerated hepatic cells were observed in rats in the CPF group (Plate VIII). Male rats in the VC+CPF group had focal areas of degenerated hepatic cells (Plate IX), while no observable microscopic lesion occurred in the female rats. Focal areas of degenerated hepatic cells were observed in rats in the VE+ CPF group. No microscopic lesion occurred in the liver of rats in the VC+VE+CPF group.

Pancreas- There was no observable microscopic lesion in rats in the control group (Plate X). Massive degeneration of both the exocrine and the islet cells of the pancreas occurred in rats from the CPF group (Plate XI). There were few areas of degenerative changes in the exocrine and islet pancreatic cells in rats from the vitamins-pretreated groups (Plate XII).

Kidneys- Fewer areas of degenerated glomeruli and renal tubules were observed in rats in the control group. Rats exposed to CPF only had degenerated and atrophied glomeruli, obliterated Bowmans capsule and degenerated renal tubules (Plate XIII). Fewer areas of degenerative changes were observed in the glomeruli and renal tubules of rats in the VC+CPF (Plate XIV), VE+CPF and VC+VE+CPF groups.

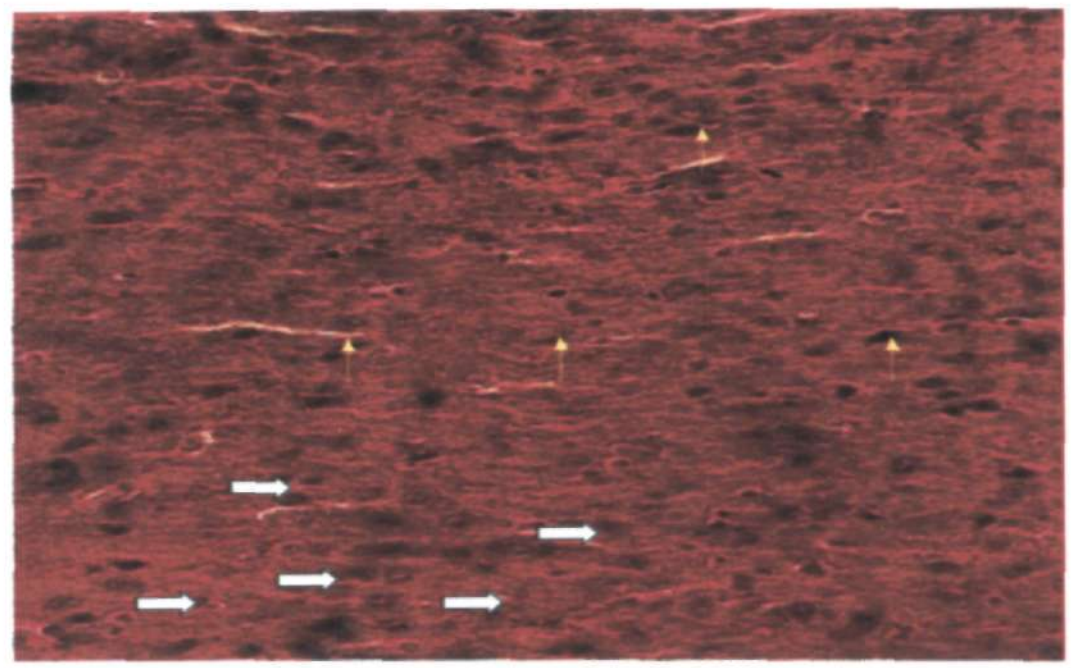


Plate I: Section of brain of rats dosed with soya oil (single arrows show intact neurones) and few areas of degenerated neurones (block arrows). H&E x400



Plate II: Section of cerebrum of rats dosed with chlorpyrifos only showing degenerated neurones (single arrow), area of microabscess (block arrow), vacuolated glial cells (lightning bolts). H&E x400

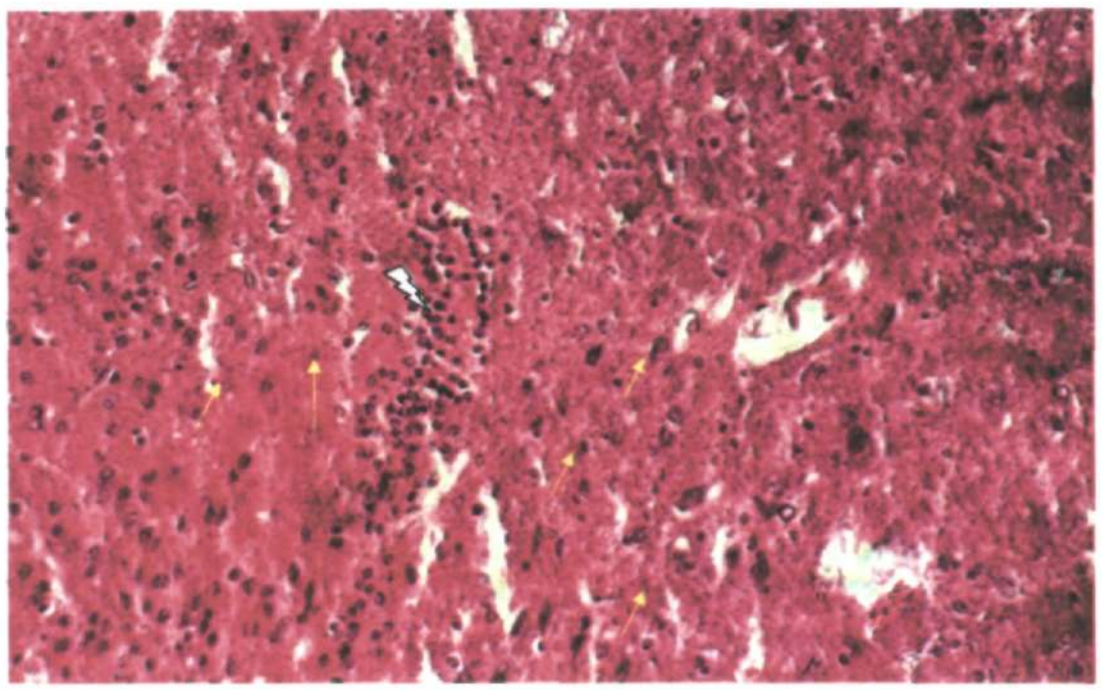


Plate III: Section of the cerebrum of rats exposed to CPF only showing degenerate neurones (single arrow), gliosis (lightning bolt) H&Ex400

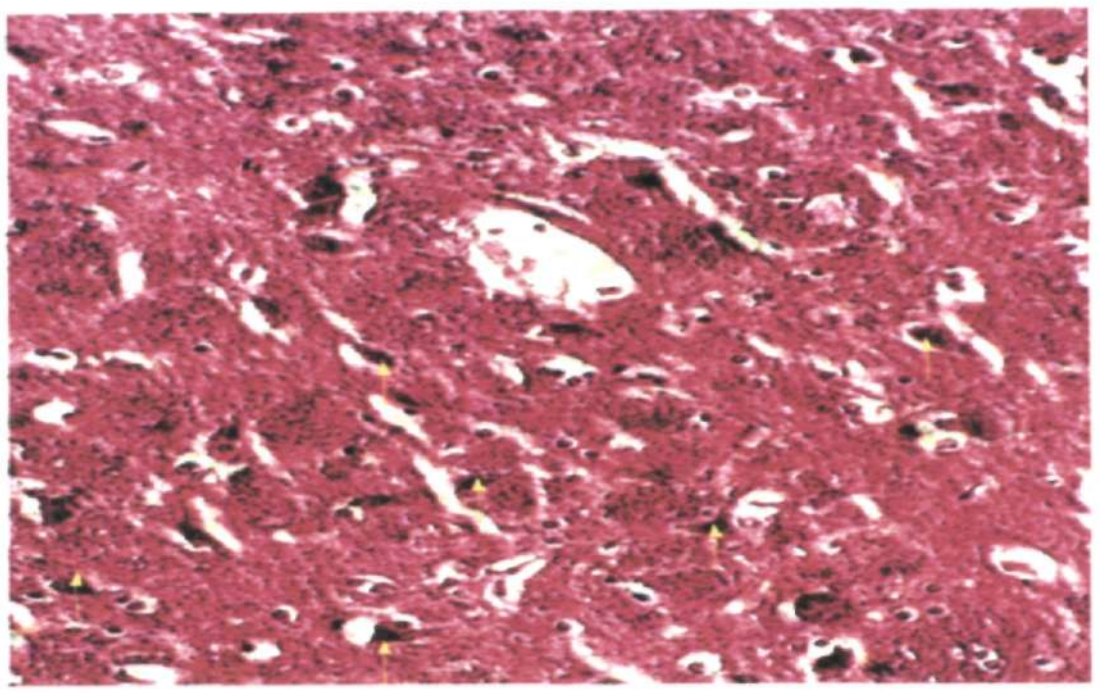


Plate IV: Section of cerebrum of rat pretreated with vitamin E before exposure to chlorpyrifos showing numerous viable neurones (single arrow) H&Ex400

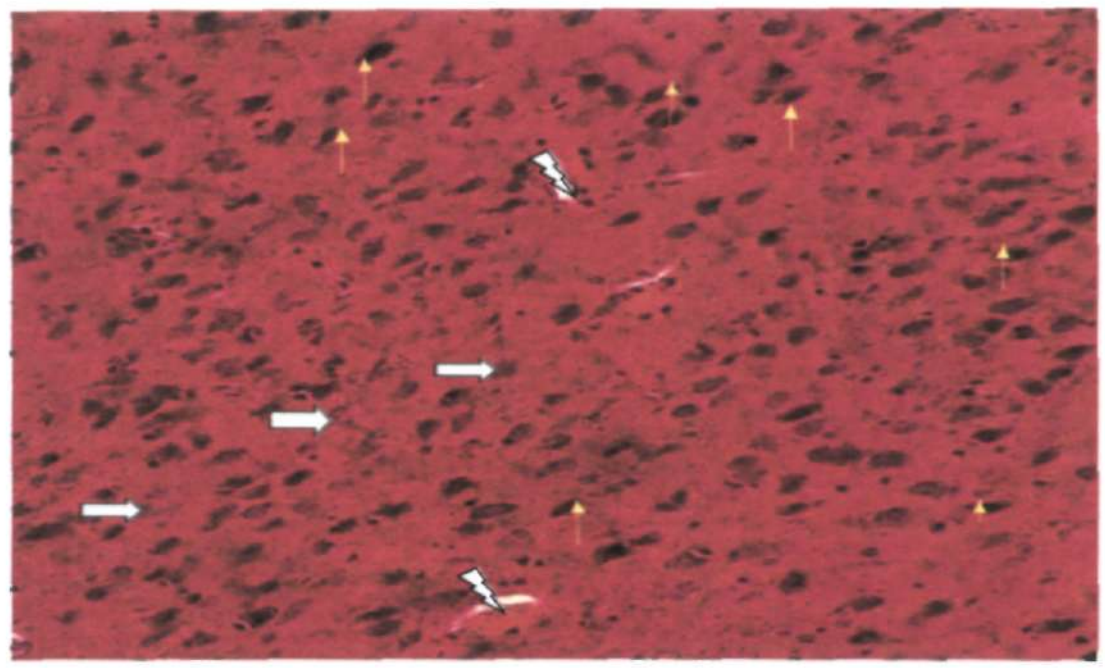


Plate V: Section of the cerebrum of rats pretreated with combination of vitamins C and E showing numerous viable neurones (single arrows), few degenerated neurones (block arrows) congested blood vessels (lightning bolt) x400

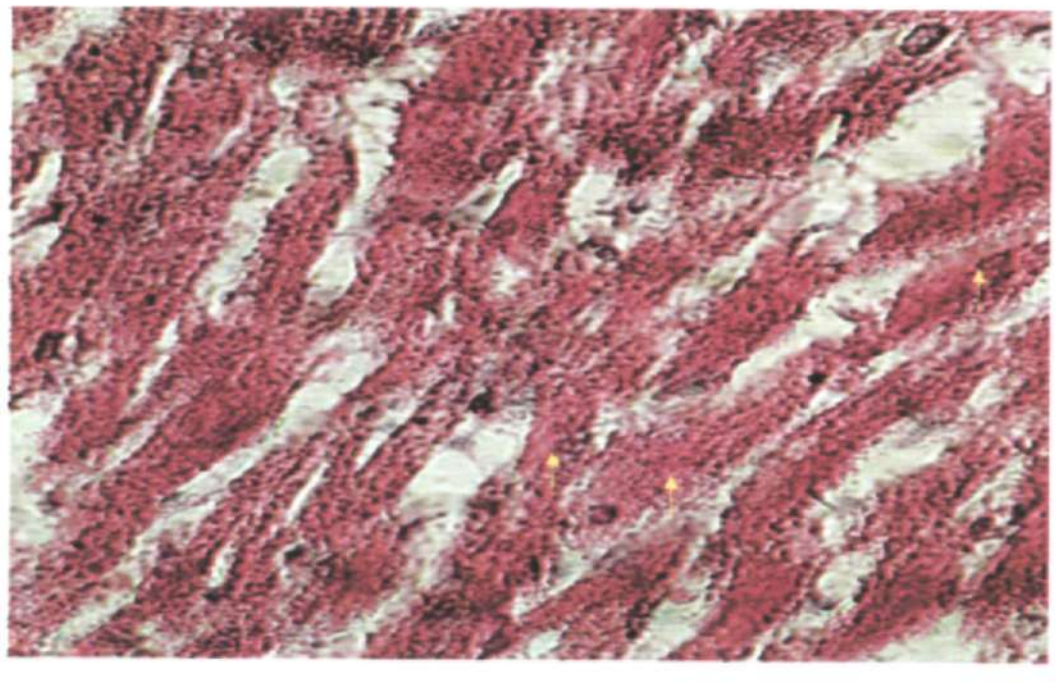


Plate VI: Section of the heart of rats exposed to CPF only showing degeneration of cardiac myocytes (single arrows) H&Ex400

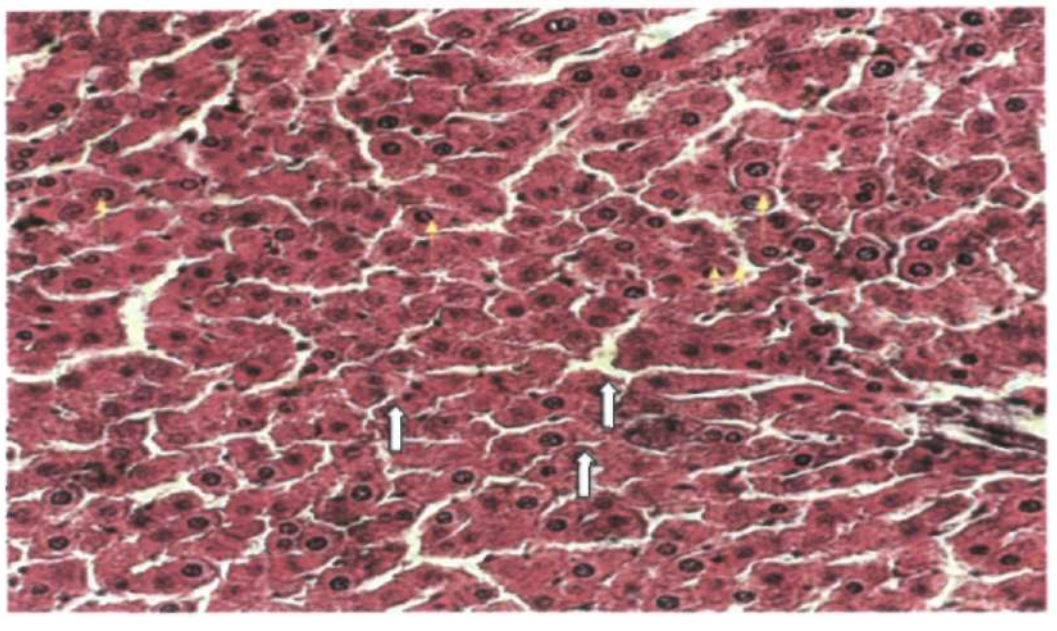


Plate VII: Section of liver of rats exposed to soya oil only showing numerous viable hepatocytes (single arrow), normal sinusoidal space and hepatic architecture, and few areas of degenerated hepatocytes (block arrows). H&Ex400.

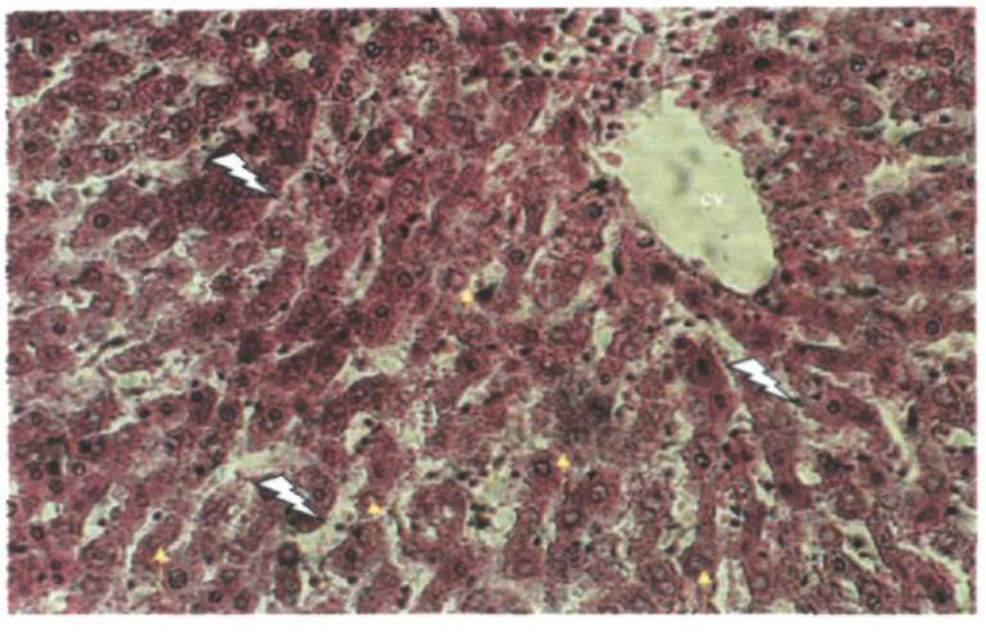


Plate VIII: Section of liver of rats exposed to chlorpyrifos only showing degenerated hepatocytes (single arrows), dilated sinusoid (lightning bolt) and intact hepatic architecture and central vein (cv). H&E x400

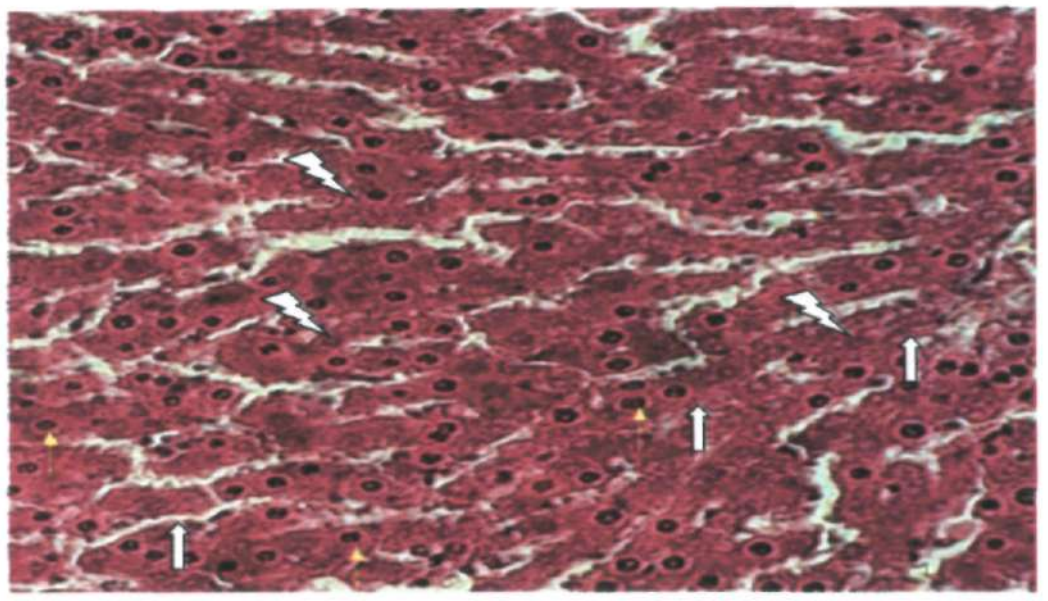


Plate IX: Section of the liver of rats pretreated with vitamin C before exposure to chlorpyrifos showing numerous viable hepatocytes (single arrows), intact hepatic architecture and sinusoids (block arrows) and fewer areas of hepatic degeneration (lightning bolts) H&Ex400

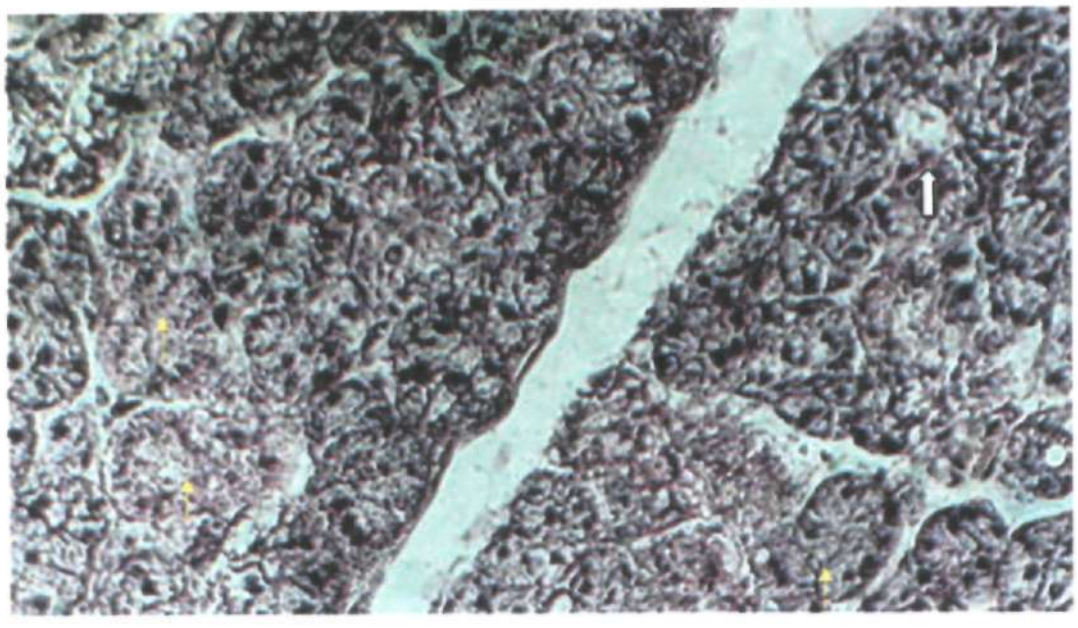


Plate X: Sections of the pancreas of rats given soya oil only showing viable exocrine (single arrows) and islet cells (block arrow) H&Ex 400

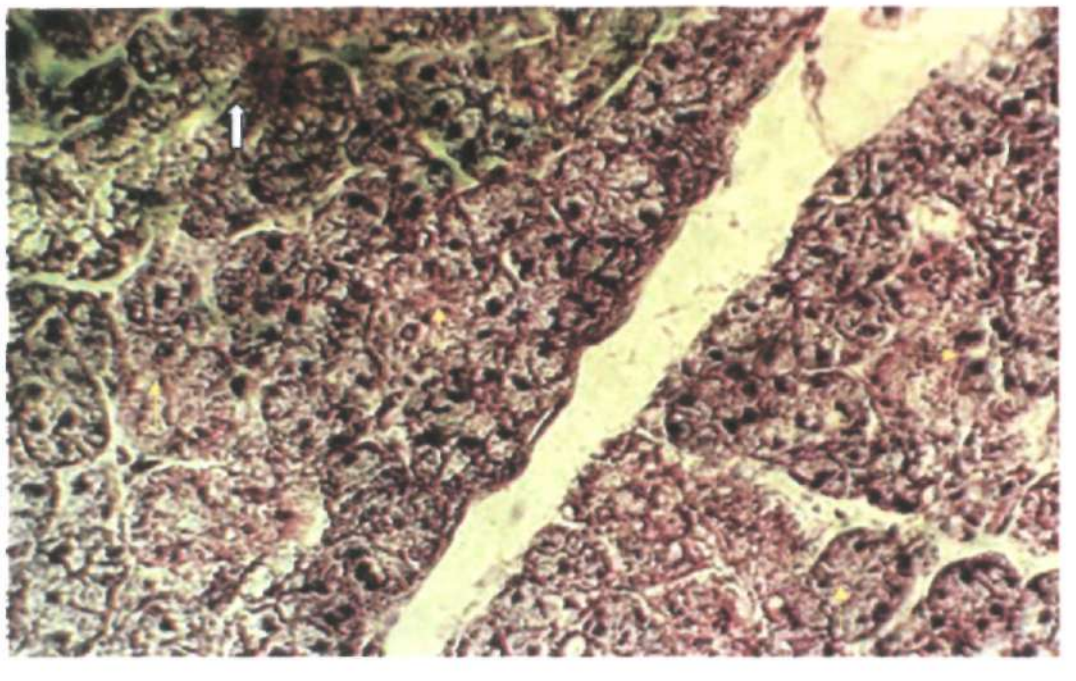


Plate XI: Section of pancreas exposed to chlorpyrifos only showing degenerated exocrine pancreas (single arrow) and islet cells (block arrow). H&Ex400

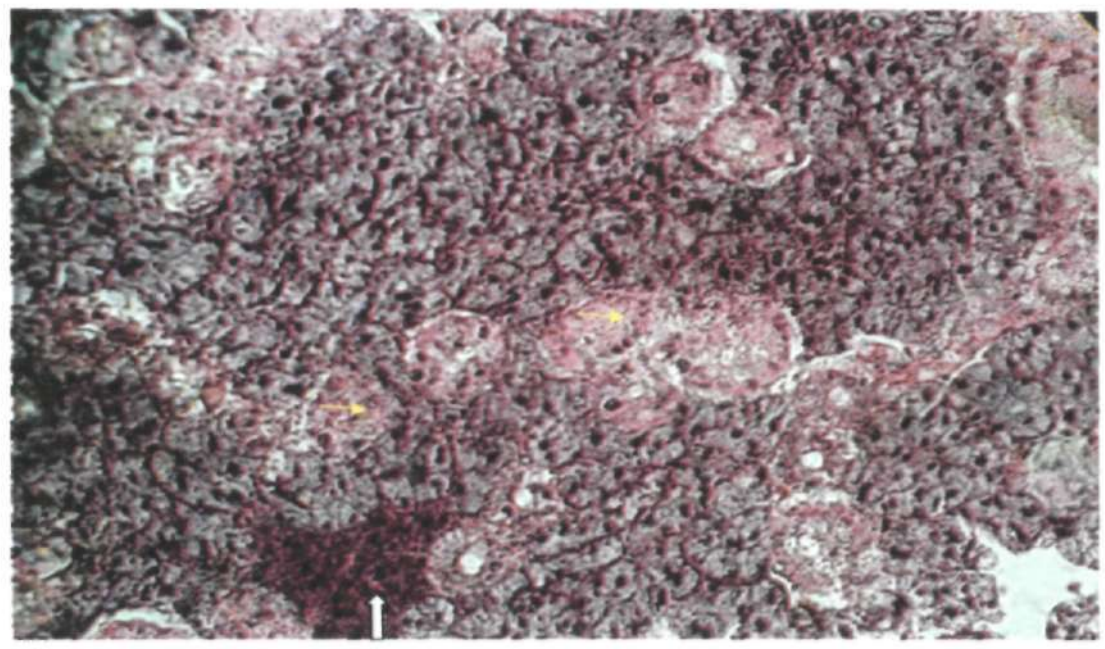


Plate XII: Section of the pancreas of rats pretreated with vitamin C before exposure to chlorpyrifos showing some degenerative areas in the exocrine pancreas (single arrow) and viable pancreatic islet (block arrow). H&Ex 400

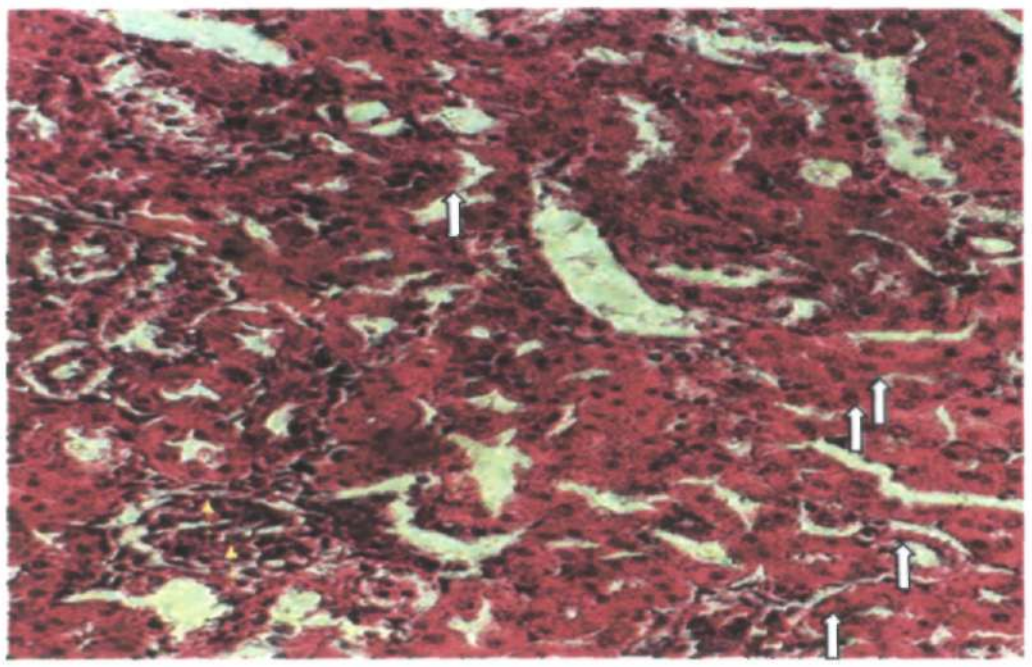


Plate XIII: Section of the kidneys of rats exposed to chlorpyrifos only showing degeneration of glomeruli and adhesion of the glomerulus to the Bowman's capsule (single arrows) and renal tubules (block arrows). H&Ex400

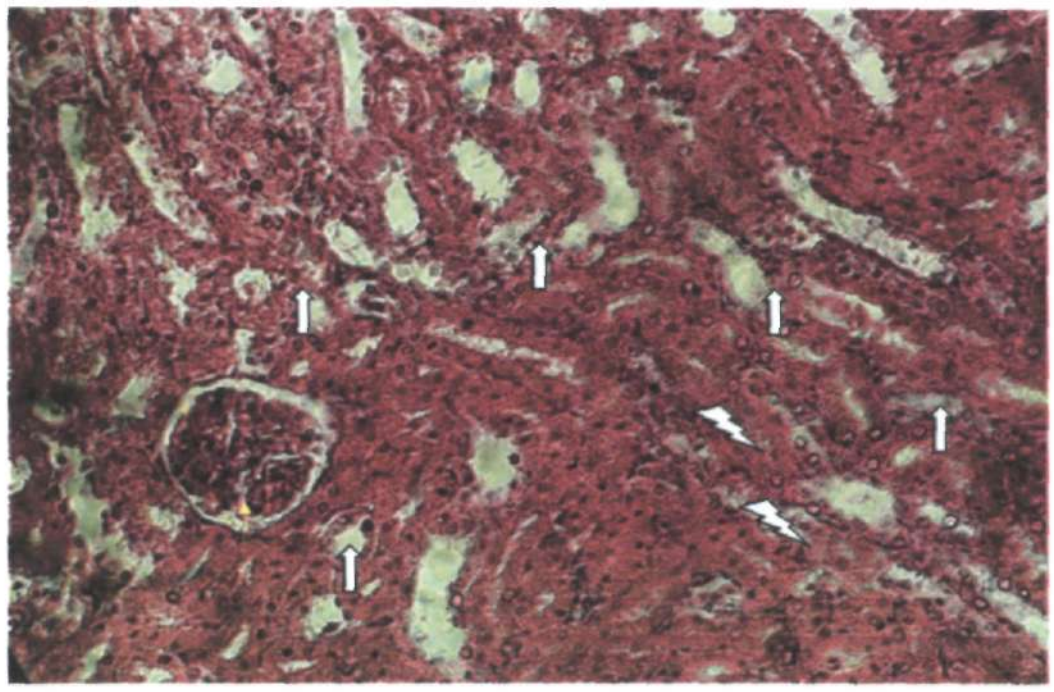


Plate XIV: Section of the kidney of rats pretreated with vitamin C prior to administration of chlorpyrifos showing normal glomeruli (single arrow) and renal tubules (block arrows) and few areas of degenerated renal tubules (lightning bolts). H&Ex400

CHAPTER FIVE

5.0 DISCUSSION

5.1 Median lethal dose (LD_{50})

The median lethal dose (LD_{50}) of 85 mg/kg obtained in the present study fell within the range of 82-270 mg/kg reported from previous studies (Gosselin *et al.*, 1984; US EPA, 1984; Berg, 1986), indicating that CPF is moderately toxic. The acute clinical signs obtained during the LD_{50} evaluation demonstrated that CPF caused muscarinic, nicotinic and central cholinergic effects, which are characteristic signs observed in OP poisoning. The huddling observed following CPF exposure may be due to induction of hypothermia (Gordon *et al.*, 1997; Smith and Gordon, 2005). Although the mechanism responsible for hypothermia is not known, it has been suggested that the response is mediated via cholinergic muscarinic pathways in the CNS (Gordon and Yang, 2000).

5.2 Chronic toxicological study

5.2.1 Clinical signs

The observed clinical manifestations of CPF poisoning in the rats exposed to CPF only were ocular discharges and conjunctivitis. The ocular discharges have been demonstrated as part of the muscarinic signs observed following exposure to OP insecticides. The ocular signs were due to inhibition of AChE by CPF, resulting in accumulation of ACh in the muscarinic cholinergic receptors. However, rats pretreated with vitamins C and/or E did not manifest any clinical sign, indicating that the vitamins may be involved in protection of neurones from AChE inhibition. The result confirmed the findings of Ambali *et al.* (2006; 2007) that vitamins C and E

ameliorated CPF-induced toxic signs in mice. Malkovics *et al.* (1983) have shown that cholinesterase inhibiting action of OP is compensated by vitamin E, while Yavuz *et al.* (2004) showed that vitamins C and E enhance the restoration of cholinesterase activity.

5.2.2 Body weight

The present study revealed a progressive increase in body weight of rats in all the groups throughout the study period, except those exposed to CPF only that did not manifest the rapid increase in body weight gain obtained in rats in the other groups. The rats exposed to CPF had a relatively lower weight gain compared to those in other experimental groups, although the difference in weight gain in animals exposed to CPF only when compared to the control was insignificant. This observation was similar to those recorded by Mollelo *et al.* (1980), Corley *et al.* (1989) and Joshi *et al.* (2007) in rats, and Ambali *et al.* (2007) in mice. Earlier studies have demonstrated a dose-dependent change in body weight following repeated exposure to CPF, and rats exposed to high doses recorded a decline in body weight (Crown *et al.*, 1985; Davies *et al.*, 1985; Barna-Lloyd *et al.*, 1990; Yano *et al.*, 2000; Terry *et al.*, 2003). Rats in vitamin-pretreated groups showed a sharp progressive rise in weight gain compared to those exposed to CPF only, indicating the role of oxidative stress in the mechanism of CPF-induced alteration in weight gain. The pretreatment of rats with a combination of vitamins C and E showed a better improvement in percentage weight gain than even the control. The lack of rapid progression in body weight gain obtained in rats exposed to CPF only compared to the other groups may be due to induction of lipid peroxidation depleting the body tissue mass. Besides, CPF-oxon, the more toxic metabolite of CPF, has been shown to inhibit cholesterol ester hydroxylase, resulting in the elimination of normal response to stress (Civen *et al.*, 1977). It has been shown that chronic exposure to CPF

5.2.3 Open-field test

5.2.3.1 Motor (Locomotor) activity

The pattern of motor activity recorded in rats dosed with CPF only showed a general reduction demonstrating a decrease in exploration and/or increased anxiety. Motor activity which represents a broad class of behaviour involving coordinated participation of sensory, motor and integrative processes (OECD, 2004) have been shown to be significantly impaired by chronic exposure to CPF in the present study. The observed decrease in motor activity in rats exposed to only CPF confirmed findings that motor functions, including locomotor activity, are impaired following repeated CPF exposure in rats (Pope *et al*., 1992; Mattson *et al*., 1996; Moser, 1995; Moser *et al*., 1997) and chicks (Al-Badrany and Mohammad, 2007). Similarly, Shankar *et al*. (1993) demonstrated a slight reduction in motor activity by week 4 with subsequent recovery in a 13-week neurotoxicity study. Sanchez-Amate *et al*. (2001) demonstrated CPF-induced anxiogenic activity in rats. Studies have associated decrease in motor activity due to CPF exposure to inhibition of AChE (Nostrandt *et al*., 1997; Moser, 2000; Timofeeva and Gordon, 2002), and that about 70% AChE inhibition must be observed before the decrease is manifested (Sanchez-Amate *et al*; 2001, 2002; Sanchez-Santed *et al*., 2004). Therefore, reduced motor activity in CPF exposure has been attributed to central activation of cholinergic motor inhibitory system (Timofeeva and Gordon, 2002). Carr *et al*. (2001) attributed reduced mobility observed in OP poisoning partly to damage in the peripheral musculature, probably due to necrosis of skeletal muscle fibre. Muscle necrosis has been observed following exposure to the OP insecticide, isofenphos and the insecticide metabolite, paraoxon (Dettbarn, 1984; Calore *et al*., 1999). The severity of the necrosis is thought to be dependent on the level and duration of the inhibition of AChE (Carr *et al*., 2001). Although the present study did not evaluate the AChE

causes vacuolization of the *zona fasciculata* (Barna-Lloyd *et al.*, 1990, Yano *et al.*, 2000), the region of the adrenal cortex responsible for the elaboration of cortisol and corticosterone required for both immediate and long-term responses to stress. Therefore, the effect of CPF on pattern of body weight changes observed in the present study may be due to oxidative stress, adrenal-mediated stress and possibly cholinergic mechanism-mediated stress.

The results obtained demonstrated that male animals had better weight gains than their female counterparts in groups dosed with CPF only and in those pretreated with vitamin E and combination of vitamins C and E, although the weeks in which this difference became manifested was earlier in the rats exposed to CPF only. Rats from the control group and those pretreated with vitamin C did not show any difference in body weight between male and female animals. The result agreed with the observation by Gordon *et al.* (1997) that the female rats are generally more susceptible to CPF poisoning than the males. The result obtained in the present study contradicted the finding of Young and Grandjean (1988) that the male rats showed a consistent decrease in body weight compared to the females following chronic exposure to CPF. Barna-Lloyd *et al.* (1990) also observed a more severe vacuolization of *zona fasciculata* in the male rats than females. No concrete explanation may be adduced on the cause of the contradictions and sex difference to body weight gain observed in the present study in rats exposed to CPF only. Similarly, the reason why the male rats pretreated with vitamin E and a combination of vitamin C and E showed better weight gain than the females towards the end of experimental period requires further investigations.

5.2.3 Open-field test

5.2.3.1 Motor (Locomotor) activity

The pattern of motor activity recorded in rats dosed with CPF only showed a general reduction demonstrating a decrease in exploration and/or increased anxiety. Motor activity which represents a broad class of behaviour involving coordinated participation of sensory, motor and integrative processes (OECD, 2004) have been shown to be significantly impaired by chronic exposure to CPF in the present study. The observed decrease in motor activity in rats exposed to only CPF confirmed findings that motor functions, including locomotor activity, are impaired following repeated CPF exposure in rats (Pope *et al.*, 1992; Mattson *et al.*, 1996; Moser, 1995; Moser *et al.*, 1997) and chicks (Al-Badrany and Mohammad, 2007). Similarly, Shankar *et al.* (1993) demonstrated a slight reduction in motor activity by week 4 with subsequent recovery in a 13-week neurotoxicity study. Sanchez-Amate *et al.* (2001) demonstrated CPF-induced anxiogenic activity in rats. Studies have associated decrease in motor activity due to CPF exposure to inhibition of AChE (Nostrandt *et al.*, 1997; Moser, 2000; Timofeeva and Gordon, 2002), and that about 70% AChE inhibition must be observed before the decrease is manifested (Sanchez-Amate *et al*; 2001, 2002; Sanchez-Santed *et al.*, 2004). Therefore, reduced motor activity in CPF exposure has been attributed to central activation of cholinergic motor inhibitory system (Timofeeva and Gordon, 2002). Carr *et al.* (2001) attributed reduced mobility observed in OP poisoning partly to damage in the peripheral musculature, probably due to necrosis of skeletal muscle fibre. Muscle necrosis has been observed following exposure to the OP insecticide, isofenphos and the insecticide metabolite, paraoxon (Dettbarn, 1984; Calore *et al.*, 1999). The severity of the necrosis is thought to be dependent on the level and duration of the inhibition of AChE (Carr *et al.*, 2001). Although the present study did not evaluate the AChE

level, the low-dose CPF administration may be adequate to cause significant inhibition of this enzyme, even though it did not result in significant clinical manifestations. Another possible mechanism of reduced limb mobility in animals dosed with CPF may be due to its ability to inhibit fatty acid amide hydrolase (FAAH), a sensitive target for many OP insecticides. Quistad *et al.* (2001) have shown that inhibition of brain FAAH observed in mice dosed with diazinon, an OP insecticide, may be responsible for reduced mobility. Rats pretreated with vitamins C and/or E showed a pattern indicating substantial improvement in motor activity compared to those in the CPF groups. This indicates that oxidative stress may be playing some roles in the pathophysiology of comparative reduction of motor activity observed in the CPF-treated animals. Generally, the present study has shown lack of sex difference in the response of rats to motor activity in all the groups except in those pretreated with vitamin E where females showed an improved motor activity over the males by day 5, week 3 and 6. The reason for this observation is not known and does require further investigation.

5.2.3.2 Frequency of rearing

The decrease in frequency of rearing recorded with increased duration of the study in rats repeatedly exposed to CPF only agreed with the observation of Terry *et al.* (2003) who obtained a dose-dependent alteration in frequency of rearing following repeated CPF exposure in rats. Acording to Bouwknecht and Paylor (1990), rearing reflects adaptive strategy of animals to explore their environment. It also reflects responses to environmental novelty and emotional states such as stress levels in rodents (Gironi Carnevale *et al.*, 1990; Sadile, 1996). Rearing can also be used to evaluate anxiety in animals, with high frequency indicating low anxiety level (Brown *et al.*, 2007). Thiel *et al.* (1998) has shown that rearing provides a good measure of

habituation than even the locomotor activity. In addition, it has been shown that rearing responses observed upon repeated exposures to the same environment are strongly influenced by inter-individual differences in habituation learning, a variety of pharmacologic and toxicologic agents, and cholinergic activity in the fore-brain and hippocampus (Terry *et al.*, 2003). Therefore, the decreased frequency of rearing observed in animals exposed repeatedly to CPF only demonstrated decreased exploratory behaviour, increased level of anxiety, increased stress level, decreased habituation learning and, probably, increased cholinergic activity in the fore-brain and hippocampus. The increase in cummulative frequency of rearing observed in groups of rats pretreated with either vitamins C or E or combination of both vitamins reflects the role played by oxidative stress in CPF-induced decrease in frequency of rearing, anxiogenic effect and reduced habituation learning. Indeed, the significant increase in the cummulative frequency of rearing in group pretreated with a combination of vitamins C and E over the control further demonstrated synergism when both vitamins are co-administered. The fact that the frequencies of rearing in male and female rats did not significantly differ shows that sex does not play a significant role in rearing frequency among all the experimental groups.

5.2.3.3 Frequency of grooming

The present work revealed a pattern showing increase in the frequency of grooming in rats treated with CPF only compared with the control and the vitamins pretreated groups. Grooming behaviour is a displacement response that is displayed in a novel environment (Espejo, 1997). Therefore, it is expected that grooming should decrease with repeated exposure to the same testing apparatus. Inability to show a decrease in frequency of grooming as demonstrated in rats repeatedly dosed with only CPF further reaffirmed the ability of this organophosphate insecticide

to impair habituation and spatial learning or increase level of anxiety. Rats pretreated with vitamins C and/or E showed a trend of general decrease in the frequency of grooming in a time-dependent manner. This again reaffirmed the role of oxidative stress in the pathophysiology of CPF-induced anxiogenic effect and impairment of habituation and spatial learning.

5.2.3.4 Frequency of stretch-attends posture

The general pattern in the frequency of stretch-attends posture recorded in CPF group in the present study revealed that of an increase, , although an initial decrease was observed. Stretch-attends postures are "risk assessment" behaviour which indicates that the animal is hesitant to move from its present location to a new position (Blanchard *et al.*, 2001). Therefore, a high frequency of this posture reflects a higher level of anxiety, impairment of habituation and spatial learning. The increased frequency of stretch-attends posture in rats exposed to CPF only in the present study further confirmed the anxiogenic effect of this pesticide. However, pretreatment with vitamins C and/or E have been demonstrated by this study to modulate the increased frequency of stretch-attends posture induced by CPF, with rats pretreated with vitamin E showing the best modulatory effect. The frequency of stetch-attends posture observed in the vitamins-pretreated groups was lower than even the control. This further showed that oxidative stress plays an important role in the anxiogenic responses, and impairment of habituation and spatial learning induced by repeated CPF administration.

5.2.3.5 Frequency of defaecation

The present study also demonstrated an increase in the frequency of defaecation (increase in number of faecal boli) in rats in the CPF group compared to those in the control and vitamins C

and/or E pretreated groups. This further confirmed the anxiogenic effect of CPF since frequency of defaecation may be used to measure anxiety. Although the validity of defaecation as a measure of anxiety has been questioned (Lister, 1990), Hall (1934) described defaecation and urination as indices of anxiety in rodents. This is because autonomic nervous system is activated in a noxious environment, resulting in increased defaecation. Bindra and Thompson (1953) argued that there is no significant relation between fearfulness, urination and defaecation as measured in open-field test. However, the same author agreed that defaecation and urination in a novel environment are signs of emotionality, not equated with fearfulness and/or timidity. The results of the present study seem to agree, when correlated, with those obtained using other anxiety-measuring devises. Therefore, defaecation may be used not only as an indication of anxiety, but also that of habituation and spatial learning. Rats from the vitamins pretreated groups, especially those pretreated with a combination of vitamins C and E demonstrated a consistent decrease in the frequency of defaecation. The finding reaffirms the role of oxidative stress in the pathophysiology of anxiogenic response and impairment of habituation, and spatial learning induced by CPF poisoning.

5.2.3.6 Frequency of urination

The fact that the frequency of urination was found to be generally higher in the CPF groups than in the other groups agreed with the finding of Yano *et al.* (2000) where increased urinary perineal soiling was observed in rats exposed to prolonged high dose of CPF. The results of the present study also demonstrated the anxiogenic effect of CPF. Rats pretreated with vitamin C and/or E generally experienced a decrease in frequency of urination compared to those exposed to CPF only, and this observation further lent credence to the role of oxidative stress in

anxiogenic, and impairment of habituation and spatial learning induced by CPF, and that urination may conveniently be used as an index of anxiety in an open-field assessment.

The open-field assessment has shown that CPF impaired locomotive and exploratory behaviour, increased anxiety and impaired habituation and spatial learning. Exposure to stressors influnces the neurobehavioural profile of an organism and can precipitate anxiety-like syndrome (Chakraborti *et al.*, 2008). The effect of CPF on anxiety agreed with the observation that workers exposed to OPs showed significantly higher anxiety level than the unexposed population (Rodnitzky *et al.*, 1975). Similarly, Brazilian workers chronically exposed to CPF and acephate have been shown to have increased generalzed anxiety disorder (Salvi *et al.*, 2003). The increased anxiogenic response experienced in rats repeatedly exposed to CPF only may reflect the ability of the pesticide to stimulate cytokines release (Gordon and Rowsey, 1999; Rowsey and Gordon, 1999). It has been shown that cytokines are molecules that relay inflammatory and immune messages to the brain (Carvajal *et al.*, 2005), and they are involved in anxiety-like responses and psychiatric stress syndrome (Kronfol and Remick, 2000; Dantzer, 2001; Anisman and Merali, 2003). The OP-exposed soldiers in Gulf war developed persistent psychiatric syndrome that closely correspond to the psychological and behavioural sequelae of a cytokine-mediated sickness response (Ferguson and Cassaday, 1999). A study has also shown CPF-induced activity in cytokine-related areas of the brain as measured by cfos expression (Carvajal *et al.*, 2005). The ability of either vitamins C or E or a combination of both vitamins to ameliorate the increased anxiogenic response and decreased habituation and spatial learning in rats elucidate the role of oxidative stress in the aetiopathogenesis of these behavioural abnormalities induced by repeated exposure to CPF.

5.2.4 Forepaw grip time

The present study has also shown a significant reduction in forepaw grip time, reflecting deficit in forepaw motor strength following chronic CPF exposure in rats. The result agreed with the finding obtained in an earlier study which showed reduction in hindlimb grip strength following repeated CPF administration in rats (Terry *et al.*, 2003). The reduction in grip time observed in rats dosed with CPF only may be related to impairment of motor strength, probably resulting from the ability of the pesticide to anterograde decrease axonal transport, which has been reportedly observed in repeated CPF exposure (Terry *et al.*, 2007). In addition, the reduced forepaw grip time may be the result of reduced neuronal viability associated with impaired microtubule synthesis and/or function (Prendergast *et al.*, 2007). It has also been postulated that disruption of kinesin-dependent intracellular transport may account for some of the long-term effects of organophosphates on the peripheral and central nervous system (Gearharta *et al.*, 2007). Reduced hand strength (Miranda *et al.*, 2004) and loss of muscle strength (Steenland *et al.*, 2000) have been observed in humans following prolong exposure to OPs. It has been shown that chronic CPF exposure at levels associated with appreciable inhibition of B-esterases produced adverse dose-effects on peripheral nerve electrophysiology suggestive of sub-clinical neuropathy (Albers *et al.*, 2007). Relationship has also been established between higher OP exposure and the development of chronic fatigue syndrome (Tahmaz *et al.*, 2003). The effect of CPF on grip time observed in the present study may involve not only the peripheral centres, but also higher centres in the brain. Pretreatment with vitamins C and/or E caused significant improvement in the CPF-impaired forepaw grip time partly during the study period. However, with increasing duration of CPF exposure, the protective ability of the antioxidant vitamins on CPF-impaired reduction in forepaw grip time waned such that by week 16 there was no

difference in the deficit between the CPF and the vitamins pretreated groups except in rats from the VC+VE+CPF group, which still maintained some level of motor strength. The time-dependent protective effect exhibited by either antioxidant vitamin C or E may be due to increase in lipid peroxidation on the peripheral nerves and the relevant areas of the brain due to continued CPF exposure. This eventually overwhelmed the antioxidant status of the rats. Peripheral neuropathy may likely occur during FR damage to the nerves (Traber *et al.*, 1987). The continued improvement in forepaw grip time shown by rats pretreated with the combination of vitamins C and E reflected the synergistic ability when both vitamins are combined. Therefore, the present study has shown that oxidative stress plays some essential role in the CPF-induced deficit in forepaw grip time.

5.2.5 Excitability score

The present study revealed a progressive decrease in the excitability score of animals dosed with CPF only. Excitability score reflects the state of alertness of the animals showing the state of sensorimotor reflex and neuromuscular coordination of the animals. Therefore, low excitability score observed in CPF group reflected a deficit in sensorimotor reflex and inability of the rats in the group to maintain neuromuscular coordination. This deficit in sensorimotor reflex may be due to impairment of neuronal activity in certain region of the brain particularly in the cerebral cortex. Pretreatment with vitamins C and/or E significantly improved the excitability score, with the combination of both vitamins being most effective. This finding demonstrates the role of oxidative stress in the mechanism of CPF-induced neurobehavioural changes. Vitamins C and/or E may be involved in preventing the lipid peroxidation caused by prolonged CPF exposure in the cerebral cortex and other brain regions. Therefore, the improvement in excitability score

observed in rats pretreated with the antioxidant vitamins may be due to the reduction in lipid peroxidation. The finding in this study corroborates the results obtained by Ayo *et al.* (2006), who demonstrated the ability of vitamin C to ameliorate low excitability score induced by road transportation stress in goats. Therefore, the improvement in the excitability score in rats pretreated with antoxidant vitamins C and/or E showed that oxidative stress plays an important role in the mechanism of impairment of excitability observed in group exposed to CPF only. Besides, vitamin C has been shown to be involved in the synthesis of neurotransmitter such as norepinephrine, which may increase brain excitability (Balz, 2003; Naidu, 2003). Similarly, vitamin C has been shown to activate the nervous system especially the sympathetic nervous system (Chervyakov *et al.*, 1977), which may have contributed to the improvement in excitability scores observed in rats pretreated with vitamin C.

5.2.6 Inclined plane

The poor performance of rats exposed to CPF on inclined plane demonstrated that OP insecticide impaired sensorimotor performance and neuromuscular coordination. The result agreed with that of Abou-Donia *et al.* (2002) who showed that sarin, an OP warfare agent impaired the inclined plane performance in rats. Pretreatment with vitamins C and/or E was shown in the present study to ameliorate the poor performance on inclined plane induced by repeated exposure to CPF. This further demonstrates the involvement of oxidative stress or lipid peroxidation to the mechanism of impairment of sensorimotor performance and neuromuscular coordination as evidenced in the poor performance on inclined plane observed in rats exposed to CPF only.

5.2.7 Ladder walk

The lower ladder score characterized by lower number of missed rungs observed in rats chronically exposed to CPF indicates that the legs of the rats were frequently being held stationary above the rungs for a relatively longer period. This observation demonstrated difficulty in the ability of the rats exposed to CPF only to move fast through the obstacle, and hence a deficit in locomotor activity. The locomotor deficit observed was dependent on the duration of exposure, with worst locomotor performance recorded in week 14 compared to week 7. The results agreed with the previous findings that slowness of movement is one of the extrapyramidal symptoms (Parkinsonism) observed in humans exposed to non-specific agricultural pesticides, which increased with the duration of exposure (Ritz and Yu, 2000; Alavanja *et al.*, 2004). Thus, the locomotor deficit observed in the present study is part of the sensorimotor deficits occurring in animals chronically exposed to CPF. The amelioration of the locomotor deficits manifested in the improvement of ladder walk test and characterized by increase in the number of missed rungs in rats pretreated with vitamin C and/or E demonstrated the important role played by oxidative stress in locomotor deficit induced by CPF. The finding showed for the first time that the administration of the antioxidants may be of value in decreasing or eliminating the risk of adverse effects of chronic CPF poisoning.

5.2.8 Forelimb-placing response

The study revealed a significant deficit in forelimb-placing response in animals dosed with CPF. The evaluation of visual-placing response in animals repeatedly exposed to CPF showed a certain degree of duration-dependent response. Thus, the number of animals in the CPF groups that showed deficit in visual forelimb-placing response was higher in week 8 than week 12 or 16.

The improvement in visual-placing response observed in this group of animals by week 12 and 16 was probably due to recovery from sensorimotor deficits to this neurobehavioural response. The fact that the overall percentage of animals exposed to CPF only that had a rating of 0 was significantly lower than those in the control revealed the ability of CPF to impair visual-placing response. Although pretreatment with vitamin C resulted in a significant decrease in overall percentage of rats with rating of 0 compared to the control, the number of animals in the VC+CPF group with this rating was more than those in group administered CPF only. Pretreatment with either vitamin E or a combination vitamins C and E which resulted in significant improvement in the overall mean percentage of animals that had rating of 0 demonstrated the role of antioxidant in ameliorating the deficit in visual-placing reponse induced by chronic CPF administration.

Similarly, majority of the rats dosed with CPF only demonstrated a poor forelimb tactile-placing response by week 4, with subsequent improvement observed in the following weeks. The significant decrease in the overall percentage of animals dosed with CPF only that had a rating of 0 showed the ability of this chemical to impair forelimb tactile-placing response. Although, pretreatment with vitamins C and E caused a significant increase in the overall percentage of rats that showed deficit in tactile-placing response compared to the control, the percentage of rats with such deficit was lower compared to those exposed to CPF only. Pretreatment with a combination of vitamins C and E caused significant improvement in the number of rats with improved tactile-placing response. This showed the role of oxidative stress in the impairment of the tactile-placing response caused by chronic exposure to CPF and the synergistic role played by co-administration of vitamins C and E in ameliorating this sensorimotor deficit.

The study also revealed a significant increase in the number of animals exposed to CPF only that showed deficit in proprioceptive-placing response by week 4. The reduction in the number of animals showing this deficit in subsequent weeks demonstrated the ability of CPF to induce some form of tolerance. The significant increase in cumulative percentage of animals showing deficit in proprioceptive-placing response in group exposed to CPF only showed the ability of this chemical to impair sensorimotor performance. Fikes (1992) observed deficits in proprioceptive placing in cats following repeated CPF exposure. Although, pretreatment with vitamin C was also shown to cause cumulative increase in the number of animals that showed deficit in proprioceptive placing response, this number was significantly less compared to those in group exposed to CPF only. Pretreatment with vitamin E and a combination of vitamins C and E caused significant reduction in the cumulative percentage of animals that showed deficit in proprioceptive-placing response. This further demonstrated the role of oxidative stress in CPF-iduced impairment of proprioceptive-placing response.

The improvement observed in the animals administered with CPF only in their proprioceptive and tactile forelimb-placing by week 8 and 16 may be due to some form of recovery or the development of tolerance to the chronic effect of the chemical. Abou-Donia *et al.* (2002) have shown that repeated exposure to low-level sarin, either alone or in combination with pyridostigmine, resulted in poor forelimb-placing responses, similar to the results obtained in the present study. The poor forelimb-placing response observed in rats exposed chronically to CPF in the present study demonstrated the ability of this organophosphate compound to cause sensorimotor deficits following repeated exposure. The response may be due to lesions in the rats' somatic sensorimotor cortex and corticospinal tract, two regions of the brain shown to

control limb-placing (Hicks and D'Amato, 1975; Abou-Donia *et al.*, 2001). In addition, studies have shown that low-level CPF exposure impaired the electroencephalography (EEG) measured in the frontal lobe (Korsak and Sato, 1977), a brain region important in sensorimotor function (Abou-Donia *et al.*, 2001). Pretreatment of rats with either vitamin C or E or a combination of both vitamins has been shown in the present study to significantly improve the forelimb-placing response of rats chronically exposed to CPF. This result further lend support to other observations in the present study that oxidative stress plays a significant role in sensorimotor performance deficits elicited by repeated exposure to CPF. The brain has been shown to be more vulnerable to injury by lipid peroxidation (Naffah-Mazzacoratti *et al.*, 2001). The antioxidant vitamins, apparently, ameliorated the oxidative damage to the sensorimotor cortex, corticospinal tract and frontal lobes caused by repeated exposure to CPF.

5.2.9 Orienting to vibrissae touch

The present study has also shown poor response to vibrissae touch in rats chronically exposed to CPF only by weeks 4, 8 and 12, but not week 16. The peak of this poor orienting to vibrissae touch occurred in week 8, when 90% of the rats did not orient to vibrissae touch. The improvement in orienting to vibrissae touch in rats dosed with CPF only by week 16 showed that the rats were capable of recovering from this neurobehavioural response with time due to induction of tolerance to some of the neurotoxic effect of this chemical. Orienting to vibrissae touch is a neurobehavioural test used to evaluate the response of animals to a stimulus. Reduced response rates (Bushnell *et al.*, 1993; 1994; Cohn and MacPhail, 1997; Maurissen *et al.*, 2000) have been observed following CPF exposure in rats. The effect of CPF on orienting to vibrissae touch further supports the ability of the OP insecticide to impair sensorimotor performance.

Although the main mechanism of the sensorimotor deficit observed was not investigated in the presnt study, it has been shown that this may be related to the impairment of anterograde and retrograde axonal transport (Terry *et al.*, 2003), possibly from accumulation of tubulovessicular profiles within the axons (Abou-Donia and Lapadulla, 1990). The deficit obtained in the present study may also be due to reduction in nerve conduction velocities (Gotoh *et al.*, 2001), resulting from decreased neuronal viability caused by impaired microtubules synthesis or function (Prendergast *et al.*, 2007). The improvement in the response to orienting to vibrissae touch in rats pretreated with vitamin C and/or E clearly showed that oxidative stress plays some important role in the toxic mechanisms of impairment of sensorimotor performance caused by chronic exposure to CPF. Lipid peroxidation is an index of irreversible neuronal damage of cell membrane phospholipids (Freitas *et al.*, 2005). Therefore, the reduction in level of neuronal lipid peroxidation following pretreatment with antioxidant vitamins C and/or E may be responsible for the improved sensorimotor performance following chronic CPF exposure.

5.2.10 Postural reflex

The present study showed that repeated administration of CPF evoked the impairment of postural reflex. 100% of the animals in group dosed repeatedly with CPF only showed consistent flexion of forelimb by week 7, while 90% showed same by week 14. The significant increase in the number of rats with a rating of 2 in this group showed the ability of CPF to impair postural reflex. This result further demonstrates the ability of CPF to impair sensorimotor performance. This finding agreed with the result obtained by Abou-Donia *et al.* (2002) who observed a deficit in postural reflex in rats repeatedly exposed to sarin. Workers applying termiticide have also been shown to have poorer performance on postural sway test (Steenland *et al.*, 2000). The

significant increase in the number of animals that showed improvement in postural reflex in groups pretreated with antioxidant vitamins C and/or E showed that oxidative stress plays an important role in the toxic mechanisms of damage to the brain, particularly the cerebellum and cerebral cortex.

5.2.11 Surface righting reflex

The study has also demonstrated impairment of surface righting reflex in rats chronically exposed to CPF only. The increased reflex righting time observed in CPF group reflected impairment in neuromuscular coordination and sensorimotor reflex. There was a duration-dependent effect of CPF on the righting reflex. The improvement in righting reflex by week 16 over that observed in week 12 reflected some form of recovery, occurring with increased duration of exposure. This may be due to the development of some form of tolerance to the OP chemical. However, on the whole, the study demonstrated significant delay in reflex righting time in rats chronically exposed to CPF, as reflected by a significant decrease in the number of animals with a rating of 0 compared to the control. This finding agreed with that observed by Dam *et al.* (2000) where impairment of righting reflex was recorded in neonatal rats exposed to chlorpyrifos. The present study showed the ability of the CPF to interfere with sensorimotor performance and neuromuscular coordination. This might be due to interference with mictotubule synthesis or action in the axons (Prenderghast *et al.*, 2007). The antioxidant vitamins C and/or E showed tremendous ability to ameliorate the impairment of reflex righting time caused by prolong exposure to CPF. The group pretreated with the combination of both vitamins C and/or E demonstrated a very significant improvement in their reflex righting time. This further demonstrates the synergistic effect of the combination of both vitamins C and E. Thus,

the present study has for the first time showed that oxidative stress plays a significant role in the impairment of reflex righting induced by chronic CPF exposure.

5.2.12 Beam-walk

The result showed a lowered beam-walk score characterized by increase in the width (decreased beam-walk length) where rats repeatedly exposed to CPF only slipped from the beam-walk apparatus. The depreciation in beam-walk score progressed with increased duration of exposure, with much deficit observed in week 14 than in week 7. This observation in rats exposed to only CPF showed the ability of the OP insecticide to impair sensorimotor performance and coordinated gait. Abou-Donia *et al.* (2002) observed similar result following repeated exposure of rats to sarin. Beam-walking performance is an integrated form of behaviour necessitating pertinent level of consciousness, memory, sensorimotor and cortical functions mediated by the cortical area (Abou-Donia *et al.*, 2001). Deficits in beam-walk score may be suggestive of injury to the cortex (Abou-Donia *et al.*, 2001).

Norepinephrine has been strongly proposed as playing a significant role in beam-walk deficits caused by cortical injury (Boyeson *et al.*, 1992; Goldstein, 1995). It facilitates the recovery from locomotor deficit by alleviating injury-induced decrease and turnover of norepinephrine levels in the cortex (Boyeson and Feeney, 1990; Kikuchi *et al.*, 2000). Norepinephrine which originates from *locus coerulus* is widely distributed in the cerebral cortex, hippocampus, cerebellum and spinal cord (Abou-Donia *et al.*, 2001). Therefore, it is probable that the chronic CPF exposure caused injury to all these areas and possibly other parts of the brain, which might have caused alteration in the noradrenergic and other catecholaminergic pathways.

The pretreatment with vitamins C and/or E improved the beam-walk score significantly, with rats pretreated with the combination of both vitamins showing the best improvement. This further showed the synergistic effect of the combination of both vitamins. This observation further emphasized that oxidative damage to the relevant portion of the brain, particularly the cortex and *locus coerulus* may be partly responsible for the beam walk deficit in rats exposed to CPF only. Therefore, the improved beam-walk score observed in groups pretreated with the antioxidant vitamins may be due to reduced lipid peroxidation in the brain. Besides, vitamin C plays an essential role in the formation of norepinephrine from dopamine (Iqbal *et al.*, 2004), which eventually increases the availability of norepinephrine essential in the successful performance of beam walk.

5.2.13 Effect of treatments on cognition

The present study has shown that repeated exposure to CPF significantly impaired learning and memory. This result agreed with many earlier studies which demonstrated that repeated low-level exposure to CPF impair cognition in rats (Bushnell *et al.* 1991; 1994; Cohn and MacPhail, 1997; Prendergast *et al.*, 1997, 1998, 2007; Stone *et al.*, 2000, Moser *et al.*, 2005). In addition, studies in humans have shown persistent cognitive deficits in farmers and pesticide applicators repeatedly exposed to OPs but are symptom-free (Steenland *et al.*, 2000; Dick *et al.*, 2001). The impairment of cognition observed in rats dosed with CPF only may be due to alteration in ACh metabolism. Since ACh has been demonstrated to be involved in cognition, agents such as OPs which alter ACh metabolism may interfere with this role. Many studies have linked central cholinergic system to synaptic plasticity, learning and memory processes (Overstreet, 1984; Bakin *et al.*, 1996; Baskerville *et al.*, 1997; Sachdev *et al.*, 1998). It is believed that OP

compounds play a role in memory loss by producing cholinergic dysfunction at the level of the synapse (Carr and Chambers, 1991). Although, the present study did not measure the level of AChE inhibition, some of the muscarinic cholinergic signs observed in rats exposed to CPF only indicate mild AChE inhibition. Therefore, it is possible that the repeated low-level CPF exposure may cause some level of impairment of AChE in addition to alteration in brain cholinergic pathways. Low-level CPF exposure has been shown to induce protracted deficits in information processing and cognitive functions, consequent from functional changes in brain cholinergic pathways resulting from alteration in bidirectional fast axonal transport (Terry *et al.*, 2007). Apart from its effect on short-term memory, long-term exposure to OPs in humans has been shown to increase reaction times for long-term memory and information processing (London *et al.*, 1997). Overstimulation followed by depression of cholinergic receptors, downregulation of muscarinic receptors (Chaudhuri *et al.*, 1993) or decreased white matter of the brain (which has been observed in methylparathion poisoning) (Nagymajtenyi *et al.*, 1988) following repeated exposure may be involved in memory impairment encountered following CPF exposure. However, other studies have shown the ability of CPF to induce cytotoxic effects directly on the hippocampal cells via the induction of apoptosis, irrespective of its effect on AChE (Terry *et al.*, 2003). Studies have also shown that CPF affects hippocampus-dependent spatial memory in rats (Levin *et al.*, 2001, 2002; Terry *et al.*, 2003). Repeated administration of CPF has been shown to impair water maze task (Terry *et al.*, 2003), a learning paradigm dependent upon intact hippocampal function (Morris *et al.*, 1982). Therefore, the impairment of learning and memory observed in the CPF group might also be due to induction of apoptosis in the brain, particularly in the hippocampus. Apoptosis has been described as the toxic end-point of CPF neurotoxicity in the brain (Caughlan *et al.*, 2004). It induces structural changes in the brain that may lead to

functional deficits, including memory and learning task (Caughlan *et al*., 2004). In addition to its effect on the hippocampus, CPF and CPF-oxon have been shown to induce apoptosis in rat cortical neurone independent of AChE inhibition (Caughlan *et al*., 2004). Inhibition of the mitochondrial activity by CPF may be an early marker of apoptotic death (Caughlan *et al*., 2004). Apoptosis may have been responsible for the massive degenerative changes in the neurones and glial cells in the cerebral cortex observed in this study in rats exposed to CPF only. Apart from providing nutrients and oxygen to neurones, glial cells also provide support and insulate one neurone from another, destroy pathogens, remove dead neurones and modulate neurotransmission (Wikipedia, 2008d; Wolosker *et al*., 2008). Degenerative changes in the neurones leads to functional deficits as it relates to neurotransmission and brain activities. Therefore, the combined effect of CPF on glial cells and neurones may have been partly responsible for the cognitive deficits observed in this study. CPF has also been shown to cause increased phosphorylation of Ca^{2+}/cAMP response element binding protein (Schuh *et al*., 2002), a protein critical to several forms of use-dependent synaptic plasticity and transcription-dependent forms of memory (Bailey *et al*; 1996; Barth *et al*., 2000; Mc Caugh, 2000; Viola *et al*., 2000; Josselyn *et al*., 2001). Hyperglycaemia observed in rats exposed to CPF may have contributed to the cognitive deficit observed in this study. Cognitive dysfunctions have been observed in diabetic human patients (Miller and Root, 1922; Schoenle *et al*., 2002; Biessels *et al*., 2003; Ryan *et al*., 2003; Brands *et al*., 2006; Weinger *et al*., 2008; Kodl and Seaquist, 2008) and rat models (Winocur *et al*., 2005). Hyperglycaemia has been proposed as a cause cognitive deficits through end organ damage to the brain resulting from increases in ROS, which could then lead to increased polyol pathway activation, increased formation of advanced glycation end products, activation of PKC and increased glucose shunting in the hexosamine pathway

(Browlee, 2005; Kodl and Seaquist, 2008). In addition, alteration in neurotransmitter function has been shown to contribute to cognitive deficit in diabetic model (Kamal *et al.*, 1999).

The present study has also shown the role played by oxidative stress as evidenced by increased lipid peroxidation in the brain, and the learning and memory impairment elicited by repeated exposure to CPF. The brain is highly susceptible to oxidative damage due to its high metabolisable oxygen demand, high level of oxidants such as iron and low level of antioxidants (Halliwell and Gutteridge, 1999; Naffa-Mazzacoratt *et al.*, 2001). Various cell stress-inducing signals such as oxidative stress, environmental stress and toxic chemical insults preferentially activate several signaling pathways such as c-jun NH_2 terminal protein kinase (JNK), p38 mitogen-activating protein kinase (MAPK) and extracellular signal-regulated protein kinase (ERK)1/2 involved in the regulation of neuronal apoptosis (Xia *et al.*, 1995; Davis, 2000). CPF has been shown to induce the activity of some of these apoptotic pathways (Caughlan *et al.*, 2004), especially the activation of JNK and ERK1/2.

Free radicals have also been implicated in apoptotic death of cells (McConkey *et al.*, 1994; Corcoran *et al.*, 1994), an important factor involved in CPF-induced impairment of memory and learning. Besides, $OH^{\cdot}$ has been shown to cause significant reduction in AChE activity in the rat brain (Tsakiris *et al.*, 2000; Iwalokun *et al.*, 2004). Therefore, antioxidant vitamins exerted protective effect on rats with learning and memory impairments, induced by chronic exposure to CPF due to a reduction in the level of brain lipid peroxidation. The results showed that the low level of FR obtained in rats pretreated with the antioxidant vitamins enhanced the dimunition of FR-induced alteration in AChE activity and, consequently, its restoration in the brain. In

addition, antioxidants have been shown to inhibit apoptosis (Stoian *et al.*, 1996; Knight, 2000). The ability of antioxidant vitamins C and E to inhibit apoptosis may have been responsible for the milder degenerative lesions observed in the neurones and glial cells observed in the vitamins-pretreated groups. Increased glutamatergic activity has also been implicated in the memory and learning deficit shown in CPF-treated rats. Although the proximate toxic mechanism of CPF-induced memory and learning impairments were not investigated in the present study, a study has shown that CPF increases glutamatergic activity by increasing the level of NR2A and NR2B subunits of NMDA receptor, resulting in neuronal death, and consequent impairment of learning and memory (Gultekin *et al.*, 2007). The glutamate toxicity, which is a major contributor to pathologic cell death within the nervous system have been shown to be mediated by ROS (Coyle and Puttfarken, 1993), apparently originating from the mitochondrial electron transport chain (Tan *et al.*, 1998). High glutamate levels block cystein uptake via the amino acid transporter X_c^- and impairs reduced glutathione (GSH) cell homeostasis (Sen *et al.*, 2000). The induction of oxidative stress by glutamate have been demonstrated to be the primary cyotoxic mechanisms in various models such as C_6 glial cells (Kato *et al.*, 1992; Han *et al.*, 1997), PC-12 neuronal cells (Froissard *et al.*, 1997; Pereira *et al.*, 1999), immature cortical neuronal (Murphy *et al.*, 1990) and oligodendroglial cells (Oka *et al.*, 1993). Antioxidants such as α-tocopherol and α-lipoic acid have been shown to protect cells against glutamate-induced cytotoxicty (Murphy *et al.*, 1989, 1990; Miyamoto *et al.*, 1989; Kato *et al.*, 1992; Han *et al.*, 1997). Therefore, the ability of antioxidant vitamins C and/or E to modulate oxidative stress protects the brain neurones from glutamate cytotoxicity, and this mechanism may be partly responsible for the amelioration of learning and memory deficits observed in the groups pretreated with both vitamins C and/or E. Besides, certain regions of the CNS such as the hippocampus, an important organ critical for

memory are sensitive to oxidative stress due to their low level of antioxidants, particularly vitamin E, relative to other brain regions (Abel and Hannigan, 1995). Furthermore, the amelioration of CPF-induced hyperglycaemia by the antioxidant vitamins C and E may have been partly responsible for the improved cognitive functions in rats exposed to CPF only.

5.2.14 Effects on haematological parameters

5.2.14.1 Effects on packed cell volume, haemoglobin concentration and red blood cell counts

The study showed a significant reduction in PCV of rats chronically exposed to CPF only. This finding agreed with the results obtained by Barna-Lloyd *et al.* (1990, 1991) in rats. However, the result on PCV contradicted the finding of Ambali *et al.* (2007) in mice, who obtained an increase in PCV attributed to diarrhoea. However, diarrhoea was not observed in the present study. The result obtained showed that pretreatment with vitamins C and/or E ameliorated the low haematocrit induced by chronic repeated CPF exposure.

The significant reduction in total RBC count in rats chronically expo\sed to only CPF agreed with earlier works by Barna-Lloyd *et al.* (1990, 1991) and Yano *et al.* (2000), but contradicted the increase in RBC count obtained by Ambali *et al.* (2007) in mice apparently due to diarrhoea resulting in haemoconcentration. However, pretreatment with vitamins C and/or E significantly increased the RBC count.

The significant reduction in Hb value obtained in rats repeatedly exposed to CPF only was in consonant with the results obtained by Barna-Lloyd *et al.* (1990,1991) in rats, and contradicted

those obtained by Ambali *et al.* (2007) in mice. Pretreatment with vitamin C and/or E was also shown in the present study to ameliorate the CPF-induced haemoglobin deficit.

The ability of chronic repeated CPF exposure to cause significant deficits in RBC and its associated parameters obtained in the present study in rats agreed with the results obtained in pesticide applicators, which revealed a decreased level of PCV, RBC and Hb (Patil *et al.*, 2003). Also, the decreased RBC and its associated parameters observed in the present study may be due to the ability of the pesticide to impair the function of haematopoietic organs. It has been shown that CPF is toxic to the bone marrow causing some chromosomal aberration (Anon, 2008). In addition, the anaemia observed in rats exposed to CPF may be due to kidney damage observed in this study, resulting in decreased renal functional mass thereby reducing the production of erythropoietin involved in bone marrow stimulation of erythrocyte production. Furthermore, study by Goel *et al.* (2006) demonstrated the ability of CPF to cause a decrease in serum concentration of iron, an essential element in the production of haemoglobin. It has been suggested that pesticide residues may interfere with several processes involved in haeme biosynthesis. Therefore, the development of anaemia may be related to interference with Hb biosynthesis and shortening of the lifespan of circulating erythrocytes (Ray, 1992).

CPF has also been shown to cause alteration in topographical morphology of the erythrocytes, prominently resulting in the formation of anisocytosis (Goel *et al.*, 2006), a feature that may accelerate RBC destruction. Furthermore, the present study has also associated chronic repeated CPF exposure to increased erythrocyte membrane fragility (see section 4.2.10.5), which further makes the RBC more prone to damage and finally destruction. Therefore, the culmination of all

the above-stated factors may be partly or wholly responsible for the anaemia observed in rats repeatedly exposed to CPF only. The anaemia may further exacerbate the CPF-induced oxidative stress since the RBC is a major site of production of antioxidant defense, especially the intracellular enzymatic antioxidants such as SODs, CATs, GSH-Px and GSH (Traber and Kamal-Eldin, 2007). The erythrocyte membranes, with their high PUFA concentration, are more susceptible to oxidative damage (Acharya *et al.*, 1991; Kumerova *et al.*, 1998).

The pretreatment of rats with vitamins C and/or E has shown that the vitamins ameliorated the CPF-induced anaemia observed in this study. This finding showed that oxidative stress may be playing some role in CPF-induced anaemia. In its reduced form, vitamin C has been shown to improve the absorption of iron from the gut (Wardlaw, 1999; Iqbal *et al.*, 2004), thereby increasing the serum concentration of iron essential for haeme synthesis. This is by facilitating the reduction of ferric iron to the ferrous form (Sayers *et al.*, 1973). Besides, vitamin C has been shown to be beneficial in the management of anaemia (Gastaldello *et al.*, 1995). The results of the present study demonstrated that vitamins C and E are effective antioxidants, preventing CPF-induced FR damage to some organs such as the kidneys essential for the elaboration of erythropoietin. The antioxidant vitamins, when administered alone or in combination, have also been shown to reduce the CPF-induced oxidative stress-mediated erythrocyte fragility, thereby prolonging the RBC lifespan. Vitamin E has been shown to enhance the stability of the membranes of the blood cells, the erythrocytes, leucocytes and platelets and, therefore, provides protection from FR attack (Pinelli-Saavedra, 2003). The result showed that administration of antioxidant vitamins C and/or E in CPF treated rats consolidated the erythrocyte membrane integrity, and thus reduces the degree of haemolysis.

5.2.14.2 Effects on white blood cell count

The study has also revealed leucopaenia due to neutropaenia in rats chronically exposed to CPF. Goel *et al.* (2006) observed leucopaenia in rats, attributed to lymphopaenia. The observed leucopaenia in the present study completely agree with the finding of Ambali *et al.* (2007) who recorded leucopaenia due to neutropaenia in mice repeatedly exposed to CPF. Neutrophils when activated play an essential role in FR-mediated cell injury through the extracellular release of superoxide FR (McCord *et al.*, 1994), which is cytotoxic also to the neutrophils resulting in destruction and, consequently, its decrease (Ambali *et al.*, 2007). Rabideau (2001) has shown that pesticides are toxic to the cells of the immune system through the induction of necrosis and apoptosis.

Pretreatment with vitamins C and/or E ameliorated the CPF-induced leucopaenia in rats. Pretreatment with a combination of vitamins C and E showed a better amelioration of the CPF-induced leucopaenia than when either of the vitamins was administered alone, demonstrating synergism of the combination of both vitamins C and E. The amelioration of leucopaenia may be due to prevention of CPF-induced FR-mediated destruction of neutrophils by the antioxidant vitamins. Besides, vitamin C has been shown to be actively transported into leukocytes (Heinecke, 2004), which enables neutrophils to accumulate the vitamin some 25-fold over plasma levels (Levine *et al.*, 1996; Food and Nutrition Board, 2000). Therefore, this selective absorption and concentration of vitamin C by neutrophils prevents its FR-mediated destruction. In addition, antioxidants have been shown to inhibit apoptosis (Stoian *et al.*, 1996; Knight, 2000), one of the molecular mechanisms involved in CPF-induced leucopaenia.

5.2.14.3 *In vitro* erythrocyte osmotic fragility

The present study has, for the first time, demonstrated an increase in erythrocyte osmotic fragility following chronic CPF exposure in rats, apparently due to impairment of erythrocyte membrane integrity induced by increased oxidative damage to the erythrocyte membrane (Stern, 1986; Wagner *et al*., 1988). Erythrocyte membranes which are highly vulnerable to lipid peroxidation are rich in polyunsaturated fatty acids. Besides, the erythrocytes contain haeme proteins (cytochromes and haemoglobin) and high level of transition elements (Cu^{2+}, Fe^{2+}) which act as prooxidant. The high level of transition elements (Cu^{2+}, Fe^{2+}) increases the susceptibility of the erythrocyte membrane to oxidative attack, leading to increased lipid peroxidation in conditions of high oxygen concentration or impaired or deficient antioxidant defence (Urano, 1993; Combs, 1998; Surai, 2001). Thus, CPF ethyl, which has been shown to induce lipid peroxidation and reduces the antioxidant defence systems in the erythrocyte membranes (Gultekin *et al*., 2001) may be involved in increasing the susceptibility to oxidative stress, thereby enhancing FR attack on the membranes and resulting in their destruction (Urano, 1993; Combs, 1998; Surai, 2001). The significant damage to the nephrons and renal tubules observed in rats exposed to CPF only may have increased the erthrocyte fragility, since serum lipid abnormalities is a common feature of nephrotic syndrome, which alters membrane lipid composition of RBC resulting in increased sensitivity to lipid peroxidation (Clemens and Bursa-Zanetti, 1989). Traber and Kamal-Eldin (2007) stated that the iron liberated from the damaged erythrocytes generate further oxidative stress. The increase in the *in vitro* erythrocyte osmotic fragility in rats exposed to CPF only confirmed the observations that *in vitro* erythrocyte osmotic fragility test can be used as an indirect method of evaluating lipid peroxidation in animals (Chihuailaf *et al*., 2002; Dariyerli *et al*., 2004). Besides, the increased erythrocyte osmotic

fragility observed in rats exposed to CPF may be due to hyperglycaemia, which also increases membrane lipid peroxidation (Jain, 1989). The amelioration of the erythrocyte osmotic fragility by antioxidant vitamins C and/or E further confirmed the role of oxidative stress in the toxic mechanism of erythrocyte damage observed following the chronic CPF exposure. The antioxidant vitamins demonstrated varying degree of protection against erythrocyte osmotic fragility at various NaCl concentrations and it seems paradoxical that vitamin C showed stronger protective effect on erythrocyte fragility than vitamin E and the combination of vitamins C and E at 0.3% Nacl. This was because vitamin C is a hydrophilic antioxidant naturally incapable of penetrating the erythrocyte lipid membrane to prevent lipoperoxidation (Niki, 1991; Sato *et al.*, 1995). The higher but insignificant increase in erythrocyte fragility observed in VC+VE+CPF group at 0.3% NaCl concentration over those in VC+CPF and VE+CPF may be due to overwhelming presence of the antioxidants beyond the existing demand of the RBC. Studies have shown that in large concentration antioxidants manifest some prooxidant effect. Vitamin C has the ability to regenerate α-tocopherol from the α-tocopheroxyl radical produced during antioxidant activity of the latter. This contributes to a significant increase in α-tocopherol level, which further increases the tocopheroxyl radical level and overwhelm the α-tocopherol regeneration capacity of vitamin C. This results in toxic build-up of tocopheroxyl radical causing damage to the erythrocyte membrane. Although, synergism between vitamins C and E has been widely demonstrated, this may not be the case all the time as demonstrated in this case, perhaps due to differential antioxidant demand in tissues and the dose. It is possible that the synergism between vitamins C and E may be governed by the dose of the antioxidants and the level of the already existing peroxidation in the tissue, which eventually determines the tissue antioxidant demand. Therefore, caution should be exercised when combining both vitamins C and E as

antioxidants. However, on the contrary, at 0.5% NaCl concentration, vitamin E and a combination of both vitamins C and E showed better protective effect on erythrocyte membrane than vitamin C. Vitamin E is a lipid soluble antioxidant and this property enhances its protection from oxidative damage by scavenging excess FR produced by oxidative stress, thus enhancing the stability of the erythrocyte membrane (Pinelli-Savedra, 2003). Uzum *et al.* (2006) have shown the ability of vitamin E to decrease erythrocyte osmotic fragility in human patients on haemodialysis and peritoneal dialysis. Similarly, Gultekin *et al.* (2001) have shown that administration of melatonin, vitamins C and E inhibits the oxidative damage to RBC induced by CPF ethyl in rats. Furthermore, Kraus *et al.* (1997) have shown that supplementation of vitamins C, E and ß-carotene decreases erythrocyte osmotic fragility and oxidative damage in zinc-deficient rats. The amelioration of CPF-induced hyperglycaemia and nephrotoxicity in rats pretreated with antioxidant vitamins C and/or E may be partly responsible for the reduced haemolysis in the vitamins pretreated groups.

The results of the study showed for the first time that the *in vitro* erythrocyte osmotic fragility test is a reliable indirect index of lipid peroxidation in rats treated chronically with CPF only, and may be of diagnostic and prognostic values in chronic CPF poisoning.

5.2.15 Serum glucose

The study demonstrated a significant increase in serum glucose level of rats chronically exposed to CPF only. Elevated glucose concentration obtained in the present study agreed with that recorded in humans acutely poisoned by CPF (Aiuto *et al.*, 1993). Similarly, Montgomery *et al.* (2008) found an association between long term-exposure to OP and organochlorine insecticides

to the activation of hypothalamic-pituitary-adrenal (HPA) axis, and the sympathetic nervous system, consequently resulting in hyperglycaemia (Bateman *et al.*, 1989; Blalock, 2002; Mechanick, 2006, Rahimi and Abdollahi, 2007). The activation of HPA axis results in the elaboration of glucocorticoids from the adrenal cortex, which increases blood glucose through promotion of gluconeogenesis and impairment of insulin-mediated glucose uptake in skeletal muscles (Oda *et al.*, 1995). Similarly, stress-mediated stimulation of the sympathetic nervous system results in the increased release of catecholamines, glucagons and growth hormones, which promotes gluconegenesis, glycogenolysis, insulin resistance and the resultant hyperglycaemia (Frayn *et al.*, 1985; Hunt and Ivy, 2002; Gustavson *et al* 2003; Gearhart and Parbhoo, 2006). Hyperglycaemia observed in this study may be due to induction of oxidative stress (Rahimi and Abdollahi, 2007). Oxidative stress results in the oxidation of antioxidant molecules such as glutathione, which has to be reduced by NADPH to be biologically functional as an antioxidant. The glucose-6-phophate dehydrogenase, which uses glucose as substrate for its activation catalyzes the initial step in the pentose phosphate pathways, responsible for reduction of NADP to NADPH. The NADPH is used for the reduction of oxidized glutathione. Therefore, glucose is the source of reducing equivalents, which are involved in the recycling of oxidized cellular antioxidants such as glutathione (Sikka and Gurbuz, 2006). Similarly, OPs lower serum paraoxonase (Sozmen *et al.*, 2002) which has antioxidant role, and, therefore, exacerbating oxidative stress. The hyperglycaemia observed in OP poisoning in this study may partly result from inhibition of AChE at neuroeffector sites in the adrenal medulla, leading to hypersecretion of adrenaline, and consequent hyperglycaemia (Gupta, 1974; Rahimi and Abdollahi, 2007). Induction of oxidative stress and increased lipid peroxidation and other changes suggestive of damage to the pancreas have been reported following OP exposure (Kamath and Rajini, 2007).

However, the finding of the present study showed that pretreatment with antioxidant vitamins C and/or E resulted in apparent normalization of the serum glucose level. This showed that increased lipid peroxidation and induction of oxidative stress may be at least partly responsible for the hyperglycaemia observed in CPF poisoning. The marked reduction in degenerative changes in the pancreas of rats pretreated with antioxidant vitamins C and/or E apparently due to impairment of oxidative stress-mediated apoptotic changes may have been responsible for the amelioration of CPF-induced hyperglycaemia. Therefore, the antioxidant vitamins administered to the rats prevented CPF-induced damage to the organs responsible for glucose metabolism, especially the liver and the pancreas. In addition, vitamin C has been shown to inhibit glycosidation reactions and suppress formation of advanced glycosidation end products in plasma and tissues (Frei *et al.*, 1989; Paolisso *et al.*, 1995; Young *et al.*, 1995). Vitamin C also competes with glucose uptake into the cell and suppresses aldose reductase activity (Cunningham *et al.*, 1994)

5.2.16 Serum electrolytes

The present study also showed that low-level chronic CPF exposure did not significantly alter the electrolyte level in rats. Specifically, the non-significant change in Na^+ contradicted the finding of Ambali *et al.* (2007) that hyponatraemia occurs in mice repeatedly exposed to CPF. The difference may be attributed to dose differences and lack of diarrhoea in rats treated with CPF in the present study. However, the lack of significant difference in the concentration of K^+ and Cl^- agreed with the result obtained by Ambali *et al.* (2007) in mice. The present work also showed that chronic exposure to low-level CPF did not alter the HCO_3^-. Pretreatment with vitamins C

and/or E did not also alter significantly the serum electrolyte level. Therefore, low-level exposure to CPF and antioxidant vitamins did not alter the the electrolyte balance in rats.

5.2.17 Serum proteins

The present study also showed an insignificant reduction in the levels of total serum protein present in rats chronically exposed to low-dose of CPF. This finding contradicted the results obtained by Ambali *et al*. (2007) in mice who observed high level of serum proteins following exposure to CPF attributed to diarrhoea. However, the findings of other authors that prolonged exposure to CPF is associated with low serum proteins and albumins (Kagan, 1971; Bomhard *et al*., 1981; Schilde and Bomhard, 1984; Szabo *et al*., 1988; Patil *et al*., 2003) agreed with those observed in the present study. The marginal decrease in total protein recorded in rats from the CPF group was due to reduced serum albumin. Albumin has been shown to bind and, subsequently, hydrolyse CPF-oxon and parathion (Ortigoza-Ferado *et al*., 1984; Sultatos *et al*., 1984). *In-vitro* covalent binding of albumin to OP compounds such as diisopropylfluorophosphate, sarin and soman have been demonstrated (Murachi, 1963; Sanger, 1963; Means and Wu, 1979; Schwartz, 1982; Black *et al*., 1999). Besides binding of OP, albumin scavenges OP molecules and, therefore, reduces the amount available for reaction with AChE (Peeples *et al*., 2005). Indeed, Peeples *et al*. (2005) have proposed albumin as a biomarker of OP poisoning. Therefore, the marginal decrease in albumin observed in the present study may be partly due to albumin binding to CPF, which reduced its measurable or free form in the serum. In addition, the degenerative changes in the hepatocytes caused by prolong low-dose CPF exposure might have adversely affected the synthesis of albumin in the liver. Besides, lipid hydroperoxides, the lipid peroxidation products of PUFA, have been shown to cause protein

degradation and denaturation (Chiba and Iwata, 2002), thereby contributing to low serum protein observed in the study. Furthermore, the hyperglycaemia observed in group exposed to CPF only may be partly responsible for the marginal decrease in albumin concentration in this group. Porte and Halter (1981) observed that hypoalbuminaemia resulting from hyperalbuminuria is a common problem in diabetic animals, apparently due to diabetic nephropathy from oxidative stress induction (Lee *et al.*, 2007). Hyperglycaemia recorded in rats exposed to CPF only induced oxidative stress, which caused oxidation of protein sulphyryls that may have resulted in activation of PKC (Craven *et al.*, 1997). The activation of PKC may have initiated a cascade of events that led to increased synthesis of glomerular transforming growth factor (TGF)-β, which has been implicated as one of the important mediators of glomerular hypertrophy and mesangial expansion in glomerular injury and diabetes (Sharma and Ziyadeh, 1994). The resulting glomerular hypertrophy and mesangial expansion eventually resulted in hyperproteinuria and hyperalbuminuria (Craven *et al.*, 1997), which may have been partly responsible for the hypoproteinaemia and hypoalbuminaemia observed in rats exposed to CPF only in this study. Furthermore, it has been shown that in diabetes mellitus, there is reduced albumin gene transcription (Wanke and Wong, 1991) via reduction in the binding of hepatic nuclear factor 1 to the site B of the albumin promoter (Barrera-Hernandez *et al.*, 1996), which may have partly contributed to the hypoalbuminaemia observed in rats exposed to CPF only.

Pretreatment of the rats with vitamin C or E and the combination of both vitamins ameliorated the deficit in total serum protein and albumin caused by CPF, through reduction of oxidative stress-induced hepatic degenerative changes. The amelioration of CPF-induced apparent hypoalbuminaemia by antioxidant vitamins C and/or E may also be due to their ability to

increase the activity of paraoxonase, especially PON 1 (Jarvik *et al.*, 2002), which aids in the detoxification of the OP compounds (Shih *et al.*, 1998). The increased activity of paraoxonase by vitamins C and/or E may have sparing effect on albumin, since they will compete with each other for binding with the CPF and subsequent detoxification. Furthermore, the amelioration may have resulted from the protection offered by the antioxidant vitamins on CPF-induced liver damage, which ultimately spared the vitamins-pretreated rats from hypoalbuminaemia. The ability of antioxidant vitamins to protect against PUFA oxidation by CPF-induced FR may also further contribute in sparing the proteins from degradative effect of lipid hydroperoxides, and thereby contribute to the amelioration observed in vitamins C and/or E pretreated groups. Vitamin C has been shown to decrease slit pore density (number of slit pores per underlying glomerular basement membrane length) in diabetic rats (Lee *et al.*, 2007), thereby resulting in amelioration of CPF-induced hyperproteinuria and hyperalbuminuria. Vitamin C and E have been shown to ameliorate CPF-induced hyperglycaemia in this study, which might have reduce hyperglycaemic-mediated apoptotic damage to the kidneys (Allen *et al.*, 2003). This may have eventually led to reduced proteins and albumin loss in the urine, resulting in apparently normal serum proteins and albumin concentrations recorded in vitamins pretreated animals. Furthermore, the antioxidant vitamins C and E prevented activation of PKC via prevention of sulphydryl protein oxidation, thereby inhibiting the production of glomerular TGF-β (Craven *et al.*, 1997), ultimately preventing hyperproteinuria and hyperalbuminuria. The ability of vitamin C to compete with glucose uptake into the cell and suppress aldose reductase activity (Cunningham *et al.*, 1994) may lead to suppression of PKC-dependent increases in TGF-β by limiting *de novo* synthesis of diacylglycerol (Craven *et al.*, 1997).

The present study also revealed an insignificantly higher globulin concentration in the serum of rats exposed to CPF only. This finding agreed with that observed by Subbotina and Bellonozhko (1968) following exposure to an OP pesticide, sevin. The result, however, contradicted those obtained by Szabo *et al.* (1988) following repeated CPF exposure in rats. The increased globulin production in CPF-treated rats may be due to induction of immunological abnormalities, since many pesticides have been shown to be toxic to the immune system through the induction of cytotoxicity (Corcoran *et al.*, 1994; Rabideau, 2001). Indeed, some of the chronic neurotoxic effects of OP pesticides may be due to their ability to induce the formation of antibodies to nervous tissue. Antibodies to myelin basic protein, neurofilament triplet protein, and glial fibrillary acidic proteins have all been exhibited after OP exposure (McConnel *et al.*, 1999). Other antibodies to smooth muscle, parietal cells, brush borders, and thyroid follicles as well as antinuclear antibodies have been demonstrated (Thrasher *et al.*, 1993). All these antibodies may be responsible for the increased globulin observed in the present study. Pretreatment with antioxidant vitamins C and/or E was also shown to further increase the serum globulin. The result agreed with the findings that vitamin C stimulates the immune system by enhancing T-cell proliferation which ultimately assists the B-cells to synthesize immunoglobulin, especially during infection (Campbell *et al.*, 1999; Naidu, 2003).

5.2.18 Serum uric acid

The study revealed an insignificantly lower level of uric acid in the serum of rats chronically exposed to CPF compared to the control. Low level serum uric acid has been associated with liver or kidney damage. Similarly, pretreatment with vitamins C and/or E did not significantly alter the serum uric acid concentration. Specifically, rats pretreated with vitamin C showed slight

elevation in the level of serum uric acid compared to control and the other vitamin pretreated group. Elevation of uric acid has been associated with obstruction of kidney since uric acid is a product of purine metabolism, which must be excreted by the kidney.

5.2.19 Serum urea

It has been shown in the present study that chronic low-dose CPF exposure in rats marginally increased the concentration of serum urea. This finding is in agreement with the result obtained by Ambali *et al.* (2007). The marginal increase in serum concentration of urea in rats exposed to CPF indicates kidney damage. The low urea concentration observed in the serum of rats pretreated with antioxidant vitamins C and/or E showed the ability of these vitamins to ameliorate kidney damage induced by low-level CPF exposure. Histopthological evaluation of the kidney revealed that groups pretreated with antioxidant vitamins significantly reduce pathological lesions induced by CPF. This further emphasized the role of oxidative stress in CPF-induced kidney damage and the ameliorative effect of antioxidant vitamins C and/or E.

5.2.20 Serum creatinine

Serum creatinine was significantly elevated in rats exposed to chronic low-dose CPF compared to the control. This observation agreed with the finding of Ambali *et al.* (2007) in mice. The elevation of creatinine may be as a result of pathological lesions in the muscle (Quintanilla, 1982) and kidneys, since elevated creatinine has been shown to be associated with renal insufficiency and renal obstruction (Ambali *et al.*, 2007; Wikipedia, 2008b). Glomerular and renal tubular degenerative changes observed in rats exposed to CPF only in this study agreed with the findings of Oncu *et al.* (2002a) observed following exposure to CPF ethyl in rats.

However, pretreatment with antioxidant vitamins C and/or E caused even a slightly lower level of creatinine than even the control, which also under normal condition was subjected to low-grade lipid peroxidation resulting from normal metabolic processes. Although the implication of a slightly lowered creatinine level observed in the vitamins-pretreated group is not known, it may reflect the protective effect of antioxidant vitamins C and E against lipid peroxidation arising from normal metabolic activities and those from chronic low-dose CPF exposure which caused damage to both muscle and kidneys. The present study showed a less severe degree of degenerative changes in the kidneys of rats pretreated with vitamins C and/or E, which may have contributed to the apparent normalization of creatinine concentration in the present study.

5.2.21 Serum liver enzymes

The significantly higher level of AST activity observed in rats chronically exposed to CPF compared to the control revealed the ability of this OP insecticide to cause damage to the liver and/or the muscle. Although AST is less specific for liver disease since its high level may also signal damages to the cardiac and skeletal muscles, RBC, kidney and brain tissue (Wikipedia, 2008a), it has been suggested that increased AST activity in the serum is a sensitive marker of liver damage, even if the damage is of a subclinical nature (Kauppinen, 1984; Meyer and Harvey, 1998). Since histopathological findings in the present study revealed damages to the cardiac, kidney and brain tissues of rats exposed to CPF only, the source of the AST cannot be attributed to liver damage only.

Similarly, chronic exposure to CPF only resulted in significant elevation of the activity of ALT, a more sensitive marker of liver damage (Ballantyne, 1988). This shows that chronic exposure to

CPF caused damage to the hepatocytes. ALT and AST are enzymes produced by the hepatocytes, where they are involved in the metabolism of amino acids and synthesis of proteins. In dying or damaged cells, these enzymes then leak into the blood stream. The elevation in ALT activity level observed in the present study in rats chronically exposed to CPF agrees with the findings of Yoshida *et al.* (1985), who observed a slight increase in the level of ALT following repeated CPF exposure in mice. In addition, elevation of serum ALT and AST has been observed following chronic exposure of rats and mice to OP insecticides (Gomes *et al.*, 1999). Similarly, results obtained from a study involving pesticide sprayers have shown high liver enzyme activities in the serum of these subjects (Patil *et al.*, 2003). The result obtained in the rats treated with CPF only contradicted the findings of Szabo *et al.* (1988) and Barna-Lloyd *et al.* (1990) in rats, and that of Ambali *et al.* (2007) in mice. These studies reported low activity level of AST and ALT following repeated CPF exposure. High ALT activity observed in the rats exposed to CPF only in the present study may be due to degenerative changes and increased lipid peroxidation in the hepatocytes. The present study showed that pretreatment with antioxidant vitamins C and/or E resulted in a non-significant decrease in the level of AST and ALT. This demonstrates the protective effect of antioxidant vitamins C and/or E on CPF-induced liver and/or muscle damage. Histopathogical findings in vitamins-pretreated groups revealed milder degree of hepatic degeneration, demonstrating some form of hepatic protection. Therefore, it may be inferred that oxidative stress plays a significant role in CPF-induced liver and/or muscle damage, and that pretreatment with antioxidant vitamins C and/or E ameliorated the damage.

Although there is no significant change in the level of ALP activity in rats chronically exposed to CPF only compared to the control. However, the activity of ALP is slightly higher in the CPF

group compared to the control. Increased ALP activity is not only associated with biliary tract obstruction and liver pathology, but it is also associated with bone, placenta, renal or intestinal damage. Based on the present result, chronic CPF exposures in rats may have caused some pathologic damage to all or any of these organs. However, this finding agreed with the result obtained by Ambali *et al.* (2007) following subchronic CPF exposure in mice. Pretreatment with vitamins C and/or E resulted in a non-significant decrease in ALP activity compared to the control, with a greater decrease observed in group pretreated with vitamin C. This again showed the protective role of antioxidant vitamins.

The study also showed a marginal but non-significant increase in the level of GGT in groups exposed to CPF compared to the control. GGT is an enzyme that is liver-specific. It is produced solely by the liver, where it is involved in transportation of amino acids and peptides into the cells, and it also takes part in glutathione metabolism. GGT is a good indicator of hepatobiliary disease (Stojević *et al.*, 2008). The main use of GGT is to confirm if the elevations in ALP levels is of hepatic aetiology, since the two enzymes have been termed cholestatic liver enzyme because of their ability to rise in inflamed or damaged bile duct. Its activity in serum is higher when the epithelia of the bilious channels in the peripheral regions are damaged, in necrotic changes of bile channels, bile obstructions, chollangiohepatitis, cirrhosis and fatty liver (Kramer and Hoffman, 1997). The relatively higher GGT obtained in rats exposed to CPF only indicate that the higher ALP activity in this group resulted significantly from lesions in the liver and bile duct. However, the low GGT activity level obtained in rats pretreated with vitamin C and/or E demonstrated the protective effect of antioxidant vitamins on liver and bile duct damage induced by chronic low-dose CPF exposure.

On the whole, the study showed an increased activity of liver enzymes such as AST, ALT, ALP and GGT in rats exposed to CPF only. This observation agreed with the degenerative changes in the hepatocytes of rats exposed to CPF only in the present study. The hepatic lesions may have resulted from increased lipid peroxidation observed in rats exposed to CPF only. However, the aparent normalization of the liver enzymes observed in rats pretreated with antioxidant vitamins C and/or E agreed with aparent normalization of lipid peroxidation index and mild pathologic changes in the hepatocytes of rats in these groups. This observation further demonstrated the role of oxidative stress in CPF-induced hepatic damage and the ameliorative role of antioxidant vitamins C and/or E

The fact that there was no sex difference in the level of the liver enzymes among the different groups shows that the response of the animals to the vehicle (soya oil), CPF and the antioxidant vitamins pretreatment on liver enzymes were not sex related.

5.2.22 Serum creatine kinase

The slight decrease in CK activity in rats chronically exposed to CPF may be an indication of liver or bone damage, which agrees with the finding that lowered CK level occurred in humans suffering from alcoholic liver disease and rheumatoid arthritis (Wikipedia, 2008c). Apart from its presence in the blood mostly as isozyme CK-M, another isozyme of CK (CK-B) is also present in the brain and spinal cord. CK present in neurones, astrocytes and oligodendrocytes (Manos *et al*., 1991) and is believed to play a role in the continuous replenishment of ATP from phosphocreatine in these cells (Wyss *et al*., 1992). Low CK activity has also been reported in the

serum of a primigravida female following a suicidal attempt with CPF ethyl (Sebe *et al.*, 2005). The result further supports the finding that some neurotoxic agents such as ethylene oxide, bromopropane and acrylamide suppress CK activity in the brain and blood of rats (Matsuoka *et al.*, 1990; 1996; Kohriyama *et al.*, 1994; Wang *et al.*, 2002), but contradicts the observation that repeated inhalation exposure of cats to CPF resulted in elevated CK (Jaggy and Oliver, 1990). Furthermore, suicidal attempt with CPF equally resulted in an increased CK activity (Aiuto *et al.*, 1983). The relationship between the CK isozyme in the brain (CK-B) and those in the serum or plasma (CK-M) is not exactly known, but a study has shown that the plasma CK-M level paralleled CK activity (Wang *et al.*, 2002). Since the present study did not measure the level of CK in the brain, the decreased CK activity observed in the group exposed to CPF only is strongly attributed to CK-M. Pretreatment with vitamins C and E was shown to marginally increase the CK activity, but a marginal increase over the control was observed in the groups pretreated with both antioxidant vitamins. This showed that co-administration of vitamins C and E abolished the CK alteration induced by CPF exposure. Therefore, oxidative damage to the liver and/or bone responsible for low CK activity was ameliorated by antioxidant vitamins, especially the combination of vitamins C and E.

5.2.23 Thyroid hormones

The non-significant reduction in the level of TSH in rats chronically exposed to CPF compared to the control, indicate that the chemical may cause some level of alteration in TSH concentration. The decrease in TSH may be due to impairment in the stimulation of its synthesis due to damage to the hypothalamus, which is the site of production of TSH releasing hormone (TRH) responsible for stimulation of hypophysis to synthesize or release TSH. The low TSH

may also have arisen from damage or impairment in the activity of the hypophysis, or due to the exhaustion of its production arising from continuous stimulation of thyroid gland as a result of hypothyroidism from low T_3 and T_4. The slight decrease in serum T_3 observed in the group chronically exposed to CPF only may be due to low T_4 level observed in the group, since T_4 has to be converted to T_3 for the biological effect of the hormone to be manifested. In addition, the low T_3 may be due to deficient production of deiodinase enzyme, which plays a significant role in the conversion of T_4 to the more metabolically active T_3, perhaps due to CPF-induced pathological damage to the liver source of the enzyme. The low level of T_4 observed in CPF group concurred with the result obtained by Rawlings *et al.* (1998) who showed low T_4 following repeated exposure of ewes to CPF. Similarly, Satar *et al.* (2005) observed hypothyroidism following exposure to methamidophos, an OP compound in rats. Studies in male pesticide formulators chronically exposed to various pesticides, including chlorpyrifos indicated impairment in thyroid function (Zaidi *et al.*, 2000). Similarly, study on some Danish greenhouse workers exposed to pesticide showed reduction in serum T_4 (Toft *et al.*, 2006). Studies have shown that a number of environmental chemicals suppress T_3 and T_4 levels by upregulating metabolic enzymes leading to their increased glucuronidation and subsequent excretion (Brouwer *et al.*, 1998b). The hypothyroidism observed in the present study in group exposed to low-dose CPF only may have arisen from the inability of the thyroid gland to secrete enough T_4 due to damage to the thyroid acinar or as a consequence of low TSH, resulting in reduced stimulation of the thyroid gland to produce T_4.

The significance of lower thyroid hormone concentrations observed in the CPF group cannot be overemphasized, especially as it relates to the neurobehavioural, cognitive and some biochemical

aspects of this study. Hypothyroidism may result in slowness of movement, calcification of basal ganglia and signs of Parkinsonism (Adler, 1999), which are related to some of the signs observed in rats chronically exposed to CPF in the present study. Impaired memory is also a feature of hypothyroidism (Wikipedia, 2008f). Hypothyroidism has also been associated with increased erythrocyte osmotic fragility (Dariyerli *et al.*, 2004), a feature observed in the present study in rats exposed to CPF only. On the whole, pretreatment with antioxidant vitamins C and/or E significantly ameliorated the hypothyroidism induced by low-dose chronic CPF exposure. This may be due to protection offered by the antioxidant vitamins from the oxidative damage to the relevant organs such as the hypothalamus, the hypophysis or the thyroid gland.

5.2.24 Serum lipid profiles

The reduction in serum TC observed in rats chronically exposed to low-dose CPF in the present study contradicted the results obtained by Yoshida *et al.* (1985) in mice and Barnard-Lloyd *et al.* (1990) in rats, who obtained an increased TC following repeated exposure to CPF. Hypocholestrolaemia was, however, observed in repeated exposure to OP insecticides such as acephate (Choudhari and Chakraharti, 1984), dichlorvos (Ryhanen *et al.*, 1984) and diazinon (Ibrahim and El-Gamal, 2003). The low cholesterol level observed in CPF-treated group in the present study was attributed to low HDL-c, apparently due to liver damage and severe anaemia. In addition, a previous study has shown the ability of OP to phosphorylate and inhibit the activity of hydroxy-methylglutaryl CoA reductase, the key enzyme essential in cholesterol production (Ryhanen *et al.*, 1984). Moreover, the decrease in serum TC in the CPF-treated group may be due to organophosphate-induced stimulation of the LDL receptors which increase the clearance of cholesterol from circulation (Brown *et al.*, 1981). In addition, the low TC observed in the CPF

group may have resulted from the ability of the OP insecticide to excessively activate lecithin cholesterol acetyltransferase (LCAT), an enzyme that catalyses the esterification of plasma or serum cholesterol. This is because the excessive activation of this enzyme may cause increased esterification of serum TC, which eventually enhances the lipid-carrying capacity of the lipoprotein (Stein, 1987). Furthermore, hypocholestrolaemia has been found to be associated with anaemia (Rifkin and Gale, 1967; Westerman, 1975; Schilling *et al.*, 2006). This has been attributed to decreased *in vivo* cholesterol synthesis in anaemic individuals (Au and Schilling, 1986). Therefore, anaemia observed in CPF-treated animals may have contributed to hypocholestrolaemia in this group of animals. The study has for the first time showed that pretreatment with antioxidant vitamins C and/or E ameliorated the CPF-induced hypocholestrolaemia. This may be due to prevention of damage caused by CPF to the liver, thereby normalizing cholesterol metabolism. In addition, severe anaemia observed in CPF-treated animals, which has been reported as a cause of hypocholesterolaemia, was alleviated by the administration of antioxidant vitamins C and/or E.

The study also revealed a slightly lower HDL-c in the serum of rats dosed with CPF only compared to the control. The low-HDL-c has also been reported in rats dosed repeatedly with low-dose acephate (Choudhari and Chakraharti, 1984), and diazinon (Ibrahim and El-Gamal, 2003). HDL is mainly synthesized in the liver and intestinal cells. It plays an important role in cholesterol efflux from tissues and carries it back to the liver for removal as bile acids (Shakoori *et al.*, 1988). It has been established that the elevated serum or plasma HDLs levels have antiatherogenic effect (McGill *et al.*, 1981), whereas the reduced levels are associated with an increased risk for coronary artery disease (Stein, 1987). It has been shown that the esterification

of free plasma cholesterol takes place in plasma HDL, where the reaction is catalyzed by the enzyme LCAT which uses HDL as a substrate (Zilva *et al.*, 1988). Therefore, the low HDL observed in rats chronically exposed to low-dose CPF may be as a result of activation of LCAT by CPF. In addition, the low HDL-c may also be due to damage to its liver source by CPF. The implication of low HDL-c observed in rats chronically exposed to CPF only indicates that prolonged exposure to this insecticide may increase the risk of individuals developing coronary heart disease.

In addition, memory deficits observed in rats exposed to CPF only may be due to the low HDL-c, since a link has been established between level of HDL and the onset of dementia, with individuals showing high HDL-c less likely to have dementia (Katzman *et al.*, 1989; Reitz *et al.*, 2005). Furthermore, a recent epidemiological study by Singh-Manoux-*et al.* (2008) has associated low HDL-c with poor memory. A number of plausible mechanisms for the memory deficits have been proposed, including its ability to regulate amyloid β protein metabolism and deposition in the brain (Reiss *et al.*, 2004) as the most prominent lipoprotein in the brain (Olesen and Dago, 2000). The low level HDL-c may also lead to neurodegenerative processes related to its antiinflammatory (Cockerill *et al.*, 2001) and antioxidant (Paterno *et al.*, 2004) activities. Besides, HDL-c plays an important role in the maturation of synapses and synaptic plasticity (Michikawa, 2003). In addtion, Wolf *et al.* (2004) have shown an association between low HDL-c and hippocampal volume. Increased level of HDL-c was observed in the group pretreated with vitamins C and/or E. It is known that HDL readily transfers vitamin E to other lipoproteins, thereby enhancing the distribution of the vitamin E to other lipoproteins (Traber and Kamal-Eldin, 2007), thus increasing vitamin E efficacy. Besides, vitamin E is highly lipid soluble and

can easily penetrate the membrane lipids to decrease lipid peroxidation. Therefore, pretreatment with vitamin E ameliorated the proatherogenic effect of CPF.

The present study has also demonstrated a marginal increase in LDL-c in rats exposed to repeated low-dose CPF compared to the control, which agreed with the observation of Ibrahim and El-Gammel (2003) in rats subjected to repeated exposure to low-dose diazinon. The increased LDL-c obtained in CPF group was accompanied by a low HDL-c, suggesting that CPF exposure promotes the deleterious conversion of some of the HDL-c to LDL-c. LDLs transport cholesterol to the arteries where it is retained by arterial proteoglycans, which initiate the formation of plaques. LDL oxidation is thought to be the first step of atherogenesis, followed by foam cell, fatty streak, and plaque formation (Nakamura *et al.*, 2008). It has been hypothesized that LDL can be transported through endothelial tight junctions and/or endothelial transcytosis from the lumen into the intima (Cancel *et al.*, 2007), in which blood antioxidants are unlikely to be available, and undergo atherogenic oxidative changes (Nakamura *et al.*, 2008). The lack of access to antioxidant by LDL leads to their oxidation. Oxidised LDLs have been shown to induce apoptosis via the induction of apoptotic genes such as FasL, Bax, p53 (Reeve *et al.*, 2007) and MAPK (Apostolov *et al.*, 2007) pathways. Therefore, the increased levels of oxidised LDLs as observed in the present study increased the susceptibility of the rats to atherosclerosis, and consequently, heart attack, stroke and peripheral vascular disease (Wikipedia, 2008e). Paradoxically, pretreatment with vitamins C and E but not combination of vitamins C and E further increased the LDL-c. Although, LDL-c is harmless until oxidized by FR (Teissedre *et al.*, 1996), it is postulated that ingesting antioxidants and minimizing free radical exposure may reduce LDL's contribution to atherosclerosis (Esterbauer *et al.*, 1991; Reaven *et al.*, 1993).

Therefore, the higher LDL-c observed in groups pretreated with antioxidant vitamins did not imply that vitamins C and E predispose the rats to increased risk of development of cardiovascular diseases, since the likelihood of its oxidation by FR is remote. Vitamin E is a major lipid soluble antioxidants occurring in the plasma especially in VLDL and LDL fractions, where it exhibits important protective role by preventing lipid peroxidation.

Furthermore, vitamin C and E have been shown to induce the formation of paraoxonase (Jarvik *et al.* 2002), a multitask protein that not only hydrolyse OPs, but also prevents the oxidation of LDLs and HDLs (Chambers, 2008) further protecting the body from development of athrosclerosis. However, it has been shown that oxidation of LDL-c can also occur via non-radical mechanisms such as direct oxidation by lipoxygenase (Noguchi *et al.*, 2002), hypochloric acid and aldehydes (Niki, 2004). This non-radical mechanism of LDL-oxidation may be an important source of concern in vitamins-pretreated animals as observed in the present study. However, it should be noted that higher contents of antiatherogenic HDL-c observed in vitamins-pretreated rats may reduce the risk of development of atherosclerosis. Besides, vitamins C and E have been shown to increase the activity of PON I (Jarvik *et al.*, 2002), which apart from aiding in the detoxicification of OPs, may also confer protection against atherosclerosis. PON I prevents the formation of oxidized LDL, inactivates LDL-derived oxidized phospholipids once they are formed and prevent oxidation of HDL phospholipids (Costa *et al.*, 2003). The reason why pretreatment with antioxidant vitamins C and E but not the combination of both vitamins resulted in increased LDL-c deserves further study.

The present study has also demonstrated a low TG in rats dosed with CPF only, and this observation agreed with those obtained in rats by Barna-Lloyd *et al.* (1990) and Choudhari and

Chakraharti (1984) in CPF and acephate poisoning, respectively. The slight hypolipaemia observed in the rats exposed to CPF only may result from liver damage or hypolipoproteinaemia (Bruss, 1997; Stojević *et al.*, 2008). This is because hypolipidaemia may result from the ability of the OP chemical to reduce the interaction of TG with the lipoproteins, especially LDL. Pretreatment with antioxidant vitamins ameliorated the CPF-induced decrease in TG. This may be due to the ability of the antioxidant to protect against oxidative damage of the liver by CPF, hence improving the entire process of lipid metabolism.

The present work has shown that chronic CPF exposure impairs lipid metabolism and demonstrated the ability of antioxidant vitamins C and/or E to ameliorate the impairment, especially by increasing the serum HDL-c. However, the study was unable to demonstrate sex difference in response of the rats to alteration in lipid metabolism among various treatment groups.

5.2.25 Lipid peroxidation

The present study demonstrated the ability of chronic CPF exposure to increase the level of lipid peroxidation in the liver, brain and the serum by increasing the concentration of TBARS, MDA. MDA is an end-product of oxidation of polyunsaturated fatty acids (Girotti, 1998; Lukaszewicz-Hussain *et al.*, 2008). The increased MDA level in animals exposed to CPF only further confirms that lipid peroxidation is one of the molecular mechanisms involved in OP insecticides-induced toxicity (Yamano and Morita 1992; Bagchi *et al.*, 1995; Vandana and Poovola, 1999; Gultekin *et al.*, 2001; Altuntas *et al.*, 2002b; Yavuz *et al.*, 2004, Lukaszewicz-Hussain *et al.*, 2008). Apart from increasing lipid peroxidation, CPF has been shown to modify endogenous antioxidants such

as SOD, CAT, GST and GSH activities (Gultekin *et al.*, 2001; Bebe and Panemagalore, 2003; Goel *et al.*, 2005; Tuzmen *et al.*, 2007, 2008; Verma *et al.* 2007; Zama *et al.*, 2007). Thus, increased lipid peroxidation resulting in organ and tissue damages was partly responsible for some of the neurobehavioural, cognitive, haematological, biochemical and tissue pathological changes induced by CPF in the present study. Goel *et al.* (2005) attributed the altered liver enzyme markers and liver histohepatic cytoarchitecture observed in rats exposed to CPF only to increased lipid peroxidation and altered antioxidant status. Tuzmen *et al.* (2008) also attributed the liver damage observed following exposure to CPF to increased lipid peroxidation and depleted antioxidant enzymes. Therefore, the altered liver enzyme activity observed in the group exposed to CPF only in the present study may be due to increased lipid peroxidation, resulting in increased apoptotic damage to the hepatocytes. This resulted in massive degenerative changes in the hepatocytes observed in group exposed to CPF only.

The increased lipid peroxidation observed in the brain of rats exposed to CPF only was, apparently, due to the ability of the OP compound to disrupt the structure and functionality of the blood-brain barrier (Parran *et al.*, 2005). Consequently, an increase in the accessibility of the brain to CPF and other neurotoxicants occurred. Some of these compounds and metabolites ordinarily would not have gained direct access to the brain. The increased lipid peroxidation may have been responsible for the degenerative changes in the neurone and glial cells, possibly through the induction of apoptosis

The present study also demonstrated that antioxidant vitamins C and/or E ameliorated the lipid peroxidation induced by chronic CPF exposure in rats. This finding agreed with the observations

made in previous studies on the protective effect of vitamins C and E against organ damage induced by OP exposure (Appenroth *et al.*, 1997; Gultekin *et al.*, 2001; Altuntas *et al.*, 2002a and b; Yavuz *et al.*, 2004). The study also demonstrated a better amelioration of CPF induced brain, liver and serum lipid peroxidation by the combination of both vitamins C and E than when either of the two vitamins was administered. Although the proximate mechanisms of the antioxidant activity of vitamins C and E are not elucidated in the present study, it has been shown that vitamins C and E are parts of the body's antioxidant system that prevent cells from oxidative attacks (Gultekin *et al.* 2001, Oncu *et al.*, 2002; Verma *et al.*, 2007). Vitamin E is the most important lipophilic antioxidant and resides mainly in the cell membranes, thus helping to maintain membrane stability (Baker *et al.*, 1986). On the other hand, vitamin C is the most important hydrophilic free radical scavenger in extracellular fluids, trapping radicals in the aqueous phase and protecting biomembranes from peroxidative damage (Haranpanhalli *et al.*, 1996; Dehghan and Dehghanan, 2006). It reduces the level of ROS both intracellularly and extracellularly, and maintains transition metals in their reduced form (Lee *et al.*, 2006). Vitamin C is an outstanding powerful antioxidant that reacts rapidly with a variety of oxidants, including the rather poorly reactive superoxide anion radical (Iheanacho *et al.*, 1993). Unlike vitamin E, vitamin C does not easily cross the blood-brain barrier because of its lipid insolubility. However, it is actively transported into the cerebrospinal fluid via the choroid plexus and is further concentrated in the neuronal cytoplasm by a second active system (Schmidt, 1990). In addition to its antioxidant effects, vitamin C is involved in the regeneration of tocopherol from tocopheroxyl radicals in the membrane (Schiff *et al.*, 1996). Thus, vitamins C and E may have interactive effects (Stoyanovski *et al.*, 1995), resulting in synergism (Frei *et al.*, 1989; Raijmakers *et al.*, 2004).

On the whole, the present study has shown that chronic exposure of CPF to rats resulted in deficits in neurobehavioural, cognitive, haematological, biochemical and tissue pathological changes. These deficits have also been shown to be partly mediated by increased lipid peroxidation induced by CPF. It is probable that CPF and its metabolic product, CPF-oxon withdraw electrons from the molecules of body tissues thereby creating an unstable configuration. The deficit affects body macromolecules such as lipids, proteins, carbohydrates and nucleic acids. The attempts by the body macromolecules to balance the electron configuration to make them acquire stability resulted in removal of electron from adjacent macromolecule. This makes the second molecule unstable, and inducing a process known as chain propagation. CPF-induced FR attack on the double bond of carbon molecules in fatty acids weakens the carbon-hydrogen bonds, resulting in its easy dissociation and generation of acetylated fatty acids (Chihuailaf *et al.*, 2002). The addition of oxygen molecule to this acetylated fatty acid resulted in the formation of unstable lipid hydroperoxides, which react with metal or metal complexes such as Fe, resulting in the propagation of the reaction (Halliwell and Gutteridge, 1985). The lipid hydroperoxides can also fragment to produce aldehyde, which may cross-link proteins and rendering them incapable of functioning as receptors or enzymes (Schmidt, 1990). The resultant effect of this is a change in membrane fluidity, permeability and function. The consequence is that of altered membrane permeability and receptor and/or enzymatic functions, responsible for the neurological and biochemical dysfunctions observed in this study. In addition, the damage caused to the liver might have further aggravated the toxicological changes observed in the group exposed to CPF only. This is because Choi *et al.* (2006) has shown that liver plays an important role in the detoxification of CPF.

The amelioration of the CPF-induced neurological and biochemical impairments observed in rats pretreated with vitamins C and/or E may be due to their ability to donate electrons to the initial biochemical events in the initiation of the FR damage resulting in their termination. This results in the stability of the biomolecules, thereby quenching the FR attack. In addition, vitamins C and E have been shown to increase the activity of paraoxonase (Jarvik *et al.*, 2002), an antioxidant enzyme, which also plays an essential role in the detoxification of the OP compound. Besides, vitamins C and E enhance the restoration of the AChE (Yavuz *et al.*, 2004), which also assist in restoring biochemical damage induced by the chronic exposure to CPF.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The present study has shown that chronic exposure of adult Wistar rats to CPF caused:

1. impairment in body weight gain compared to the soya oil-treated control.
2. reduction in motor activity and promote anxiety.
3. deficits in coordinated gaits and neuromuscular coordination.
4. reduction in sensorimotor performance, motor strength, excitability and reflexability.
5. impairment in cognition (learning and memory).
6. reduction in haematological parameters such as PCV, Hb and total erythrocyte count. It also caused marked leucopaenia due to neutropaenia.
7. increased erythrocyte osmotic fragility which made the RBC susceptible to lysis.
8. hyperglycaemia which reflects its diabetogenic potentials.
9. decrease in the level of total serum proteins apparently due to low albumin, and hyperglobulinaemia
10. significant increase in serum creatinine concentration resulting from muscle and renal damage.
11. increased activity of some serum enzymes such as ALT, AST, ALP and GGT which reflected some level of hepatic damage.
12. slight decrease in the activity of serum CK, suggestive of some level of damage to the liver and/or the bone

13. alteration in thyroid function characterised by reduction in the level of T_3, T_4 and TSH, suggestive of damage to the thyroid and/or the hypothalamus or pituitary glands.
14. alteration in lipid profiles characterized by hypocholestrolaemia, low TG, high LDL-c and low HDL-c, which on the overall suggests that chronic CPF exposure may predispose individuals to the risk of developing atherosclerosis.
15. increased lipid peroxidation in the brain, liver and serum as evidenced by increased TBARS, MDA in these organs. This shows that lipid peroxidation and oxidative stress plays an important in the haematological, biochemical and tissue pathological changes observed in rats exposed to CPF only.
16. varying degree of pathological lesions to the brain, heart, lungs, stomach, intestine, pancreas, spleen, liver and kidneys, which may have been partly responsible for the observed alterations in clincal, haematological and biochemical changes.

The study has also shown that pretreatment with antioxidant vitamins C and/or E improved the body weight changes, and ameliorated deficits in neurobehavioural and cognitive changes induced by chronic exposure to CPF. In addition, the antioxidant vitamins C and/or E to a varying degree ameliorated CPF-induced deficits in PCV, Hb, RBC and WBC. Furthermore, the study has for the first time revealed the ability of vitamins C and/or E to protect the integrity of RBC membrane as observed in amelioration of the CPF-induced increased erythrocyte osmotic fragility. Similarly, pretreatment with vitamins C and/or E protect against CPF-induced hypoproteinaemia and hypoalbuminaemia. The study has also for the first time shown the ability of viatmin C and/or E to ameliorate the hyperglycaemia induced by chronic exposure to CPF. The antioxidant vitamins C and/or E were also shown to

ameliorate CPF-induced increased activity of serum ALT, AST, ALP and GGT levels. Pretreatment with vitamins C and/or E has also for the first time been shown to ameliorate hypothyroidsm induced by chronic CPF exposure in rats. Pretreatment with antioxidant vitamins C and/or E was also shown to modulate hypocholesterolaemia, hypolipidaemia and low HDL-c associated with chronic exposure to low dose of CPF. Paradoxically, however increased serum concentration of LDL-c was observed in group pretreated with either of the two vitamins but not their combination. The study has further reaffirmed the ability of vitamins C and/or E to protect the body against CPF-induced lipid peroxidation as demonstrated by reduced concentration of MDA in the vitamins pretreated groups. Pretreatment with antioxidant vitamins C and/or E resulted in amelioration of CPF-induced pathological lesions to the brain, lungs, stomach, intestine, kidneys, pancreas, liver and spleen.

6.2 Conclusions

In conclusion, the study has shown that:

i. Chronic low-dose CPF exposure to Wistar rats caused significant alteration in neurotoxic, clinical, haematological, biochemical and tissue pathological changes,

ii. Lipid peroxidation is an important molecular mechanism involved in neurobehavioural, cognitive, haematological, biochemical and tissue pathological changes in chronic low-dose CPF exposure in rats

iii. Pretreatment with antioxidant vitamins C and/or E ameliorated CPF-induced clinical, neurotoxicological, haematologic, biochemical and tissue pathological changes in rats.

6.3 Recommendations

6.3.1 Specific recommendations

i. Attempts should be made to relate the neurological, haematological and biochemical changes observed following prolonged low-dose exposure to AChE inhibition, and evaluate the relationship between vitamins C and/or E and AChE activity.

ii. Further work should be done to relate the toxicological changes observed in this study to the antioxidant status of the animals. This will provide a better index of assessing the level of oxidative stress. Furthermore, monitoring the level of antioxidant enzymes or substances in individuals exposed to CPF and other OPs may provide basis for therapeutic interventions in alleviating OP-induced symptoms.

iii. Evaluation of biomarkers that may be used to easily identify exposure of individuals to OP compounds and OP-induced neurogenic diseases.

iv. Additional work should be carried out as to ascertain the reason why pretreatment with either vitamin C or E but not the combination of both vitamins resulted in higher LDL-c than those exposed to CPF only, and the implication of this on human and animal health.

6.3.2 General recommendations

i. Farmers, pesticide applicators, veterinary attendants, veterinarians and other users of CPF should avoid having direct contact with the OP compound by the use of effective protective clothings and masks.

ii. Government and non-governmental organizations and individuals should organize awareness campaign and sensitization of the general populace on the danger of indiscriminate use of pesticides.

iii. Livestock and arable crop farmers should be sensitised on the need to observe withdrawal periods stated by the manufacturers before turning pesticide-treated products into the market for human and animal consumption

iv. Government should have a political will and put policy in place measures that will check importation of dangerous and banned pesticides into the country through effective border policing and control. In addition, pesticides that have instructions on their labeling in foreign languages other than English language should not be allowed into the country.

v. Periodic evaluation of haematological and biochemical parameters of individuals or animals who are frequently exposed to low-dose CPF should be put in place, to enable professional counselling of the individual as it relates to exposure.

vi. Individuals who are at risk of coming in contact with CPF continuously should be pretreated with the cheap and readily available antioxidant vitamins C and E to reduce damages caused by frequent exposure to low-dose exposure.

vii. It is imperative that Nigeria should put in place effective regulatory and legal framework through the enactment of pesticide control act that will check the toxicological and environmental menace caused by pesticides.

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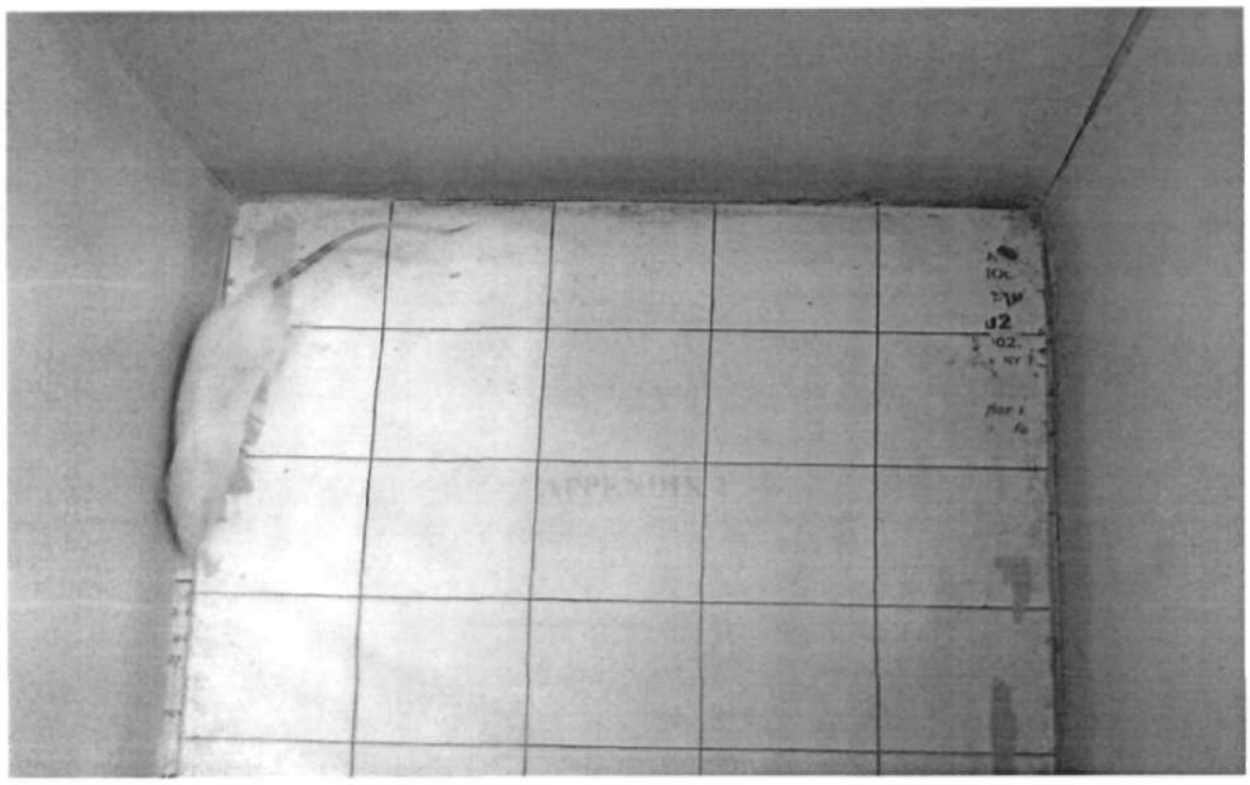
Zou, W., Yue, P., Lin, N., He, M., Zhou, Z., Lonial, S., Khuri, F.R., Wang, B. and Sun, S. (2006). Vitamin C inactivates the proteasome inhibitor PS-341in human cancer cells. *Clinical Cancer Research*, 273-280.

APPENDIX 1

Effect of treatments on haematological and biochemical changes inWistar rats

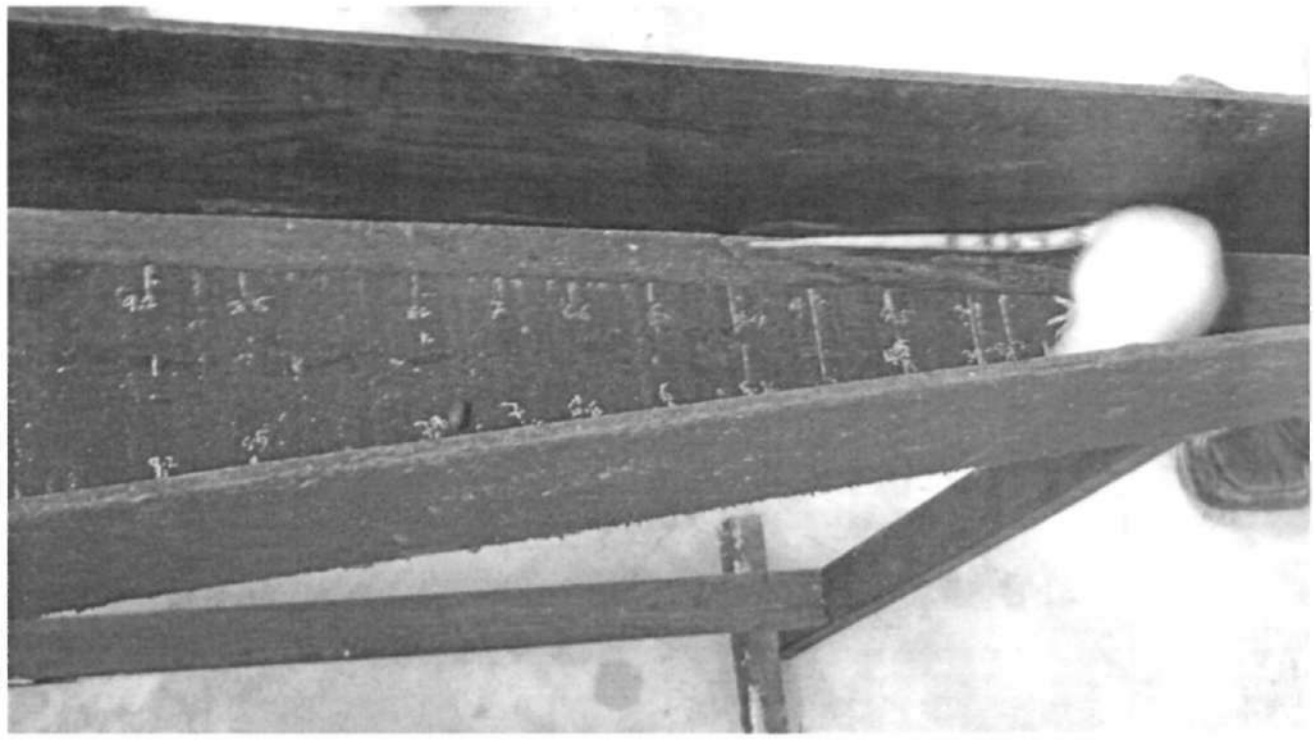
Parameters	Soya oil	Chlorpyrifos (CPF)	Vitamin C +CPF	Vitamin E +CPF	Vitamins C+E+CPF
PCV (%)	42.2 ±1.6	35.3 ± 1.1	40.2 ± 0.9	38.8 ± 1.1	43.8 ± 2.4
RBC ($x10^{12}$g/dL)	5.8 ± 0.5	3.7 ± 0.1	5.1 ± 0.1	5.0 ± 0.1	5.0 ± 0.2
Hb (g/dL)	14.1 ± 0.5	11.8 ± 0.4	13.4 ± 0.3	13.0 ± 0.3	14.6 ± 0.8
WBC (x 10^6 g/Dl)	9.8 ± 0.7	5.6 ± 0.3	7.7 ± 0.4	7.1 ± 0.3	8.7 ± 1.4
Glucose (g/dL)	4.2 ± 0.1	5.2 ± 0.3	4.6 ± 0.3	4.5 ± 0.2	4.6 ± 0.3
Na^+ (mMol/L)	140 ± 1.1	139.8 ± 1.2	141 ± 0.9	139 ± 0.7	139.8 ± 0.6
K^+ (mMol/L)	4.2 ± 0.2	4.1 ± 0.2	4.5 ± 0.2	4.3 ± 0.1	3.9 ± 0.2
Cl (mMol/L)	98 ± 1.0	99.3 ± 1.2	99 ± 1.2	98.3 ± 0.6	98.7 ± 0.7
HCO_3^- (mMol/L)	24.5 ± 1.0	24.7 ± 0.7	23.7 ± 0.9	25 ± 0.8	24.7 ± 0.6
Total protein (g/dL)	65.5 ± 2.3	61.8 ± 2.5	69.2 ± 2.7	72.2 ± 1.4	73.2 ± 2.7
Albumin (g/dL)	39.7 ± 1.6	35.0 ± 1.5	39.5 ± 1.8	38.8 ± 2.1	43.2 ± 1.3
Globulin (g/dL)	25.8 ± 1.4	27.2 ± 1.4	29.7 ± 1.8	33.3 ± 1.2	30.1 ± 2.3
Urea (mMol/L)	4.4 ± 0.2	4.6 ± 0.2	4.3 ± 0.1	4.4 ± 0.1	4.3 ± 0.2
Creatinine (mMol/L)	81.5 ± 3.6	97 ± 4.2	77.7 ± 3.8	76 ± 4.6	80 ± 2.9
Uric acid (mMol/L)	205 ± 9.6	198.8 ± 11.7	227.0 ± 11.8	201.8 ± 8.8	199.0 ± 11.2
AST (IU/L)	17.7 ± 2.1	24.0 ± 1.4	18.3 ± 1.9	20.2 ± 1.4	16.7 ± 1.4
ALT (IU/L)	35.2 ± 2.4	44.5 ± 1.4	25.7 ± 3.0	30.7 ± 2.8	37.2 ± 2.0
ALP (IU/L)	65.5 ± 5.2	69.3 ± 5.5	65.7 ± 4.2	59.3 ± 3.3	61.2 ± 2.4
GGT (IU/L)	39.8 ± 3.3	41.7 ± 1.6	32.8 ± 3.3	36.8 ± 2.6	35.7 ± 2.7
CK (IU/L)	48.8 ± 2.7	46.5 ± 2.7	47.3 ± 3.5	47.3 ± 3.8	49 ± 3.2
TSH (ng/ml)	3.7 ± 0.3	3.5 ± 0.2	3.3 ± 0.3	3.3 ± 0.2	3.9 ± 0.2
T3 (ng/ml)	1.7 ± 0.1	1.5 ± 0.1	1.5 ± 0.2	1,6 ± 0.2	1.7 ± 0.1
T4 (ng/ml)	70.5 ± 2.2	65.8 ± 3.1	79.8 ± 3.9	75.3 ± 5.5	73.3 ±3.9
TC (mg/dL)	4.5 ± 0.2	4.3 ± 0.1	4.8 ± 0.3	4.9 ± 0.4	4.6 ± 0.2
TG (mg/dL)	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.1
HDL-c (mg/dL)	1.3 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1
LDL-c (mg/dL)	2.9 ± 0.3	3.1 ± 0.2	3.4 ± 0.3	3.4 ± 0.4	3.0 ± 0.2
Liver MDA (nmol/ mg tissue protein)	0.2 ± 0.02	0.4 ± 0.02	0.2 ± 0.02	0.2 ± 0.02	0.1 ± 0.02
Brain MDA (nmol/mg tissue protein)	0.26 ± 0.01	0.5 ± 0.01	0.27 ± 0.02	0.32 ± 0.02	0.24 ± 0.02
Serum MDA (nmol)	0.2 ± 0.02	0.3 ± 0.01	0.2 ± 0.03	0.2 ± 0.02	0.2 ± 0.02

APPENDIX 2



Appendix 2- Open-field assessment apparatus

APPENDIX 3



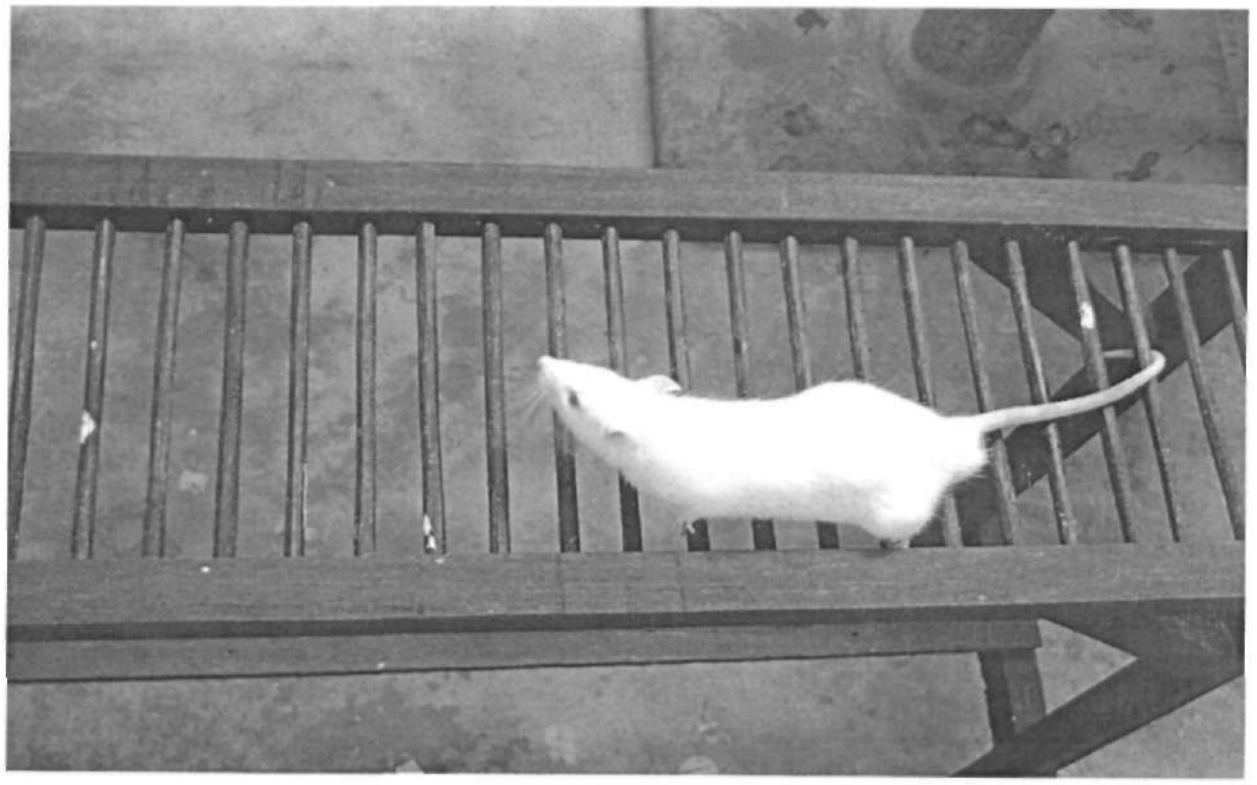
Appendix 3: Beam-Walk apparatus

APPENDIX 4



Appendix 4: Incline plane apparatus

APPENDIX 5



Appendix 5: Ladder-walk apparatus

APPENDIX 6



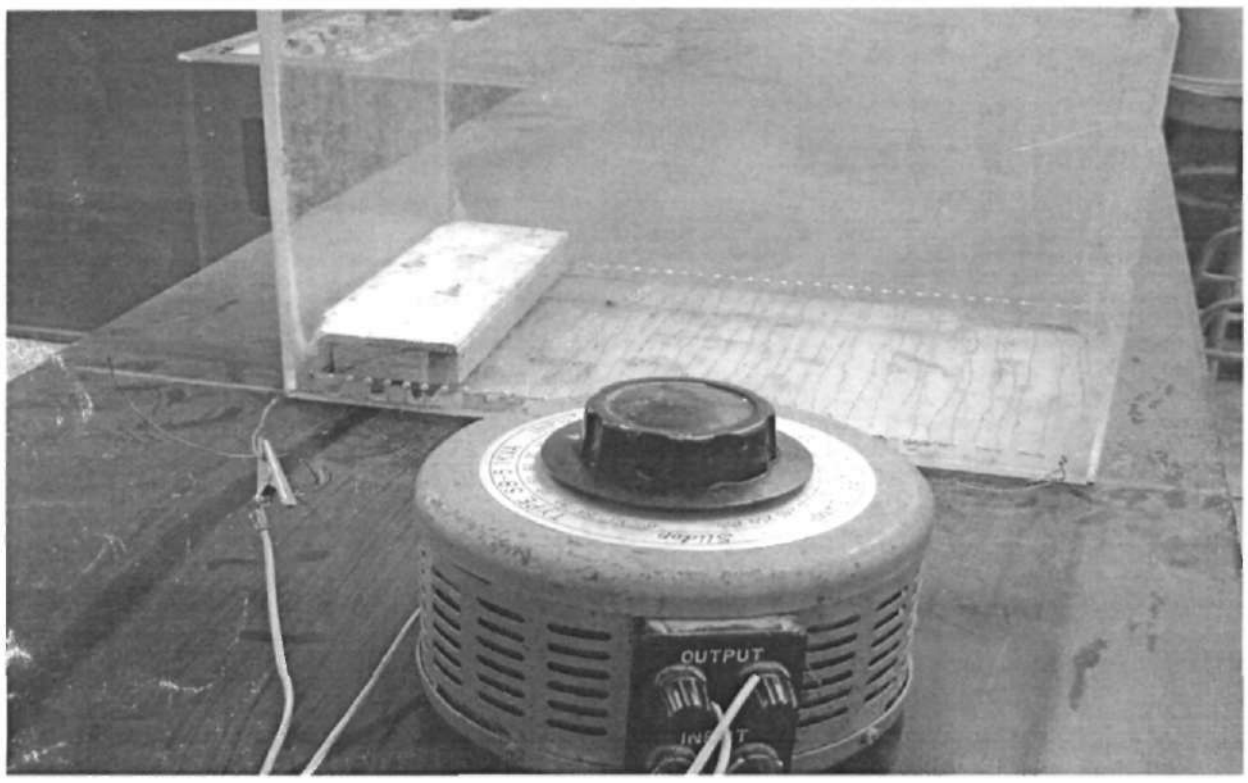
Appendix 6: Forelimb grip time assessment apparatus

APPENDIX 7



Appendix 7: Step-down inhibitory avoidance learning instrument

APPENDIX 8



Appendix 8: Step-down inhibitory avoidance learning instrument with a voltage regulator