AMELIORATIVE EFFECTS OF MELATONIN ON SUBACUTE CHLORPYRIFOS-INDUCED REPRODUCTIVE TOXICITY IN MALE WISTAR RATS

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FACULTY OF VETERINARY MEDICINE,
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

NOVEMBER, 2010

DECLARATION

I declare that this thesis entitled 'Ameliorative Effects of Melatonin on Subacute Chlorpyrifos-Induced Reproductive Toxicity in Male Wistar Rats.' has been performed by me in the Department of Veterinary Physiology and Pharmacology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria under the supervision of Dr S. F. Ambali, Prof. J. O. Ayo and Dr B. Mohammed.

The information derived from the literature has been duly acknowledged and referenced. No part of this thesis has previously been presented for the award of a degree or diploma at any university.

UMOSEN, ANGELA JECINTA AMA

Name of Student	SIGNATURE	DATE

CERTIFICATION

This thesis entitled "Ameliorative Effects of Melatonin on Subacute Chlorpyrifos-Induced Reproductive Toxicity in Male Wistar Rats." by **UMOSEN, Angela Jecinta Ama** meets the regulations governing the award of the degree of Master of Science in Veterinary Toxicology of Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

This work is dedicated to God Almighty for the strength He gave me, for His favor and blessings to me, and to my mother - Late Mrs Alberta Sophia Nkrumah Umosen, whose memory would remain in my memory till I die. I couldn't have been what I am today without her. May God bless her soul. Mother, you are greatly missed and loved. To my late cousin Esi Juliana Nkrumah Sobo, God bless your soul also. Your memory lingers on in my heart.

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ABSTRACT

Studies were conducted to determine the effect of subacute chlorpyrifos exposure on semen characteristics and hormonal, biochemical and pathological changes and the ameliorative effects of melatonin in male rats. Fourty adult male Wistar rats divided into 4 groups of 10 animals each were used for this study. Rats in group I (S/oil) served as passive control and received the soya oil (2 ml/kg). Rats in group II (Mel) (active control) were administered with melatonin (0.5 mg/kg), while those in group III (CPF) were exposed to CPF only (8.5 mg/kg ~ $1/10^{th}$ of the LD_{50}). Rats in group IV (Mel + CPF) were pretreated with melatonin (0.5 mg/kg) and then exposed to CPF (8.5 mg/kg), 10 minutes later. The regimens were administered orally by gavage once daily for a period of 28 days. During this period, the rats were observed for signs of toxicity and death. At the end of the study period, the rats were sacrificed, and sera obtained from the blood samples of the animals were analyzed for testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), triiodothyronine (T_3), thyroxine (T_4) and thyroid stimulating hormone (TSH). Testicular tissues and pituitary glands were assayed for the levels of malonaldehyde (MDA), catalase (CAT) and superoxide dismutase (SOD) and acetylcholinesterase (AChE). Testicular weight, epididymal sperm count and semen characteristics were also evaluated. The sections of the brain, testes, adrenal glands, liver and kidneys were examined for histopathological changes. The results showed that pretreatment with melatonin ameliorated the CPF induced alterations in toxic signs, epididymal sperm count, semen characteristics, testicular weight, histopathological changes and levels of testosterone, FSH, LH, T_3, T_4, TSH, MDA, AChE, CAT and SOD. In conclusion, subacute CPF-induced alterations in sex hormones and semen characteristics were ameliorated by melatonin due to its antioxidant and AChE restoration properties. Therefore, it is conceivable

that farmers, pesticide applicators and individuals who are repeatedly exposed to low-dose CPF and perhaps, other organophosphates may be protected from the pesticide-evoked reproductive toxicity by pretreatment with melatonin.

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Chapter 1

INTRODUCTION

Pesticides are a heterogeneous group of chemicals developed to control a variety of pests. They are used extensively all over the world, and it is almost impossible for anyone to avoid daily exposure to low-levels of several pesticide residues (Joshi *et al*., 2007). The use of pesticides in modern era has increased due to the compelling needs to increase food production for the increasing human and animal populations, and to improve public health (Ambali, 2009). It has led to the improvement of human and animal nutrition through greater availability, longer storage life and lower cost of food and high productivity (Joshi *et al*., 2007). However, these economic and health benefits are achieved with potential risk and adverse health effects on humans, animals and the environment (Joshi *et al*., 2007). Organophosphate (OP) insecticides constitute one of the most widely used pesticides and their application is an integral part of modern agriculture. Recently, evidence of adverse health effects due to low-level exposure to OPs has begun to emerge (Peiris-John and Wickremasinghe, 2008).

Chlorpyrifos (CPF; 0,0 – diethyl 0-3,5,6-trichloro-2-pyridyl thiophosphonate) is a broad spectrum OP pesticide used against a wide variety of insects, including fleas, wasps, bees, cockroaches and agricultural pests (Eaton *et al*., 2008). It is a chlorinated OP synthesized by Dow Chemical Company in USA and introduced into the American market in 1965. It is marketed under various names such as Dursban,® Lorsban,® Dorsan,® Dowco® (Zayed *et al*., 2003; Eaton *et al*., 2008). CPF has been reported to cause male and female genital deformities (ENDS, 1999). It exerts negative effects on semen production in the testes and decreases testosterone concentration in rats (Mikhail, 1979; Sherman, 1995; Joshi *et al*.,

2007). Its principal mechanism of toxicity is due to inhibition of acetylcholinesterase (AChE), resulting in the accumulation of the neurotransmitter, acetylcholine (ACh) at the nerve endings and the neuromuscular junctions (Vishwananthan and Srinivassan, 1964). Besides, CPF exerts its toxic effects via oxidative stress induction (Altuntas *et al*., 2004; Ambali *et al*., 2007, 2010a - d). It causes the generation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) as well as significant changes in either antioxidant pools or ROS/RNS-scavenging enzyme system, or both (Halliwell and Gutteridge, 1992; Banerjee *et al*., 1999; Beuret *et al*., 2005; Sharma *et al*., 2005). There is growing experimental evidence, both *in vitro* and *in vivo* linking male infertility and defective sperm function to oxidative-nitrative stress (Rajesh Kumar *et al*., 2002; Wang *et al*., 2003).

Animal studies have shown that antioxidants protect tissue from CPF-induced toxicity (Gultekin *et al*., 2001; Ambali *et al*., 2007, 2010a-d). Antioxidants are agents, vitamins and other nutrients that protect cells from damage caused by free radicals. *In vitro* and *in vivo* studies have shown that antioxidants prevent the free radical damage that is associated with cancer and heart disease (Noguchi *et al*., 2000; Valko *et al*., 2005).

Melatonin (N-acetyl-5-methoxytryptamine), the principal secretory product of the pineal gland, produced during the dark phase of the circadian cycle, is a highly conserved antioxidant molecule (Hastings *et al*., 1989; Reiter, 1991). It is a lipophilic hormone that easily passes through cell membranes and barriers (Mennendez-Pelaez *et al*., 1993; Shida *et al*., 1994; Costa *et al*., 1995). Melatonin along with its metabolites has been shown to scavenge ROS or RNS (Costa *et al*., 1995). This cascade reaction makes melatonin highly

effective, even at low concentrations, in protecting cells from oxidative stress (Tan *et al*., 2007).

1.1 Statement of Research Problem

There is increasing concern about the impact of the environment on public health, including reproductive health. Recent scientific findings on human reproduction have shown that the occurrence of infertility has grown from 50% in the 1960s to 60% in the present day, especially in industrialized countries (Oehninger, 2001). The significant increase in testicular abnormalities over a relatively short period of time is apparently due to environmental rather than inheritable factors (Giwercman *et al*., 1993). A large number of chemical agents have been reported to exert negative effects on the reproductive performance of animals (Afifi *et al*., 1991; Sánchez-Peña *et. al*., 2004; Saradha and Mathur, 2006), and they include environmentally toxic chemicals such as pesticides (Cox, 1994).

Organophosphates represent one of the most widely used world-wide pesticides, accounting for about 50% of the global insecticidal use (Casida and Quistad, 2004). Therefore, OPs are one of the insecticides implicated in the increasing cases of infertility in both man and animals (Peiris-John and Wickremasinghe, 2008). Although the main mechanism of OP toxicity is related to AChE inhibition, the induction of oxidative stress is increasingly being implicated, especially at doses that do not inhibit AChE (Slotkin, 2005; Ambali *et al*., 2010a - d). In fact, elevated ROS levels have been detected in up to 40% of semen samples obtained from infertile men (Rajesh Kumar *et al*., 2002; Wang *et al*., 2003). Exposure to many types of environmental contaminants, including pesticides, enhances this oxidative

damage by increasing the generation of free radicals and/or decreasing antioxidant potential, in the body (Wang *et al*., 2003).

Despite advances in diagnosis, the causes of subfertility in humans and animals remain largely unknown. Poor semen quality and low level of female and male reproductive hormones have been suggested as causes of subfertility among humans and animals exposed to low levels of OPs (Peiris-John and Wickremasinghe, 2008). Subfertility may occur due to male factors, such as reduced quality and quantity of sperm, and female factors, such as ovulation disturbances and tuboperitoneal disorders (Peiris-John and Wickremasinghe, 2008). In general, poor semen quality and low male and female reproductive hormone levels have been suggested as the likely reasons for subfertility among people exposed to low levels of OPs (Peiris-John and Wickremasinghe, 2008).

1.2 Justification for the Study

Pesticides have been of great value to man in the control of many pests. Despite its usefulness in human affairs, its application is associated with numerous adverse effects not only on humans and animals, but also on the environment. Moreover, the increasing development of resistance by pests to the existing pesticides has led to the synthesis and development of more potent ones, leading to increase proliferation of pesticides with consequent adverse health outcomes (Ambali, 2009). In many developed countries, the use of many pesticides have been banned or severely restricted, based on concerns about their safety to the ecosystem. This has made the developing countries a haven for sales and application of banned or restricted pesticides (Eaton *et al*., 2008). There is an increasing concern about the impact of the environment on public health, including reproductive

ability. Reports have shown a significant decrease in quality of human semen (Nelson and Bunge, 1974; Murature *et al*., 1987; Bendvold, 1989). Environmental chemical contaminants, including OP pesticides, have been largely implicated (Cox, 1994).

In Nigeria, there is limited knowledge on the effect of OP pesticide on male reproduction. The nearly ubiquitous and daily exposure of human and animal populations to OP pesticides, including CPF, have raised great concern about their propensity to elicit adverse reproductive effects at exposures that are undetected because of the absence of systemic signs of intoxication (Mileson *et al.*, 1998; Colborn, 2006; Costa, 2006; Meeker *et al.*, 2006). Lack of awareness on the danger of low-dose exposure, poor legislation, porous borders, illiteracy among users, lack of enforcement of withdrawal period and poor labeling are some of the factors that are responsible for the increasing prevalence of pesticide exposure in Nigeria (Ambali, 2009). In addition, application of safety measures such as the use of protective masks, gloves and boots by pesticide users in developing countries is generally minimal or non-existent. This is because safety measures are unaffordable (Kishi, 1995). Hence, the danger posed by OP insecticide will continue to be with us for a long time.

Therefore, measures aimed at mitigating the adverse health impact caused by low-dose OP exposure are, therefore, of great concern. Since it has been established that oxidative damage is one of the molecular mechanisms involved in OP poisoning (Bagchi *et al*., 1995; Ambali *et al*., 2007, 2010a - d; Tuzmen *et al*., 2007, 2008), it is conceivable that the administration of the antioxidant, melatonin may reduce the negative effects caused by low-

dose CPF exposure on reproduction in animals. Although the effects of CPF on reproductive hormones and sperm quality have been evaluated, there is paucity of information on the protective role of melatonin on CPF-induced toxicity. Moreover, the inadvertent low-dose exposure to OPs warrants the consideration of new clinical approaches to their detection and antidotal therapy (Taylor *et al*., 2007).

1.3 General Aim of the Study

The aim of the study was to evaluate the effect of subacute CPF exposure on some thyroidal and reproductive hormones, semen characteristics and pathological changes, the role of oxidative stress and the ameliorative effect of melatonin.

1.4 Specific Objectives of the Study

The specific objectives of the study were to evaluate the:

1. Effect of subacute CPF exposure on biochemical and histopathological changes and thyroid and some reproductive hormonal concentrations in male Wistar rats.
2. Effect of subacute CPF exposure on epididymal sperm count, motility and morphology in Wistar rats.
3. Role of oxidative stress in the subacute CPF-induced alterations of histoarchitecture, thyroid and reproductive hormonal concentrations, biochemical and semen characteristics in Wistar rats.

4. Ameliorative effect of melatonin on subacute CPF-induced alterations in biochemical and histopathological parameters, semen characteristics, thyroid and reproductive hormonal concentrations in Wistar rats.

1.5 Research Hypotheses

1. Subacute CPF exposure does not alter histoarchitecture, thyroid and reproductive sex hormone concentrations in male Wistar rats.

2. Subacute CPF exposure does not alter semen characteristics in Wistar rats.

3. Oxidative stress does not play a role in the subacute CPF-induced alteration of thyroid and sex hormones, semen characteristics and pathological changes in Wistar rats.

4. Administration of melatonin does not ameliorate the subacute CPF-induced changes in thyroid and sex hormone concentrations, semen, oxidative and pathological characteristics in Wistar rats.

Chapter 2 LITERATURE REVIEW

2.1 Pesticides

Pesticides are agents intended for destroying, repelling, or mitigating any pests (Xavier *et al*., 2004). They include herbicides, fungicides, acaricides, insecticides and also substances intended for use as plant regulators, defoliant or desiccant (Jaga and Dharmani, 2003). Pesticides occupy a unique position among the many hazardous chemicals that man and animals encounter daily (Ashraf and Abd El-Aty, 2008). Exposure to pestcides is a global public health issue. The World Health Organization (WHO) has estimated that each year there are some 3,000,000 cases of acute pesticide poisonings, with 220,000 deaths (He, 2000). The majority of these incidents occur in developing countries, particularly in Africa, Asia, and Central and South America (Jaga and Dharmani, 2003).

The use of pesticides has increased rapidly in the last fifty years due to intensification of farming in order to obtain higher yields (Xavier *et al*., 2004). However, over-dependence on chemicals not only resulted in a high cost of production, but also irreparable damage to the environment and long term health problems to humans and animals (Xavier *et al*., 2004). Pesticide levels in the environment are likely to be higher in developing countries than in agricultural environments in developed countries, due to uncoordinated regulation and monitoring. Moreover, indiscriminate use of pesticides is mainly a developing world phenomenon (Van der Hoek *et al.*, 1998).

In developed countries, the safe use of pesticides causing minimum harm to humans and the environment is ensured by the availability of technology, infrastructure, regulation and economic support (Van der Hoek *et al.*, 1998).

2.2 Historical Perspectives

The use of pesticides dates back to ancient times, to before 500BC, with the initial use of sulphur by the Egyptians for the control of insect pests and prevention of damage to crops (Delaplane, 2000). However, man's great dependence on them for pest control is very recent, dating essentially from World War II with the first use of dichlorodiphenyl-trichloroethane (DDT) in 1942 (Clementi *et al.,* 2007). Since the discovery and use of tetraethyl pyrophosphate (TEPP) in the late 1930s, thousands of pesticides have been synthesized and distributed worldwide in order to combat the menace of pests. However, this has led to the consequent deleterious effect on man, animals and the environment (Clementi *et al.,* 2007).

2.3 Definition and Classification of Pesticides

Pesticides are characterized on the basis of their function into insecticides, herbicides, rodenticides, fungicides, miticides, chemosterilants, repellant and fumigants (Cope *et al*., 2004).

2.3.1 Insecticides

These are substances used to kill insects. In the earlier days, inorganic agents like cyanide were used, but were later found to be toxic to humans and animals (Cope *et al*., 2004).

2.3.2 Herbicides

Herbicides are used for weed control in agriculture, right-of-ways, and gardens. They are designed to kill plants rather than animals (Cope *et al*., 2004).

2.3.3 Rodenticides

They are chemical substances used for killing rodent pests such as rats, mice, in order to prevent them from damaging food, crops and other valuable materials (Murphy, 1975; Cope *et al*., 2004).

2.3.4 Fungicides

Fungicides are used extensively in agriculture and around homes and structures to control mold and mildew. They are used to kill or inhibit growth of fungi that cause economic damage to crops or ornamental plants or endanger the health of domestic animals or humans (Cope *et al.*, 2004).

2.3.5 Miticides

These are compounds used specifically against mites and ticks (Cope *et al*., 2004).

2.3.6 Chemosterilants

These substances inhibit the reproduction of insect pests rather than kill them directly (Cope *et al*., 2004).

2.3.7 Repellants

These substances, like the chemosterilants, impair the reproducibility of the pests and repulse them rather than have lethal effects on them (Cope *et al*., 2004).

2.4 Uses of Pesticides

The use of pesticides has helped to improve human nutrition through greater availability, longer storage life, control of vector and vector-borne diseases and lower cost of food production as well as productivity (Eaton *et al*., 2008). However, these economic health benefits are not achieved without potential risk and possible adverse health effects to humans, domesticated animals and the environment (Eaton *et al*., 2008).

2.5 Health Effects

Pesticides are probably the most frequently deliberately released toxic chemicals into the environment (Clementi *et al.,* 2007). They are chemicals that are biologically active by design; this explains the possibility of damaging human health (JPR, 1999). Pesticides and related chemicals originating from human activity or agricultural farming are discharged directly or indirectly into the receiving environments (Clementi *et al.,* 2007). The presence of these chemicals in the environment has become a global issue. Human pesticide poisonings and illnesses are clearly the highest price paid for all pesticide uses (EPA, 1992; Pimentel, 2005).

Several studies have shown that there is carcinogenic threat related to the use of pesticides, especially among farm workers and pesticide applicators, who are often exposed to them (Pimentel and Hart, 2001). Certain pesticides have been shown to induce tumours in laboratory animals, and there is some evidence that suggest similar effects occur in humans (Colborn *et al*., 1996). Many pesticides are also estrogenic – they mimic or interact with the hormone estrogen – linking them to increase in breast cancer among some women (Colborn *et al.,* 1996).

Other chronic health effects of pesticides include neurological effects; there is some evidence that pesticides can cause sensorimotor disturbances as well as cognitive deficits such as memory loss, language problems and learning impairment (Hart and Pimentel, 2002); respiratory problems, for example, asthma, chronic sinusitis, and/or chronic bronchitis (Weiner and Worth, 1972) and reproductive effects (Hart and Pimentel, 2002). For example, some pesticides have been found to cause testicular dysfunction or sterility

(Colborn *et al*., 1996) and also decrease human sperm counts (Pimentel and Hart, 2001). Pesticides at low concentrations may act as blockers of sex hormones, causing abnormal sexual development, abnormal sex ratios, and unusual mating behavior (Pimentel and Hart, 2001). They can also interfere with other hormonal processes such as thyroid and its influence on bone development (Ewing, 1999). Pesticides can cause adverse effects by interfering with the body's hormones or chemical messengers (Zaheer Khan and Francis, 2005). Hormones play a crucial role in guiding normal cell differentiation in early life forms, and so exposure to endocrine disrupting substances can alter the normal process of development (Zaheer Khan and Francis, 2005). The major types of chronic health effects of pesticides include neurological effects, respiratory and reproductive effects, and cancer (Waddell *et al*., 2001).

The difficulty in measuring health effects arising from environmental pesticide contamination and in controlling exposure as a result of its rapidly increasing use has been highlighted (McConnell *et al.,* 1999). Human and animal pesticide poisonings and illnesses are clearly the highest adverse effects of all pesticide uses (EPA, 1992).

2.6 Organophosphates

Many pesticides classified by the WHO as highly or extremely toxic are in widespread use. Among these are the OPs (WHO/UNEP, 1990; Wesseling *et al*., 1993; Ngowi *et al*., 2001). Organophosphate compounds are the most widely used group of insecticides globally (Casida and Quistad, 2004). They were first recognised in 1854, but their general toxicity was not established until the 1930s (Minton and Murray, 1988).

Organophosphate compounds are a diverse group of chemicals used in both domestic and industrial settings. Examples of OPs include: insecticides (malathion, parathion, diazinon, fenthion, dichlorvos, chlorpyrifos, ethion), nerve gases (soman, sarin, tabun, VX), ophthalmic agents (echothiophate, isoflurophate), and antihelmintics (trichlorfon) (DPR, 2000).

2.6.1 Historical perspectives

Organophosphates were first discovered in the early 1800s, when Lassaigne reacted alcohol with phosphoric acid. It was later shown to be toxic in 1938 by a group of German chemists where they were introduced as nerve poisons or chemical warfare agents during World War II, and later they were developed into less potent chemicals (Sullivan and Blose, 1992; Ecobichon, 1996).

Organophosphate pesticides are synthetic in origin and are normally esters, amides, or thiol derivatives of phosphoric, phosphonic, phosphorothioic, or phosphonothioic acids. They contain both carbon and phosphorus atoms (Abou-Donia, 1994). Most are only slightly soluble in water and have a high oil-water partition coefficient and a low vapour pressure (Abou-Donia, 2003).

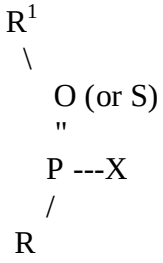


Figure 2.1: General structure for organophosphorus compounds (Abou-Donia, 2003)

Where R^1 and R^2 are usually simple alkyl or aryl groups, both of which may be bonded directly to phosphorus (in phosphonates), or linked via – O - , or – S – (in phosphates). The group X can be any one of a variety of substituted and branched aliphatic, or heterocyclic groups (Abou-Donia, 2003).

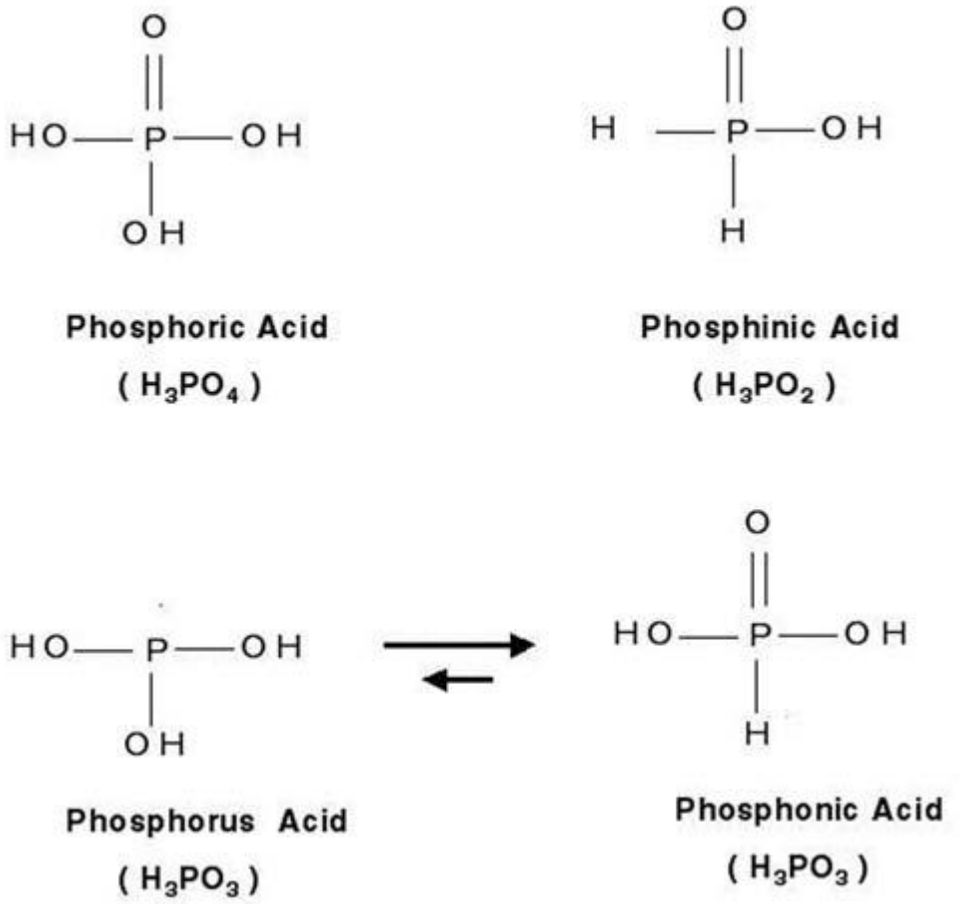


Figure 2.2: Chemical structures of phosphoric acid, phosphinic acid, phosphorus acid, and phosphonic acid (Abou-Donia, 1994)

2.6.2 Uses of organophosphates

Organophosphate compounds are used to destroy insects such as fleas, lice, flies, and mosquitoes (Schenker *et al.*, 1992). These chemicals are used for commercial purposes, for pest control on crops in agriculture and on livestock, and for domestic use, that is, by

individuals in their own homes (Schenker *et al.*, 1992; Sullivan and Blose, 1992). They are additionally used as industrial fluids, flame retardants, therapeutics and nerve gas agents (Abou-Donia, 2003). This compound has gained popularity worldwide in preference to organochlorines because of their lack of persistence and hence less damaging to the environment. The OPs break down more rapidly than do organochlorines in the environment. Therefore, they have a milder environmental impact, hence are safer and less persistent (Schenker *et al.*, 1992; Sullivan and Blose, 1992). In view of this factor, they have largely replaced organochlorine insecticides, which can bioaccumulate and biomagnify (Schenker *et al.*, 1992). However, studies have shown that OP compounds cause a much greater acute toxicity than organochlorine compounds and can cause reproductive failure following repeated exposure (Pope, 1999).

2.6.3 Organophosphate exposure

The recent increase in the agricultural use of OP pesticides has been reported to have adverse health impacts. Studies on health effects of low-level exposure to OPs have documented self-reported symptoms affecting various systems (Ohayo-Mitoko *et al.*, 2000; Smith *et al.*, 2003), including the nervous system (Beach *et al.*, 1996; Peiris-John *et al.*, 2002) and the respiratory system (Peiris-John *et al.*, 2005). Hence, organophosphate exposure is a major public health issue in terms of health, morbidity, health care, and general safety from toxicity (Fenske *et al.*, 2002). Exposures to OPs occur via inhalation, absorption into the skin and ingestion (Sullivan and Blose, 1992; Ecobichon, 1996).

A simple classification of exposure to OPs is: (a) occupational exposure and (b) environmental or non-occupational exposure (Muldoon and Hodgson, 1992; Sullivan and Blose, 1992). Unintentional, accidental, or suicidal poisoning can occur in both occupational and non-occupational exposures to organophosphates (Goozner *et al*., 2002; Langley and Sumner, 2002; Jaga and Dharmani, 2003).

2.6.3.1 *Occupational exposure*

Workers are exposed to pesticides in their workplaces as a result of their occupation (Jaga and Dharmani, 2003). Irrespective of whether the job involves pesticide use, the presence of the chemical in the work environment constitutes an occupational exposure (Jaga and Dharmani, 2003). Workers who handle OPs directly are at higher risk of exposure than those who do not handle pesticides directly (Schenker *et al*., 1992; Sullivan and Blose, 1992). Some examples of occupational exposure occur amongst agricultural workers, manufacturing industry workers, pest exterminators, greenhouse workers and florists, office workers, health care workers, veterinary employees, pathologists performing autopsies, store employees and Gulf War veterans (Jaga and Dharmani, 2003).

2.6.3.2 *Non–occupational or environmental exposure*

This type of exposure occurs in any place where the exposure to pesticides is not a result of a person's job or occupation (Jaga and Dharmani, 2003). It is observed in general population in places other than workplaces (Muldoon and Hodgson, 1992). Individuals are exposed by the entry and spread of the chemicals into the environment (Schenker *et al*., 1992; Ecobichon, 1996). They include residential exposure (resident use, exterminator use), dietary exposure,

accidental exposure, agricultural worker take-home exposure, close proximity to farms, aerial spraying, public places, contaminated organ donor and suicidal (intentional) poisoning among others (Jaga and Dharmani, 2003).

2.6.4 Mechanism of toxicity and health effect

2.6.4.1 *Acute cholinergic syndrome*

Organophosphates are tailor-made to inhibit acetylcholinesterase (AChE), an enzyme essential in humans and other animal species (Abou-Donia, 2003; Bentacourt and Carr, 2004). These compounds cause cholinergic neurotoxicity by disrupting the cholinergic system that includes AChE and its natural substrate, the neurotransmitter acetylcholine (Ach) (Abou-Donia, 2003). The OP insecticides bind with the AChE at the neuromuscular junction and deactivate or inhibit the activity of the enzyme by irreversible phosphorylation (Schenker *et al.,* 1992). This results in elevated levels of ACh at the muscarinic receptors, nicotinic receptors in autonomic ganglia, and receptors in the central nervous system (CNS) (Schenker *et al.,* 1992; Kwong, 2002). When the active site of AChE is inactivated by phosphorylation of the serine hydroxyl group, ACh released from nerve terminals accumulates and over-stimulation of muscarinic and nicotinic receptors occurs (Singh and Sharma, 2000).

High dose exposure to OP insecticides causes acute intoxication as a result of increased activity of ACh at muscarinic, nicotinic and CNS receptors (Jaga and Dharmani, 2003). Excess ACh initially causes excitation, and then paralysis of cholinergic transmission, resulting in some or all of the cholinergic symptoms, depending on the dose, frequency, duration and route of exposure, as well as other factors such as concomitant exposure to

other chemicals and individual sensitivity and susceptibility (Sams *et al*., 2000). The muscarinic symptoms include gastro-intestinal upset, diarrhoea, salivation, bronchospasm, miosis, urination, sweating, lacrimation, bradycardia and fasciculation (Schenker *et al*., 1992; Sullivan and Blose, 1992, Singh and Sharma, 2000). Muscle weakness and paralysis as well as hypertension and tachycardia may occur following activation of nicotinic receptors during acute intoxication, (Schenker *et al*., 1992; Sullivan and Blose, 1992). Central nervous system (CNS) effects may also occur from severe intoxication, causing depression, emotional irritability, cognitive impairment, convulsions and coma (Singh and Sharma, 2000).

2.6.4.2 *Intermediate syndrome*

Intermediate syndrome has been considered as a major contributing factor of OP-related morbidity and mortality because of its frequent occurrence and probable consequence of respiratory failure (Yang and Deng, 2007). This syndrome occurs 24 to 96 hours following the acute cholinergic crisis, and it results from presynaptic and postsynaptic neuromuscular dysfunction (Jaga and Dharmani, 2003). This toxicity is suspected to be due to the accumulation of ACh at the nicotinic neuromuscular junction, causing a depolarizing block (He, 2000). Despite a high incidence, the pathophysiology that underlies intermediate syndrome remains unclear (Yang and Deng, 2007).

Proposed mechanisms of intermediate syndrome include different susceptibility of various cholinergic receptors, muscle necrosis, prolonged acetylcholinesterase inhibition, inadequate oxime therapy, downregulation or desensitization of postsynaptic acetylcholine receptors,

failure of postsynaptic acetylcholine release, and oxidative stress-related myopathy (Yang and Deng, 2007).

The clinical manifestations affect conscious patients without cholinergic signs, and involve the muscles of respiration, proximal limb muscles, neck flexors, facial muscles (Ecobichon, 1996; He, 2000; Yang and Deng, 2007) and muscles innervated by motor cranial nerves (Yang and Deng, 2007).

The treatment of intermediate syndrome is mainly supportive. Nevertheless, because intermediate syndrome generally concurs with severe organophosphate toxicity and persistent inhibition of AChE, early aggressive decontamination, appropriate antidotal therapy, and prompt institution of ventilatory support should be helpful in ameliorating the magnitude and/or the incidence of intermediate syndrome (Yang and Deng, 2007). With appropriate therapy that commonly includes artificial respiration, sensory functions characteristically remain normal and full recovery is evident in 4-18 days (Singh *et al*., 2003; Yang and Deng, 2007).

2.6.4.3 *Organophosphate – induced delayed polyneuropathy*

Organophosphate–induced delayed polyneuropathy (OPIDP) is a neurodegenerative disorder caused by a severe exposure to OPs, characterized by a delayed onset of prolonged ataxia and upper motor neurone spasticity as a result of a single or repeated exposure to organophosphorus esters (Johnson, 1975; Smith *et al*., 2003). OPIDP is a dying back axonopathy, characterised by sensory and motor symptoms that develop days to weeks after

poisoning (Lotti, 1992). In addition to the damage in peripheral nerves, OPIDP may also involve damage to the spinal cord and medulla oblongata (Lotti, 1992). The syndrome has been correlated with the inhibition of neuropathy target esterase (NTE), a neurotoxic esterase in the CNS, and not the AChE enzyme (Singh and Sharma, 2000).

Common features of this syndrome are CNS and peripheral nervous system (PNS) effects of a mixed sensory-motor neuropathy (Cavanagh and Patangia, 1995). The neuropathological lesion is a central-peripheral distal axonopathy caused by a chemical transection of the axon (known as Wallerian-type degeneration), followed by myelin degeneration of distal portions of the long and large-diameter tracts of the CNS and PNS (Cavanagh and Patangia, 1995). The clinical features are predominantly motor neuropathy, primarily manifesting as numbness and weakness of the lower extremities, followed by progressive ascending weakness of limb muscles (Lotti and Moretto, 2005; Yang and Deng, 2007). Symptoms are characterized by progressive distal muscle weakness, ataxia in lower limbs, flaccid paralysis, and spasticity (Schenker *et al*., 1992; He, 2000; Singh and Sharma, 2003). Although there may be some functional recovery, motor neurones may permanently lose function (Jokanovic *et al*., 2002). The extent of delayed polyneuropathy is determined by the differences in structural activity of different OP insecticides (Ecobichon, 1996).

2.6.4.4 *Organophosphate-induced chronic neurotoxicity (OPICN)*

This disorder has been variously referred to in the literature as: “chronic neurobehavioural effects,” (Yokoyama *et al*., 1998), “chronic organophosphate induced neuropsychiatric disorder (COPIND) (Jamal, 1997), amongst others. Recent studies have shown that large toxic doses of organophosphorus compounds cause early convulsive seizures and subsequent

encephalopathy, leading to the necrotic death of brain neuronal cells, whereas small doses produce delayed apoptotic death (Abou-Donia, 2003). This indicates that OPICN may result from exposure to large acutely toxic or small subclinical doses of OP compounds. Damage is present in both the (peripheral nervous system) PNS and (central nervous system) CNS, with greater involvement of the latter (Abou-Donia, 2003). Within the brain, neuropathological lesions are seen in various regions, including the cortex, hippocampal formation and cerebellum (Abou Donia, 2003). The lesions are characterized by neuronal cell death, resulting from early necrosis or delayed apoptosis. Neurological and neurobehavioural alterations are exacerbated by concurrent exposure to stress or to other chemicals that cause neuronal cell death or oxidative stress (Ray and Richards, 2001). Since CNS injury predominates, improvement is slow and complete recovery is unlikely (Tabershaw and Cooper, 1966; Nishiwaki *et al*., 2001; Abou Donia, 2003).

Studies on the effects of exposure to OP compounds over the past half century have shown that chronic neurological and neurobehavioural symptoms include headache, drowsiness, dizziness, anxiety, apathy, mental confusion, restlessness, labile emotions, anorexia, insomnia, lethargy, fatigue, inability to concentrate, memory deficits, depression, irritability, confusion, generalized weakness and tremors (Dille and Smith, 1964).

2.6.5 Organophosphates and reproduction

A number of chemicals commonly encountered in the environment have been associated with altered endocrine function in animals and humans, and exposure to some endocrine disrupting chemicals may result in adverse effects on reproduction, foetal/child development,

metabolism, neurological function, and other vital processes (Diamanti-Kandarakis *et al*., 2009). Interest in the potential adverse reproductive effects of these compounds has heightened in recent years as a result of studies which show that several OP compounds, including dimethoate, methyl parathion, malathion, dichlorvos, chlorpyrifos and dimethyl-methylphosphate impair fertility, suppress libido, deteriorate semen quality and cause testicular degeneration following repeated exposure (Krause and Homola, 1974; Krause, 1977; Haas *et al.,* 1983).

There is increasing evidence to suggest an association between environmental exposure to certain agricultural pesticides and adverse reproductive outcomes in men and women exposed to OPs (Peiris-John and Wickremasinghe, 2008). The consequences of exposure manifest at different times, some involving a long latency (Peiris-John and Wickremasinghe, 2008). It is assumed that some pesticides such as amitraz, some organophosphate pesticides and dithiocarbamates have an influence on the gonadotrophic hormones, including interferences with the release of luteinizing hormone (LH) (Dankwardt, 1998). The disturbances of the hormone balance may then lead to changes in the gonads such as lowered testicular and ovarian weight (Dankwardt, 1998). Many pesticides, like OP compounds, dithiocarbamates, copper fungicides and some pyrethroids also influence spermatogenesis and the number and quality of sperms (Dankwardt, 1998). Some pesticides have been reported to impair female reproduction and the development of offspring (Dankwardt, 1998). Reproductive toxins exert their effects at different stages of the male reproductive system (Dankwardt, 1998). Some influence the CNS, the autonomic nervous system and/or the pituitary-testicular axis, causing sexual dysfunction (Traina *et al*., 1994). Furthermore, some chemicals may have a direct toxic effect on the Sertoli and Leydig cells and/or the germinal

cells, and others could affect the accessory genital organs, causing biochemical alterations of the seminal plasma (Eliasson, 1985).

Reports on male factors leading to subfertility suggest adverse effects of occupational exposure to OPs on semen quality (Padungtod *et al*., 2000; Swan *et al*., 2003; Kamijima *et al*., 2004; Yucra *et al*., 2006). These effects include reduced seminal volume, percentage motility, percentage of sperm with normal morphology, and seminal zinc concentration (a marker of prostate function), as well as significantly increased seminal pH, percentage of immature sperm morphology, and leucocyte concentration (Peiris-John and Wickremasinghe, 2008).

A review of occupational exposure to pesticides, not exclusively OPs, suggests an increased risk of specific morphological abnormalities in sperm, including a decreased sperm count per ejaculate and a reduced percentage of viable sperm (Hanke and Jurewicz, 2004). Although studies on the impact of OP exposure on reproductive hormone levels have been reported, the toxicological and epidemiological significance of these findings is unclear (Hanke and Jurewicz, 2004). Studies among male agricultural workers have shown significantly reduced serum LH and testosterone levels (Yucra *et al.,* 2006).

Studies have suggested that OP exposure disrupts the hypothalamic—pituitary endocrine function and indicate that FSH and LH are the hormones mostly affected in males (Recio *et al*., 2005). Other studies have shown that exposure to OPs increases LH and FSH levels and decreases testosterone level (Padungtod *et al*., 1998). Exposure of experimental animals to

OPs has shown a significant reduction in brain AChE activity, LH and progesterone levels and egg production (Gupta *et al.*, 1984; Rattner and Michael, 1985). In addition, OPs have been shown to cross the placental barrier and, thus, potentially affect the developing foetus (Astroff and Young, 1998).

There are several possible mechanisms for the antigonadal actions of OPs: they may exert a direct inhibitory action on the testes or they may affect the pituitary, causing changes in gonadotrophin concentrations and, thus, subsequent spermatogenic impairment or they may change the concentration of neurotransmitters (Sarkar *et al*., 2000). The main toxic action of OP is phosphorylation of proteins (Pina-Guzman *et al*., 2005). Chemical changes in sperm nuclear proteins (protamines), which pack DNA during the last steps of spermatogenesis, is part of the cause of male reproductive toxicity (Bustos-Obregon *et al*., 2005; Pina-Guzman *et al*., 2005).

2.7 Chlorpyrifos

Chlorpyrifos (CPF, O,O-diethyl-*O*-(3,5,6-trichloro- 2-pyridyl) (figure 2.3) is a broad spectrum OP insecticide, utilized extensively in agriculture and for residential pest control throughout the world (Whyatt *et al.*, 2007). It has been commercially used since the 1960s, particularly for the control of foliar insects of cotton, paddy fields, pasture and vegetable crops (Singh and Walker, 2006). The wide-spread use of CPF in agriculture has increased public concern on potential human health risks. This may result from acute or chronic dietary exposure to CPF residues in food (Singh and Walker, 2006).

2.7.1 Physical properties

Chlorpyrifos is a white crystal-like solid with a strong odour that does not mix well with water, so it is usually mixable with oily liquids before it is applied to crops or animals (Whyatt *et al.,* 2007).

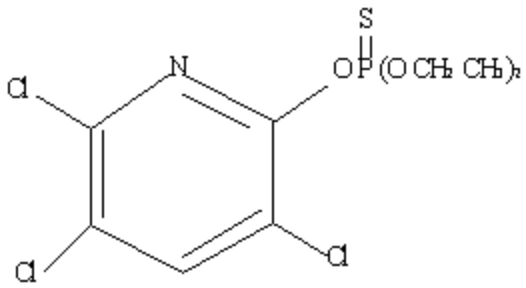


Figure 2.3: Chemical structure of chlorpyrifos

2.7.2 Uses of chlorpyrifos

Chlorpyrifos is a broad spectrum OP insecticide/acaricide which has been widely used in the home and on the farm to control a variety of insects. First introduced into the non-crop specialty market, it was marketed in the late 1960s to control pests in turfgrass and ornamentals, and to control indoor pests (Racke, 1993). CPF was first registered for termiticide use in the United States in 1980 (Racke, 1993). Products are available for both professional pest control workers and home owners. In the home, CPF has been used to control cockroaches, fleas, and termites; it is also an active ingredient in some pet flea and tick collars. On the farm, it is used to control ticks on cattle and as a spray to control crop pests. In 1997, CPF was voluntarily withdrawn from most indoor and pet uses (Racke,

1993). However, the United States Environmental Protection Agency officially banned some of the indoor or residential uses of CPF in 2000 (Racke, 1993).

Exposure pathways include ingestion, inhalation and dermal exposure. Dietary exposures to trace levels of CPF on food products appear to be the major source of non-occupational exposures to CPF (Eaton *et al.*, 2008). Breathing, ingesting, or touching CPF can cause neurological effects, stemming from the inhibition of cholinesterase, an enzyme necessary for the proper transmission of nerve impulses. Symptoms include excessive salivation and tearing, uncontrolled urination, weakness, nausea, vomiting, diarrhoea, headaches, pinpoint pupils, confusion and dizziness (Eaton *et al*., 2008). At higher doses, tremors, convulsions or respiratory paralysis may occur, sometimes leading to coma and death (Eaton *et al*., 2008). Other effects of exposure to CPF include changes in behaviour or sleeping pattern, mood changes, and effects on the nerves and/or muscles in the limbs, which may appear as odd sensations such as numbness or tingling, or as muscle weakness (Agency for Toxic Substances and Disease Registry (ATSDR), 1998).

2.7.3 Metabolism

The major site of CPF metabolism is the liver, where it is rapidly bioactivated (desulphurated) by a P-450-dependent monooxygenase to chlorpyrifos oxon (Sultatos and Murphy 1983) (Figure 2.4). The oxon is 300-400 times more potent at inhibiting brain acetylcholinesterase than the parent compound (Huff *et al*., 1994). The rate of detoxification of the oxon is also rapid (Sultatos and Murphy, 1983). Thus, it is rare to find either the parent

compound or the oxon in body fluid samples (Sultatos and Murphy, 1983; Nolan *et al*., 1984), except in very high exposures (Nolan *et al.*, 1984).

The proposed mechanism for cytochromes P450-mediated (CYP450) bioactivation of OP proceeds via a phosphooxythiran intermediate result in either a desulphuration reaction, generating the oxon or a dearylation reaction that detoxifies OP to hydrolysis products (Sams *et al*., 2000) (Figure 2.4). Excretory products identified are [Cl]-3,5,6- trichloro-2-pyridyl phosphate (75–80%), 3,5,6-trichloro-2-pyridinol (TCP) (15–20%), and traces of CPF, suggesting deethylation is a significant metabolic pathway in rats (Clegg and Van Gemert, 1999). Toxicokinetics of the major metabolite - 3,5,6-trichloro-2-pyridinol (TCP) - indicates absorption, distribution and excretion to be similar to those of the parent compound following oral administration, with excretion being mainly in the urine with small amounts in the faeces (Clegg and Van Gemert, 1999).

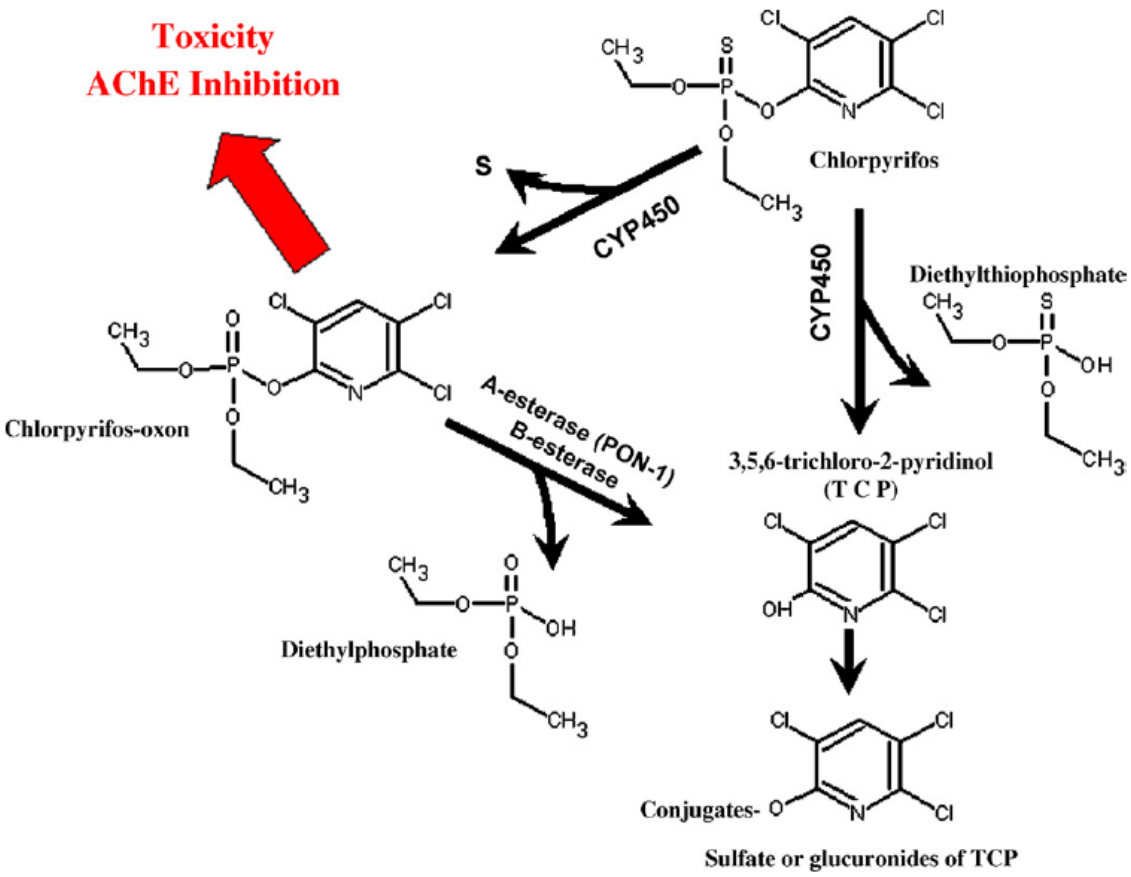


Figure 2.4: Metabolic pathway for chlorpyrifos (Reproduced from Clegg and Van Gemert, 1999).

2.7.4 Mechanism of toxicity and health effects

The primary mechanism of action of CPF is to inhibit AChE by their oxygenated metabolites (oxons), due to the phosphorylation of the serine hydroxyl group located in the active site of the molecule (Sultatos, 1991). CPF after ingestion is subjected to microsomal oxidation in the liver, resulting in the formation of chlorpyrifos-oxon, which is a potent inhibitor of AChE in the peripheral tissues and CNS (Sultatos, 1991). The main target of CPF is AChE, which hydrolyzes Ach in cholinergic synapses and in neuromuscular junctions (Moser and Padilla, 1998). Failure to degrade ACh released into neuronal synapses of the CNS and at myoneural junctions results in continued repeated synaptic transmission (Clegg and Van Gemert, 1999).

Chlorpyrifos has moderate acute toxicity. Clinical (cholinergic) signs are not observed in laboratory animals without substantial inhibition of brain AChE activity (Clegg and Van Gemert, 1999). Signs of acute oral poisoning by CPF are typical of cholinesterase-inhibiting compounds. These cholinergic responses, observed in severe AChE inhibition include excessive glandular secretions (salivation, lacrimation, rhinitis), miosis, bronchoconstriction, vasodilation, hypotension, diarrhoea, nausea, vomiting, urinary incontinence, and bradycardia associated with muscarinic receptor stimulation. Tachycardia, mydriasis (dilation of the pupil), muscle fasciculations, cramping, twitching, muscle weakness, muscle paralysis, and hypertension are associated with nicotinic receptor stimulation. CNS toxicity includes respiratory depression, anxiety, insomnia, headache, tremor, lack of coordination, salivation, lacrimation, decreased body temperature, apathy (Clegg and Van Gemert, 1999), drowsiness, dizziness, loss of concentration, confusion, tremors, convulsions, and coma (ATSDR, 1998; Aaron and Howland, 1998). These effects usually appear within a few minutes to 24 hours after exposure, depending on the extent and route of exposure. In non-fatal exposures, the effects are usually transient, with rapid and complete recovery following cessation of exposure (Aaron and Howland, 1998).

In vitro studies have shown numerous potential molecular targets for CPF, besides AChE. They include cytotoxicity, effects on macromolecule synthesis (DNA, RNA, proteins), interactions with neurotransmitter receptors, interactions with signal transduction pathways, effects on neuronal differentiation, interactions with various enzymes, other neurochemical effects (e.g., neurotransmitter release or uptake), and other effects (e.g. oxidative stress, effects on microtubules) (Eaton *et al.*, 2008).

Although the most important feature of acute CPF toxicity is related to irreversible inhibition of AChE, toxicity has also been observed at doses that do not inhibit AChE (Neishabouri *et al.*, 2004; Slotkin, 2005). Therefore, other mechanisms have been associated with CPF-induced toxicity. Among the associated mechanisms, the induction of oxidative stress has received considerable attention. It has been reported that oxidative stress could be an important component to the mechanism of toxicity of CPF (Gultekin et al., 2000; Altuntas *et al*., 2004; Ambali *et al*., 2007, 2010a - d). Recent findings have indicated that toxic manifestations induced by OPs may be associated with an enhanced production of ROS such as O_2^-, $OH^{\cdot}$ and H_2O_2, which enhance the oxidative process and induce lipid peroxidative damage in cell membranes. This toxicant produces marked deterioration of oxidative stress biomarkers; glutathione, antioxidant enzymes and antioxidant potential (Gultekin *et al*., 2001; Verma *et al*., 2007).

2.7.5 Chlorpyrifos and reproduction

Despite recent curtailment of production for domestic use [U.S. Environmental Protection Agency (EPA), 2000], exposures of pregnant women and children to CPF remain virtually ubiquitous in both agricultural and urban communities (Curl *et al*., 2002; Enrique *et al*., 2002; Fenske *et al*., 2002; Berkowitz *et al*., 2003). Studies have suggested that animals exposed to pesticides have a greater risk of adverse reproductive outcomes, including embryonic and foetal death (Joshi *et al*., 2007).

Occupational studies have shown significant associations for maternal as well as paternal exposure to chemicals like chlorpyrifos and adverse reproductive outcomes (Joshi *et al*., 2007). Specifically, anti-ChE compounds have been implicated in the following adverse outcomes: changes in hormone levels such as adrenocorticotropic and follicle-stimulating hormones; impaired semen quality and concentration; increased risk of spontaneous abortion and congenital defects resulting in fetal death; and altered birth parameters such as low birth weight and birth length with home and agricultural exposure to OPs like CPF (Joshi *et al*., 2007). Studies have also shown that CPF induced a marked reduction in epididymal and testicular sperm counts in exposed males and a decrease in serum testosterone concentration (Joshi *et al*., 2007). Also, histopathological examination of testes following CPF exposure shows a mild to severe degenerative changes in seminiferous tubules (Joshi *et al*., 2007). Thus, CPF is a suspected endocrine disruptor that interferes with the natural function of estrogen, androgen and thyroid hormones (Eaton *et al*., 2008).

2.8 Oxidative Stress

The term 'oxidative stress' was first coined by Sies (1985) to account for the imbalance between ROS and the antioxidant opposing forces. Oxidative stress therefore is said to be caused by an imbalance between the production of reactive oxygen and the biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage (Wolff *et al*., 1986). The fact that oxygen is ubiquitous in aerobic organisms has led to the concept of the oxygen paradox; namely the fact that this life-supporting molecule is also a precursor to the formation of harmful ROS. ROS can damage virtually any biological molecule in its vicinity, including DNA, essential proteins, and membrane lipids (Wolff *et*

al., 1986); and if ROS formation exceeds the capacity of antioxidant, it will lead to cell dysfunction (Giaccia *et al*., 2004; Giordano, 2005).

All forms of aerobic life are subjected constantly to oxidant pressure from molecular oxygen and also ROS, produced during the biochemical utilization of O_2 and prooxidant stimulation of O_2 metabolism (Routledge *et al*., 1994; Guetens *et al*., 2002). Many active intermediaries, such as electrophiles and free radicals are produced during physiological and pathological processes (Marnett *et al*., 2003). ROS are free radicals that contain the oxygen atom (Marnett *et al*., 2003). ROS is a collective term, which includes oxygen radicals [superoxide ($O_2^{\cdot}$)-, hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), and alkoxyl ($RO^{\cdot}$)] and certain non-radicals that are either oxidizing agents or easily converted into radicals, such as hypochlorus acid (HOCl), ozone (O_3), peroxynitrite anion ($ONOO^{-}$), singlet oxygen, and H_2O_2 (Guetens *et al*., 2002). They are very small molecules that include oxygen ions and peroxides and can be either inorganic or organic. The ROS are highly reactive due to the presence of unpaired valence shell electrons (Marnett *et al*., 2003). They are to some extent unstable and react with other molecules (Tkaczyk and Vízek, 2007).

ROS formed as a natural by-product of the normal metabolism of oxygen have important roles in cell signaling (Tkaczyk and Vízek, 2007). They are generated in normal aerobic metabolism in mitochondria, which are the main site of production of radicals (Jarvinen, 2001). In the cytosol and plasma membrane, ROS are formed by NADPH oxidase, cytochrome P450 oxidase and xanthine oxidase (Jarvinen, 2001). This reaction may lead to the generation of other, sometimes even more reactive molecules (Jarvinen, 2001). ROS are

generated as a result of one- electron reduction of molecular oxygen, leading to the production of superoxide, O_2^- (Equation 1)

$$O_2 + e^- \rightarrow O_2^- \quad (1)$$

Superoxide radical (O_2^-) is unstable and quickly undergoes another reduction to hydrogen peroxide (H_2O_2), either spontaneously or in a much faster reaction catalysed by superoxide dismutase (SOD) (Equation 2) (Tkaczyk and Vízek, 2007).

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

Hydrogen peroxide (H_2O_2) is relatively stable and can migrate from its site of origin; therefore, it is capable of affecting many important cellular molecules. It can also turn into highly reactive hydroxyl radical $OH^{\cdot}$ (Fenton's reaction, catalysed by free iron). The hydroxyl radical may be formed when H_2O_2 reacts with iron or copper ions (Equation 3) (Tkaczyk and Vízek, 2007). This reaction is known as Fenton reaction.

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

In Haber-Weiss reaction, hydroxyl radical is generated from O_2^- and H_2O_2 (Jarvinen, 2001).

$$H_2O_2 + O_2 \xrightarrow{Cu^{2+}/Fe^{2+}} OH^{+} + OH^{-} + O^2 \quad (4)$$

2.8.1 Oxidative stress and its role in mechanism of disease

Cellular injury caused by ROS is associated with their ability to impair cellular structures by membrane lipoperoxidation and DNA strand breaks and function due to changes in enzymatic activity and signalling (Tkaczyk and Vízek, 2007). Cellular and organismal decline is, in part, believed to be a consequence of oxygen and nitrogen-based reactants, which persistently damage macromolecules throughout the life-time. The resulting accumulation of damaged molecules eventually and seriously compromise essential functions of cells, leading to their death. Excessive cellular loss causes deterioration of organ function and inevitably to the demise of the organism. The sequence of events is known as free radical theory of ageing (Reiter *et al.,* 2002). Indeed, it has been shown that the action of free radicals could increase the risk of diseases such as cancer and heart problems, and could accelerate ageing (Marnett *et al.,* 2003).

Reaction of ROS with lipid membrane, rich in polyunsaturated fatty acids will cause lipid peroxidation. Malondialdehyde (MDA) is the end-product of lipid peroxidation and can be measured by thiobarbituric acid test (TBARS test) (Wills, 1987; Hermes-Lima, 2004; Halliwell and Gutteridge, 2007). Tissue lipid peroxidation is a degradative phenomenon as a consequence of free radical production and propagation, which affects mainly polyunsaturated fatty acids, and it is strongly involved in the pathogenesis of several diseases, such as diabetes, cancer as well as toxicity associated with drugs and ageing (Rada and Leto, 2008). The high content of polyunsaturated fatty acids in membranes and continuous exposure to high concentrations of oxygen and iron in haemoglobin make erythrocytes highly susceptible to oxidative injury (Hebbel, 1986; Clemens and Waller, 1987).

The brain is deficient in oxidative defence mechanisms and, hence, is at greater risk of damage mediated by ROS, resulting in molecular and cellular dysfunction (Gupta *et al*., 2003). ROS can increase the permeability of the blood-brain barrier, alter tubulin formation, and inhibit the mitochondrial respiration. If unchecked, it can lead to a geometrically progressing lipid peroxidation (Gilman *et al*., 1993).

The cell has several mechanisms to deal with the effects of oxidative stress, either by repairing the damage (damaged nucleotides and lipid peroxidation by-products) or by directly diminishing the occurrence of oxidative damage by means of enzymatic and non-enzymatic antioxidants (Gultekin *et al.*, 2001). Organisms use scavenger enzymes and metal ion-sequestrating agents such as superoxide dismutase, catalase, glutathione peroxidase, vitamin C and glutathione, together comprising the primary defence, as protection against ROS (Guetens *et al*., 2002). Enzymatic and non-enzymatic antioxidants also scavenge free radicals and ROS, and, therefore counteract oxidative stress (Gultekin *et al.*, 2001).

2.8.2 Oxidative stress and reproduction

Oxygen-derived species, such as the superoxide radical ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2), are generated in all aerobic cells (Fridovich, 1986; Halliwell and Gutteridge, 1992). The excessive generation of these species by endogenous or exogenous sources (e.g., redox-cyclic drugs, ionizing radiation, and xenobiotics) may cause damage to cellular DNA through a variety of mechanisms (Von Sonntag, 1987; Halliwell and Aruoma, 1991).

Oxidative DNA damage by free radicals and ROS have been associated with the pathology of numerous diseases such as neurodegenerative diseases (Gibson and Huang, 2004), vascular disease (Evans *et al*., 2004; Madamanchi *et al*., 2005), cancer, diabetes, periodontal diseases (Pinzani *et al*., 1998; Agarwal and Allamaneni, 2004) and ageing (Halliwell and Gutteridge, 1989). ROS have been shown to cause reproductive cell death, and human infertility (Agarwal and Saleh, 2002; Aitken and Baker, 2004; Agarwal *et al*., 2005; Baker and Aitken, 2005). Oxidative damage to spermatozoa, induced by excessive production of free radicals or impairment of the natural antioxidant mechanisms, has been identified to affect sperm function and even cause male infertility (Agarwal *et al*., 2005). Spermatozoa generate ROS in physiological amounts, which play a role in sperm functions during sperm capacitation, acrosome reaction and oocyte fusion (Hammadeh *et al*., 2009). However, ROS must be continuously inactivated to keep their levels low in order to maintain normal cell function and avoid ulterior damage (Agarwal *et al*., 2003; O'Flaherty *et al*., 2006). In addition, damaged spermatozoa are likely to exacerbate the ROS generation (Hammadeh *et al*., 2009).

During excessive ROS production or malfunction of the native antioxidant production mechanisms, free oxidants cause problems by subjecting several tissues to oxidative stress (Agarwal and Said, 2003; Balercia *et al*., 2003; Aitken, 2005). Although ROS have been widely implicated in the aetiopathogenesis of both female infertility (Agarwal and Saleh, 2002; Agarwal *et al.,* 2005) and male subfertility and impaired sperm function, their exact role is still not clear (Hammadeh *et al.*, 2009). Spermatozoa require short exposures to low amounts of free radicals to become hyperactive. Moreover, due to a high polyunsaturated

fatty acid content, human sperm plasma membranes are highly sensitive to ROS-induced lipid peroxidation; thus, decreasing membrane fluidity (Hammadeh *et al*., 2009). This results in increased lipid peroxidation, decreased sperm motility, viability, function and ultimately lead to infertility (Hammadeh *et al*., 2009). A shift in the levels of ROS towards pro-oxidants in semen can induce oxidative stress on spermatozoa (Hammadeh *et al*., 2009). Spermatozoa are particularly susceptible to OS-induced damage for many reasons, such as a high content of polyunsaturated fatty acids (Aitken, 1995; Agarwal, et al., 2003), low concentrations of scavenging enzymes and glutathione levels, high production of free radicals resulting from mitochondrial respiration, and deficient DNA repair mechanisms (Wang *et al*., 2003). Excessive ROS production may result in impaired steroidogenesis and spermatogenesis by several mechanisms, including DNA damage, lipid peroxidation and protein oxidation (Astiz et al., 2009)

Studies have shown that there is a protective action of antioxidants against the deleterious effects of ROS on cellular lipids, proteins and DNA (Hammadeh *et al.*, 2009). Seminal plasma and sperm possess an antioxidant system to scavenge ROS and prevent ROS-related cellular damage. Under normal circumstances, there is an appropriate balance between oxidants and antioxidants (Aitken, 1995; Hammadeh *et al*., 2009).

2.9 Antioxidants

The term antioxidants originally were used to refer specifically to chemicals that prevent the consumption of oxygen (Matill, 1947). Historically, in the late 19th century, extensive study was devoted to the use of antioxidants in important industrial processes, such as the

prevention of metal corrosion, the vulcanization of rubber, and the polymerization of fuels in the fueling of internal combustion engines (Matill, 1947). Recently, great attention has been given to antioxidants by a reason of their medical use. This is because antioxidants perform an important role in the maintenance of the integrity of the living organisms and the association of many human diseases with oxidative stress (Koleckár *et al*., 2007).

Antioxidants are quickly oxidized and, hence, are reducing agents. They prevent oxidative reactions by scavenging ROS. Some of the antioxidants are soluble in water (hydrophilic) or in lipid (hydrophobic) (Curtis *et al*., 1996). Hydrophilic antioxidants act in cytosol and blood plasma, while the hydrophobic antioxidants act within the cell membrane and protect it from lipid peroxdation (Curtis *et al*., 1996). Antioxidants are chemicals that offer up their own electrons to the free radicals, thus preventing cellular damage. When an antioxidant neutralizes a free radical, it becomes inactive (Curtis *et al*., 1996), therefore, the need to continuously supply the body with antioxidants (Evans and Halliwell, 2001). Antioxidants function by coordinating and balancing systems to protect tissues and body fluids from damage by reactive oxygen/nitrogen/chlorine species, whether produced physiologically or as a response to inflammation, infection or disease (Evans and Halliwell, 2001).

2.9.1 Mechanism of action of antioxidants

Naturally occurring antioxidants, of high or low molecular weight, can differ in their composition, their physical and chemical properties and in their mechanism and site of action (McCord and Fridovich, 1969). They can be divided into enzymatic and non-enzymatic antioxidants or exogenous and endogenous antioxidants.

Enzymatic antioxidants, such as SOD, catalase, and glutathione peroxidase (GPx), attenuate the generation of ROS by removing potential oxidants or by transforming them into relatively stable compounds (McCord and Fridovich, 1969). SOD, which was discovered in the late 1960s (McCord and Fridovich, 1969), catalyzes the transformation of the superoxide radical into hydrogen peroxide, which can then be further transformed by the enzyme catalase into water and molecular oxygen (Equation 5) (McCord and Fridovich, 1969; Tkaczyk and Vizek, 2007).

$$\underset{\text{Oxygen}}{O_2} \rightarrow \underset{\text{Superoxide}}{O_2^{\cdot}} \xrightarrow{\text{SOD}} \underset{\text{Hydrogen Peroxide}}{H_2O_2} \xrightarrow{\text{catalase}} \underset{\text{Water}}{H_2O} \qquad (5)$$

This enzymatic pathway is involved in the detoxification of ROS (McCord and Fridovich, 1969; Tkaczyk and Vizek, 2007).

While superoxide anion in itself is not particularly reactive, it can reduce transition metal ions such as iron, and it is converted to one of the most reactive radicals - the hydroxyl radical (Ursini *et al.*, 1982). Thus, elimination of superoxide can attenuate the formation of the harmful $OH^{\cdot}$. GPx reduces lipid peroxides (ROOH), formed by the oxidation of polyunsaturated fatty acid, to a stable, non-toxic molecule - hydroxyl fatty acid (ROH). Together with phospholipases, GPx can also convert phospholipid hydroperoxides into phospholipid hydroxide (Ursini *et al.*, 1982).

Two principle mechanisms of action have been proposed for activity of antioxidants (Sies, 1997). The first is a chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present in the system. The second mechanism involves removal of ROS/RNS initiators (secondary antioxidants) by quenching chain-initiating catalysts (Sies, 1997).

2.9.2. Exogenous antioxidants

The exogenous antioxidants are introduced into the body to help supplement the endogenous/enzymatic antioxidants which are normally compromised during oxidative stress (Halliwell, 2001). The most widely studied dietary antioxidants are vitamin C, vitamin E and beta-carotene. Vitamin C is capable of neutralizing reactive oxygen species in the aqueous phase before lipid peroxidation is initiated. Vitamin E is the most effective chain-breaking antioxidant within the cell membrane, where it protects membrane fatty acids from lipid peroxidation. Beta-carotene and other carotenoids also provide antioxidant protection to lipid-rich tissues. Flavonoids also have antioxidant activity and exhibit several biological effects (Evans and Halliwell, 2001).

2.9.3 Endogenous antioxidants

The biological systems have evolved with endogenous defence mechanisms to help protect against free-radical-induced damage. GPx, catalase and SOD are antioxidant enzymes, which metabolize toxic oxidative intermediates. They in turn require micronutrients as co-factors such as selenium, manganese, zinc, iron and copper for optimum catalytic activity and effective antioxidant defence mechanisms (Halliwell and Gutteridge, 1992; Halliwell, 2001).

Superoxide dismutase, catalase and GPx are three primary enzymes, involved in direct elimination of ROS ($OH^{\cdot}$, $O_2^{\cdot}$, H_2O_2) whereas glutathione reductase, glucose-6-phosphate dehydrogenase, and cytosolic glutathione-S-transferase are secondary enzymes, which help in the detoxification of ROS by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione and NADPH, necessary for optimum functioning of the primary antioxidant enzymes (Vendemiale *et al.,* 1999; Singh *et al*., 2003).

Due to the inefficiency of endogenous defence systems and existence of some pathophysiological situations, ROS can be produced in excess, and increasing amounts of dietary antioxidants will be needed for diminishing the cumulative effect of oxidative damage over an individual's life span (Sun *et al*., 2002). Glutathione, ascorbic acid, alpha-tocopherol, NADPH, selenium, mannitol, melatonin, beta-carotene, biluribin, benzoate, the iron-bonding transferring, among others, play a homoestatic or protective role against ROS, produced during normal cellular metabolism and after active oxidation insult (Evans and Halliwell, 2001).

One of the body's endogenous antioxidants is melatonin, produced by the pineal gland. The pineal gland, once considered a part of the body without any known significant function, gradually has gained importance when melatonin was found to be the factor regulating sleep behaviour. Now the antioxidant property has made melatonin an important molecule in research (Gupta *et al*., 2003).

2.10 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) (Figure 2.5), the chief secretory product of the pineal gland was first isolated from bovine pineal tissue and chemically characterized by Lerner *et al.* (1958) and Lerner and Case (1959). The synthesis of melatonin in the pineal gland exhibits a pronounced circadian rhythm. Its concentration in the body is typically lower during the day and reaches maximal levels at night in the darkness (Fellenberg *et al.*, 1980; Claustrat, *et al.*, 1995; Tomas-Zapico and Coto-Montes, 2007). Intravenous, intranasal, transdermal and oral administrations of melatonin have all been reported to increase circulating melatonin levels (Zhdanova, *et al*., 1996; Benes *et al*., 1997). Melatonin is highly lipophilic as well as aqueous soluble (Cavallo and Hassan, 1994; Shida, *et al*., 1994). Its synthesis within the rat pineal gland is seriously affected by light signals, received through the eyes. It is naturally synthesized from the amino acid tryptophan via synthesis of serotonin (Tomas-Zapico and Coto-Montes, 2007).

It is a ubiquitous molecule with many functions, ranging from its involvement in the neuroendocrine system, mainly in reproductive physiology, its circadian rhythm control and sleep processes, and more recently, its antioxidant properties as well as in the immune system (Guerrero and Reiter, 2002; Carrillo-Vico *et al*., 2006), and in the tumour growth (Blask, 1993).

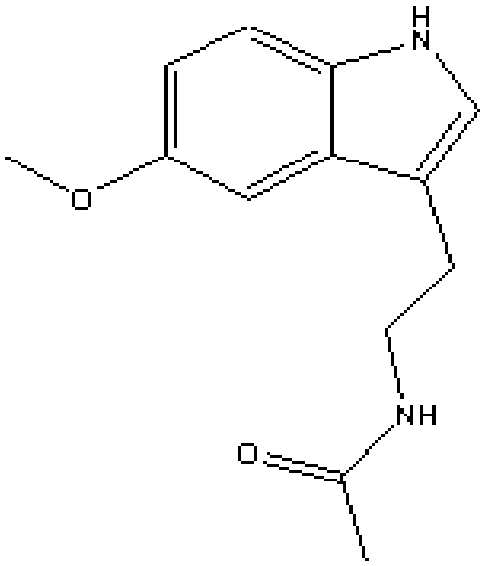


Figure 2.5: Chemical structure of melatonin ($C_{13}H_{16}N_2O_2$)

2.10.1 Antioxidant effect of melatonin

Ianas *et al.* (1991) were the first to suggest that melatonin may function as an antioxidant. It has been documented that melatonin reduces oxidative damage in all varieties of macromolecules under conditions where free radicals products are produced (Blanchard *et al*., 2000; Karbownik and Reiter, 2000; De Almedia *et al.,* 2003). Melatonin has also been shown to stimulate enzymes, involved in metabolizing reactive oxygen intermediates, thereby further increasing its ability to protect against free radicals (Pablos *et al*., 1998). Melatonin is closely associated with the cellular antioxidant defence and this action is developed at two levels: 1) as a direct antioxidant, due to its ability to act as a free radical scavenger; and 2) as an indirect antioxidant, since it is able to induce and / or increase the activity and expression of the main antioxidant enzymes ((Tomas-Zapico and Coto-Montes, 2007).

A good antioxidant must fulfil a series of characteristics: large distribution within tissues, cells and subcellular compartments, ability to cross morphologically barriers and quick transport into the cells (Karbownik and Reiter, 2000). Physical chemistry properties of melatonin allow this molecule to penetrate into all cells and cellular compartments in the organism, since it is highly soluble in lipids and partially in water (Ceraulo *et al*., 1999; Leon *et al*., 2004).

Melatonin represents one of the most important immune-enhancing substances in the body; and, as a free radical scavenger, it protects all body and brain cells against genetic damage considered to be a precursor to cancer (Gulcin, 2008). Melatonin regulates chronobiological and reproductive systems and mammary gland functions. Its other functions include free radical scavenging and DNA repair via immunomodulation, body weight control and the promotion of wound healing (Gulcin, 2008). Mammalian skin and hair follicles are not only melatonin targets, but also sites of extrapineal melatonin synthesis (Fischer, 2008).

2.10.2 Melatonin and disease conditions

Antioxidants have attracted a great deal of attention as potential agents for preventing age-related free radical-based diseases (Halliwell, 2001; Harman, 2002). The ageing process itself is believed to be a result of the persistent accumulation of molecular debris resulting from the un-ending mutilation by free radicals (Harman, 1998; 1999). Melatonin has the capability of diminishing the destruction of DNA, proteins and lipids that occur as a result of their reactions with ROS and RNS (Reiter *et al*., 2004). Melatonin can easily cross cell membranes and the blood-brain barrier (Hardeland, 2005). Diminution in melatonin

signalling could partly explain the prevalence of insomnia in older people. The shortening of the circadian period, occurring in elderly persons, may cause an increase of the oxidative stress that accelerates these processes, associated with ageing; for example, neurodegenerative disease (Floyd and Hensley, 2002; Mariani *et al.*, 2005).

Studies have shown that the melatonin's antioxidant abilities may help reduce the severity of Alzheimer's disease, which involves inflammation and free-radical damage in the brain, and protect the brain against cellular changes (Masilamoni, 2008). This is also applicable in the case of Parkinson's disease (Mariani *et al.*, 2005; Masilamoni, 2008). Increased LPO is one of the main causes of diabetic complications since the possible sources of oxidative stress and damage to proteins in diabetes include free radicals generated by autoxidation reactions of sugars, and sugar adducts to protein and by autoxidation of unsaturated lipids in plasma and membrane proteins (Baynes, 1991; Baydas *et al*., 2002). Furthermore, Baydas *et al* (2002) showed that melatonin is able to reduce MDA concentrations in diabetic rats.

2.10.3 Melatonin in chemical poisoning

Melatonin was found to protect against damage from formaldehyde-induced oxidative renal damage and neurotoxicity (Zararsiz, 2007), tetrachloride-induced changes (Ogeturk, 2004), arsenite-induced peripheral neuropathy (Lin, 2009), deltamethrin (pesticide)-induced nerve cell damage (Guo, 2008) and 2-Bromopropane-induced testicular toxicities (Huang, 2008). Alonso-Gonzalez (2008) found that melatonin helped to prevent cancers that were a result of cadmium contamination. In a review of the effects of melatonin, Reiter (2008) showed that it ameliorated the extensive free radical-mediated damage that ensued following exposure to a

wide variety of environmental insults, including toxic prescription drugs, neural toxins, herbicides and metals.

The toxicity of OP compounds is mediated by generation of ROS. These toxic molecules can be counteracted by antioxidants such as vitamins C and E, spin traps, melatonin and low-molecular weight thiols. The latter compounds can also increase the synthesis of glutathione, which can both ameliorate the OP-induced oxidative stress and enhance OP detoxification (Pena-Llopis, 2005; Ranjbar *et al*., 2005). Melatonin was shown to be more effective than vitamins C and E in reducing molecular damage that occurs under high free-radical generating conditions (Montilla *et al*., 2001). Its unique intracellular distribution and cascade of scavenging actions brought about by its metabolites also contribute to its high efficacy (Montilla *et al*., 2001).

2.10.4 Metabolism and pharmacokinetics of melatonin

Endogenous melatonin synthesized by the pineal gland is released quickly into the circulation and then into other body fluids, including cerebro-spinal fluid (Rousseau *et al*., 1999), saliva and bile (Tan *et al*., 1999). Melatonin levels in the bile and cerebro-spinal fluid are several times higher than that in the serum. Serum melatonin half-life is estimated to be between 30-60 minutes and first-pass metabolism in the liver results in a clearance rate of 90 percent (Claustrat *et al*., 1986). Hepatic enzymes convert melatonin to 6-hydroxymelatonin, 70% of which is subsequently bound to sulphate and 6% bound to glucuronide and excreted in the urine. Moreover, orally administered melatonin is rapidly absorbed and its bioavailability ranges from 10-56%. The half-life of exogenous melatonin is 12-48 minutes (DeMuro *et al.,* 2000).

2.11 Antioxidant Mechanism of Melatonin

Melatonin is an antioxidant that can easily cross cell membranes and the blood-brain barrier (Hardeland, 2005). It is a powerful free radical scavenger, as it is able to remove H_2O_2, $OH^{\cdot}$, $ONOO^-$, singlet oxygen, O_2 and LOO. The large subcellular distribution of melatonin facilitates its ability to protect lipids in the cellular membranes, protein in the cytosol and DNA in the nucleus from free-radical damage (Reiter *et al*., 2004). Melatonin is able to increase the activity and / or the expression of the main antioxidant enzymes such as SOD, CAT, GPx and glutathione reductase (Reiter *et al*., 2005). It reduces lipid peroxidation *in vivo* more effectively than either vitamin C or E (Gitto, *et al*., 2001; Montilla, *et al*., 2001). Unlike other antioxidants, melatonin does not undergo redox cycling which is the ability of a molecule to undergo reduction and oxidation repeatedly. Redox cycling may allow other antioxidants (such as vitamin C) to regain their antioxidant properties. Melatonin, on the other hand, once oxidized, cannot be reduced to its former state because it forms several stable end-products upon reacting with free radicals. Therefore, it has been referred to as a terminal (or suicidal) antioxidant (Tan *et al.,* 2007). Its capacity to absorb free radicals extends at least to the quaternary metabolites of melatonin, a process referred to as "the free radical scavenging cascade". This is not true of other, conventional antioxidants (Tan *et al*., 2000).

Chapter 3 MATERIALS AND METHODS

3.1. Experimental Animals

Fifty two adult male Wistar rats were obtained from the Animal House of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria. They were housed in cages in the Laboratory of the Department and fed pellets, prepared from growers mash, maize bran and groundnut cake at a ratio of 4:2:1 and water was provided *ad libitum*. The rats were preconditioned for two weeks in the laboratory prior to the commencement of the experiment.

3.2 Chemical Acquisition and Preparation

Commercial grade CPF, Termicot® (Sabero Organics, Gujarat, India), which is a 20% emulsifiable concentrate was obtained from a reputable Agrochemical Store in Zaria, and it was reconstituted in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria) to make 1% stock solution. Melatonin tablet (5 mg, NATROL, Inc. Chatsworth, USA) was dissolved in 10 ml of distilled water, to make a 0.5 mg/ml suspension, daily before administration.

3.3 Determination of Median Lethal Dose (LD_{50})

The median lethal dose (LD_{50}) was determined according to the method of Lorke (1983). This was evaluated in two phases. Phase 1 involved 9 rats, which were divided into three groups (I, II, III) of three animals each. The animals in group 1 were administered with CPF at 100 mg/kg, while those in group II were dosed at 200 mg/kg; group III animals were given at 400 mg/kg orally. The rats were monitored for death and signs of toxicity over a 48-hour period. Based on the result obtained from phase 1, the evaluation progressed to the second phase, which comprised three groups of one animal each. All the animals were dosed

with a reconstituted CPF equivalent to 100 mg/kg, 90 mg/kg and 80 mg/kg and equally monitored for death and signs of toxicity over a 48-hour period. The LD_{50} was then calculated based on the method of Lorke (1983).

3.4 Subacute Reproductive Toxicity Study

Forty adult male Wistar rats, 7-8 weeks of age and weighing between 101-132 g were used for this phase of the study. They were weighed and divided into four groups, consisting of 10 rats per group. Rats in Group I served as the passive control and were dosed with the vehicle (soya oil, 2 ml/kg). Rats in group II were dosed with melatonin dissolved in water (0.5 mg/kg) (Gultekin *et al.*, 2006), while those in group III were given CPF only (8.5 mg/kg or $1/10^{th}$ of the LD_{50}). Rats in group IV were pretreated with melatonin (0.5 mg/kg), and CPF (8.5 mg/kg) was administered after 10 min. All the regimens were administered orally by gavage once daily for a period of 28 days. During the period, rats were observed for toxic signs and death. In addition, weekly body weight changes were recorded using a digital weighing balance (Citizen Scales (1) PVT. Ltd).

At the end of the study period, the rats were sacrificed by jugular venesection after light ether anaesthesia and blood samples were collected from each rat into test tubes. Thereafter, the blood was allowed to clot. The samples were then centrifuged at 800 × g for 10 min to obtain the serum samples used for analysis of testosterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroxine (T_4), triiodothyronine (T_3) and thyroid-stimulating hormone (TSH).

3.5 Effect of Treatments on Testicular Tissue and Pituitary Gland Lipoperoxidation

The level of thiobarbituric acid reactive substance, malonaldehyde (MDA) as an index of lipid peroxidation was evaluated in the testicular tissue and pituitary gland 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 MDA. The testes and pituitary gland from each animal in all the groups were rapidly removed, weighed using a standard weighing scale and then homogenized in a known volume of ice-cold phosphate buffer to obtain a 10% homogenate, which was then centrifuged at 6,000 x g for 10 minutes to obtain the supernatant. The supernatant was then used to assess the level of protein and MDA in the samples. Briefly, MDA concentration in the supernatant of testicular and pituitary gland homogenates were evaluated as follows: 2.5 ml of 100 g/L trichloroacetic acid solution was added to 0.5 ml of supernatant from both the testicular and pituitary gland homogenate in a centrifuge tube and placed in a boiling water bath for 15 minutes. After cooling under tap water for 5 minutes, the mixtures were then centrifuged at 1,000 x 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 (100^{o}C) water bath for 15 min. The solution was cooled under tap water and the absorbance measured using a UV spectrophotometer at 532 nm. The MDA concentration in both the testes and pituitary gland was calculated by the absorbance coefficient of MDA-TBA complex 1.56 x 10^{5}/cm and expressed in nmol/mg of protein. The protein content of the sample was determined using the Lowry method (1952).

3.6 Determination of Concentration of Follicle-Stimulating Hormone, Luteinizing Hormone and Testosterone

The concentrations of serum FSH, LH and testosterone were assayed using enzyme immunoassay (EIA) ELISA kit (Syntron Bioresearch Inc., Carlsbad, U.S.A.) as stated by the manufacturer.

3.7 Determination of Antioxidant Enzymes in Testicular and Pituitary Glands

The testes and pituitary glands were carefully collected and homogenized and 1% phosphate-buffered solution was added. The samples were collected and stored. Catalase and superoxide dismutase activities were measured using commercial kits (Northwest Diagnostics of Virginia, U.S.A.).

3.8 Determination of Serum Thyroid Hormones

Thyroid hormones (T_3, T_4 and TSH) concentrations were assayed using enzyme immunoassay (EIA) kit (Syntron Diagnostics, U.S.A.).

3.9 Evaluation of the Effect of Treatments on Pituitary Gland and Testicular Acetylcholinesterase Activity

Acetylcholinesterase activity was determined by the method of Ellman *et al*. (1961), using acetylthiocholine iodide as a substrate. Briefly, the pituitary gland and testes of each animal were weighed using the mettle weighing balance (Mettler® P161, Mettler instrument AG, CH806, Geifensee, Zurich, Switzerland) and then homogenized in cold (0–4 °C) 20 mM phosphate-buffered saline, incubated with 0.01M 5,5-dithio-bis(2-nitrobenzoic acid) in 0.1 M phosphate-buffered saline, pH 7.0. Incubations were allowed to proceed at room temperature for 10 min. Then, acetylthiocholine iodide (0.075 M in 0.1 phosphate-buffered saline, pH 8.0) was added to each tube, and absorbance at 412 nm was measured

continuously for 30 min. Each organ in every group was prepared in triplicate and acetylcholinesterase activity calculated based on the rate of colour change per minute, using the extinction coefficient of 1.36×10^4 expressed as nanomoles/min/mg protein.

3.10 Determination of Some Sperm Characteristics and Epididymal Sperm Count

The testes were removed, and semen carefully collected from the caudal epididymis onto a slide. Percentage of mass motile spermatozoa was assessed immediately under light microscope at x 100 as described by Rota *et al.* (1995). The epididymal sperm count was evaluated using the right caudal epididymis, which was carefully removed, dried out with blotting paper and immediately homogenized in 1 ml of 0.5% formol saline. 1 ml of the aliquot was diluted at 1:200 using erythrocyte diluting pipette. Counting was done using an improved Neubaeur counting chamber under light microscope at the magnification of x 400 as described by Yarube *et al*. (2009). Sperm morphology was assessed after staining the semen with eosin-nigrosin as described by Michael *et al*. (2008).

3.11 Gross and Histopathological Examinations

Postmortem gross examination was conducted, and the observed lesions recorded. Tissue sections of pituitary and testicular glands were fixed in 10% Bouins solution and processed as described by Luna (1960) and the lesions observed microscopically were recorded. Sections of the liver, kidneys, pancreas, thyroid and adrenal glands were also processed and stained with Haematoxylin and Eosin (H and E).

3.12 Statistical Analysis

Values obtained were expressed as mean ± SEM and subjected to statistical analysis using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison

test. Graphpad Software, San Diego, California, USA (www.graphpad.com) was used for the analyses. Values of $P < 0.05$ were considered significant

CHAPTER 4 RESULTS

4.1 Determination of Median Lethal Dose (LD_{50}) for Chlorpyrifos

The rats in all the groups died within the 48-hour period of monitoring. Prior to their death, they showed signs of toxicity, which included hyperexcitability, mild muscle tremors, arching of the back, pilorection, increased urination, lacrimation, dyspnea, weakness and death. Based on this result, a second phase of this study was carried out. It comprised of three groups of one rat each. The animals were dosed with CPF at 100 mg/kg, 90 mg/kg and 80 mg/kg respectively. The result is shown in Table 4.1.

Based on the dose-response, the LD_{50} was calculated based on the method of Lorke (1983) as,

$$\frac{90 + 80}{2} = 85 \text{ mg/kg.}$$

Therefore, the LD_{50} was 85 mg/kg.

Table 4.1: Summary of mortality pattern in phase two of the median lethal dose determination

Dose	No of rats used	No of death / No of rats used
100 mg/kg	1	1/1
90 mg/kg	1	1/1
80 mg/kg	1	0/1

4.2 Subacute Reproductive Toxicity Study

4.2.1 Effect of subacute administration of soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin on testicular malonaldehyde (MDA) concentration

There testicular MDA concentration in the CPF group was significantly higher ($P < 0.01$) when compared to S/oil, Melatonin or Melatonin+CPF group. There was no significant change ($P > 0.05$) in the MDA concentration in the Melatonin+CPF group when compared to either S/oil or Melatonin group (Figure 4.1)

4.2.2 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on pituitary gland malonaldehyde concentration

There was a significant increase ($P < 0.01$) in the concentration of testicular MDA in the CPF group when compared to S/oil, Melatonin or Melatonin+CPF group. There was no significant change ($P > 0.05$) in the MDA concentration in the Melatonin+CPF group when compared to either S/oil or Melatonin group (Figure 4.2).

4.2.3 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on testicular superoxide dismutase activity

The effect of subacute exposure to soya oil, CPF and/or melatonin on testicular SOD level is shown in Figure 4.3. There was no significant change ($P > 0.05$) in activity level of SOD between the groups. However, CPF group had the lowest concentration decreasing by 11% compared to the S/oil group. The Mel+CPF group showed a comparatively higher (6%) testicular SOD activity level compared to the CPF (17%) and S/oil (5%) group but was comparatively lower (11%) than the Mel group.

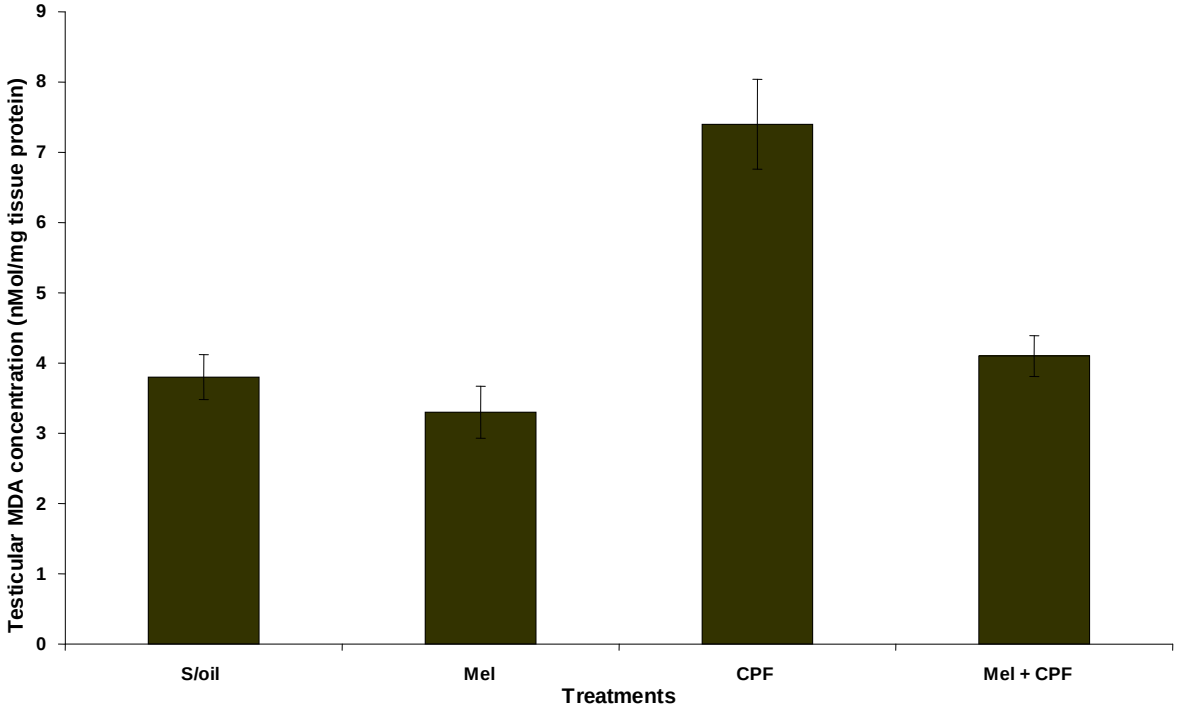


Figure 4.1: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlor pyrifos (CPF) on testicular malonaldehyde (MDA) concentration in Wistar rats (n=5)

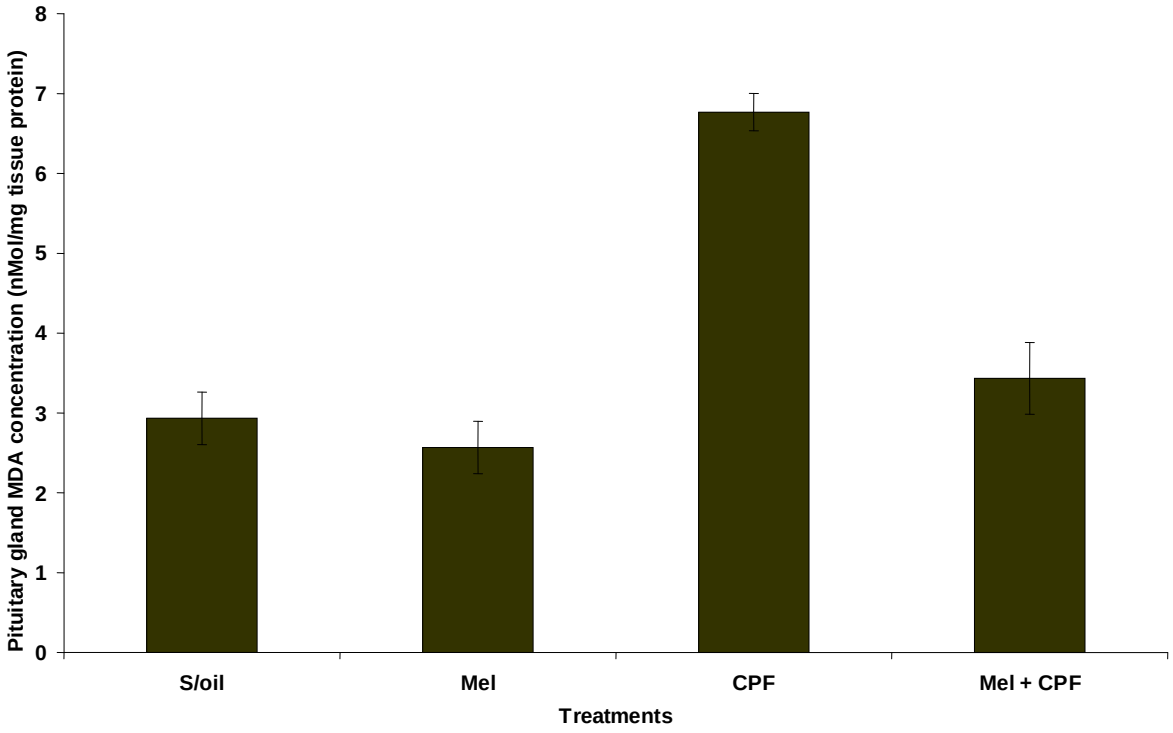


Figure 4.2: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on pituitary gland malonaldehyde (MDA) concentration in Wistar rats (n=5)

4.2.4 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on pituitary gland superoxide dismutase activity

There was no significant change ($P > 0.05$) in pituitary gland activity level of superoxide dismutase between the groups. However, CPF group had the lowest SOD activity decreasing by 36% compared to the S/oil group. The Mel + CPF group showed a comparatively higher (18%) pituitary gland SOD activity compared to the CPF group but was comparatively lower than those in S/oil (32%)and Mel (32%) groups (Figure 4.4).

4.2.5 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on testicular catalase activity

The effect of treatments on testicular CAT activity level is shown in Figure 4.5. There was a significant change ($P < 0.05$) in testicular activity of CAT in CPF groups when compared to the Mel group, but not significant ($P > 0.05$) when compared to S/oil or Melatonin + CPF group. However, CPF group had the lowest CAT activity decreasing by 27% compared to the S/oil group. The Mel + CPF group showed a comparatively higher testicular catalase level (26%) compared to the CPF group but it was comparatively lower than those in S/oil (2%) and Mel (11%) groups.

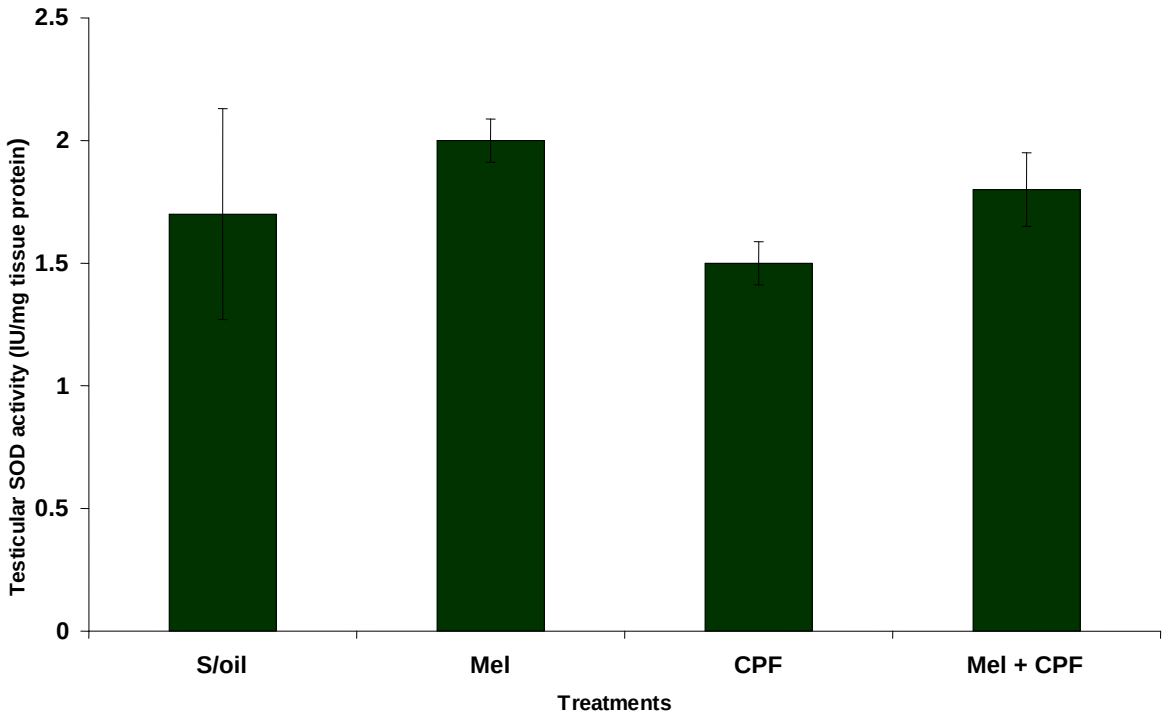


Figure 4.3: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on testicular superoxide dismutase (SOD) activity in Wistar rats (n=5)

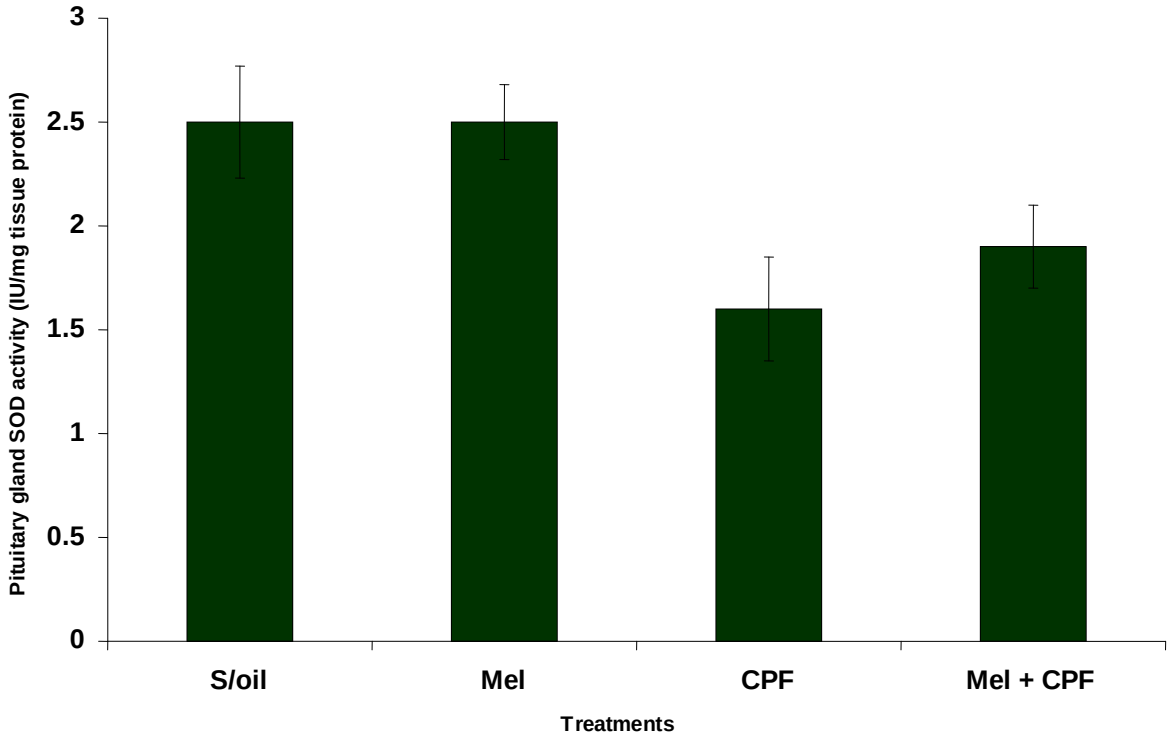


Figure 4.4: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on pituitary gland superoxide dismutase (SOD) activity in Wistar rats (n=5)

4.2.6 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on pituitary gland catalase activity

There was no significant change ($P > 0.05$) in pituitary gland CAT activity between the groups. However, CPF group had the lowest concentration decreasing by 7% compared to the S/oil group. The Mel + CPF group showed a comparatively higher pituitary gland CAT activity (4%) compared to the CPF group but it was comparatively lower than that of the S/oil group (4%) and the same with that of the Mel group (Figure 4.6).

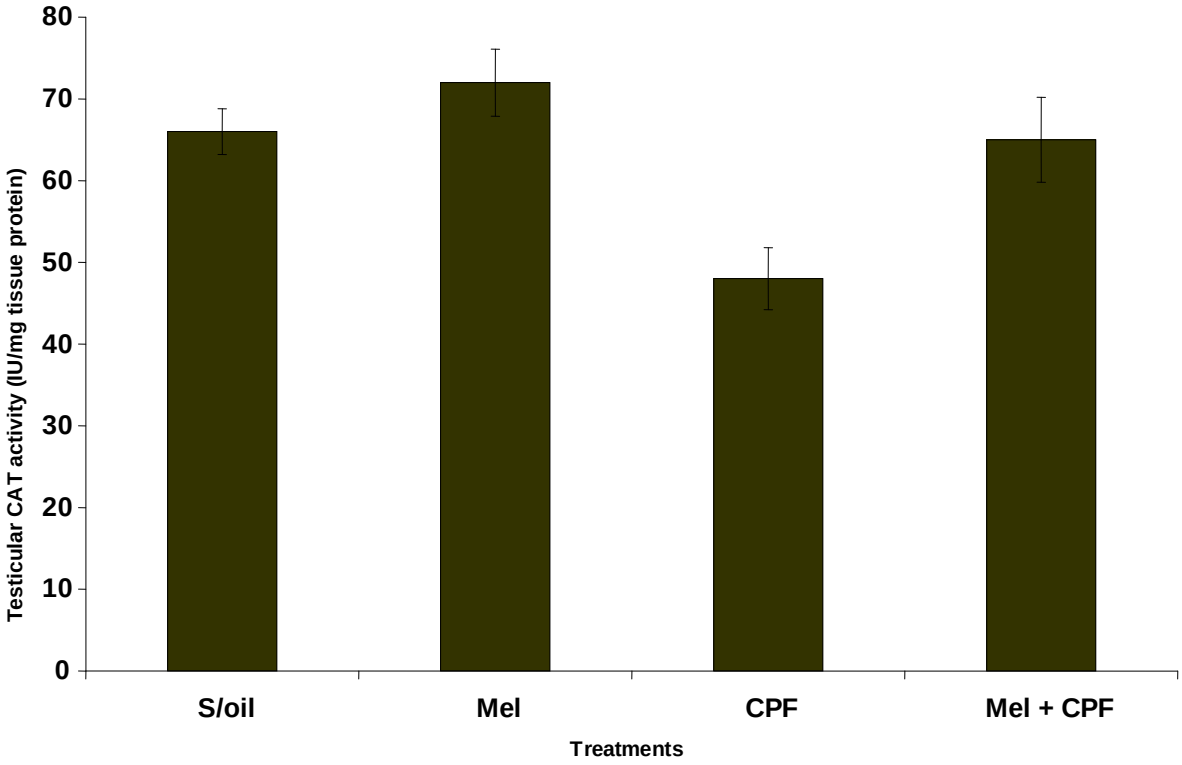


Figure 4.5: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on testicular catalase (CAT) activity in Wistar rats (n=5)

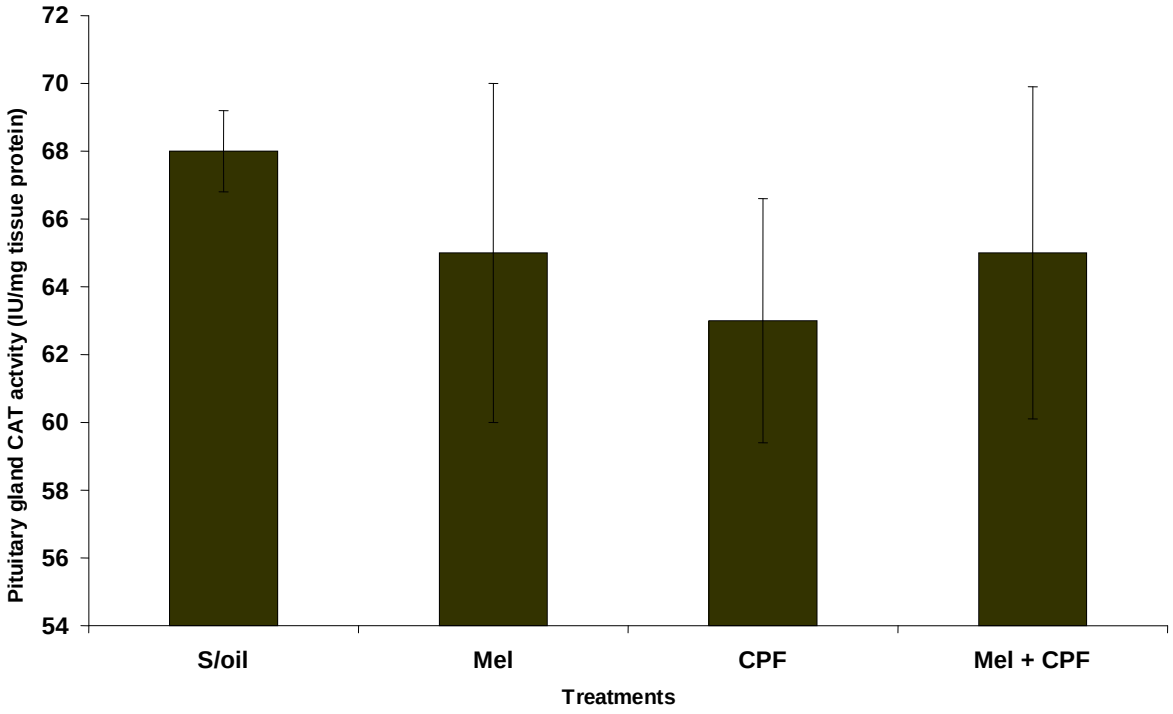


Figure 4.6: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on pituitary gland catalase (CAT) activity in Wistar rats (n=5)

4.2.7 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on testicular acetylcholinesterase activity

There was no significant change ($P > 0.05$) in testicular AChE activity between the groups. However, CPF group had the lowest activity decreasing by 12% compared to the S/oil group. The Mel + CPF group showed a comparatively higher testicular AChE activity compared to the CPF (34%), Mel (19%) and S/oil (26%) groups (Figure 4.7)

4.2.8 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on pituitary gland acetylcholinesterase activity

The effect of subacute exposure to soya oil, CPF and/or melatonin on pituitary gland acetylcholinesterase activity is shown in Figure 4.8. There was no significant change ($P > 0.05$) in pituitary gland AChE activity between the groups. However, CPF group had the lowest concentration decreasing by 22% compared to the S/oil group. The Mel + CPF group showed a comparatively higher pituitary gland AChE activity compared to the CPF (34%), Mel (2%) and S/oil (16%) groups (Figure 4.8)

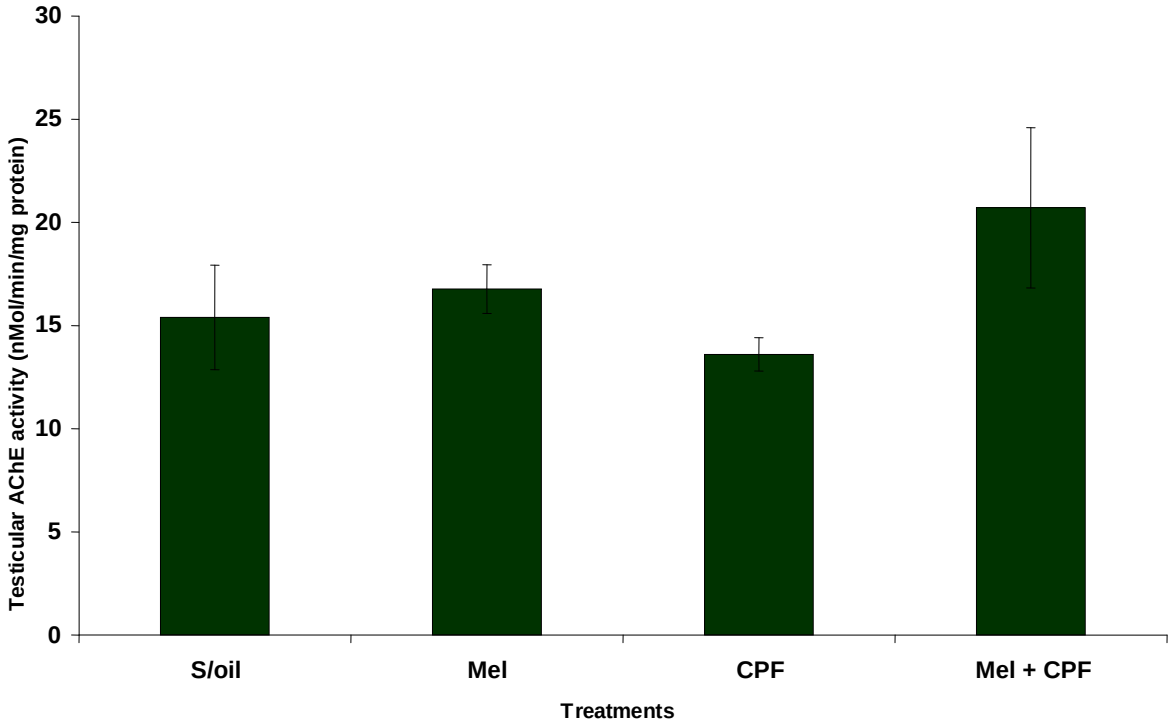


Figure 4.7: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on testicular acetylcholinesterase (AChE) activity in Wistar rats (n=5)

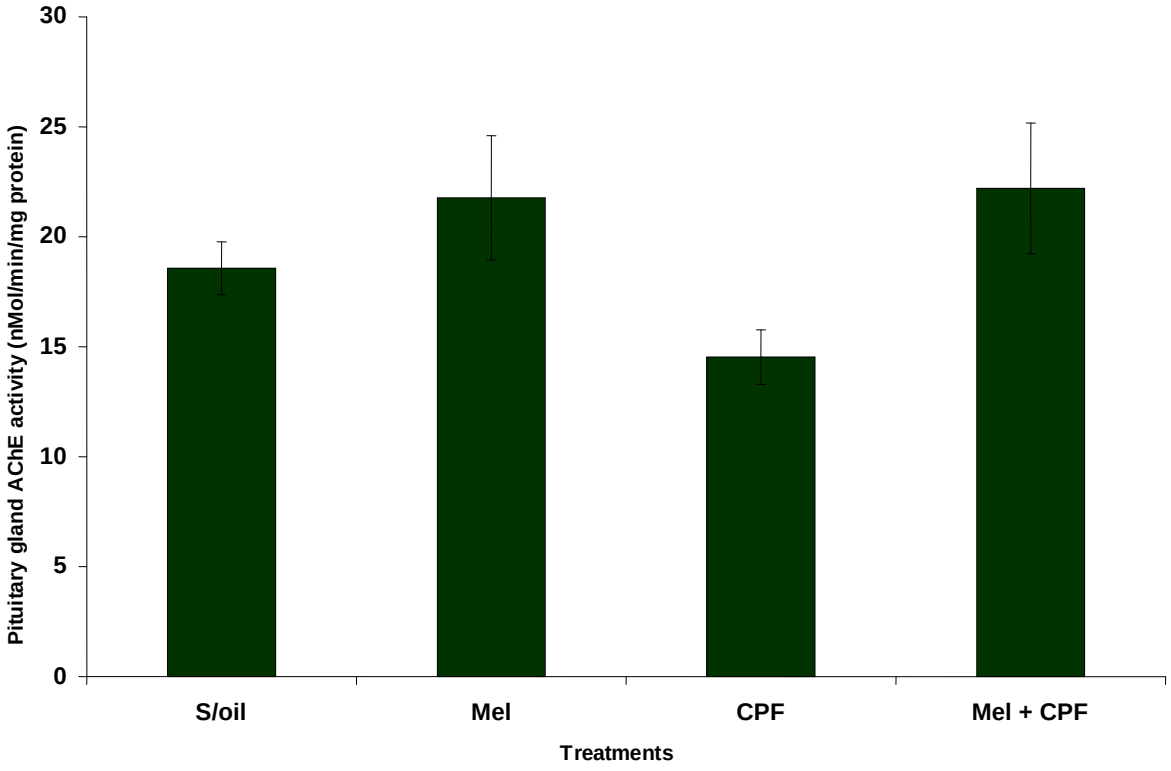


Figure 4.8: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on pituitary gland acetylcholinesterase (AChE) activity in Wistar rats (n=5)

4.2.9 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on follicle-stimulating hormone

The effect of subacute exposure to soya oil, CPF, and/or melatonin on serum FSH concentration is shown in Figure 4.9. There was no significant change ($P > 0.05$) in FSH concentration between the groups. However, Melatonin group had the highest concentration increasing by 20% compared to the S/oil. However, Mel + CPF group showed a comparatively lower concentration (11%) compared to the Melatonin group (20%) but it was comparatively higher than those in the CPF group (5%).

4.2.10 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on luteinizing hormone

The effect of subacute exposure to soya oil, CPF and/or melatonin on serum LH concentration is shown in Figure 4.10. There was no significant change ($P > 0.05$) in luteinizing hormone concentration between the groups. However, CPF group had the lowest concentration decreasing by 11% compared to the S/oil group. The Mel + CPF group showed a comparatively higher serum LH concentration (17%) compared to the CPF group and even when compared to those in S/oil and Mel group (13%).

4.2.11 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on testosterone

The effect of subacute exposure to soya oil, CPF and/or melatonin on serum testosterone concentration is shown in Figure 4.11. There was no significant change ($P > 0.05$) in testosterone concentration between the groups. However, CPF group had the lowest concentration decreasing by 16% compared to the s/oil group. The Mel + CPF group showed

a comparatively higher serum testosterone concentration (6%) compared to the CPF group but it was comparatively lower than those in the S/oil and Mel (15%) group.

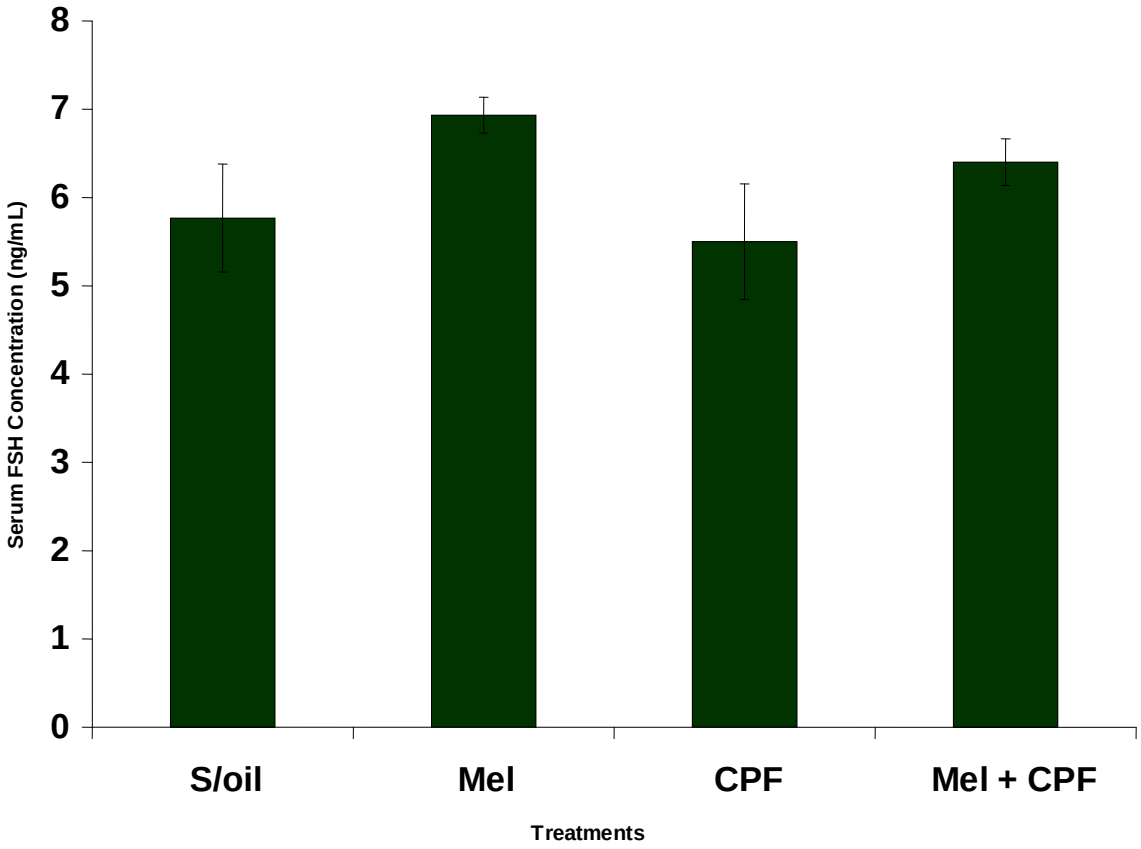


Figure 4.9: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on follicle stimulating hormone (FSH) concentration in Wistar rats (n=5)

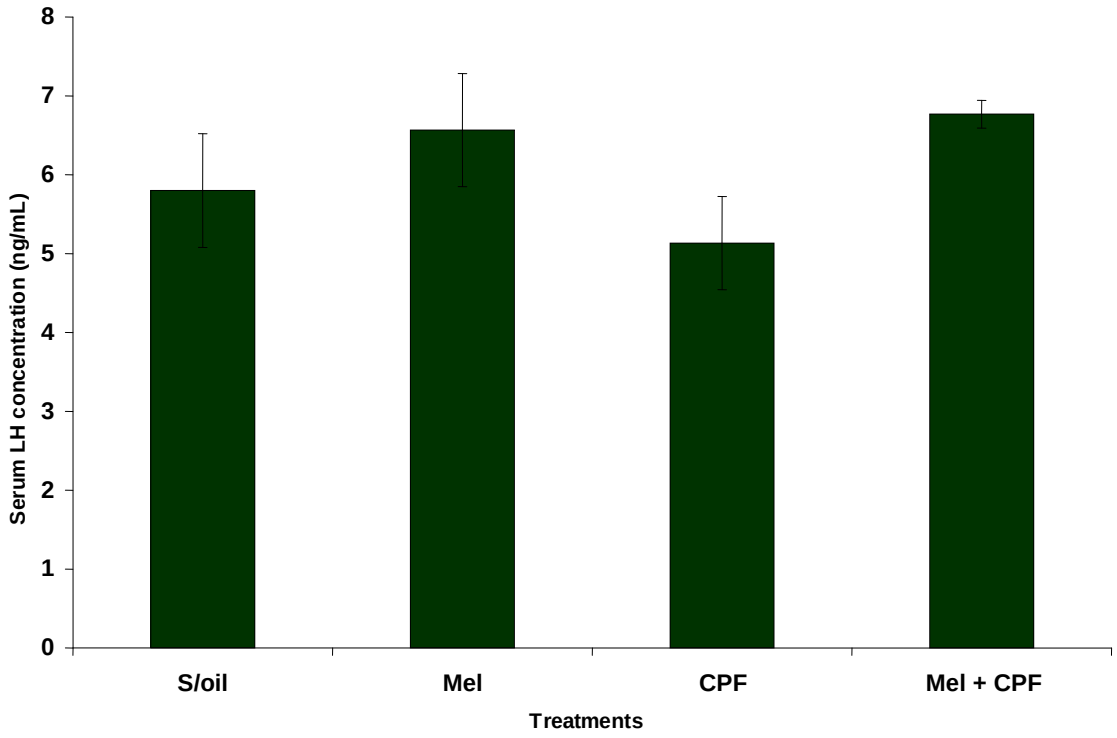


Figure 4.10: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on luteinizing hormone (LH) concentration in Wistar rats (n=5)

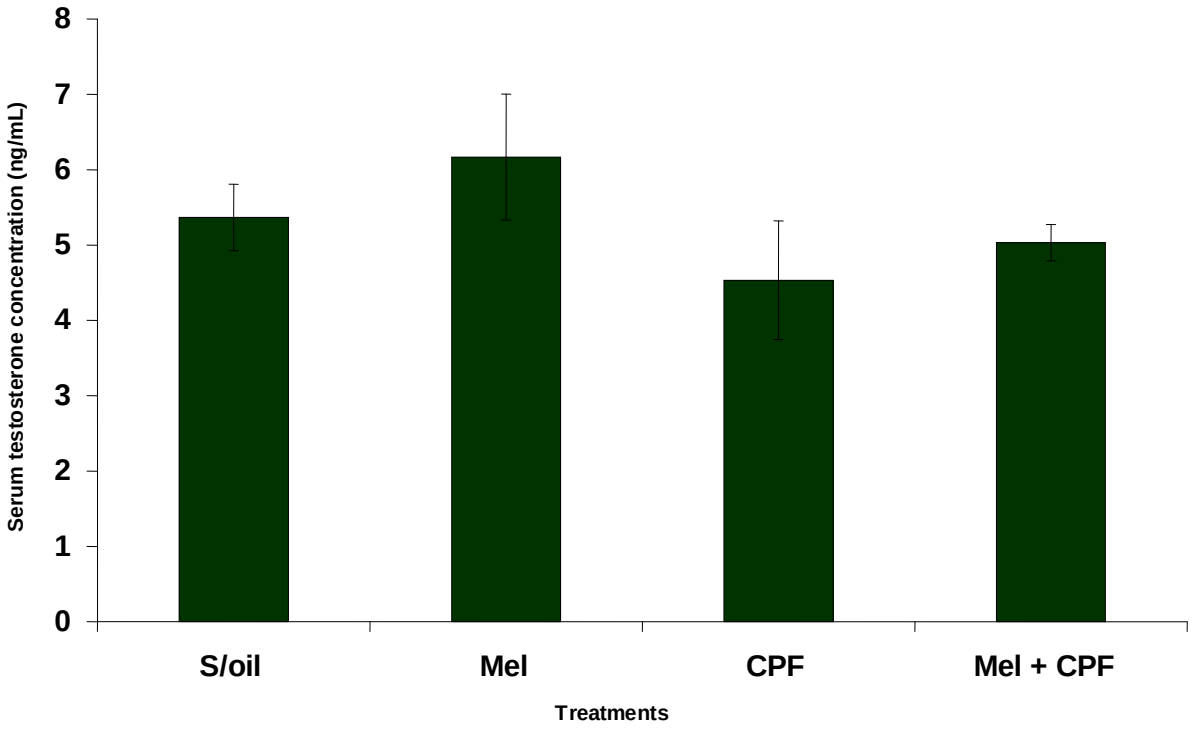


Figure 4.11: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on testosterone concentration in Wistar rats (n=5)

4.2.12 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on serum thyroid hormones

The effect of subacute exposure to soya oil, CPF and/or melatonin on serum T_3 concentration is shown in Figure 4.12. There was no significant change ($P > 0.05$) in the concentration of serum T_3 between the groups. However, the CPF (13%) and Mel + CPF (13%) group had a higher concentration compared to that of the Mel group (24%) and a comparatively lower T_3 serum concentration to S/oil (15%).

There was a significant change ($P < 0.05$) in serum T_4 hormone concentration in the CPF treated group when compared to the Mel group. However, there was no significant change ($P > 0.05$) in serum T_4 concentration in the CPF group when compared to S/oil or Mel + CPF. However, CPF group had the lowest concentration decreasing by 18% compared to the S/oil group. The Mel + CPF group had a 4% increase in serum T_4 hormone concentration compared to the CPF and S/oil group, but was comparatively lower than that of the Mel group (22%) (Figure 4.13).

The effect of treatments on serum TSH hormone concentration is shown in Figure 4.14. There was no significant change ($P > 0.05$) in serum TSH concentration between the groups. However, CPF group had a lower concentration decreasing by 15% compared to the S/oil group. The Mel + CPF group showed a comparatively lower serum TSH concentration (16%) compared to the CPF (1%), S/oil (19%) and the Mel (14%) group.

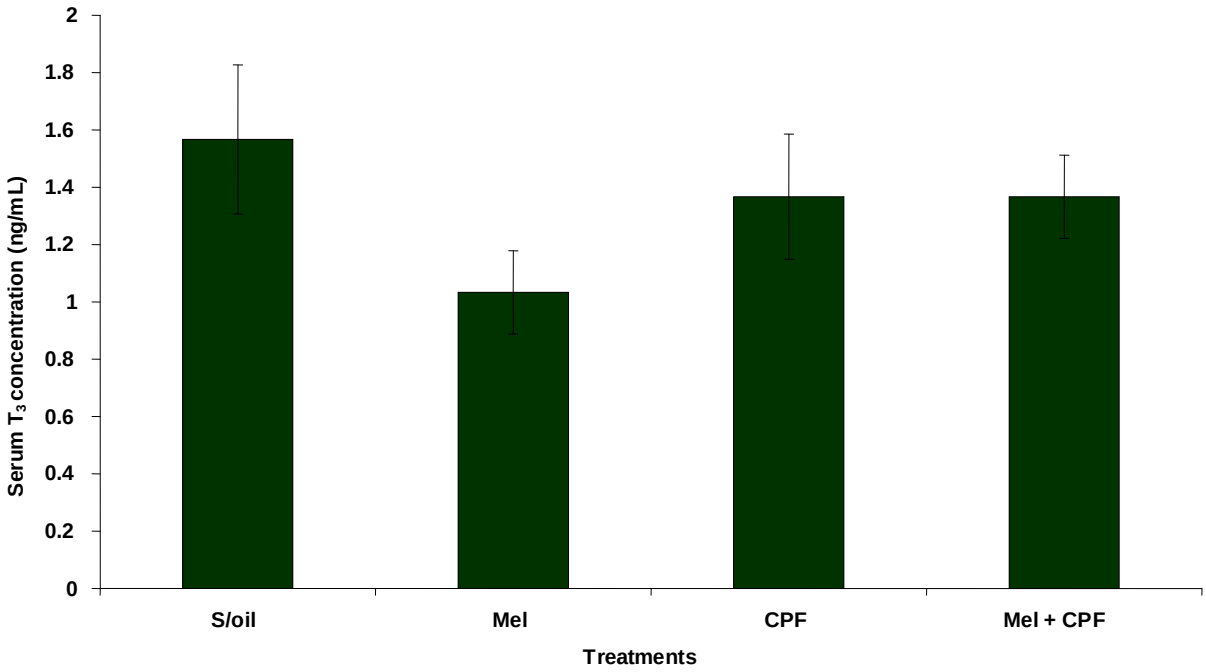


Figure 4.12: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on serum triiodothyronine (T_3) concentration in Wistar rats (n=5)

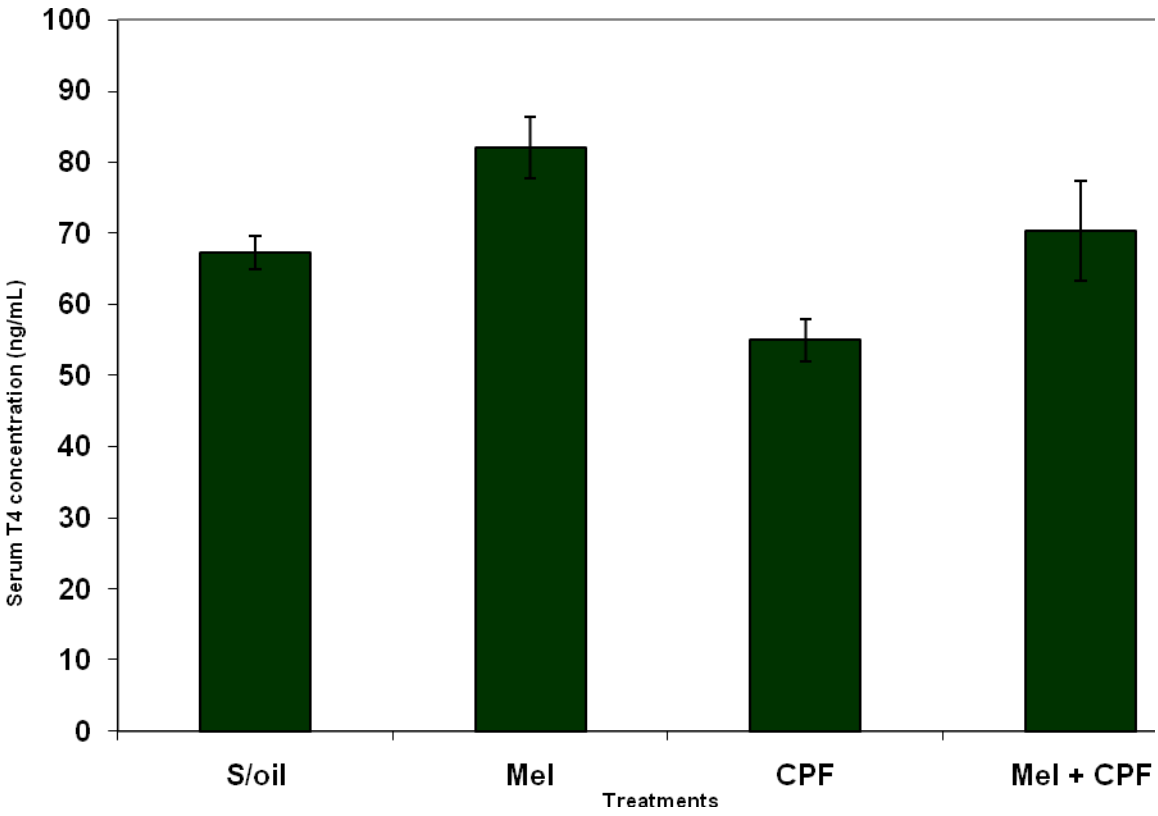


Figure 4.13: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on serum thyroxine (T_4) concentration in Wistar rats (n=5)

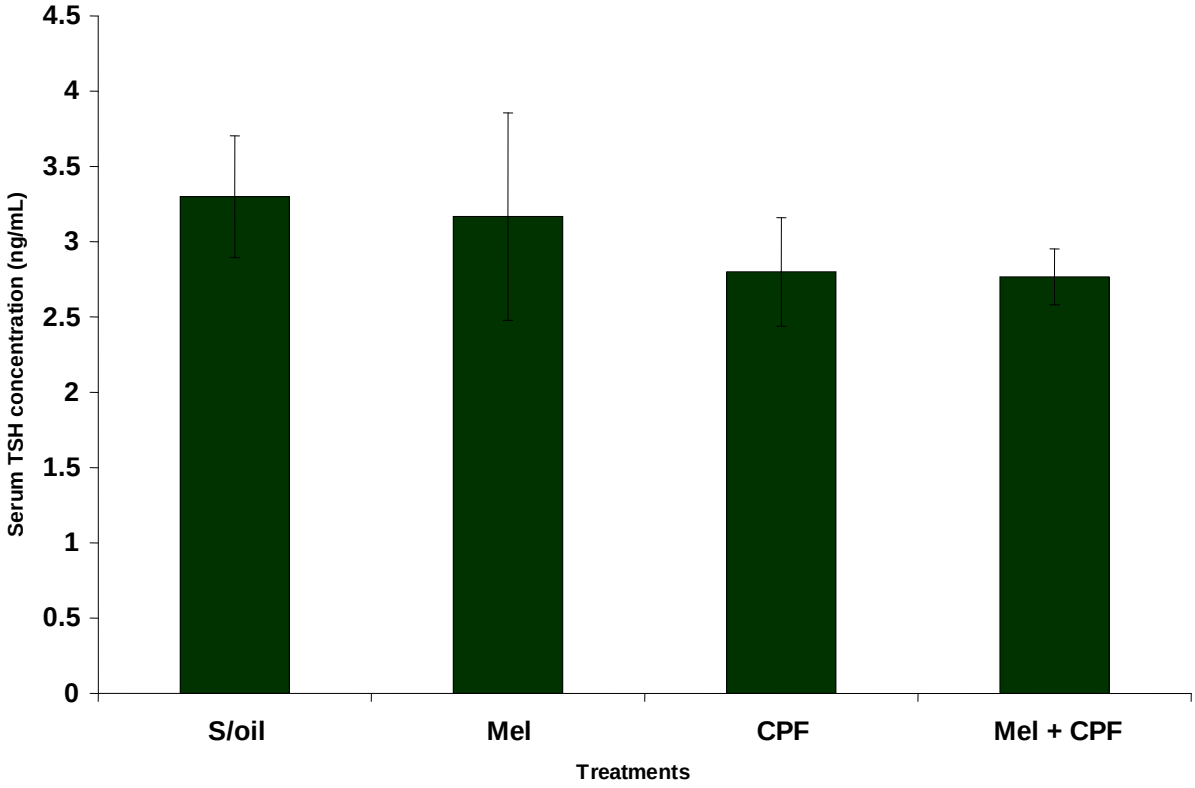


Figure 4.14: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on serum thyroid stimulating hormone (TSH) concentration in Wistar rats (n=5)

4.2.13 Evaluation of mean weekly body weight changes

The effect of treatments on weekly body weight changes is shown in Figure 4.15 and Figure 4.16. Rats in all the groups showed a progressive weight changes throughout the duration of the study. However, the body weight changes of rats in the CPF group were comparatively lower than the other groups. The cumulative body weight changes in the S/oil, Mel, CPF and Mel+CPF groups increased by 34%, 29%, 20% and 24% respectively (Figure 4.15).

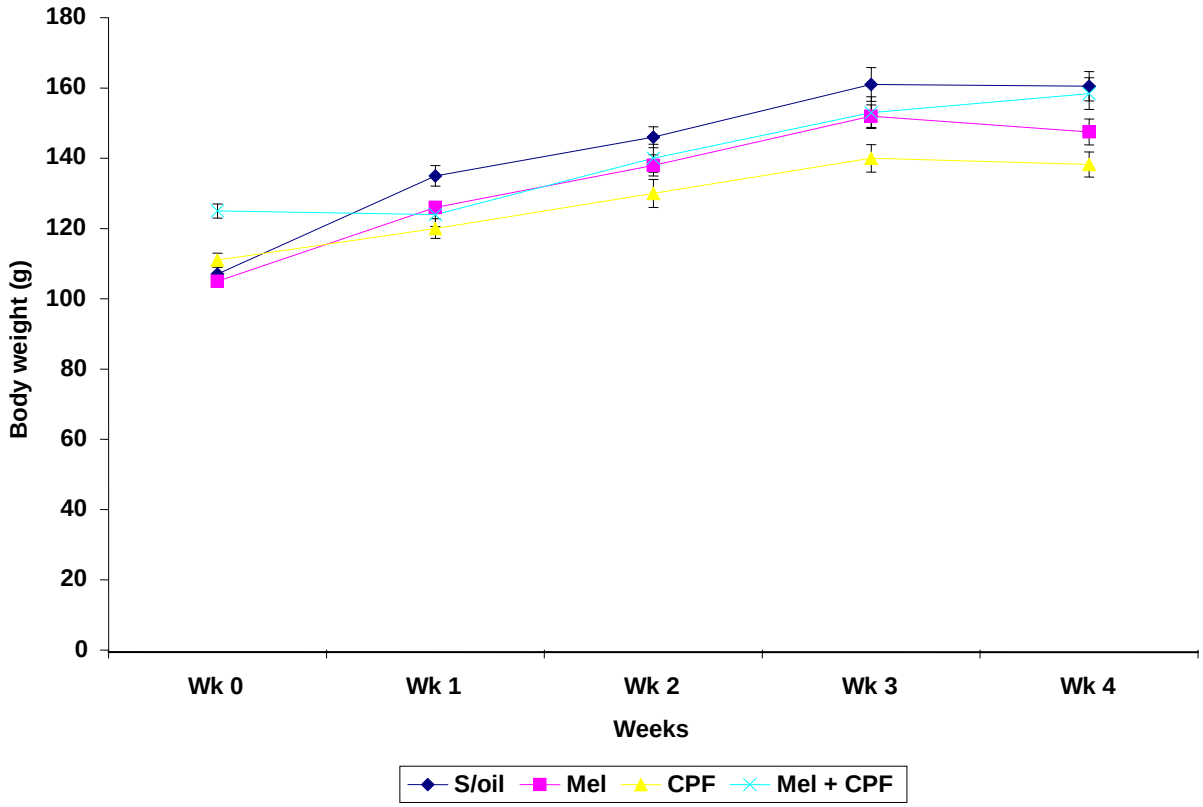


Figure 4.15: Dynamics of body weight changes in Wistar rats dosed with soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin (Mel) following subacute exposure

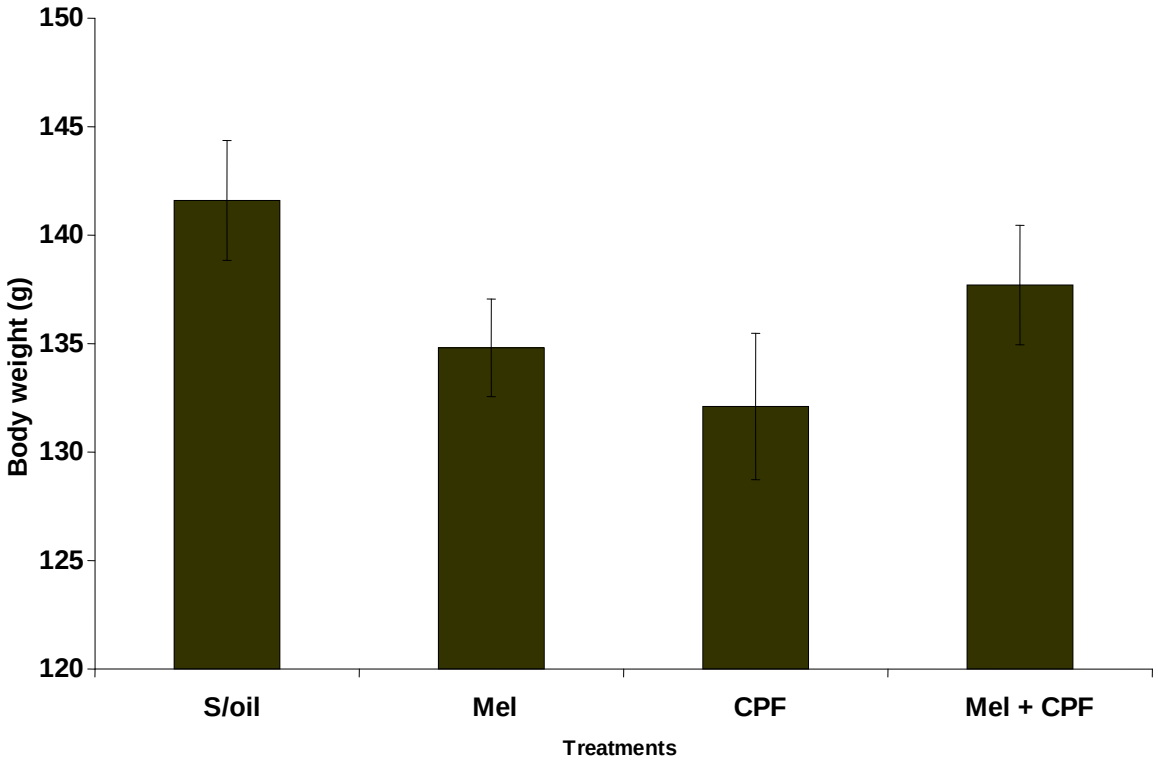


Figure 4.16: Cummulative body weight changes in Wistar rats dosed with soya oil (S/oil), chlorpyrifos (CPF) and/or melatonin (Mel) (n=5)

4.2.14 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on testicular weight

The effect of subacute exposure to soya oil, CPF and/or melatonin on testicular weight is shown in Figure 4.17. There was no significant change ($P > 0.05$) in testicular weight between the groups. However, CPF group had the lowest testicular weight decreasing by 22% compared to the S/oil group. The Mel + CPF group showed a comparatively higher testicular weight (22%), compared to the CPF group, with no difference when compared with the S/oil and Mel group.

4.2.15 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on sperm concentration

The effect of treatments on sperm concentration is shown in Figure 4.18. There was a significant decrease in sperm concentration in the CPF group when compared to either S/oil ($P < 0.01$), Mel ($P < 0.05$) or Mel + CPF group ($P < 0.05$). The CPF group had the lowest sperm concentration decreasing by 81% compared to the S/oil group. The Mel + CPF group showed a comparatively higher sperm concentration (31%) compared to the CPF group, but it was comparatively lower than that in the S/oil group and at *par* with the Mel group (31%).

4.2.16 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on sperm motility

The effect of subacute exposure to soya oil, CPF and/or melatonin on sperm concentration is shown in Figure 4.19. There was a significant decrease in sperm motility in CPF when compared to either S/oil ($P < 0.001$), Mel ($P < 0.01$) or Mel + CPF ($P < 0.01$) group. However, CPF group had the lowest sperm motility decreasing by 60% compared to the S/oil group. The Mel + CPF group showed a comparatively higher sperm motility (51%)

compared to the CPF group but it was comparatively lower than those in the S/oil (23%) and Mel (3%) group.

4.2.17 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on sperm morphology

Chlorpyrifos treated group had the least number of dead sperm cells and a relatively high number of live sperm cells, although they had the highest number of dead head and bent tail (morphological abnormalities) sperm cells when compared to the rest of the groups. The soya oil group had the least number of live sperm cells and the highest number of dead sperm cells. They also had the highest number of free tail and cut tail abnormalities when compared to the other groups as shown in Table 4.2.

4.2.18 Effect of subacute administration of soya oil, chlorpyrifos and/or melatonin on histopathological changes in wistar rats

Kidneys- The kidneys of rats in the S/oil and melatonin groups had no apparent lesions. Focal areas of necrosis of renal tubular epithelial cells and hemorrahages of the glomeruli and intertubular spaces, with collapse of renal tubules were observed in the kidneys of rats exposed to CPF only. Rats in Mel+CPF group also had mild necrosis of tubular epithelium and hemorrhages of intertubular spaces.

Liver: The liver of rats in the S/oil and melatonin groups had no apparent lesions. There was loss hepatic histoarchitecture and diffuse necrosis and vacuolation of hepatocytes, with hemorrhage into the hepatic parenchyma in the CPF group. The liver of rats in the Mel+CPF group showed dilated and congested sinusoid, with focal areas of necrosis of the hepatocytes, and mild hemorrhages into the liver parenchyma

Adrenal glands: There was congestion of adrenal medulla with hemorrhages in the *zona reticularis* of rats exposed to CPF only (Plate III). The adrenal glands of rats exposed to Mel+CPF were also congested but with milder hemorrhages in the *zona reticularis* (Plate IV).

Testis: There was no apparent lesion in the testis of rats in S/oil group (Plate V) while the melatonin group had degeneration of spermatogenic cells, hemorrhage and disruption of seminiferous tubules into the tubular lumen (Plate VI) and The rats in the CPF group had necrosis of primary spermatogenic cells, with reduced number of cell layers of spermatogenic cells and the seminiferous tubules were devoid of spermatids (Plate VII). The testicular tissue of rats in the Mel+CPF group was also congested, with reduced number of cell layers of spermatogenic cells and the seminiferous tubules have very few elongated spermatids (Plate VIII).

Brain- The brain of rats in the S/oil (Plate IX) and Melatonin (Plate X) groups had no apparent lesion. The rats in the CPF group showed degenerative changes in the neurons and the glial cells (Plate XI). The neurons and glial cells of rats in the Mel+CPF group had very few degenerative changes (Plate XII).

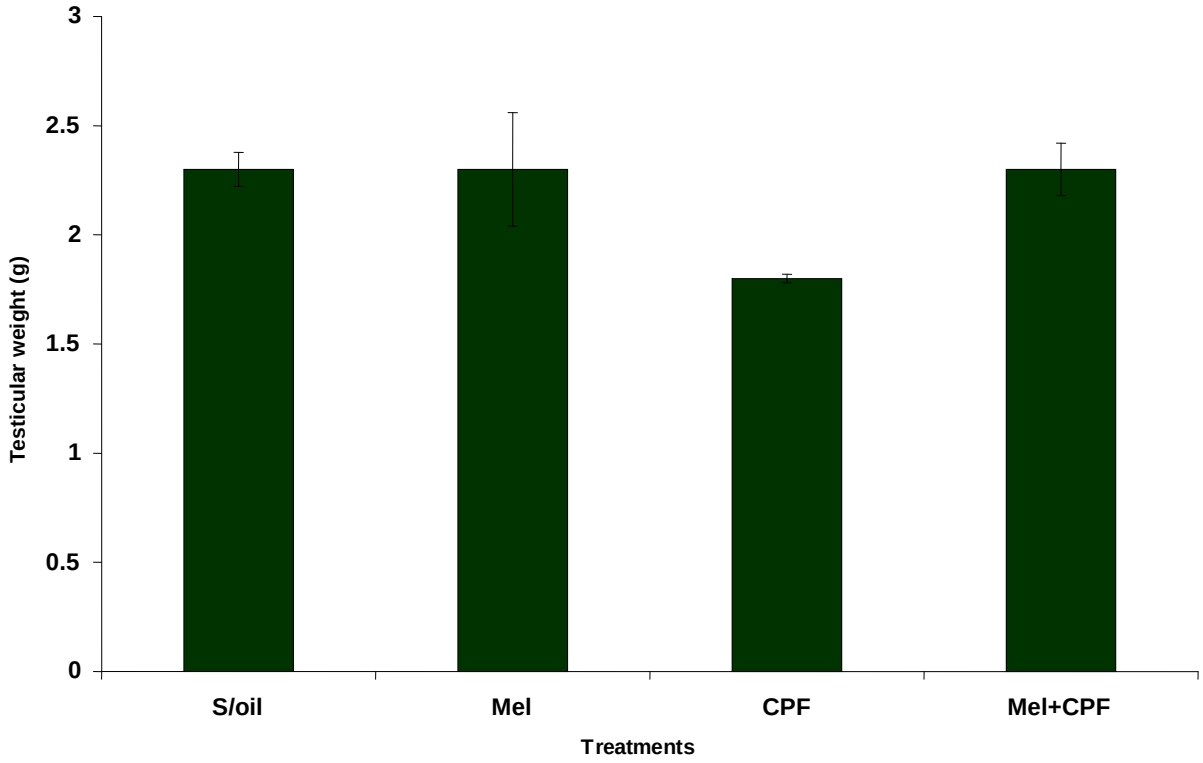


Figure 4.17: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on testicular weight in Wistar rats (n=5)

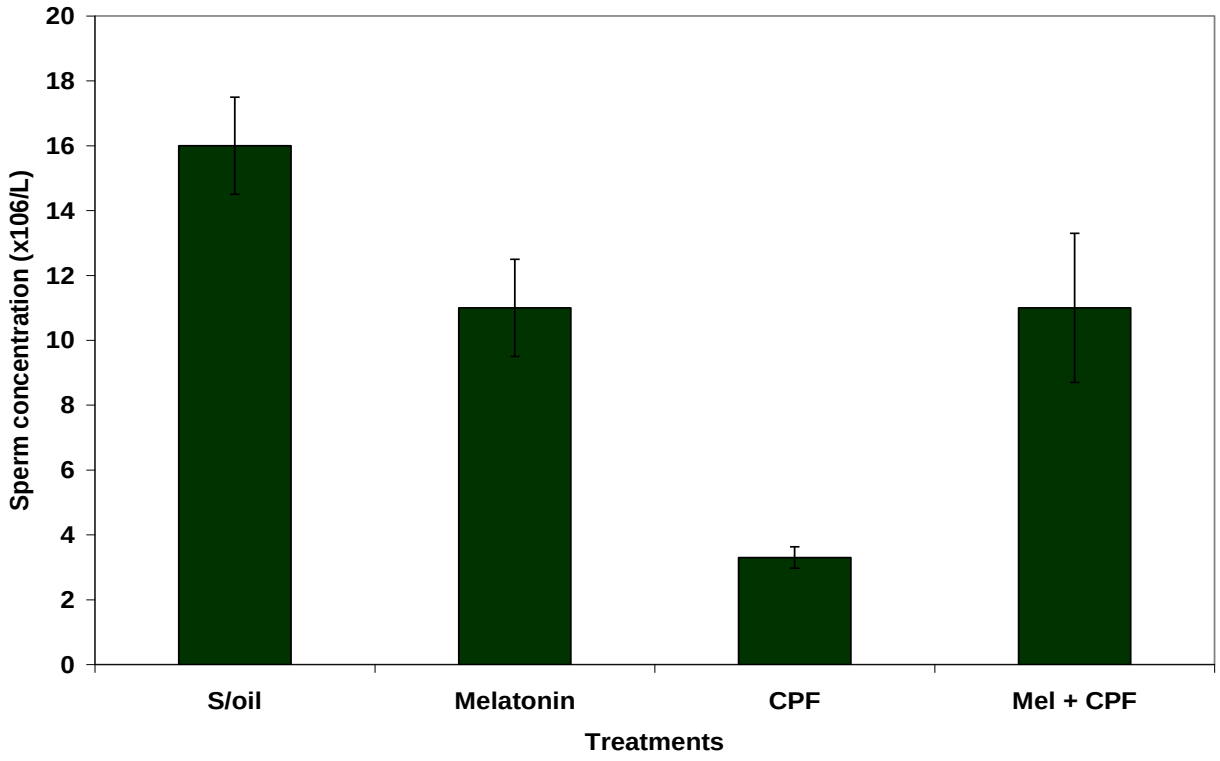


Figure 4.18: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on sperm concentration in Wistar rats (n=5)

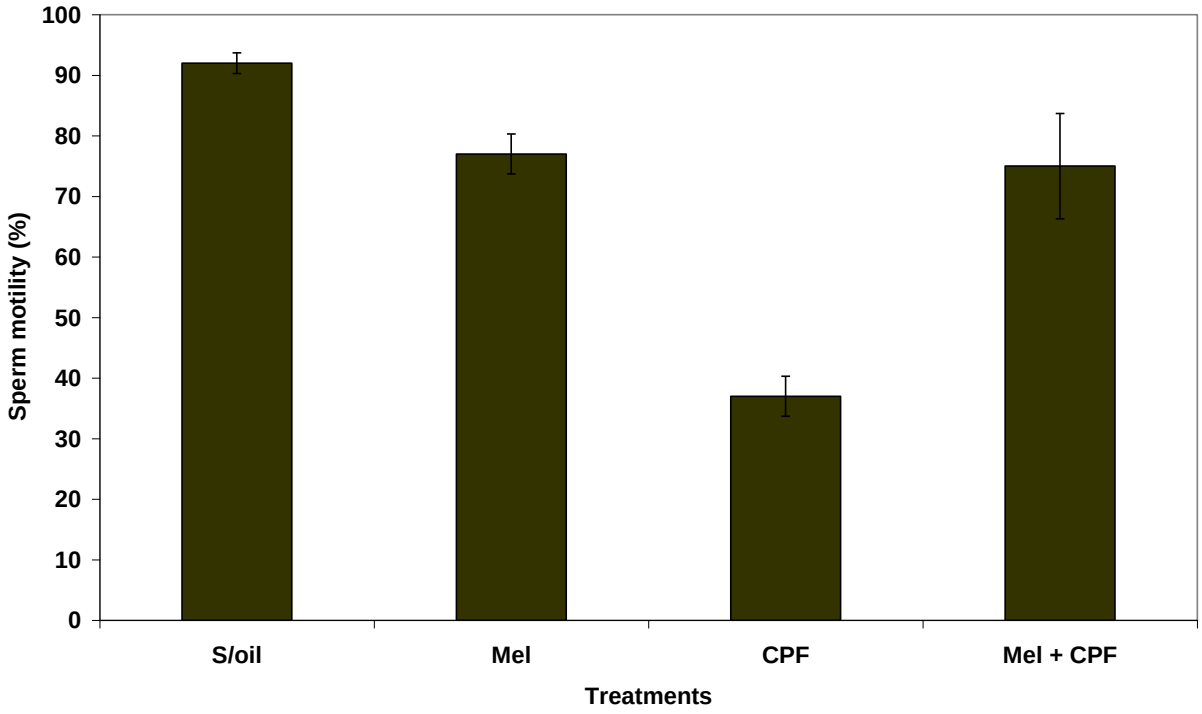


Figure 4.19: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on sperm motility in Wistar rats (n=5)

Table 4.2: Effect of subacute exposure to soya oil (S/oil), melatonin (Mel) and/or chlorpyrifos (CPF) on sperm morphology in Wistar rats

Parameters (%)	S/oil	Melatonin	CPF	Mel + CPF
Live sperm cells	79 ± 8.7	90 ± 2.3	88 ± 4.3	88 ± 8.0
Dead sperm cells	21±8.7	10±2.3	9.0±2.5	12±8.0
Dead head	11.00±1.2	13.33 ±2.4	21.67 ±1.5	20.67 ±1.5
Free tail	25.67± 5.4	18.67 ±4.1	23.67 ±3.8	19.50± 0.5
Cut tail	23 ± 7.0	20 ± 5.3	19 ± 1.5	15 ± 1.8
Bent tail	16.00 ± 1.7	13.33 ± 2.3	17.67 ± 2.4	10.67 ± 1.2

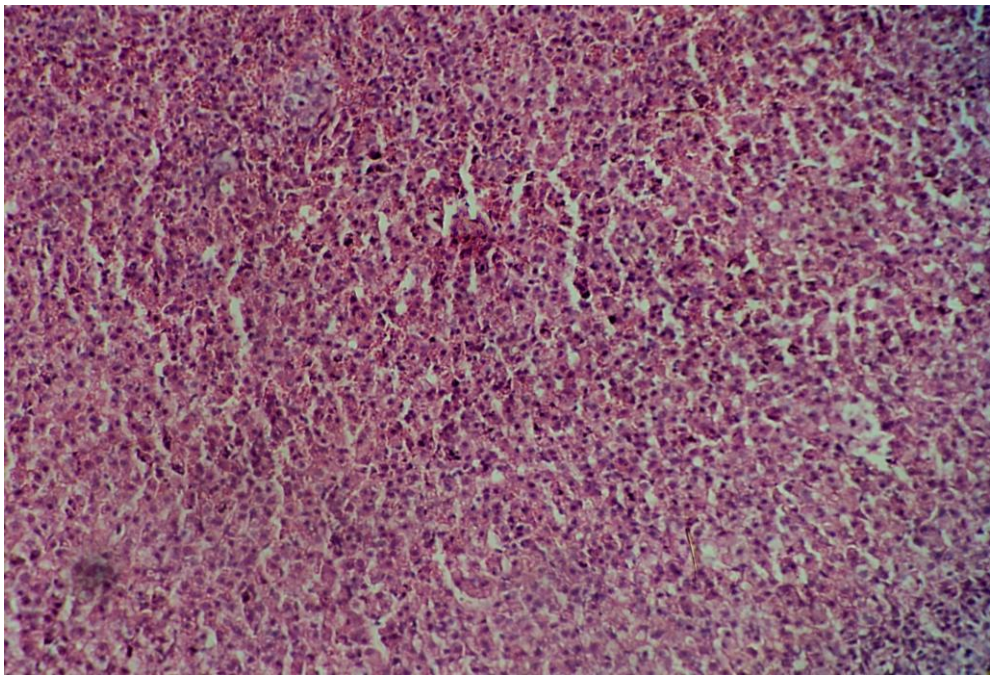


Plate I: Photomicrograph of a section of the adrenal gland of a Wistar rat exposed to subacute soya-oil administration showing no apparent histopathological lesion (H&E x 400)

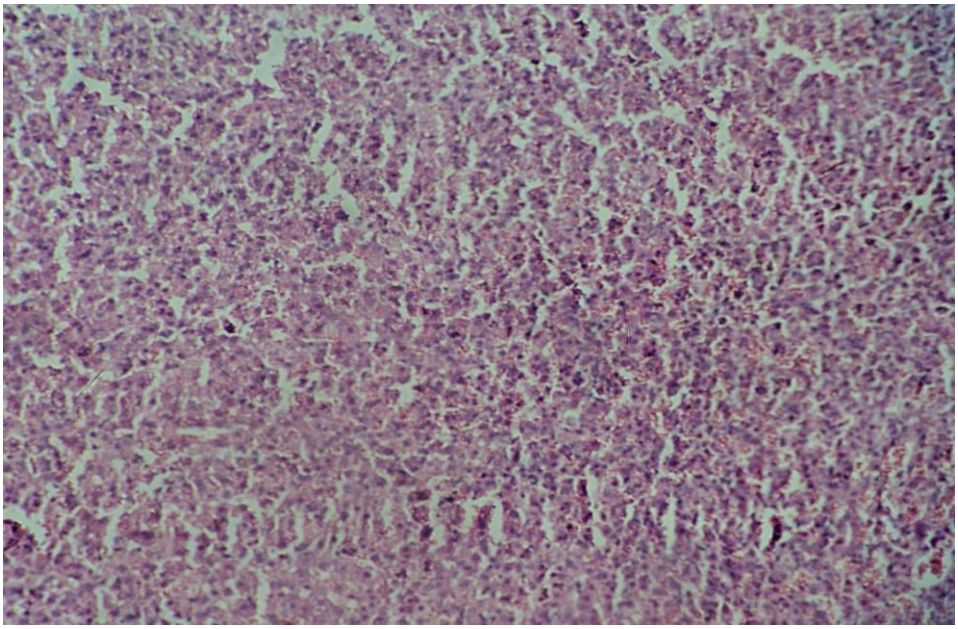


Plate II: Photomicrograph of a section of the adrenal gland of a Wistar rat exposed to subacute melatonin administration showing no apparent histopathological lesion (H&E x 400)

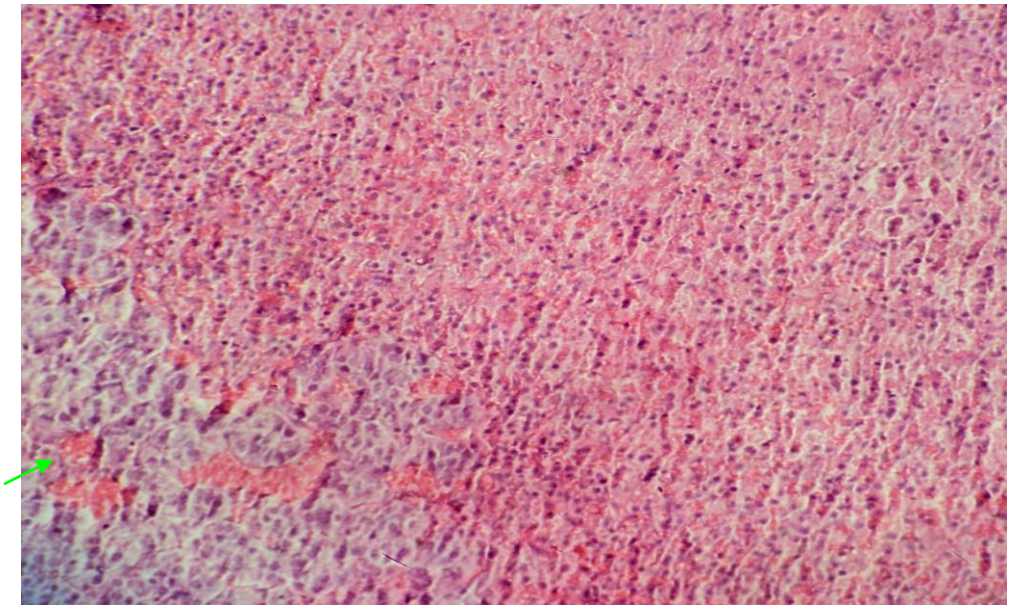


Plate III: Photomicrograph of a section of the adrenal gland of a Wistar rat exposed to subacute chlorpyrifos administration showing hemorrhages in the zona reticularis (arrow) (H&Ex 400)

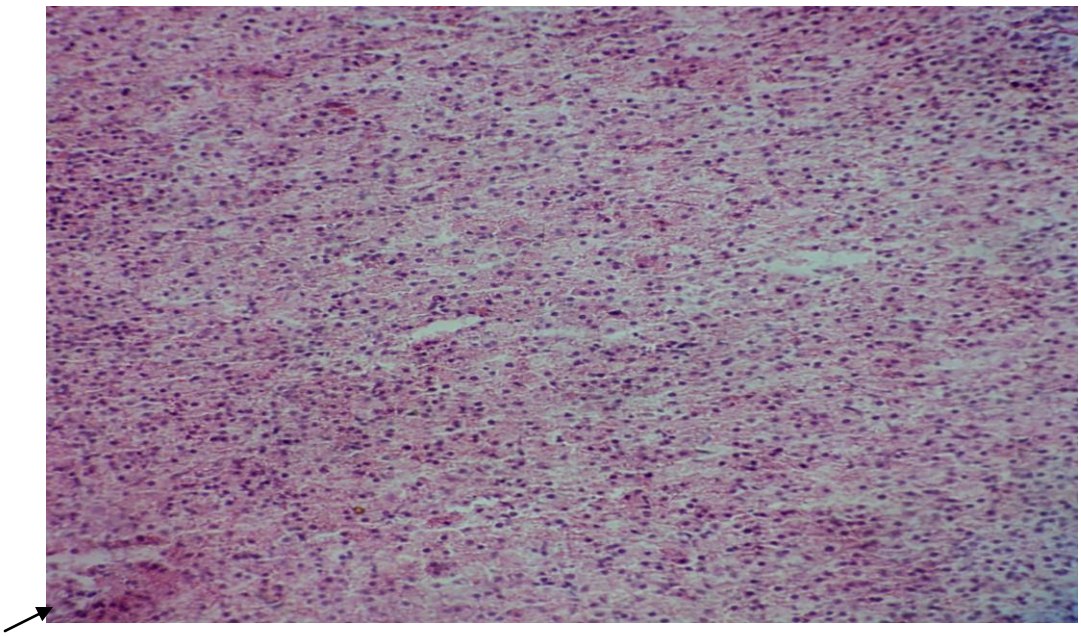


Plate IV: Photomicrograph of a section of the adrenal gland of a Wistar rat exposed to subacute melatonin+chlorpyrifos administration showing mild haemorrhages in the *zona reticularis* (arrow) (H&E x 400).

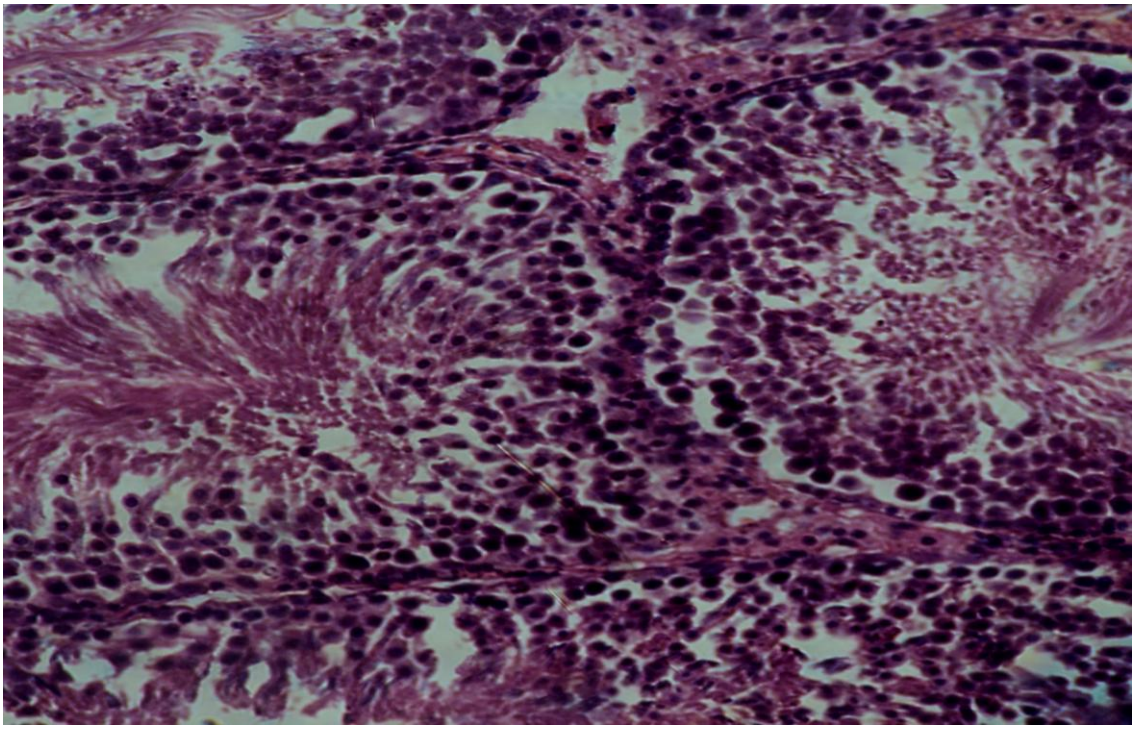


Plate V: Photomicograph of a section of the testis of a Wistar rat exposed to subacute soya oil administration showing no apparent histopathological lesion (H&E x 400).

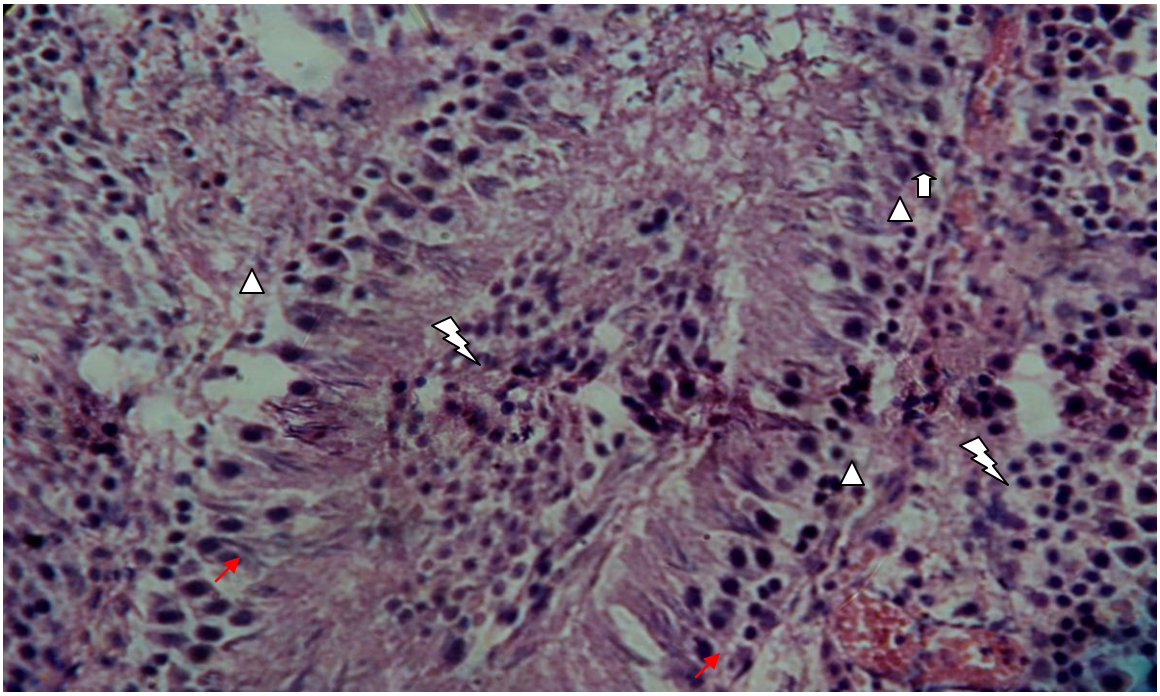


Plate VI: Photomicrograph of a section of the testis a Wistar rat exposed to subacute melatonin administration showing necrosis of spermatogenic cells (line arrows), disruption of spermatogenic cells and movement into the lumen (thunderbolts), loss of integrity of the seminiferous tubules (triangular arrows) and haemorrhage in intertubular spaces (block arrows) (H&E x 400)

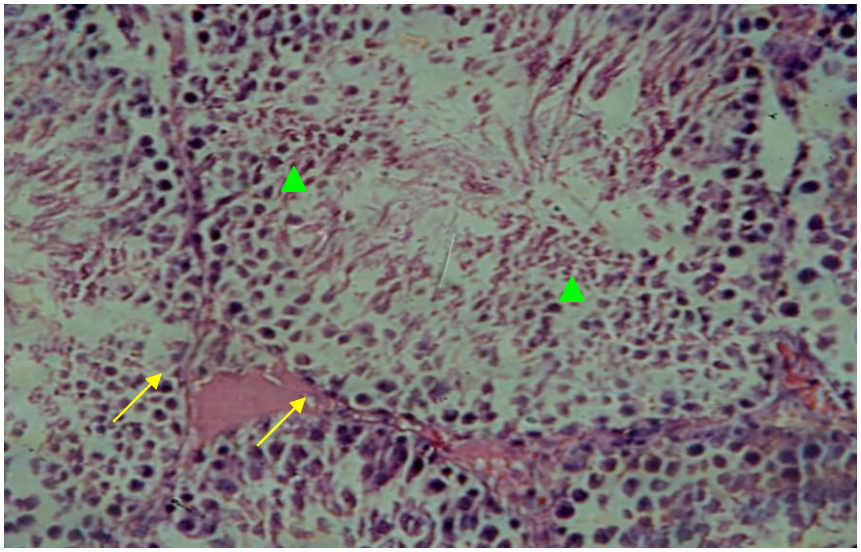


Plate VII: Photomicrograph of a section of the testis of a Wistar rat exposed to subacute chlorpyrifos administration showing necrosis of spermatogenic cells (reduction in cell layers) (line arrows) with seminiferous tubules devoid of spermatids (triangular arrow) (H&E x 400)

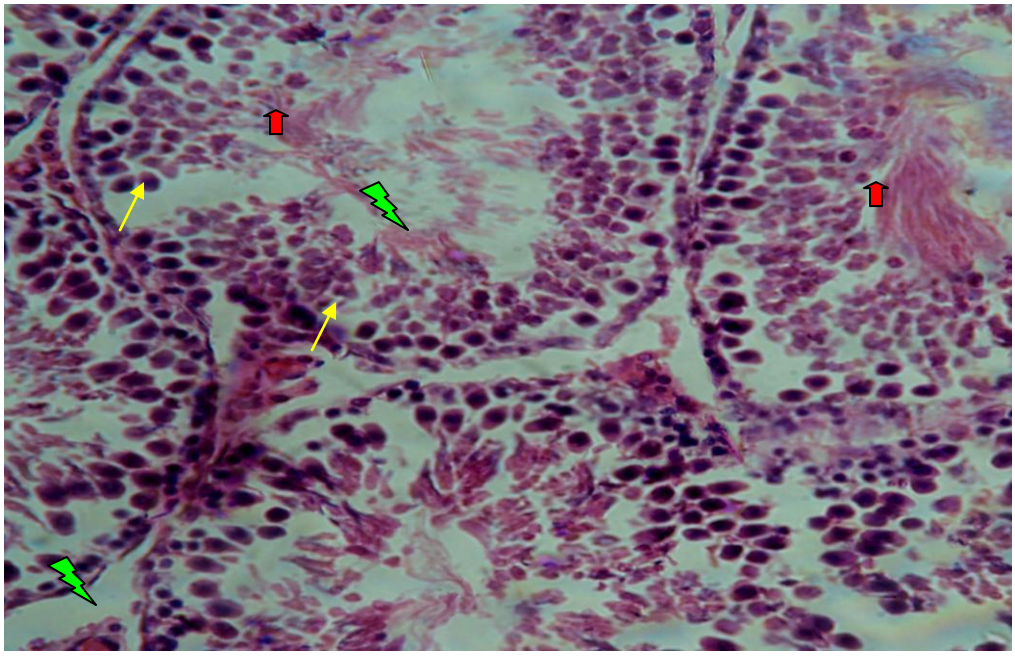


Plate VIII: Photomicrograph of a section of the testis of a Wistar rat exposed to subacute melatonin+chlorpyrifos administration showing reduced number of cell layers of spermatogenic cells (line arrows) with seminiferous tubules showing a few number of elongated spermatids (lightning bolts) in the lumen (block arrows) (H&E x 400).

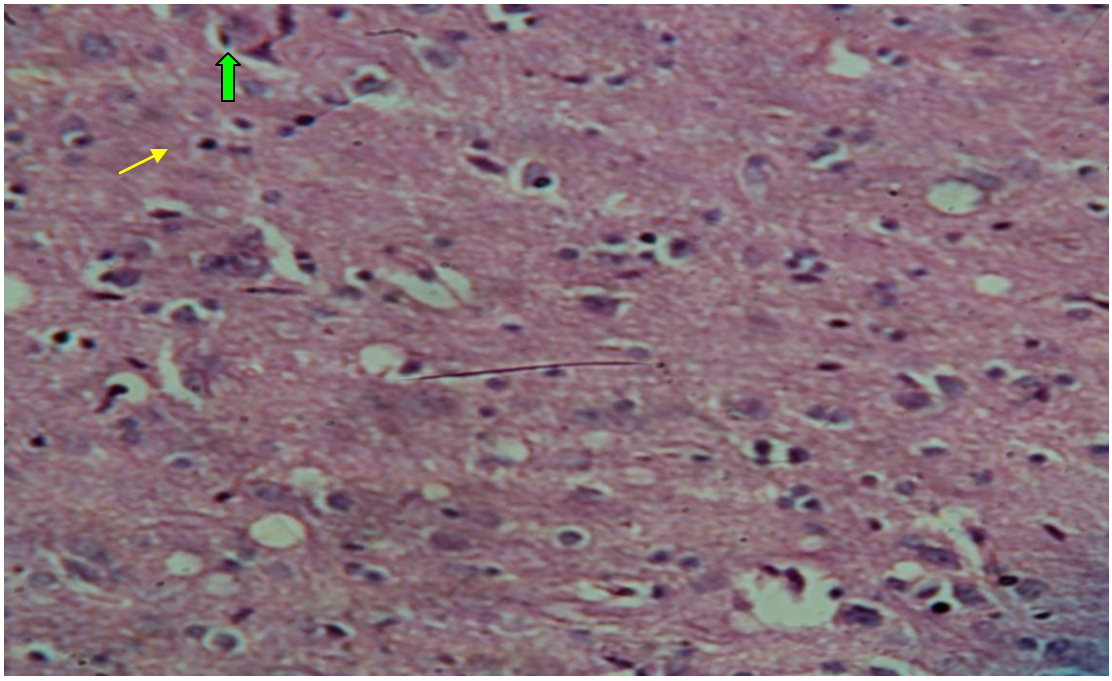


Plate IX: Photomicrograph of a section of the brain of a Wistar rat exposed to subacute soya oil administration only showing apparently normal neurons (block arrows) and glial cells (line arrows) ((H&E x 400).

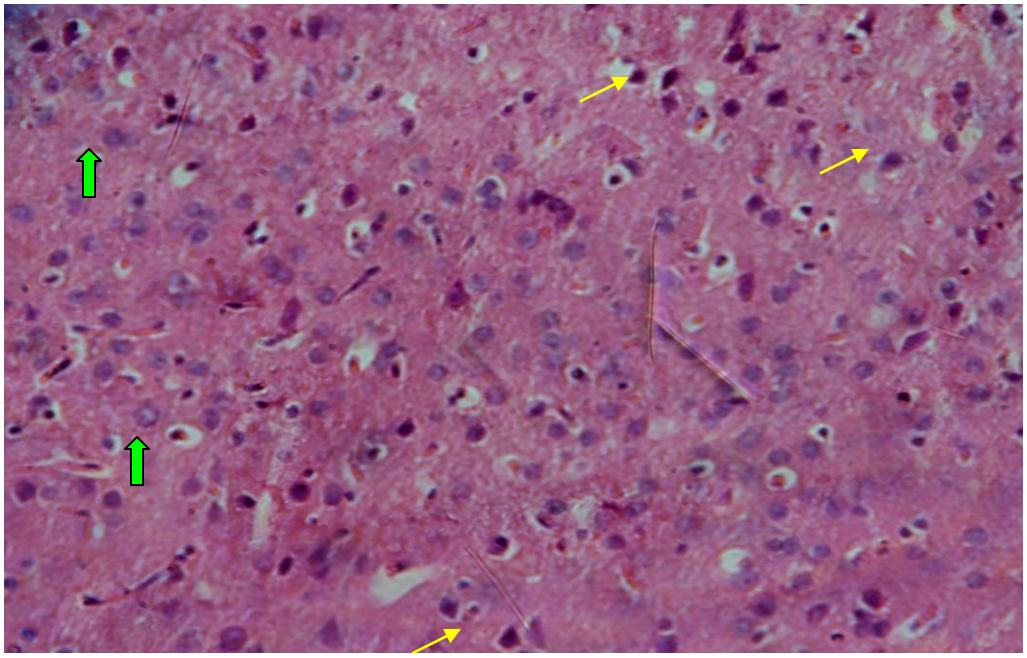


Plate X: Photomicrograph of a section of the brain of a Wistar rat exposed to subacute melatonin administration only showing apparently normal neurons (line arrows) and glial cells (block arrows) (H&E x 400).

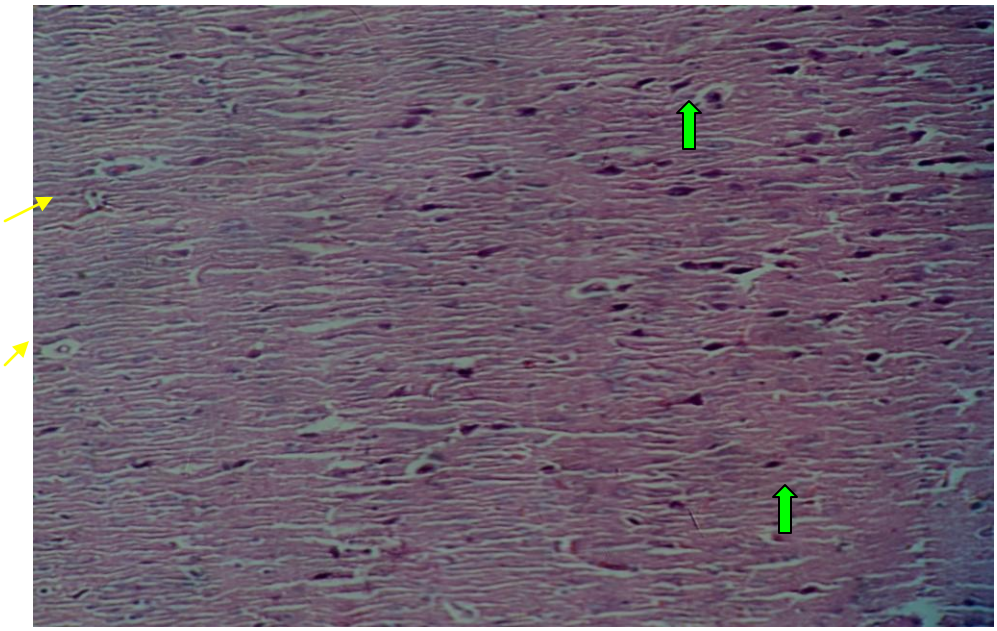


Plate XI: Photomicrograph of a section of the brain of a Wistar rat exposed to subacute chlorpyrifos administration showing degeneration of neurons (block arrows) and glial cells (line arrows) (H&E x 400).

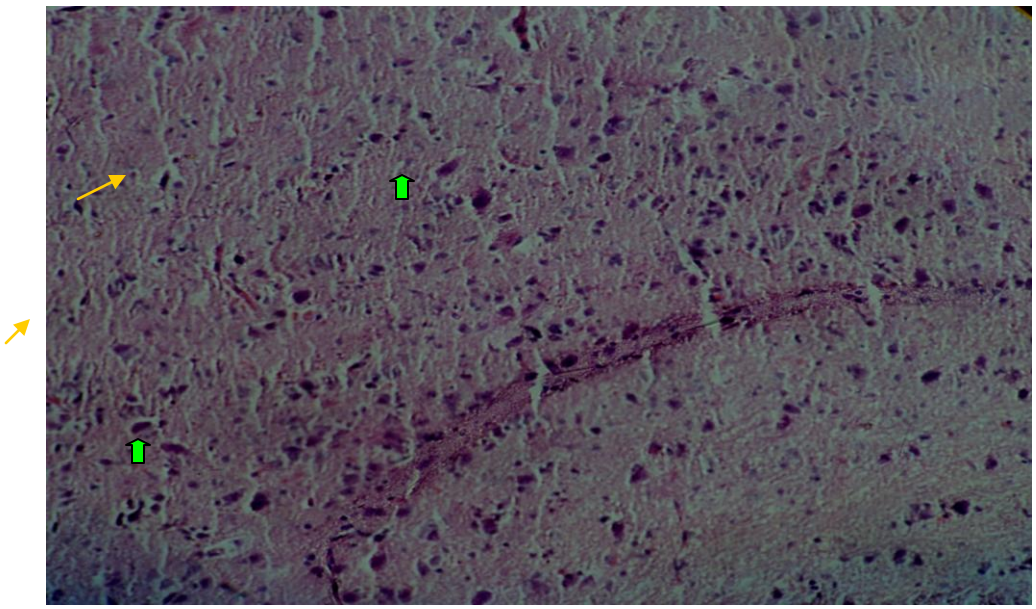


Plate XII: Photomicrograph of a section of the brain of a Wistar rat exposed to subacute melatonin+chlorpyrifos administration showing apparently viable neurons (block arrows) and glial cells (line arrows) (H&E x 400).

CHAPTER 5 DISCUSSION

The study had shown that CPF increased the concentration of MDA in both the pituitary gland and the testes. Earlier studies have also shown increase in MDA concentration following CPF exposure (Gultekin *et al.*, 2000; Verma *et al.*, 2007; Ambali *et al.*, 2010b). MDA is a by-product of lipoperoxidation resulting from interaction of oxygen radicals with polyunsaturated fatty acids residues in membrane phospholipids that damages proteins and DNA (Krishnamoorthy *et al*., 2007). Thus, the high MDA contents of both the pituitary and the testicular tissues in the CPF group are indications of the level of lipoperoxidative changes, reflecting alteration in structural and, consequently functional status of the organs. The present study showed that pretreatment with melatonin comparatively decreased MDA concentration, indicating its antioxidant. This result further confirmed the established findings that melatonin, the most important secretory product of the pineal gland, is a free-radical scavenger and antioxidant molecule. Melatonin has been found to protect cells, tissues and organs against oxidative damage induced by a variety of free radical generating agents (Reiter *et al*., 2002a; Tomas-Zapico and Coto-Montes, 2007). Melatonin acts as a direct free radical scavenger (Tan *et al*., 1993) and as an indirect antioxidant via its stimulatory actions on antioxidative enzymes (Reiter *et al*., 2000; Rodriquez *et al*., 2004). The radical-scavenging potency of melatonin is much greater than that of the most important endogenous radical scavenger, glutathione, and the classical hydroxyl radical scavenger, mannitol (Poeggeler *et al.*, 1994). Melatonin has also been reported to alter the activities of enzymes that improve the total antioxidative defence capacity of the organism; that is, the activities of SOD, glutathione reductase, GPx, glucose 6-phosphate dehydrogenase, and nitric oxide synthase (Reiter *et al*., 2002b). Its hydrophilic and lipophilic nature also aids in

its free-radical scavenging and antioxidant actions. Its lipophilic nature enhances the easiness at which it crosses the morphophysiological barriers such as the blood–brain barrier, and enters cells and subcellular compartments (Reiter *et al*., 2005). It distributes in all cell compartments, being especially high in the nucleus and mitochondria (Menendez-Pelaez *et al*., 1993; Martin *et al*., 2000). Furthermore, several metabolites such as 6-hydroxymelatonin and *N*-acetyl-*N*-formyl-5-methoxykynurenamine that are formed when melatonin neutralizes damaging reactants are themselves scavengers, suggesting that there is a cascade of reactions that greatly increase the efficacy of melatonin in blocking oxidative damage. Other processes which may contribute to melatonin's ability to reduce oxidative stress include stimulation of GSH synthesis, an important antioxidant which is in high concentrations within cells. GSH reduces electron leakage from the mitochondrial electron transport chain, which decreases free-radical generation, and limits cytokine production in inflammatory processes, actions that also lower toxic reactant generation. It also exerts synergistic effects with other classical antioxidants such as vitamins C and E, and glutathione (Reiter *et al.*, 2004). All these factors, apparently, enhanced the antioxidant status recorded in the group pretreated with melatonin.

The present study also showed a decrease in the activities of both pituitary and testicular SOD in the CPF group compared to the other groups. SOD, which catalyzes the dismutation of O_2^- to H_2O_2 is the first line antioxidant defence in the body. The decreased activity in the CPF group may be due to decreased synthesis, increased degradation or even outright inactivation of the enzyme. SOD is the most important antioxidant enzyme in the spermatozoa where it plays a key physiological role in epididymal sperm capacitation

(Bauché *et al*., 1994). Therefore, the low SOD activity in the testes recorded in the CPF group may also indirectly reflect the SOD activity status in the spermatozoa, resulting in impairment of sperm capacitation in the group. The increase in both pituitary and testicular SOD activities recorded in the group pretreated with melatonin demonstrated the ability of the antioxidant agent, melatonin to protect the SOD from the ravaging effect of CPF-induced ROS, apparently through boosting of the antioxidant reserves in both the testis and the pituitary gland.

The study also showed a comparative decrease in CAT activities in both pituitary gland and the testes in the CPF group. CAT is known to neutralize H_2O_2 to H_2O and O_2. The decline in the activity of CAT observed in CPF-treated rats may be due to the reduced conversion of $O2^{\bullet-}$ to H_2O_2 by SOD, leading to the accumulation of $O_2^{\bullet-}$. This accumulation of $O2^{\bullet-}$ has been shown to inhibit the activity of CAT (Kono and Fridovich, 1982) as $O_2^{\bullet-}$ converts ferroxy state of CAT to ferryl state, which is an inactive form of the CAT (Freeman and Crapo, 1982). Several studies have shown that CPF reduced the activities of both SOD and CAT (Verma *et al*., 2007; Astiz *et al*., 2009). Melatonin's improvement of CAT activity was demonstrated by its ability to boost the endogenous antioxidant reserve and protect CAT from the ROS induced by CPF. The lipoperoxidative changes and the low antioxidant status, as evidenced by low level of activity of SOD and CAT and high MDA concentration in the CPF group, were indicative of oxidative stress. The oxidative stress may partly be responsible for the alteration in the synthesis of some hormones and other substances essential for sustaining reproductive activity, culminating in the low sperm count recorded in the CPF group.

The study also revealed a comparatively lower AChE activity in the testis and pituitary gland of rats in the CPF group, indicating a higher level of AChE inhibition in the group. The alteration in the AChE activity may be partly responsible for the depreciating level of gonadotrophins and even spermatogenesis recorded in the CPF group. The result is in agreement with the finding that OPs act as neuroendocrine disruptors by its AChE inhibitory action, and that it increases ACh activity in the brain (Herken and Neubert, 1953). The increase in ACh activity in both the brain and testicular tissue may alter the levels of other neurotransmitters such as dopamine, noradrenaline and 5-hydoxytryptamine (Sarkar *et al.*, 2000). This may have been involved in neuroendocrine disruption and, consequently fertility problems following OP exposure. Thus, pretreatment with melatonin improved the AChE activity. Indeed, some antioxidants such as vitamin C and E have been shown to increase AChE activity following its inhibition by OPs (Yavuz *et al*., 2004; Ambali *et al*., 2010d). The restoration of AChE activity by melatonin was, therefore, partly responsible for the improvement in the levels of gonadotrophins and sperm concentration recorded in the Mel+CPF group.

The low FSH concentration in the CPF treated rats obtained in the present study was in agreement with the finding of Zidan (2009). This decrease in FSH concentration may be due to the ROS-induced effect of the OP insecticide on the pituitary gland and hypothalamus. The effect of this was the impairment in GnRH synthesis and low FSH output by the hypothalamus and pituitary gland, respectively. Similarly, the low FSH output may be due to low AChE activity in the pituitary gland.

The present study showed a relative decrease in serum LH concentration in the CPF group compared to the other groups. Similar result has also been recorded by Zidan (2009) following CPF-methyl exposure in Wistar rats. The decrease in LH concentration may be due to CPF-evoked oxidative damage to the pituitary gland, characterized by increased MDA concentration, low specific CAT and SOD activities, and whole brain degenerative changes. In addition, the oxidative damage to the hypothalamus, the centre responsible for the secretion of gonadotrophin-releasing hormone (GnRH) which stimulates LH and FSH release by the pituitary gland may partly play a significant role in the LH deficits in the CPF group. Furthermore, low AChE activity in the pituitary gland may block nerve impulses, thereby affecting pituitary gland activity, including LH release. OP insecticides such as CPF, fenthion, fenithrithion and dimethoate have been shown to act like androgen receptor antagonists or suppressors of gene expression related to gonadotrophin synthesis (LH and FSH) or steroidogenesis (Walsh *et al*., 2000; Gore, 2001; Kitamura *et al*., 2003). Besides, the results of the present study agree with the finding that CPF suppressed androgenic activity (Kang *et al*., 2004; Jeong *et al.*, 2006) in rats. CPF has also been demonstrated to profoundly affect hypothalamic GnRH gene expression and subsequently reduced LH and FSH concentration (Padungtod *et al*., 1998; Guven *et al*., 1999; Gore, 2001). Recio *et al*. (2005) showed that LH and FSH are mostly affected by OP-induced hypothalamic-pituitary disruption. The improvement in LH concentration in the melatonin pretreated rats was due to the antioxidant effect of the agent, protecting the pituitary gland and the hypothalamus from CPF-induced oxidative changes.

The comparatively low testosterone concentration observed in the CPF group has been reported in previous studies (Joshi *et al.*, 2007; Zidan, 2009). Similarly, Meeker *et al*. (2004, 2006) have shown inverse relationship between concentrations of testosterone and that of 3,5′- trichloropyridinol, the metabolite of CPF in humans. This decrease in serum testosterone concentration may be due to low serum LH concentration in the CPF-treated rats. This is because circulating LH is responsible for maintaining normal testosterone concentration (Ellis and Desjardins, 1982) by stimulating Leydig cells to release testosterone (Sarkar *et al*., 2000). Similarly, the degenerative changes in the testes of rats partly due to CPF-induced oxidative stress apparently reduced the ability of Leydig cells to secrete adequate quantity of testosterone. Krause (1977) reported that the decreased testosterone might be due to direct damage to Leydig cells or to a lowered stimulation of these cells by LH. Also, it has been shown that OPs cause decline in serum steroid hormone levels by increasing steroid catabolism and elimination or directly inhibit steroid hormone production (Civen and Brown, 1974). OPs have also been shown to inhibit steroidogenesis in the adrenal cells (Chattopadhyay *et al.*, 2005). The cumulative effect of the responses was a decrease in the concentrations of steroidal hormones, LH, FSH and testosterone. Thus, the improvement in testosterone concentration observed in the group pretreated with melatonin may be due to relative increase in serum LH and the relative improvement in the testicular, hence, histoarchitecture of Leydig cells demonstrated in this group.

The results of the present study which showed a relative decrease in the concentrations of T_3, T_4 and TSH in the CPF group agree with the finding of Rawlings *et al*. (1998) who recorded low T_4 concentration in ewe following CPF exposure. Similarly, the results of studies conducted by Zaidi *et al*. (2000) in male pesticide formulators chronically exposed to

various pesticides, including CPF indicated impairment in thyroid function. A reduction in serum T_4 was also recorded in some Danish greenhouse workers exposed to pesticide (Toft *et al*., 2006). Although the reason for the low thyroid hormone activity was not known precisely, oxidative stress may have partly been involved. Indeed, Ambali (2009) recorded high concentration of MDA in the thyroid gland of rats chronically exposed to CPF, indicating lipoperoxidative damage to thyroid acinar. Thyroid gland has also been shown to be a sensitive target to CPF developmental exposure (De Angelis *et al*., 2009). There is ample evidence that thyroid hormone is essential to the normal development of testes in the neonate (Cooke *et al*., 1994; Hardy *et al*., 1996). Studies involving other OPs such as profenofos (El-kashoury and El-far, 2004) have shown reduction in thyroid hormone levels. The low thyroid hormones levels in the CPF group may be partly responsible for the low sperm motility and concentration (Meeker *et al*., 2004). Hypothyroid men have been reported to have lower sperm motility than euthyroid controls (Corrales–Hernandez *et al*., 1990), and thyroxine (T4) replacement in these individuals has been reported to improve sperm motility (Kumar *et al*., 1990).

The body weight dynamics showed a progressive increase in body weight gain throughout the four weeks of exposure, with a comparatively lower body weight gain in the CPF group. The observation in this study that prolonged exposure to CPF did not cause significant change in cumulative body weight gain agrees with the results obtained in earlier studies of Mollelo *et al*. (1980) and Corley *et al*. (1989). However, the fact that relatively lower cumulative body weight changes were recorded in the CPF group compared to the other groups showed that the OP compound caused some adverse body weight changes. Yoshida *et al.* (1985) demonstrated a dose-dependent decrease in body weight gain following a 28-day-

feeding study. Similarly, many other studies demonstrated that prolonged CPF exposure caused a decrease in body weight gain in laboratory rodents (Yano *et al*., 2000; Ambali *et al*., 2007*;* Akhtar *et al*., 2009). Pretreatment with melatonin progressively increased the weight gain. The cumulative body weight change in the melatonin pretreated group was comparatively higher than that recorded in the CPF group. This fact has shown that oxidative damage is in part involved in the mechanisms of adverse body weight changes in CPF poisoning. Apart from oxidative stress, the adverse body weight changes recorded in the CPF group may be related to cholinergic stress (Ambali *et al*., 2007), and also adrenal-mediated stress, caused by the inhibition of the enzyme cholesterol ester hydrolase by CPF oxon, the active metabolite of CPF (Civen *et al*., 1977; Kousba *et al*., 2004). Unlike the result obtained in melatonin pretreated group, rats exposed to melatonin only recorded a relative decrease in body weight compared to S/oil and, paradoxically, the melatonin+CPF groups. Melatonin has been shown to induce loss in body weight in diet-induced obesed rats, perhaps due to increased metabolic profile and improved insulin sensitivity, without altering food intake (Prunet-Marcassus *et al*., 2003).

The clinical signs observed in the CPF treated groups were consistent with cholinergic symptoms associated with cholinesterase inhibition. CPF inhibit AChE, the enzyme that hydrolyzes the neurotransmitter ACh. The resultant accumulation of ACh in the synaptic cleft precipitates a “cholinergic crisis”. However, oxidative stress has also been shown to complement cholinergic crisis in OP-induced acute toxicosis (Vidyasagar *et al*., 2004). The reduction in the severity of signs of toxicity observed in rats pretreated with melatonin may be due to AChE restoration potentials of the antioxidant, exhibited in the present study.

Similarly, the antioxidant properties may be involved in the restoration of toxic signs in melatonin-pretreated group.

The comparatively lower testicular weight recorded in the CPF group demonstrates the adverse effect of CPF on testicular weight changes. This result agreed with those recorded by Joshi *et al.* (2007). The adverse weight changes were, apparently, due to testicular degenerative changes arising from CPF-evoked oxidative damage. Similarly, Colborn *et al.* (1996) showed that impairment in the release of LH by OPs was responsible for the gonadal changes. Furthermore, spermatogenic arrest and inhibition of steroid biosynthesis in Leydig cells may have contributed to the reduced testicular weight (Kaur and Mangat, 1980; Sujatha *et al*., 2001). In the present study, pretreatment with melatonin has been shown to ameliorate the decrease in testicular weight induced by CPF, probably due to antioxidant and tissue protecting properties of melatonin. Similarly, melatonin pretreatment reduced the testicular degenerative changes induced by CPF. The improvement in CPF-induced lipoperoxidative and neuronal degenerative changes in the brain and, consequently, the pituitary gland by melatonin resulting in improvement in the elaboration of LH may have played a role in the apparent increase in testicular weight changes in this group.

Sperm count is one of the most important factors that affect fertility (Bett *et al.*, 1996). The present study has shown a low caudal epididymal sperm count in the CPF group compared to the values obtained in other groups. This may be due to low FSH, LH and, subsequently, testosterone concentrations in the CPF group. The degenerative changes in the seminiferous tubules, decreased number of spermatogenic cells and reduced number of viable spermatozoa confirmed the low sperm count in the CPF group. Testosterone is required for differentiation of sex organs and production of sperms (Watanabe *et al*., 1986). Maintaining

serum LH concentration is critical for initiating and supporting spermatogenesis and even fertility (Pidoux *et al*., 2007; Fattahi *et al*. 2009), hence the lowered serum LH in the CPF group may be involved in the impairment of spermatogenesis. FSH is vital to reproduction because it enhances the production of androgen-binding protein by the Sertoli cells of the testes, by binding to FSH receptors on their basolateral membranes (Walter, 2003) and is critical for the initiation of spermatogenesis. Infact, circulating FSH has long been considered a valuable marker for Sertoli cell function and spermatogenesis (Jensen *et al.*, 1997). Sertoli cells, the major epithelial components of the seminiferous epithelium, are essential for the control of spermatogenesis, because they supply nutrients which ensure germ cell proliferation and differentiation (Saunders, 2003). Many Sertoli cell functions are, however, regulated by FSH (Simoni *et al.*, 1999). In the testis, spermatogenesis takes place in the tubular compartment, and steroidogenesis in the interstitial compartment. Despite being anatomically separated, both compartments are closely connected with each other in a paracrine manner. The integrity of both compartments is necessary for quantitative and qualitatively normal sperm production (Brinkworth and Handelsam, 1997).

Spermatogenesis is a complex process of cell proliferation and differentiation that occurs in the seminiferous tubules (Sokol *et al*., 1994). The seminiferous epithelium is a complex and highly dynamic tissue in which germ cells proliferate, undergo meiosis, and differentiate into spermatozoa (Timmons *et al.*, 2002). The degeneration of the seminiferous tubules observed in the present study either due to the direct effect of CPF or indirectly due to oxidative damage to the seminiferous tubules, especially the Sertoli cells and/or the entire testes may play a significant role in the reduced sperm count recorded in the CPF group.

Effect of the toxicant on the Sertoli cells is crucial to sperm formation since it is involved in the control of spermiation, and any perturbation will cause epithelial disorganization and subsequent atrophy (Bardin *et al*., 1988). Therefore, the low sperm count in the CPF group may be due to suppression of gonadotrophin (Sinha *et al*., 1995), and the direct and indirect effects (due to oxidative damage) on the testicular elements such as seminiferous tubules, especially the Sertoli and Leydig cells. In addition, CPF-induced oxidative damage directly to the spermatozoa may alter its genomic integrity, even with rupture of DNA strands (Shen *et al*., 1999).

Excessive ROS production also results in impaired steroidogenesis and spermatogenesis by several mechanisms, including DNA damage, lipid peroxidation and protein oxidation (Prahalathan *et al*., 2006). Melatonin was able to improve the CPF-induced sperm count deficit, perhaps due to its antioxidant effect in both the pituitary gland and the testis. The improvement in the sperm count in rats pretreated with melatonin may be due to reduced damage to the seminiferous tubules and increase in the number of apparently viable spermatozoa in the tubular lumen, observed histologically when compared to the changes observed in the CPF group, probably due to the antioxidant effect of melatonin. Paradoxically, however, the sperm count of rats in group exposed to melatonin only was comparatively lower than the value recorded in the S/oil group. Melatonin has been shown to have effects on the testicular metabolism in adult rats, suggesting that it plays some role in the modulation of the testicular function (Hemieda, 2003).

The result also showed a decrease in the sperm motility in group exposed to CPF only. This finding agreed with that recorded by Joshi *et al*. (2007) in mice. Meeker *et al*. (2004) demonstrated an inverse association between sperm motility and serum concentration of 3,5,6-trichloro-2-pyridinol, the metabolite of CPF. Sperm motility is an important functional sperm characteristic which is of value in predicting sperm fertilizing capacity (Aitken *et al.*, 1984), and it has been used extensively as a marker of seminal vesicle function (Schoenfled *et al*., 1979). Any perturbation on sperm motility would seriously affect fertilizing ability (Murugavel *et al.*, 1989). Marked inhibition of sperm motility recorded in the CPF group may be due to low level of ATP content (Bai and Shi, 2002). Sperm motility may be affected by altered enzymatic activities of oxidative phospholytic process. Oxidative phospholytic process is required for adenosine triphosphate (ATP) production, a source of energy for the forward movement of spermatozoa. Full ATP pool is crucial for normal spermatozoal movement; and a slight deprivation of ATP leads to reduction in motility, which may cause infertility (Tso and Lee, 1981; Bedford, 1983).

In addition, ROS, which causes lipid peroxidation of sperm cell membranes, may lead to damage of the midpiece and axonemal structure, malfunctioning of capacitation and acrosomal reaction and loss of motility, which may ultimately result in infertility (Krishnamoorthy *et al*., 2007). Similarly, CPF-induced epididymal dysfunction, which may exert a detrimental effect on various cytostructural modifications and biochemical changes during sperm maturation process, could decrease sperm count and motility (Krishnamoorty *et al.,* 2007*)*. Pretreatment with melatonin restored the CPF-evoked decrease in sperm motility. This may be due partly to the ability of the antioxidant to protect the sperm from

oxidative damage and provide relatively good environment in the epididymis for sperm maturation.

The result obtained in the present study also showed that rats exposed to CPF only had the highest number of sperm abnormality. The percentages of dead head and bent tail were comparatively higher in the CPF group than in the other groups. The percentages of sperm with free tail and cut tail were comparatively higher in S/oil group rats than that recorded in any other group. The percentage of live sperms was comparatively higher in the melatonin group compared to any other group, with rats in the S/oil group having the highest percentage of dead sperm. Sperm morphology appears to be the most informative semen measurement for discriminating between fertile and infertile men, and is better than sperm concentration in this respect (Guzick *et al*., 2001). Sperm morphology could in addition be a marker of toxic damage even in the absence of any effect on fertility (Zidan, 2009). From the morphological observations, the present study revealed that those sperms in the CPF group, then followed by S/oil group, recorded the highest morphological abnormalities, with those in the melatonin group having the least. The reason why a higher number of morphological abnormalities were recorded in the S/oil is not known and deserves further study. The present study, however, showed that pretreatment with melatonin appeared to have reduced the morphological abnormalities induced by subacute CPF exposure.

The histopathological lesions recorded in the kidneys, liver, adrenal glands, testes and the brain in the CPF group showed that the insecticide induced widespread tissue damage. The degenerative changes in the kidneys and liver had been reported in earlier studies (Goel *et al*., 2005; Ambali, 2009). The consequence of this damage is an exacerbation of tissue damage as a result of poor metabolism and excretion of the xenobiotic. The fact that the

adrenal glands have also been adversely affected by CPF exposure showed that CPF may interfere with steriodogenesis, hence impairing the production of some hormones essential in stress adaptation. The lesions observed in the testes, brain and invariably to the hypothalamus and the pituitary gland may have played a negative role in the elaboration of sex hormones essential in spermatogenesis. Although pretreatment with melatonin was able to limit the organ toxicity induced by CPF, the protection was not complete. This fact has demonstrated that oxidative stress may not be the entire molecular mechanism involved in CPF-induced cytotoxicity. However, the reduction in the lesion was evidence that oxidative stress is partly involved in CPF toxicity.

CHAPTER 6 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

The present study has shown that subacute exposure of adult male Wistar rats to CPF caused oxidative damage to the testicular tissue and pituitary glands, decreased AChE activity, serum LH, FSH, testosterone and thyroid hormones (T_3, T_4 and TSH). The exposure also caused apparent reduction in body and testicular weight, sperm concentration and motility, and increased sperm morphological abnormalities. Subacute CPF exposure also induced varying degree of lesions to the liver, kidneys, adrenal glands, testes and brain. Pretreatment with melatonin ameliorated the CPF-induced oxidative damage to the pituitary gland and testes, improved body and testicular weight and improved semen characteristics. In addition, melatonin also caused some level of reduction in the lesions to the liver, kidneys, adrenal glands, testes and the brain. Therefore, the study demonstrated that oxidative stress was involved in CPF-induced cytotoxicity and that melatonin ameliorated the toxicity.

6.2 Conclusions

In conclusion, the study has shown that:

1. Subacute CPF exposure in Wistar rats caused significant alterations in biochemical parameters, thyroid and sex hormone concentrations and histoarchitecture.

2. Subacute CPF exposure altered sperm concentrations, motility and morphology in Wistar rats.

3. Oxidative stress plays an important role in the molecular mechanism of subacute CPF-induced alterations in thyroid and sex hormones, biochemical and pathological changes and semen characteristics in Wistar rats.

4. Pretreatment with melatonin ameliorated the subacute CPF-induced alterations in biochemical parameters, thyroid and sex hormone concentrations, and semen characteristics in Wistar rats, partly due to its antioxidant and AChE restoration properties.

6.3 Recommendations

6.3.1 Specific recommendations

1. Individuals who are likely to be exposed to low-dose CPF and other organophosphates should wear protective gadgets to avert adverse reproductive consequences.

2. Direct contact of animals and man to CPF and other organophosphates should be limited. However, where contact is inevitable, individuals should be pretreated with melatonin.

3. Study to evaluate the effect of prolonged pesticide use on reproductive abilities of larger animals and humans should be conducted

4. Further study should be carried out to determine the direct effect of prolonged S/oil exposure on sperm morphology in Wistar rats.

6.3.2 General recommendations

1. Sensitization and public awareness of livestock and arable crop farmers on the need to observe the expiry date and withdrawal periods stated by the manufacturers before using pesticides or turning pesticide treated products into the market for human and animal consumption should be conducted.

2. There is the need to formulate effective pesticide legislations and strengthen the country's borders to prevent or limit dumping of expired and banned pesticides.

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