EFFECTS OF MELATONIN ON NEURO– AND REPRODUCTIVE TOXICITY INDUCED BY *IN UTERO* AND LACTATIONAL CO-EXPOSURE TO CHLORPYRIFOS AND CYPERMETHRIN IN MALE WISTAR RATS

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A PhD THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN VETERINARY TOXICOLOGY

DEPARTMENT OF VETERINARY PHARMACOLOGY AND TOXICOLOGY

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ZARIA, NIGERIA

MARCH, 2018

DECLARATION

I declare that the work in this thesis, entitled "Effects of melatonin on neuro- and reproductive toxicity induced by *in utero* and lactational co-exposure to chlorpyrifos and cypermethrin in male Wistar rats" was performed by me in the Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, under the supervision of Professors S.F. Ambali, J.O. Ayo and Dr. M.U. Kawu. The information derived from the literature has been duly acknowledged in the text and in the list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Muftau SHITTU

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CERTIFICATION

This thesis entitled "EFFECTS OF MELATONIN ON NEURO- AND REPRODUCTIVE TOXICITY INDUCED BY *IN UTERO* AND LACTATIONAL CO-EXPOSURE TO CHLORPYRIFOS AND CYPERMETHRIN IN MALE WISTAR RATS" by Muftau SHITTU meets the regulations governing the award of Doctor of Philosophy (Veterinary Toxicology) of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to Almighty Allah for being the originator and sustainer of the world and everything therein; to the loving memory of my late parents Alhaji Shittu and Mrs Raliat Shittu for their prayers and encouragement. May Allah forgive them and make Aljana Firdaus their final abode. Ameen; my family for their patience; and finally to my mentor, Professor S. F. Ambali for his trust and believe that this work can be done.

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ABBREVIATIONS AND SYMBOLS

$	–	Dollar
±SEM	–	Plus/minus standard error of mean
µl	–	Microlitre
$^1O_2$	–	Singlet oxygen
3-BPA	–	3-phenoxybenzoic acid
$3O_2$	–	Triplet-state molecular oxygen
3PBAlc	–	3-phenoxybenzyl alcohol
3PBAld	–	3-phenoxybenzaldehyde
4-F-3-PBA	-	4-Fluoro-3-phenoxybenzoic acid
4-HNE	–	Hydroxy nonenal
5HT	–	Serotonin
8-OHdG	–	8-hydroxydeoxyguanosine
ACh	–	Acetylcholine
AChE	–	Acetylcholinesterase
ACP	–	Advisory committee on pesticide
ADH	–	Alcohol-dehydrogenase
AGD	–	Anogenital distance
ALDH	–	Aldehyde-dehydrogenase
AMH	–	Anti mullerian hormone
AMPA	–	Amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	–	Analysis of variance
AR	–	Androgen receptor
AREs	–	Androgen receptor response element

ATP	–	Adenosine-5-triphosphate
BBB	–	Blood brain barrier
BBSRC	-	Biotechnology and Biological Sciences Research Council
BChE	–	Butyrecholinesterase
BHA	–	Butylated hydroxyl anisole
BHT	–	Butylated hydroxyrotoluene
cAMK	–	Cyclic adenosine monophosphate
CAMK II	–	Ca^{2+}/calmodulin-dependent protein kinase
CAT	–	Catalase
CC	–	Chlorpyrifos and cypermethrin
CCM	–	Chlorpyrifos + Cypermethrin + Melatonin
Cd	–	Cadmium
CMAP	–	Compound muscle action potential
CNS	–	Central nervous system
CO.	–	Company
COPIND	–	Chronic organophosphate-induced neuropsychiatric disorder
CPF	–	Chlorpyrifos
CPO	–	Chlorpyrifos oxon
CRE	–	cAMP-respponsive element
CRL	–	Crown-rump length
CS	–	Choreoathetosis and salivation
Cu^{+}	–	Copper
CYP	–	Cypermethrin
DCCA	-	Dichlorovinyl)-2,2-dimethyl-(1-cyclopropane) carboxylic acid

DEP	–	Diethylphosphate
DETP	–	Diethyl thiophosphate
DGC	–	Dentate granule cell
DHA	–	Docosahexaenoic acid
DNA	–	Deoxyribonucleic acid
DW	–	Distilled water
E.C.	–	Emulsifiable concentrate
EDTA	–	Ethylene diamine tetra acetic acid
EGFR	–	Epidermal growth factor receptor
ELISA	–	Enzyme linked immunosorbent assay
ER	–	Estrogen receptor
ERK	–	Extracellular signal-regulated kinase
ETC	–	Electron transport chain
F1	–	First generation
Fe	–	Iron
Fe^{2+}	–	Ferous or Iron II
FISH	–	Fluorescence *in situ* hybridisation
FSA	–	Free sialic acid
FSH	–	Follicle-stimulating hormone
FST	–	Forced swimming test
GABA	–	Gama-aminobutyric acid
GD	–	Gestation day
GDNF	–	Glial cell line-derived neurotrophic factor
GFAP	–	Glial fibrillary acidic protein

GH	–	Growth hormone
GH-IGF-1	–	Growth hormone insulin growth factor-1
GHS	–	Globally harmonized system
GnIH	–	Gonadotropin-inhibiting hormone
GPx	–	Glutathione peroxidase
GR	–	Glutathione reductase
GSH	–	Glutathione
GSK	–	Glycogen synthase kinase
GSSG	–	Oxidized glutatione
H_2O_2	–	Hydrogen peroxide
HB-EGF	–	Heparin-binding epidermal growth factor
hCG	–	Human chorionic gonadotrophin
HCL	–	Hydrochloric acid
Hg	–	Mercury
HOCl	–	Hypochlorous acid
HPG	–	Hypothalamic-pituitary-gonadal
HRP	–	Horse radish peroxidase
IL-1	–	Interlukin-1
IL-1β	–	Interleukin-1 beta
IL-6	–	Interlukin-6
IMS	–	Intermediate syndrome
INSL3	–	Insulin-like 3
JNK	–	cJun N-terminal kinase
K^+	–	Potassium ion

LD_{50}	–	Median lethal dose 50
LDL	–	Low density lipoprotein
LGR	–	Leucine-rich repeat-containing G protein coupled receptor
LH	–	Luteinizing hormone
LPO	–	Lipid peroxidation
LTD	–	Limited
M_1, M_2	–	Melatonin receptors
MAG	–	Myelin associated glycoprotein
MAO	–	Monoamine oxidase
MAPK	–	Mitogen-activated protein kinase
MCC	–	Melatonin, Chlorpyrifos and cypermethrin
MDA	–	Malondialdehyde
MEL	–	Melatonin
Mg^{2+}	–	Magnesium
Ml	–	Milliliter
mm	–	Millimetre
MPTP	–	Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	–	Messenger ribonucleic acid
MtDNA	–	Mitochondrial DNA
MTL	–	Medial temporal lobe
Na^{+}	–	Sodium
NADP	–	Nicotinamide adenine dinucleotide phosphate
NCC	–	Neutral crest cells
NDGA	–	Nordihydroguaretic acid

ng/ml	–	nanogramme per milliliter
NKCC	–	Na^{+}-K^{+}-2Cl^{-}cotransporter
NO•	–	Nitric oxide radical
NO_1	–	Nitrosonium cation
NO_2	–	Nitrite
NO_3	–	Nitrate
NOS	–	Nitric oxide synthase
NTE	–	Neuropathy target esterase
NWLSS	–	Northwest Life Sciences Specialities
O_2^{o-}	–	Superoxide anion radical
OD	–	Optical density
OECD	–	Organisation for Economic Cooperation and Development
$OH^{\bullet}$	–	Hydroxyl radical
$ONOO_2$	–	Peroxynitrite
OPIDN	–	Organophosphate-induced delayed neuropathy
OPIDP	–	Organophosphate-induced delayed polyneuropathy
P=O	–	Organophosphate with oxygen forming double bond with the central
P=S	–	Organophosphate with sulphur-forming double bond with the central
Pb	–	Lead
PBS	–	Phosphate buffered saline
PG	–	Propyl gallate
PND	–	Postnatal day
PON	–	Paraoxonase

PUFA	–	Polyunsaturated fatty acid
PYR	–	Pyrethroid
QR	–	Quinone reductase
R_1, R_2	–	Alkoxy group
RNA	–	Ribonucleic acid
RNS	–	Reactive nitrogen species
RNS	–	Repeatative nerve stimulation
ROS	–	Reactive oxygen species
S/oil	–	Soya oil
SCN	–	Suprachiasmatic nuclei
SN	–	Substantia nigra
SOD	–	Superoxide dismutase
SSR	–	Secondary sex ratio
T	–	Tremor
T_3	–	Triiodothyronine
T_4	–	Thyroxine
TBA	–	Thiobarbituric acid
TBHQ	–	Tertiary butyl hydroquinone
TCA	–	Trichloro acetic acid
TCP	–	Trichloro-2-pyridinol
TDS	–	Testicular dysgenesis syndrome
TH	–	Thyroid hormone
TH	–	Tyrosine hudroxylase
TMB	–	Tetramethylbenzidine

TNF-α	–	Tumor necrosis factor alpha
TRH	–	Thyrotrophin releasing hormone
TSH	–	Thyroid stimulating hormone
TVHQ	–	Tertiary butylated hydroxy quinone
UK	–	United Kingdom
USA	–	United States of America
USEPA	–	United States Environmental Protection Agency
UV	–	Ultraviolet
vAchT	–	Vesicular acetycholine transporter
VSSCs	–	Voltage-sensitive sodium channels
WHO	–	World Health Organization
WM	–	Working memory
X	–	Leaving group

ABSTRACT

Organophosphates and pyrethroids are common insecticides used in combination. Their use in agricultural and indoor activities has opened several windows of exposure to vulnerable non-target young animals and humans during pregnancy and breast-feeding, which may adversely affect their physical, reproductive and mental health. Oxidative stress has been implicated in pesticide-induced toxicity. The aim of the study was to investigate the ameliorative potentials of melatonin (MEL) on neurotoxic and reproductive changes provoked by *in utero* and lactational co-exposure to chlorpyrifos (CPF) and cypermethrin (CYP) in male Wistar rats. Sixty out of the sixty five pregnant rats obtained following overnight mating of seventy-two sexually-mature female nulliparous Wistar rats with 72 adult males in a 1 : 1 mating scheme were used for the study. Evidence of mating was assessed in each female animal by the presence of spermatozoa deposit or vaginal plug. Day 1 of gestation was the day copulation plugs/spermatozoa were found in the vagina. The 60 pregnant dams were thereafter divided at random into six 6 groups of 10 animals each. Group I (DW) was given distilled water (2 ml/kg), while group II (S/oil) was given Soya oil only (2 ml/kg). Group III (MEL) was dosed melatonin (0.5 mg/kg) only, while group IV (CC) was co-administered with CPF (1.9 mg/kg; $1/50^{th}$ of the LD_{50}) and CYP (7.5 mg/kg; $1/50^{th}$ of the LD_{50}); group V (MCC) was pretreated with MEL (0.5 mg/kg) and then co-exposed to CPF (1.9 mg/kg; $1/50^{th}$ of the LD_{50}) and CYP (7.5 mg/kg; $1/50^{th}$ of the LD_{50}), respectively, 30 minutes later. Group VI (CCM) was co-exposed to CPF (1.9 mg/kg; $1/50^{th}$ of the LD_{50}) and CYP (7.5 mg/kg; $1/50^{th}$ of the LD_{50}) respectively, and post-treated with MEL (0.5 mg/kg), 30 minutes later. The regimens were given to the dams by gavages once daily from gestation day 1 (GD 1) to postnatal day (PND) 21. Weekly monitoring of body weight and clinical

signs on the dam were carried out and recorded. The dams were allowed to deliver normally and some developmental/ reproductive/neurobehavioural parameters were evaluated on the FI generation male rats at different time intervals, namely: litter size and weight, number of lives and dead pups, anogenital distance (AGD), crown-rump length (CRL), time of eye and ear opening, time of testicular descent, surface and aerial righting reflex, ability to walk and time pivoting, negative geotaxis, inclined plane, ladder walk, bean walk, excitability score, forepaw grip, forced swimming and cognitive (learning and short-term memory) tests. At PND 80, F1 male rats were mated to a sexually-matured, untreated nulliparous female in a 1 : 1 mating schedule and the females were observed for reproductive indices, including mating index, live-birth index, sex ratio, viability index, gestational length index, fertility index, pregnancy (gestation) index. At the end of the experiment, the rats were sacrificed by jugular venesection after light chloroform anaesthesia. The F1 male rats were evaluated for epididymal sperm concentration, sperm motility, sperm live-dead ratio, sperm morphology, and concentrations of sex [follicle stimulating hormone (FSH), luteinising hormone (LH) and testosterone] and thyroid [triodothyronine (T_3), thyroxine (T_4) and thyroid-stimulating hormones (TSH)] hormones, and tissues' biochemical parameters (levels of malondialdehyde [MDA] concentration; activities of superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx] and acetylcholinesterase [AChE], concentration of testicular and epididymal total and free sialic acid, and epididymal fructose). Post-mortem gross and histopathological examinations of some tissues of rats were also conducted on F1 generation male rats. The results revealed alterations in litter size and weight, number of live/dead pups, anogenital distance (AGD), crown-rump length (CRL), time of eye and ear openings, time of testicular descent, surface and aerial righting reflex, ability to walk

and time pivoting, negative geotaxis, inclined plane, ladder walk, beam walk, excitability score, fore-paw grip time, forced swimming and cognition; in the group co-exposed to CPF and CPY. Mating of F1 generation male rats of dams co-exposed to CPF and CPY with untreated female rats showed alteration in mating index, live birth index, sex ratio, viability index, gestational length index, fertility index, pregnancy (gestation) index. The F1 male rats of CC group showed decreased epididymal sperm concentration, sperm motility; sperm live-dead ratio, concentrations of sex hormones levels (FSH, LH, and testosterone) and altered sperm morphology. Similarly, there were alterations in the tissue levels of MDA, thyroid hormones (T_3, T_4 and TSH), testicular and epididymal total and free sialic acid, epididymal fructose, SOD, CAT, GPx and AChE in the F1 male rats. Pre- and post-exposure treatment with MEL mitigated the pesticide-evoked developmental, reproductive, reflexogenic, neurobehavioural, biochemical, oxidative and histological changes in F1 male rats. However, MEL pretreatment was more beneficial than post-exposure treatment. The mitigation of pesticide-evoked developmental, reproductive, reflexogenic, neurobehavioural, biochemical, oxidative and histological changes by MEL was partly due to its antioxidant and free radical scavenging properties.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Organophosphates (OP) are a diverse group of highly toxic chemicals originally developed as chemical weapons before the World War II (Soltaninejad and Shadnia, 2014). They are widely used as agricultural and horticultural pesticides in human and veterinary medicines, in various public health hygiene products; and as insecticides in homes and gardens (Piao *et al*., 2004). Currently about 50-70% of global pesticides used are OP compounds, amounting to about 90 million pounds (40.5 Million kg) annually (Ojha *et al*., 2011; Mullins *et al*., 2015).

Chlorpyrifos (CPF) (*0-0*-diethyl-0-3,4,6-trichloro-2-pyridyl phosphorothionate) is one of the most widely used OP pesticides in agricultural and domestic pest control (Steenland *et al*., 2000). It was first marketed in USA in 1965, and its use has increased rapidly, in part due to banning of chlordane for termite control in 1988 (Steenland *et al.*, 2000). It is one of the most widely used OP insecticides in United States of America, with an annual cost of 8-10 million pounds in agricultural sector in 1999 (Donaldson *et al.*, 2002). Approximately 822 registered pesticide products on the United States market contain CPF (Grabusky *et al*., 2004; Mitra *et al*., 2008). However, in 2000, the United States Environmental Protection Agency (EPA) revised the risk assessment of CPF and, thereafter, decided to phase out or eliminate some of its residential uses (USEPA, 2000). Recent report by United State Environmental Protection Agency and the United Kingdom (UK) showed that CPF may be banned for agricultural use in the United States of America and other European Nations base on human health and ecological risk assessment (AgroNews, 2016; USEPA, 2016). Like other OP insecticides, the main

mechanism of systemic toxicity is due to inhibition of acetylcholinesterase (AChE), resulting in cholinergic toxicity (Eaton *et al*., 2008). However, toxicity occurs at doses that do not affect AChE activity or long after restoration of its activity (Chakraborti *et al*., 1993; Saulbury *et al*., 2009), prompting the search for other mechanisms. Oxidative stress is one of the other molecular mechanisms implicated in CPF poisoning (Ambali and Aliyu, 2012; Shittu *et al*., 2012a, b).

Pyrethroids are synthetic forms of pyrethrins. There are three types, types I, II and III differing in chemical structure and symptoms after exposure. Their mechanism of action involve the inhibition of central nervous transmission through prolongation of opening of sodium ion channels in the nerve cell membrane, although type II pyrethroids additionally inhibit gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter (Bradberry *et al*., 2005; Breckenridge *et al*., 2009). The pyrethroids have emerged as a major class of highly active insecticides due to their high bio-efficacy and relatively low toxicity when compared to organochlorine and organophosphorous insecticides (Clickman and Lech, 1982; Casida *et al*., 1983). Cypermethrin (CYP) is a class II synthetic pyrethroids with a wide range of application in agriculture, forestry, domestic, horticulture and veterinary medicine (Yavasoglu *et al*., 2006). Several studies have described the developmental effects of CYP, while its neurotoxicity has been reported in adult rodents (Bjorling-Poulsen *et al*., 2008; Sharma *et al*., 2014b; Lee *et al*., 2015b). Apart from the inhibition of central transmission through prolongation of sodium ion channels in the nerve cell membrane, the induction of oxidative stress has been implicated in its toxicity (Wielgomas and Krechniak, 2007a).

Oxidative stress is an emerging phenomenon that has been associated with different diseases, including those induced by environmental pollutants (Durackova, 2010). It is an imbalance between production of free radicals and reactive metabolites, so-called oxidants, and their elimination by protective mechanisms, referred to as antioxidative systems. This imbalance leads to damage of important biomolecules and organs with potential impact on the whole organism (Durackova, 2010). Oxidative stress has been linked to toxicity associated with OPs (Ambali *et al* 2010a-c; El-Kashoury and Tag-Eldin, 2010; Shittu *et al*., 2012a, b), pyrethroids (Wielgomas and Kretchniak, 2007b) and their mixtures (Wielgomas and Kretchniak, 2007b; El-Demerdash, 2011a,b). Naturally, the body is endowed with antioxidant systems that help in coping with the menace, posed by ROS that are normally generated during the normal body metabolic processes. However, oxidative stress ensues when the antioxidant systems are overwhelmed due to increased ROS generation beyond their capacity to neutralise them. Under this circumstance, exogenous antioxidant supplementation becomes imperative (Aggarwal *et al*., 2013).

Melatonin (N-acetyl-5-methoxytriptamine) is a hormone produced from the pineal gland. It is produced during the dark phase of the circadian cycle and is a highly conserved antioxidant molecule (Kudo *et al*., 2007; Reiter *et al*., 2010). It plays significant roles in many body processes, including control of reproductive functions, modulation of immune system activity, inhibition of tumorigenic processes and inhibition of oxidative stress (Reiter, 2000; Hardeland *et al*., 2009). Its role as an antioxidant and free-radical scavenger (Lekic *et al*., 2010), and its involvement in the enhancement of body antioxidant systems have been well documented (Lanoix *et al*., 2012).

1.2 Statement of Research Problems

Despite advances in diagnosis, the cause of subfertility in humans and animals remain largely speculative. The presence of chemicals in the environment that disrupt normal endocrine function, leading to reproductive and developmental disorders has been a source of concern to human and animal health (De Angelis *et al*., 2009; Shittu *et al*., 2013, 2014). Pesticides are among many chemical contaminants that have been implicated in the increasing prevalence of reproductive and developmental disorders (Kjeldsen *et al*., 2013; Mehrpour *et al*., 2014; Shittu *et al*., 2014; Saravi and Dehpour, 2016). Concerns with regards to developmental neurotoxicty due to pesticides have been fuelled by recent epidemiological observations that children exposed prenatally or during early postnatal life suffer from various neurological deficits (Young *et al.*, 2005; Engel *et al*., 2007; Eskenazi *et al*., 2007).

Pesticides are ubiquitous in the environment and have significant economic, environmental and public health impact (Elhalwagy and Zaki, 2009). Due to the need to combat pesticide resistance encountered following repeated application of a single chemical pesticide, the use of combinations of chemical pesticides has increased over the decades (Miodovnik and Wolff, 2011). Insecticide mixture involving the organophosphates and pyrethroids groups are among the most common chemical combinations used to stem the tides of increasing pesticide resistance and improving bioefficacy (El-Demerdash, 2011a,b). This, however, poses new challenge to humans, animals and the environment. Increasing evidence has shown that chemical combinations have detrimental effect on public health, including reproductive and developmental health (Wang *et al*., 2009a).

Exposure to pesticides, including OPs has been implicated in the decline in the sperm count of men (Dindyal, 2004) and animals (Joshi *et al*., 2007; Shittu *et al*., 2014). Decreased semen quality and low level of female and male reproductive hormones have been suggested as causes of sub-fertility in humans and animals, exposed to low levels of OPs (Peiris-John and Wickremesinghe, 2008; Shittu *et al*., 2013). Sub-fertility may occur due to many factors involving both male and female factors, such as reduced quality and quantity of sperm, ovulation disturbances and tuboperitoneal disorders (Peiris-John and Wickremesinghe, 2008). In general, poor semen quality and low hormone levels have been suggested as the likely reasons for subfertility among people exposed to low levels of OPs (Peiris-John and Wickremesinghe, 2008).

The neurobehavioural consequences of foetal or childhood pesticide exposure are a major biochemical and societal concern (Erikson and Talts, 2000; Landrigan, 2001). Along with other widely used OP insecticides, CPF is undergoing increasing scrutiny because of its developmental neurotoxic effects (Barone *et al.,* 2000; Landrigan, 2001), especially as it affects the immature brain (Pope, 1999; Barone *et al.*, 2000). It has also been shown that CPF inhibits DNA synthesis, mitosis, neural outgrowth and neuronal cell replication and differentiation (Erickson and Talts, 2000).

Although pyrethroids are often considered to be ‘safer pesticides because of their low to moderate acute toxicity to non target species, their increased use raises concerns of potential adverse effect, particularly in sensitive populations such as children (DeMicco *et al.,* 2010). Recent studies have indicated that children are exposed to pyrethroids during neonatal development raising concerns about its health implication. For example, a study found that 67% of a cohort of preschool children had detectable level of the

pyrethroid metabolite 3-phenoxybenzoic acid in their urine (Morgan *et al*., 2007). Furthermore, pyrethroid metabolites have also been found in the urine of pregnant women (Berkowitz *et al.,* 2003). The findings that the developing foetus and children are exposed to measurable levels of pyrethroids raises concern over the potential for developmental neurotoxicity (DeMicco *et al*., 2010), since it has been shown that some pyrethroids are more acutely toxic to developing animals than adults (Sheets, 2000). This is buttressed by the fact that a recent study has demonstrated that developmentally expressed isoforms of voltage-gated sodium channels, the molecular target of pyrethroids are uniquely susceptible to some pyrethroids (Meacham *et al.,* 2008). In addition, the endocrine disruption property of pyrethroids, including CYP has recently been reported (Solati *et al*., 2010). Pyrethroids, including CYP, suppress the normal gene expression, regulated by androgen (Xu *et al*., 2008). Maternal CYP exposure during lactation has been shown to impair testicular development and spermatogenesis in male mouse offspring (Wang *et al*., 2011b). In humans, evidence abounds that varieties of pyrethroids and their metabolites disrupt the function of multiple nuclear hormone receptors and have the potentials to alter endocrine and reproductive activities in humans (Du *et al*., 2010).

The increasing use of pesticide mixture, especially those involving pyrethroids and OPs to combat the menace of pesticide resistance may pose new insight into their effect on developing individuals, especially as it relates to reproductive and mental status.

1.3 Justification of the Study

In developing nations such as Nigeria, women play an important role in agriculture irrespective of their reproductive status. During pregnancy, the foetus can be exposed to

pesticides during farm works; thus, endangering the health of the unborn child (London *et al*., 2002; Bretveld *et al*., 2008). Pregnant and lactational mothers may be exposed to pesticides from various sources including air, food, water and occupational and household environments (Monsour and Mossa, 2010). These pose great threat to the health of the unborn child and the developing children since they are more vulnerable than adults to events that are unique to development, such as cell division and differentiation (Rogers and Kavlock, 2001).

Many pesticides have been tested for toxicological studies. A limited number of these studies have been done to evaluate the risk of mixture of more than one active ingredient. Presently, the large-scale use of these mixtures of pesticides is on the increase in developing countries (Committee on Toxicity of Chemicals, 2002). The introduction of these new mixtures into the environment has necessitated accurate identification of their potential hazards to human and animal health (Bolognesi, 2003). In addition, the local manufacturers formulate a mixture of pesticides ready to use. These mixtures may be more hazardous than using each of them alone (Noaishi *et al*., 2013). Pesticide mixture is increasingly used in agriculture and public health because of its high efficacy, convenience, rapidity of actions and lack of resistance by pests. This, however, poses fresh and unique challenge since their effect on health and environment is just emerging. The OPs and pyrethroids are one of the most commonly used pesticide mixture in agriculture and public health. Although epidemiological and animal model studies have shown that CPF and CYP individually cause reproductive and developmental neurotoxicity, information on the effect of *in-utero* and lactational exposure to OPs and pyrethroids combination on these two landmarks is scanty and, therefore, needs to be further investigated. This is even more pertinent in a developing

country like Nigeria that is more vulnerable to pesticide toxicity due to lack of or less stringent enforcment of pesticide use regulations; and the fact that many industrialized countries have turned the developing countries to a dumping ground for restricted or even prohibited pesticides (Mbakaya *et al.,* 1994; Ngowi *et al.,* 2001; Smith, 2001). In addition, most livestock and arable farmers, who are the end-users of these products, are not literate enough to recognize the long-term health and environmental consequences of exposure to these pesticides. The lack of adherence to withdrawal periods before turning treated animal and plant products to the market for consumption is also prevalent in these countries. Therefore, exposure to pesticides will continue to be a major problem to contend with in many of these countries (Bertolote *et al*., 2006).

Since it is impracticable to combat the menace posed by indiscriminate pesticide usage in many developing countries, measures aimed at reducing its impact on health of man and animals must, therefore, be put in place. Organophosphate and pyrethroids are the most widely used insecticide group accounting for 50-70% (Ojha *et al*., 2011; Mullins *et al*., 2015) and 25% (Shafer *et al*., 2005), respectively, of the global insecticide application. Although very few studies have evaluated the effect of co-administration of OP and pyrethroids, none of these, to the best of my knowledge, has focused on developmental neuro- and reproductive toxicities. It is, therefore, appropriate to investigate the effect of *in-utero* and lactational co-exposure to CPF and CYP on reproductive and neuro-developmental parameters of the male F1 generation. The lactational exposure becomes more pertinent, especially since several studies have shown increase in pesticide residues in milk and milk by-products in both humans and animal models (Fagnani *et al*., 2011; Kiljanek *et al*., 2013). Since oxidative stress is central to the molecular mechanisms of CPF and CYP toxicity-associated low

antioxidant reserve in the developing foetus (Shi *et al*., 2005), the study will further attempt to evaluate the role of antioxidant melatonin (MEL), in mitigating the developmental neuro- and reproductive toxicity, which may be evoked by this pesticide mixture.

1.4 General Aim of the Study

The study was aimed at evaluating the effect of MEL on developmental, neurobehavioural and reproductive toxicity evoked by *in utero* and lactational co-exposures to CPF and CYP in F1 generation male Wistar rats

1.5 Objectives of the Study

The specific objectives of the study were to evaluate the:

i. Developmental toxicity associated with *in utero* and lactational co-exposure to CPF and CYP in F1 generation male Wistar rats.

ii. Neurotoxicity associated with *in utero* and lactational co-exposure to CPF and CYP in F1 generation male Wistar rats.

iii. Reproductive toxicity associated with *in utero* and lactational co-exposure to CPF and CYP in F1 generation male Wistar rats.

iv. Role of oxidative stress in the developmental neuro- and reproductive toxicity associated with *in utero* and lactational co-exposure to CPF and CYP in F1 generation male Wistar rats.

v. Ameliorative effect of MEL on developmental, neurobehavioural and reproductive toxicity associated with *in utero* and lactational co-administration of CPF and CYP in F1 generation male Wistar rats.

1.6 Null Hypotheses

i. *In utero* and lactational co-exposures to CPF and CYP does not cause developmental, neurobehavioural and reproductive toxicity in F1 generation male Wistar rats.

ii. Oxidative stress does not play a role in the developmental, neurobehavioural and reproductive toxicity, induced by *in utero* and lactational co-exposure to CPF and CYP in F1 generation male Wistar rats.

iii. Melatonin (MEL) does not ameliorate the neuro- and reproductive toxicity, induced by *in utero* and lactational co-exposures to CPF and CYP in F1 generation male Wistar rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Pesticides

Pesticides, which are substances or mixture of substances used against pests in all developmental forms are daily contaminants of the environment, including water, soil, air and are found in human and animal tissues all over the world (Mnif *et al*., 2011). The role of pesticides in the modern day agricultural activity cannot be overemphasized, particularly in the control of pests in plants and animals, reduction of farm labour requirements, as well as improvement in farm yields and performance (Eyhorn *et al*., 2015). Pesticides are designed to protect crops against unwanted species involving weeds, insects and fungi, in order to increase crop yields and for controlling vector and vector-borne diseases in homes (Bjorling-Poulsen *et al*., 2008). The estimated annual application of pesticides is more than four million tones with only about 1% reaching the target pest (Bala *et al*., 2015).

Pesticides include insecticides, herbicides, fungicides and various other substances used to control pest (Casida, 2009). Although the definition of pesticide varies with time and countries, however, the essence of pesticide remains constant; that is, it is a substance or mixture of substances that is poisonous and efficient to target organisms and is safe to non-target organisms and environments (Zhang *et al*., 2011). However, no pesticide is able to meet this ideal definition, as toxicity is recorded in non-target organisms and the environments.

2.1.1 General health effect

Although their use has been positively acknowledged, adverse health effects of pesticide adversity is a global phenomenon, causing annually an estimated unintentional 25,000 poisoning in agricultural workers worldwide (Jeyaratnam, 1990; Alavanja, 2009). Thus, they pose potential risk to the health of animals and humans. The health challenges arising from pesticide exposure can be acute or chronic (Mamane *et al*., 2015). Acute poisoning involves exposure to a large amount of the pesticides within a short period resulting in sudden, sometimes violent syndrome that may even cause mortality. Chronic syndromes may either arise from persistent exposure to low level of pesticides over a prolonged period or those that occur after months or years following acute exposure (Eddleston *et al*., 2005; Kumar *et al*., 2010; Rastogi *et al*., 2010). The effect of pesticides such as organochlorines, OPs, and cabamates may cause an array of adverse effects, notably death, diseases and birth defects in human and animals. Specific effects include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive and developmental disorders, and disruption of the immune system (Alavanja *et al*., 2013; Shittu *et al*., 2014; Kumar *et al*., 2015a; Lake *et al*., 2015).

2.1.2 Characteristics and general effects of pesticide mixture

Humans and animals are subjected to a range of chemical exposures from the environment. Chemicals in air, water, soil and food, occupational exposures and lifestyle factors, all contribute to a complex exposure situation in everyday life activities (Silins and Hogberg, 2011; Woodruff, 2011). It has long been known that toxicity can be modified by simultaneous or sequential exposure to multiple agents in the environment. The modifications and effects brought about by exposure to chemical

mixtures may be synergistic, additive or antagonistic (Silins and Hogberg, 2011; Rodea-Palomares *et al*., 2015; Heys *et al*., 2016).

There are two main principles describing how individual chemicals in a mixture affect one another: the concept of additivity and interaction. Additivity assumes that chemicals act by the same or different modes of action, which results in dose or effect addition. Interaction assumes that individual chemicals affect toxicity of one another, either by synergism or antagonism (more or less than an additive effect) (Cassee *et al*., 1998). Examples of interactions are agent-to-agent interactions, toxicokinetic and toxicodynamic interactions (Mumtaz *et al*., 2007; Sexton and Hattis, 2007). For both models described above, there are mathematically based methods used to predict toxicity of mixtures. However, toxicity is not always simple to predict for complex mixtures. Modes of actions for some chemicals may be unknown and interacting effects may differ depending on dose and dose ratio (Spurgeon *et al*., 2010). Depending on the availability of data, different strategies for assessing the effects and risks of mixtures or combined exposures are employed for risk assessment. For example, the risk could be assessed using data of the mixture in question (whole-mixture approach), using data of a similar mixture or of individual chemicals, and employing the concept which best applies to the particular mixture (Kortenkamp *et al*., 2009).

2.1.3 Organophosphates insecticides

Organophosphate insecticides are esters, amides or thio-derivatives of phosphonic or phosphoric acid (Carullo *et al*., 2015). They attain great importance in pest control because of their rapid decomposition and less likely accumulation in the environment (Aydin *et al.*, 2011). Therefore, in practice, and more specifically, it is used to refer to

those OP compounds, which irreversibly inhibit the enzyme AChE activities (Nyberg and Cassel, 2015). They are classified into two main groups: Organophosphate (P=O) and organothiophosphate (P=S), depending on if oxygen or sulphur forms double bond with the central phosphate atom.

The OPs are among the most widely used insecticides globally and they are readily available commercially for domestic and industrial purposes (Ali and Chia, 2008). They account for over 50-70% of all insecticides applied world-wide (Casida and Quistad, 2004; Ojah *et al*., 2011). The OP exposure is a major public health issue in terms of death, morbidity, health care and general safety from toxicity (Jaga and Dharmani, 2003). The OP poisoning continues to be a major cause of morbidity and mortality in third world countries (Ojah *et al*., 2011), as a result of poor regulation, monitoring and even availability of technology and infrastructure (Jaga and Dharmani, 2003). Majority of the deaths resulting from poisoning in these nations have been due to cholinesterase-inhibiting pesticides such as organophosphates (Gbaruko *et al.*, 2009).

2.1.3.1 Chemical structure of organophosphate

Organophosphate insecticides (Figure 2.1) contain a central phosphorus atom with a double bond attached to either sulphur or oxygen, where R1 and R2 are usually simple alkyl groups which is either ethyl or methyl in structure; both of which may be bonded directly to phosphorus (in phosphonates), or linked via –O- or –S- (in phosphothionate). The –X represent a leaving group which is specific to the individual OP and can be any one of the variety of substituted and branched aliphatic or heterocyclic groups (Kazemi *et al*., 2012).

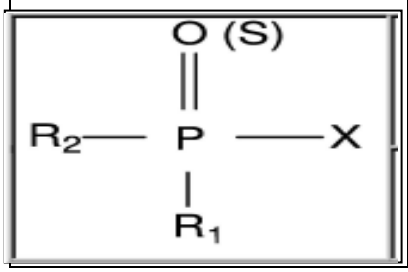
Molecular formula of organophosphorus compounds	
	R_1 and R_2 are most commonly alkoxy groups, though other chemical substitutes are also possible either oxygen or a sulphur atom could attache to the phosphorus with a double bond
	X is the so-called "leaving group", that is displaced when the OP phosphorylates acetycholinesterase (AChE), and is most sensitive to hydrolysis

Figure 2.1: General structure of organophosphate (Adapted from Carullo *et al*., 2015)

2.1.3.2 Mechanisms of action

Acute OP poisoning can manifest in three different phases; namely acute cholinergic crisis, intermediate syndrome (IMS) and delayed polyneuropathy (Yang and Deng, 2007; Morris *et al*., 2014).

Acute cholinergic syndrome occurs due to inhibition of activities of AChE in synaptic clefts, thereby deregulating the metabolism of ACh (Lionetto *et al*., 2013; Judge *et al*., 2016). ACh is a neurotransmitter critical to skeletal muscle motor neurons, peripheral parasympathetic and sympathetic neurons and multiple fibres in the central nervous system, including those that help regulate memory acquisition (Fagerlund and Ericksson, 2009). Inhibition of AChE by OP insecticides causes accumulation of ACh at the neuronal junctions and results in continued stimulation and then suppression of neurotransmission (Slotkin *et al.*, 2006; Colovic *et al*., 2013).

Some OP insecticides also affect the nervous system by inhibiting neuropathy target esterase (NTE), resulting in a rare condition known as organophosphate-induced

delayed polyneuropathy (OPIDP) (Richardson *et al*., 2015). Intermediate syndrome reported with some OP compounds has also been reported to be caused by different pathways, including 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, oxidative stress-related myopathy and changes in receptor-ligands binding kinetics (Yang and Deng, 2007; Coulson, 2015). Oxidative stress resulting from increased ROS generation and decreased tissue antioxidant potentials are increasingly been implicated in the molecular mechanism of OPs poisoning (Shittu *et al*., 2014; Surajudeen *et al*., 2014).

The biochemical mechanism of organophosphate inactivation of AChE is initiated by precursory phosphorylation at the catalytic serine residue (Colovic *et al*., 2013). The phosphorylation of AChE by an OP is synchronous with the ejection of a leaving group to yield a stable, covalent phosphoserine ester bond (Thompson *et al*., 2010). The phosphorylated AChE can usually undergo two possible post-inhibition fates:

a. Reactivation, which is the cleavage of the phosphoserine-ester bond either spontaneously (water) or mediated by oxime antidotes.

b. “Ageing” in which phosphoester bond other than the phosphoserine is cleaved to produce a phosphate oxyanion. An aged OP-AChE conjugate is considered to be irreversibly inhibited and typically unable to regain enzymatic activity (Liu *et al*., 2008).

The inhibition and post-inhibition rates and mechanism are dependent on the structure and reactivity of the phosphorous ester ligand (Liu *et al*., 2008). For example, OP-AChE conjugate that contains branched dimethyl esters generally reactivates readily,

whereas OP-AChE conjugate that contains longer or branched alkyl groups (for example, Isopropyl and isobutyl) are more prone to undergo an ageing mechanism (Masson and Lockridge, 2010).

Intermediate syndrome (IMS) occurs 24-96 hours after exposure to acute OP poisoning and is said to last from 5 to 18 days and may result, in part, from muscle necrosis (Yang and Deng, 2007; Narang *et al*., 2015). Findings by Jayawardane *et al*. (2008) suggest that IMS is a spectrum of disorder in which the initial pathological process starts with acute cholinergic state, which may or may not progress through a series of electrophysiological changes leading to respiratory failure and progressive decrement on repetitive nerve stimulation (RNS). Clinically, an association between increased blood muscle enzyme (that is, creatinine phosphokinase and lactate dehydrogensase) and the development of IMS has been demonstrated, which also supported the role of muscle injury in IMS (John *et al*., 2003). It has been further proposed that down regulation or desensitization of postsynaptic acetylcholine receptors after prolonged ACh stimulation could explain the occurrence of IMS (Radwan and El-Nabarawy, 2014).

Early studies on the mechanism of OPIDP centered on the inhibition of the esterases, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Abou-Donia, 2003) by OP esters. Subsequent studies, however, eliminated both enzymes as target for OPIDP. However, there was a proposal that a serine based enzymatic substance, neuropathy target esterase (NTE), is preferentially inhibited by some OP compounds that are capable of producing OPIDP (Lotti and Moretto, 2005). Despite numerous studies since the introduction of the concept many years ago, the NTE hypothesis has not advanced our understanding because: (a) evidence for the involvement of NTE in the development

of OPIDP is correlative (b) it has not been shown how inhibition and ageing of NTE leads to axonal degeneration (c) NTE, which is present in neuronal, non-neuronal tissues and other sensitive species has no known biochemical or physiological function (d) some OP pesticides that produce OPIDP in humans do not inhibit or age NTE (Lotti, 1992; Costa, 2006). However, the most convincing evidence against this hypothesis is the recent finding that NTE-knock out mice are sensitive to the development of OPIDP (Bus *et al*., 2003; O'Callahan, 2003; Winrow *et al*., 2003), indicating that this enzyme in not involved in the mechanism at OPIDP. However, recent studies have revealed protein kinase as targets for OPIDP (Abou-Donia, 2003). Anomalous hyperphosphorylation of cytoskeletal elements has been associated with OPIDP (Abou-Donia, 2003). Central to this hypothesis is the observation that increased aberrant protein kinase-mediated phosphorylation of cytoskeletal proteins could result in the destabilization of microtubules and neurofilaments, leading to their deregulation in the axon (Abou-Donia, 1995). Protein kinases are able to amplify and distribute signals, because a single protein can phosphorylate many different target proteins (Abou-Donia, 1995).

Small doses of OP compounds cause delayed neuronal cell death that involves ROS. The OP insecticides that cause mitochondrial damage/dysfunction also cause depletion of ATP and increased generation of ROS, which result in oxidative stress (Mishra *et al*., 2013). The ROS can cause fatal depletion of mitochondrial energy (ATP), induction of proteolytic enzymes, and DNA fragmentation, leading to apoptotic death (Borutaite, 2010; Orrenius *et al*., 2010; Circu and Aw, 2010). These results are consistent with DNA damage detected in the lymphocytes in peripheral blood of rabbits and haematopoietic bone marrow cells of mice, following exposure to the OP insecticide

CPF, diazinon; and dichlorvos respectively (Rahman *et al*., 2002; Nazam *et al*., 2013; Zafiropoulos *et al*., 2014).

The brain is highly susceptible to oxidative stress-induced injury for several reasons; (a) its oxygen requirement are high; (b) it has a high rate of glucose consumption (c) it contains large amount of peroxidizable fatty acids; and (d) it has relatively low antioxidant capacity (Floyd and Carney, 1992; Gandhi and Abramov, 2012). In addition, those brain regions that are rich in catecholamines are exceptionally vulnerable to free radical generation. The catecholamines adrenaline, noradrenaline, and dopamine can spontaneously breakdown (auto-oxidise) to free radicals, or can be metabolized to radicals by the endogenous enzymes such as monoamine oxidases (MAO). One of such region of the brain is the substantia nigra (SN), where a connection has been established between antioxidant depletion (including GHS) and tissue degeneration (Anand and Nehru, 2006).

Several studies have shown that oxidative stress is an important component of the mechanism of OP compound toxicity (Lukaszewicz-Hussain, 2010; Shittu *et al*., 2012a, 2014; Surajudeen *et al*., 2014; Fenga *et al*., 2015). Repeated daily oral doses of pesticide in rats altered the biochemical parameters and antioxidant status (Manna *et al*., 2004; Kalender *et al*., 2010). Several studies reveal that OPs may induce oxidative stress leading to generation of free radicals and alteration of antioxidant status (Lasram *et al*., 2015). Studies have shown an increasing level of MDA concentration in farm workers and applicators than in controls (Astiz *et al*., 2011). Pesticides induce a wide array of human health effects through oxidative stress causing cytogenetic damage and carcinogenicity (Gaikward *et al*., 2015).

2.2 Chlorpyrifos

Chlorpyrifos (O, O-diethyl-3, 5, 6-trichloro-2-pyridyl phosphorothioate) (CPF) (Figure 2.2) is a chlorinated OP insecticide widely used globally in the last four decades for agricultural and non-agricultural purposes. It was registered in the United States by Dow Elanco Chemical Company as early as 1965 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. It was first registered as a termiticide in the United States in 1980 (Racke, 1993) and has largely replaced chlordane, a highly toxic pesticide hitherto used for this purpose. Although CPF has been principally used as pesticide in agricultural sector, its domestic use is found to be extensive in homes when used indoors to get rid of cockroaches, fleas, spiders and flies (Lemus and Abdelghani, 2000). It is also applied on the body surface of domestic animals like sheep and horse to eradicate lice and fleas, as well as for the treatment of dog kennels against pests (Extension Toxicology Network, 1996).

Farmers are exposed to CPF by ingestion, inhalation, as well as dermal exposure during preparation of the spray solutions, loading of spray tanks and application (Poet *et al*., 2014). The trade names of the CPF include Brodan®, Detmol UA®, Dowco 179®, Dursban®, Empire®, Eradex®, Lorsban®, Paqeant®, Piridane®, Scout®, Stipend®, Termicot®, Termikill® and many others (Smith *et al*., 2009; Salyha, 2010). In the United States of America, there are over 850 registered CPF products (Grabusky *et al*., 2004; Mitra *et al*., 2008). In China alone, an annual CPF production of 200,000 metric tonnes was estimated in 2015 (John and Shaike, 2015). Due to the widespread usage, CPF has been frequently detected in air, soil, and water environments in China (Li *et al*., 2011, 2014; Zhang *et al*., 2012). Despite recent restrictions on home use in certain countries

like USA, Yemen, South Africa and many others, it remains a popular pesticide throughout the world (Mitra *et al*., 2008). It is authorised for use in about 100 countries worldwide, including; Ukraine, U.S.A., Canada, the United Kingdom, Spain, France, Italy, Japan, Australia, New Zealand, and most other developed (Salyha, 2010); and developing nations, including Nigeria. More than 50 crops, many of which are dietary staples for entire nations, are protected from insect infestation with CPF products (Salyha, 2010). In 2000, the National Centre for Food and Agricultural Policy, Washington DC, estimated that up to 3 million pounds (1.4 million kg) of CPF was being used in the home-and-garden market each year in USA (Colborn, 2006; Mitra *et al*., 2008). The United Kingdom Advisory Committee on Pesticides (ACP) have recommended that use of CPF in home and garden products be banned and have also raised concerns about the safety levels for the pesticide in food (Salyha, 2010). In 2002, the use of CPF was restricted to only agricultural applications, and all domestic use was to be completely phased out by 1st January, 2005 (Colborn, 2006); while its total ban for both agricultural and non-agricultural use by December, 2016 in the USA is under review (Davies, 2015).

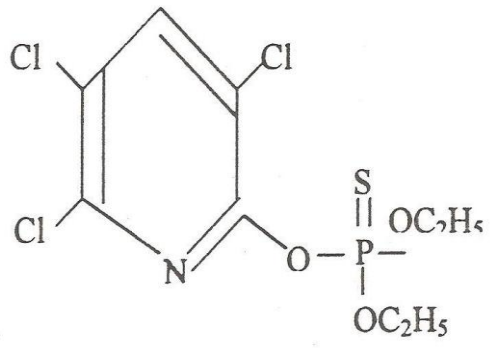


Figure 2.2: Chemical structure of chlorpyrifos (USEPA, 2000)

2.2.1 Physico-chemical properties

Chlorpyrifos with a molecular weight of 35,062 is a clear to white crystalline solid pesticide with a strong mercaptan odour and water solubility of 2 mg/L (25°C). It is soluble in benzene, acetone, chloroform, carbon disulphide, diethyl ether, xylene, methylene chloride, ethanol and methanol. Melting point of CPF is estimated to be 41.5 - 44°C; vapour pressure: 2.5 mPa (25°C); partition coefficient: 46,990; soil adsorption coefficient Koc: 6070; hydrolysis half-life: 72 days (pH 7); aqueous photolysis half-life: 29.6 days (pH 7); aerobic soil metabolism: 76.9 days; aerobic aquatic metabolism (t½): 153.8 days while the anaerobic aquatic metabolism (t½) is found to be 81.5 days (Wauchope *et al*., 1992; Barr and Angerer, 2006). It is available as granules, wettable powder, dustable powder and emulsifiable concentrate (Wauchope *et al*., 1992). It has short to moderate persistence in the environment as a result of several dissipation pathways that might occur concurrently. Primary mechanisms of dissipation include volatilization, photolysis, abiotic hydrolysis, and microbial degradation (Solomon *et al*., 2014). Volatilisation dominates dissipation from foliage in the initial 12 hour after application, but decreases as the formulation adsorbs to foliage or soil (Mackay *et al*., 2014). In the days after application, CPF adsorbs more strongly to soil, and penetrates more deeply into the soil matrix, and becomes less available for volatilization; then other degradation processes become important (Solomon *et al*., 2014).

2.2.2 Uses

Chlorpyrifos was originally used primarily to kill immature or laval stages of mosquitoes (Wu, 2000). It is an important tool in the management of a large number of pests (mainly insects and mites) and is used on a wide range of crops globally, as it is effective in controlling cutworms, corn rootworms, cockroaches, grubs, flea beetles,

flies, termites, fire ants, and lice. It is used as an insecticide on grains, cotton, field, fruit, nut and vegetable crops, as well as on lawns and ornamental plants (Solomon *et al*., 2014). It is also registered for direct use on sheep and turkeys, for horse site treatment, dog kennels, domestic dwellings, farm buildings, storage bins, and commercial establishments (Extension Toxicology Network, 1996). It acts on pests primarily as a contact poison, with some action as a stomach poison. It does not mix well with water, so it is usually mixed with oily liquids before it is applied to crops or animals. It may also be applied to crops in a capsule form (Salyha, 2010).

2.2.3 Mechanism of action

The main toxicological target for CPF is AChE, the inhibition of which results in the accumulation of acetylcholine, continual stimulation of acetylcholine receptors, and subsequent hyperactivity in the cholinergic system (Marty *et al*., 2012; Carr *et al*., 2017). Disruption of the nervous system that results is the secondary effect that causes the death of the animal. The amino acid sequence of AChE is highly conserved in animals, with the result that CPF is toxic to most groups of animals, although differences in toxicokinetics (absorption, distribution, metabolism, and excretion-ADME) account for differences in susceptibility among species (Solomon *et al*., 2014). However, many studies have demonstrated that even at low levels, CPF interferes with normal development through different cellular and molecular mechanisms beyond the inhibition of AChE (Gupta *et al*., 2010a,b). The CPF has been reported to induce neurochemical and behavioural aberrations at dosage levels that do not produce characteristic signs of cholinergic hyperactivity and induce only minimal amounts of AChE inhibition (Slotkin *et al*., 2006, 2007; Timofeeva *et al*., 2008a,b). Such

observations have led to the hypothesis that some of the developmental toxic effects of CPF occur through non-cholinergic mechanisms (Carr *et al*., 2017).

Another mechanism, proposed for toxicity of CPF is related to oxidative stress. It has been shown that acute exposure to OPs causes hypergeneration of ROS and consequently cellular lipid peroxidation (LPO) (Abdollahi *et al*., 2004a). Oxidative stress is involved in the pathophysiology of several toxicants and disease processes (Abdollahi *et al*., 2004b; Shadnia *et al*., 2005). The oxidants have a high potency in donating oxygen to the other substances making them enormously unstable and very reactive (Jowzi *et al*., 2016). Recent studies have demonstrated that CPF generates oxidative stress and lipid peroxidation in different cell types, rat models and also causes neuronal damage by elevating the production of ROS, DNA damage, and lipid peroxidation in the CNS (Verma *et al*., 2007; Geter *et al*., 2008; Saulsbury *et al*., 2009). CPF-induced apoptosis have also been reported and is said to depends on ROS and signalling pathways, such as the mitogen-activated protein kinase (MAPK) pathways, including c-Jun N-terminal kinase (JNK), p38 MAPK (p38), and extracellular signal-regulated kinase (ERK) (Lee *et al*., 2012). CPF is reported to have an unusually long effect on AChE, resulting from its fat accumulation effects, since it is more lipophilic due to gradual release from the fat tissues (Eaton *et al*., 2008). Furthermore, CPF has been shown to inhibit cyclic adenosine monophosphate (cAMP) second messenger systems of muscarinic receptors M2 and M4 in a concentration-dependent manner (Ward and Mundy, 1996; Howard *et al*., 2007).

2.2.4 Toxicokinetics

2.2.4.1 Absorption

Absorption varies with species. In humans, about 70% is absorbed after oral exposure of volunteers (Nolan *et al*., 1984). With the CPF metabolite, 3,5,6-trichloro-2-pyridinol (TCPY), the minimal dermal absorption was 1 - 3% (Nolan *et al*., 1984). CPF is rapidly absorbed and transported to the brain following oral exposure (Mendrala and Brzak, 1998).

2.2.4.2 Distribution

The highest tissue concentrations were found in the liver and kidney, but CPF did not bioconcentrate in peripheral tissues (Cochran *et al.,* 1992). When pregnant rats were dosed with CPF at 7 mg/kg per day on gestation day 14 - 18, foetal brain TCPY concentration was twice as high as the maternal brain concentration, five hours after the last dose of CPF (Hunter *et al*., 1999).

2.2.4.3 Metabolism

Chlorpyrifos is bioactivated to chlorpyrifos oxon (CPO) in the liver through cytochrome P450-mediated desulphuration **(**Figure 2.3**).** CPO is the active metabolite of CPF, and undergoes further metabolism by both A– and B-esterases to form diethylphosphate (DEP) and TCPY, which can undergo phase II sulfation and glucoronidation (Timchalk *et al*., 2007a). CPO mediates the toxic effect of CPF by binding irreversibly with AChE eliciting cholinergic hyperstimulation in the nervous system and in neuromuscular junctions (Timchalk *et al*., 2007b). Some A esterases (including CPF oxonase and paraoxanase) and carboxylesterase (β esterase) are known to play an important role in the detoxification of xenobiotics including CPF (Zhang *et al*., 2014a). Nevertheless,

detoxification of CPF is not limited to carboxylesterase or A-esterase as bovine serum albumin has been shown to inhibit the effects of CPF on DNA synthesis *in vitro* (Qiao *et al*., 2001). In a study on human volunteers, the mean half-life (t½) of CPF in the body was 29.6 hours (Nolan *et al*., 1984).

The urinary elimination profiles of the individual metabolites DEP, DETP and TCPy following exposure to CPF have been investigated in both human volunteer studies and in human poison incidences (Timchalk *et al*., 2007a). Bicker *et al*. (2005) evaluated the pharmacokinetics of DETP, DEP and TCPy simultaneously in urine samples, obtained following accidental ingestion of a commercial insecticide containing CPF. In addition to the three major CPF metabolites (that is, DETP, DEP and TCPy), twelve additional metabolites of CPF and CPF-oxon were identified.

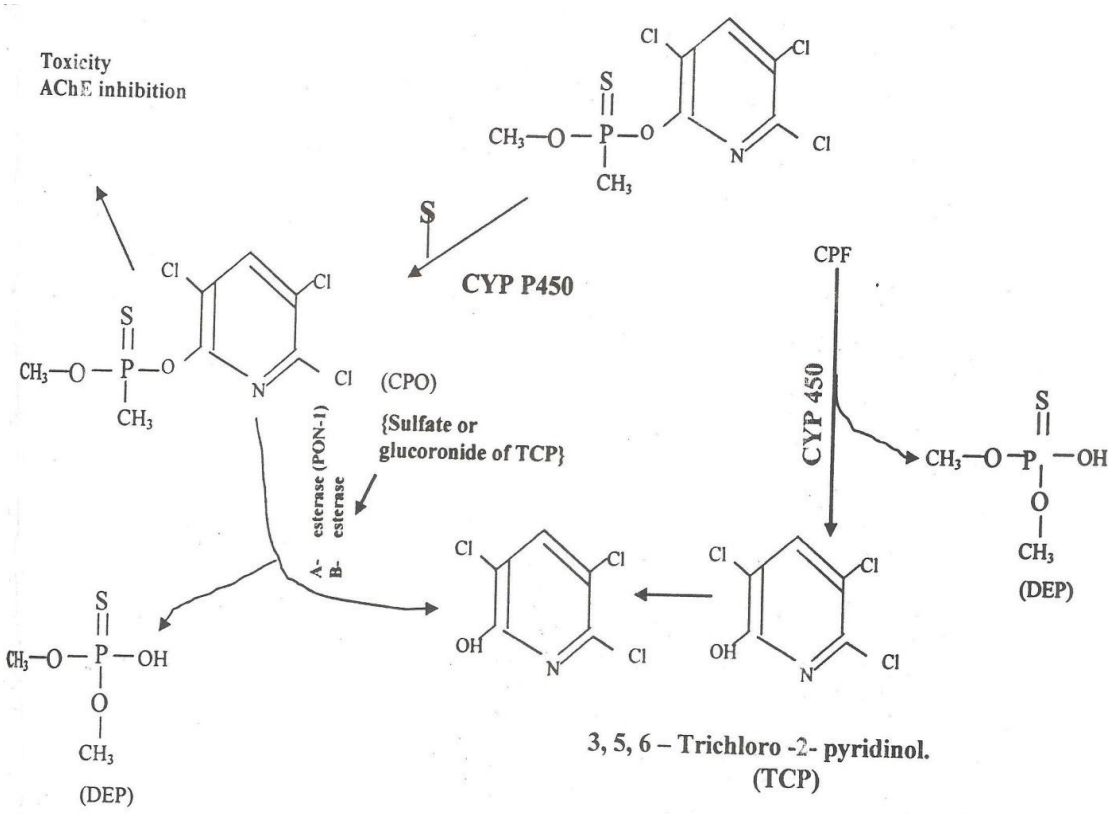


Figure 2.3: Metabolic scheme for CPF (Adapted from Timchalk *et al*. 2007b)

The main features of CPF metabolism (Figure 2.3) have been summarized by Tang *et al*. (2001), thus:

a. Activation of CPF by cytochrome P450 (CYP P450), oxidative desulphuration of the P=S moiety to P=O, resulting in the toxic intermediate CPF oxon (CPO);

b. Detoxification by CYP 450 dearylation of CPF, resulting in 3,5,6-trichloro-2-pyridinol and diethylthiophosphate.

c. Detoxification by A-esterase that hydrolyse the phosphate ester bonds of CPF and CPO to form 3,5,6-trichloro-2-pyridinol and also dietylthiophosphate (from CPF) or diethylphosphate (from CPO) and

d. Formation of glucoronide and sulphate conjugates or 3,5,6-trichloro-2-pyridinol (TCP).

2.2.4.4 Excretion

Acid labile conjugates of TCP appear to be the major urinary conjugate accounting for 10–15% of the total urinary TCP. The more minor metabolites included monoalkylated CPF and CPF-oxon, as well as a number of mono-dechlorinated CPF and CPF-oxon cysteine and glutathione conjugates (Timchalk *et al*., 2007b).

The main route of excretion in humans, rats and goats was through the urine. Approximately 70% of an oral dose was excreted in the urine of humans, while 90% was excreted in rats (Cochran *et al.*, 1992). Less than 0.1% of the dose administered to goats was found in the milk (Eaton *et al*., 2008).

2.3 Health Effects

2.3.1 Acute cholinergic syndrome

The principal toxic mechanism of CPF in humans and animals is inhibition of AChE, an enzyme responsible for degradation of ACh (Li *et al*., 2015a). ACh is a neurotransmitter present in central and peripheral neurones (Wessler and Kirkpatrick, 2008). The inhibition results in accumulation of ACh in receptors, leading to continued stimulation and subsequent cholinergic syndrome, observed as muscarinic, nicotinic and central effects (Peter *et al*., 2014). Muscarinic symptoms include salivation, lacrimation, urination, gastro-intestinal upset (emesis, diaphoresis and diarrhea), miosis, bradycardia, bronchospasm and bronchorrhea (Katz, 2015). Nicotinic effects are characterised by muscle fasciculations, cramping, weakness, and diaphragmatic failure. Autonomic nicotinic effects include hypertension, tachycardia, mydriasis, and pallor, while CNS effects manifest as anxiety, emotional instability, restlessness, confusion, ataxia, tremors, seizures and coma (Katz, 2015).

2.3.2 Intermediate syndrome

Cholinergic crisis, intermediate syndrome (IMS), and OP-induced delayed neuropathy (OPIDN) are the syndromes that are observed in OP intoxication. Intermediate syndrome following OP poisoning has been described by Senanayake and Karalliedde (1987). Approximately 20% of patients following exposure to OP pesticides may experience IMS (Karalliedde *et al*., 2006). The late onset of respiratory failure associated with IMS is a major contributor to the high morbidity, mortality, and cost of OP poisoning treatment. The IMS is clinically characterised by weakness in the cranial nerves, weakness of respiratory, neck and proximal limb muscles, and depressed deep tendon reflexes. These symptoms appear within 24 to 96 h post OP exposure and result

in two types of paralysis. The type 1 paralysis responds to atropine treatment, while type 2 does not. Duration of the IMS varies from a few days to several weeks (Van den Neucker *et al*., 1991). Mechanisms underlying the IMS are not fully known. Delayed and persistent AChE inhibition, muscle necrosis seen as electromyographic changes, down regulation or desensitization of post-synaptic ACh receptors, failure of post-synaptic ACh release, and oxidative stress-related myopathy are involved in IMS. Toxicokinetic factors, such as a high lipid-solubility, duration of AChE inhibition and metabolite excretion, evolution of alterations on repetitive nerve stimulation (RNS), type and frequency of muscle lesions are used to estimate the probability of occurrence of IMS (Abdollahi and Karami-Mohajeri, 2012).

Clinical and experimental electrophysiological studies in the IMS show that subclinical electrophysiological abnormalities are common and progressive. Studies demonstrate a repetitive firing following a single stimulus, gradual reduction in twitch height or compound muscle action potential (CMAP), followed by an increase in repetitive stimulation (decrement–increment phenomenon); and continued reduction in twitch height or CMAP with repetitive simulation (Karalliedde *et al*., 2006; Jayawardane *et al*., 2009).

2.3.3 Organophosphate-induced delayed polyneuropathy

Organophosphate-induced delayed polyneuropathy (OPIDP) is a rare toxicity resulting from exposure to certain organophosphorus (OP) esters (Datta and Bhattacherjee, 2013). It is characterised by distal degeneration of some axons of both the peripheral and central nervous systems occurring 1- 4 weeks after single or short-term exposures. Cramping muscle pain in the lower limbs, distal numbness and paraesthesia are

common signs, followed by progressive weakness, depression of deep tendon reflexes in the lower limbs and, in severe cases, in the upper limbs (Lotti and Moretto, 2005; Jokanovic *et al*., 2011). Signs include high-stepping gait associated with bilateral foot drop and, in severe cases, quadriplegia with foot and wrist drop as well as pyramidal signs. In time, there might be significant recovery of the peripheral nerve function but, depending on the degree of pyramidal involvement, spastic ataxia may be a permanent outcome of severe OPIDP (Lotti and Moretto, 2005). Human and experimental data indicate that recovery is usually complete, especially in the young (Lotti, 1992; Jokanovic *et al*., 2011). At onset, the electrophysiological changes include reduced amplitude of the compound muscle potential, increased distal latencies and normal or slightly reduced nerve conduction velocities. The progression of the disease, usually over a few days, may lead to non-excitability of the nerve with electromyographical signs of denervation (Jokanovic *et al*., 2011).

Nerve biopsies have been performed in a few cases and showed axonal degeneration with secondary demyelination. Neuropathy target esterase (NTE) is thought to be the target of OPIDP initiation (Johnson, 1975). The ratio of inhibitory powers for acetylcholinesterase and NTE represents the crucial guideline for the aetiological attribution of OP-induced peripheral neuropathy. In fact, pre-marketing toxicity testing in animals select OP insecticides with cholinergic toxicity potential much higher than the level that cause OPIDP. Therefore, OPIDP may develop only after very large exposures to insecticides, causing severe cholinergic toxicity (Lotti and Moretto, 2005).

2.3.4 Organophosphorus ester-induced chronic neurotoxicity

Various epidemiological studies have demonstrated that individuals exposed to a single large dose, or small sub-clinical doses of OP have developed a chronic neurotoxicity that persists for years after exposure and is distinct from both cholinergic and OPIDP effects (Peter *et al*., 2014). This disorder has been variously referred to in the literature as: "chronic neurobehavioural effects" (Yokoyama *et al*., 1998); "chronic organophosphate-induced neuropsychiatric disorder (COPIND) (Ghimire and Parajuli, 2016), "neuropsychological abnormalities" (Ross *et al.,* 2010b); "chronic central nervous effects of acute OP pesticide intoxication" (Rosenstock *et al*., 1991); "delayed neurologic behavioural effect of subtoxic doses" (Scremin *et al*., 2003).

Organophosphate-induced chronic neurotoxicity (OPICN) is produced by exposure to large, acutely toxic- or small sub-clinical doses of OP compounds. Clinical signs which continue for a prolonged period, ranging from weeks to years after exposure, consists of neurological and neurobehavioural abnormalities (Abou-Donia, 2003). Damage is present in both the peripheral nervous system and central nervous system, with greater involvement of the later (Abou-Donia, 2003).

Within the brain, neuropathological lesions are seen in various regions, including the cortex, hippocampus formation, and cerebellum. These lesions are characterised by neuronal cell death resulting from early necrosis or delayed apoptosis. Neurological and neurobehavioural alterations are exacerbated by concurrent exposure to stress from other chemical that cause neuronal cell death or oxidative stress (Abou-Donia, 2003). Since CNS injury predominates, improvement is slow and complete recovery is unlikely. Individuals with a history of acute OP exposure showed increased incidence of depression, irritability, confusion, and social isolation (Savage *et al.*, 1988). Such

exposure resulted in decreased verbal attention, visual memory motoricity, and affectivity (Munoz-Quezada *et al*., 2013; Fiedler *et al*., 2015). It has been reported that exposure to OP requiring medical treatment was associated with a persistent deficit in neuropsychological functions (Rosenstock *et al*., 1991; Terry, 2012; Stallones and Beseler, 2016). In addition, persistent long-term cognitive dysfunction and defects in concentration, word finding and short-term memory in individuals exposed to low sub-clinical levels of the OP insecticide, CPF have been reported (Kaplan *et al*., 1993).

2.3.5 Reproductive and developmental toxicity

Organophosphates are among the most widely used synthetic insect pesticides. The widespread use of OPs has stimulated research into the possible existence of effects related with their reproductive toxicity (Joshi *et al*., 2007). CPF, an OP has been reported to have adverse effects on the reproductive system of rats (Shittu *et al*., 2013; Ikpeme *et al*., 2016). Miranda-Contreras *et al*. (2013) showed that OP and carbamate have the potential to cause chronic occupational exposure to these pesticides on male reproductive function, leading to damage to sperm chromatin, decreased semen quality and alterations in reproductive hormones. Adverse reproductive effect from exposure to Chlorpyrifos-ethyl has been noted to passist 90 days post exposure in rats (Kenfack *et al*., 2015). CPF induces severe testicular damage and results in reduction in sperm count and thus, affect fertility (Joshi *et al*., 2007). It has been documented that CPF causes an inhibitory effect on the secretion of pituitary hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and a decrease in testosterone biosynthesis in testes of rats (Shittu *et al*., 2014).

Although some OPs are undergoing increasing scrutiny and restriction because of their propensity to elicit developmental neurotoxicity (Barone *et al*. 2000; Rice and Barone 2000; Landrigan 2001; Casida and Quistad, 2004; Slotkin, 2004), these compounds comprise 50% of all insecticide use worldwide; and exposure of the human population continues to be nearly ubiquitous (Casida and Quistad, 2004). Originally, it was thought that the adverse effects on brain development reflected the same basic mechanism that underlies systemic toxicity, namely, cholinesterase inhibition and consequent cholinergic hyperstimulation (Pope 1999). However, evidence accumulating over the past decade implicates a host of other mechanisms that depend instead upon the direct targeting of events specific to the developing brain (Barone *et al*. 2000; Rice and Barone 2000; Slotkin 2004). CPF, the most investigated OP insecticide, has been shown to disrupt the basic cellular machinery that controls the patterns of neural cell maturation and the formation and activity of synapses; exclusive of the effects on cholinesterase, which are mediated instead by its metabolite, chlorpyrifos oxon (Barone *et al*. 2000; Casida and Quistad 2004; Gupta 2004), which may likely be shared by other OPs (Whyatt *et al*. 2002; Slotkin, 2004).

2.3.6 Mutagenicity and carcinogenicity

Genotoxicity and mutagenicity of pesticides for nontarget organisms and their influence on ecosystems are of worldwide concern (Li *et al*., 2015a). Experimental studies have proven CPF induced genotoxicity (Ali *et al*., 2008, 2009; Abdelaziz *et al*., 2010; Khanna *et al*., 2011), sister-chromatid exchanges (Amer and Aly, 1992; Ali *et al*., 2009), and chromosomal loss (Woodruff *et al*., 1983). In addition, CPF-induced mitotic abnormalities and cytotoxicity has been reported (Roy *et al*., 1998). Also, a dose-dependent increase in DNA damage in the liver and brain of rats following both acute

and chronic exposure to CPF was noted at high doses of 50 and 100 mg/kg body weight (25% and 50% of rat LD_{50}), via the intramuscular injection route (Mehta *et al.*, 2008; Ojha *et al*., 2013).

2.3.7 Teratogenicity

Rauh *et al*. (2012) reported that CPF is associated with birth defects in children exposed prenatally to CPF presented as overt changes in the brain cortical structure, including reduced cortical thickness, which correlated with cognitive deficits. Ambali *et al*. (2009) reported that CPF affects conception and causes pre-implantation losses in a dose-dependent manner in Swiss albino mice. Similarly, in a study by Tian *et al*. (2005), exposure of pregnant mice to doses of CPF below those that cause significant maternal toxicity induced teratogenic and embryotoxic effect, manifested as increased and decreased absence of thoracic vertebrae and the number of caudal vertebrae, respectively.

2.3.8 Other effects

Immunologic abnormalities such as an increased expression of the CD5, CD8 surface markers and T-lymphocyte (Navarro *et al*., 2001; Nakadai *et al*., 2006) have also been reported following exposure to CPF.

2.4 Chlorpyrifos and Oxidative Stress

Although CPF induced toxicity occur mainly through inhibition of AChE, other mechanisms including induction of oxidative stress have also been implicated (Ahmed *et al*., 2010; Shittu *et al*., 2012a). This is especially true as the objective signs of toxicity can occur in the absence of detectable AChE inhibition (Aldridge *et al*., 2005c). Several

studies have demonstrated induction of oxidative stress by CPF in rats (Verma *et al*., 2009; Shittu *et al*., 2012a, 2014; Uchendu *et al*., 2014; Smida *et al*., 2017) and humans (Jang *et al*., 2015). Exposure to CPF can differentially modify endogenous antioxidants like superoxide dismutase (SOD), and glutathione, which can lead to the development of oxidative stress in some tissues (Chiapella *et al*., 2013). Oxidative stress has also been implicated in the pathogenesis of deficits in several tissues in the body. Oxidative stress induced by chlorpyrifos has been identified in cell and animals experiments (Saulsbury *et al*., 2009; Ma *et al*., 2013). CPF induces endoplasmic reticulum stress in JEG-3 cells; however, these cells are able to attenuate it, down-regulating the levels of the pro-apoptotic protein p53 (Reyna *et al*., 2017). Significant increase of urinary 8-hydroxydeoxyguanosine (8-OHdG), a urinary oxidative stress biomarker was observed on farmers involed in CPF spraying, which indicates a potential oxidative damage in farmers (Wang *et al*., 2016).

2.5 Pyrethroids

They are a class of synthetic insecticides. Their chemical structure is based on naturally occuring pyrethrins, which are found in the flowers of *Chrysanthemum cinearaefolum* (Saillenfait *et al*., 2015). They are known to have a high selectivity for insects (Wolansky and Harrill, 2007) and are a class of neurotoxic pesticides registered for use in agricultural and indoor applications. Use of pyrethroids has continuously increased during the past years (Casida and Quinstad, 1998), and is presently being evaluated for many applications and as a possible replacement for some of the OPs, carbamate or organochlorine insecticides (Ross *et al*., 2010a). They combine a high toxicity to insects with a low toxicity to mammals and their mammal (oral) to insect (topical) toxicity ratio (LD_{50} rat/LD_{50} insect) is much higher than that of the other major classes of insecticides.

Comparison of toxicities of insecticides with toxicity ratios (selectivity factors) of 16 (carbamate), 33 (OPs), 91 (organochlorines) and 4500 (pyrethroids) indicate the relative safety of pyrethroids (Perger and Szadkowski, 1994).

2.5.1 Chemical structure of pyrethroids

The basic pyrethroid structure consists of an acid and an alcohol moiety, with an ester bond. Changes have been progressively introduced to increase their insecticidal potency and decrease their sensitivity to air and light. Most pyrethroids are chiral molecules and exist as mixtures of enantiomers (Saillenfait *et al*., 2015) and contain cycloproane carboxylic acid moieties (or an equivalent group) lined to aromatic alcohols through a central ester (or ether) bond (Figure 2.4). Changes in the basic pyrethroid structure are geared toward attaining increased insecticidal potency or photostability, which may result in changes in pyrethroid activity in non-target species (Soderlund *et al*., 2002). A notable example of such modification is the addition of a α-cyano group to the alcohol moiety, which is responsible for superior insecticidal activity; and potency of approximately one order of magnitude in acute lethality studies in rodents (Wolansky and Harrill, 2007). Apart fron the α-cyano group, other inclusions to the fundermental acid and alcohol moieties of prethroids include variations in the identity and position of halogenated and hydrophobic chemical groups, and the stereochemical arrangement of the group (Naumann, 1998)

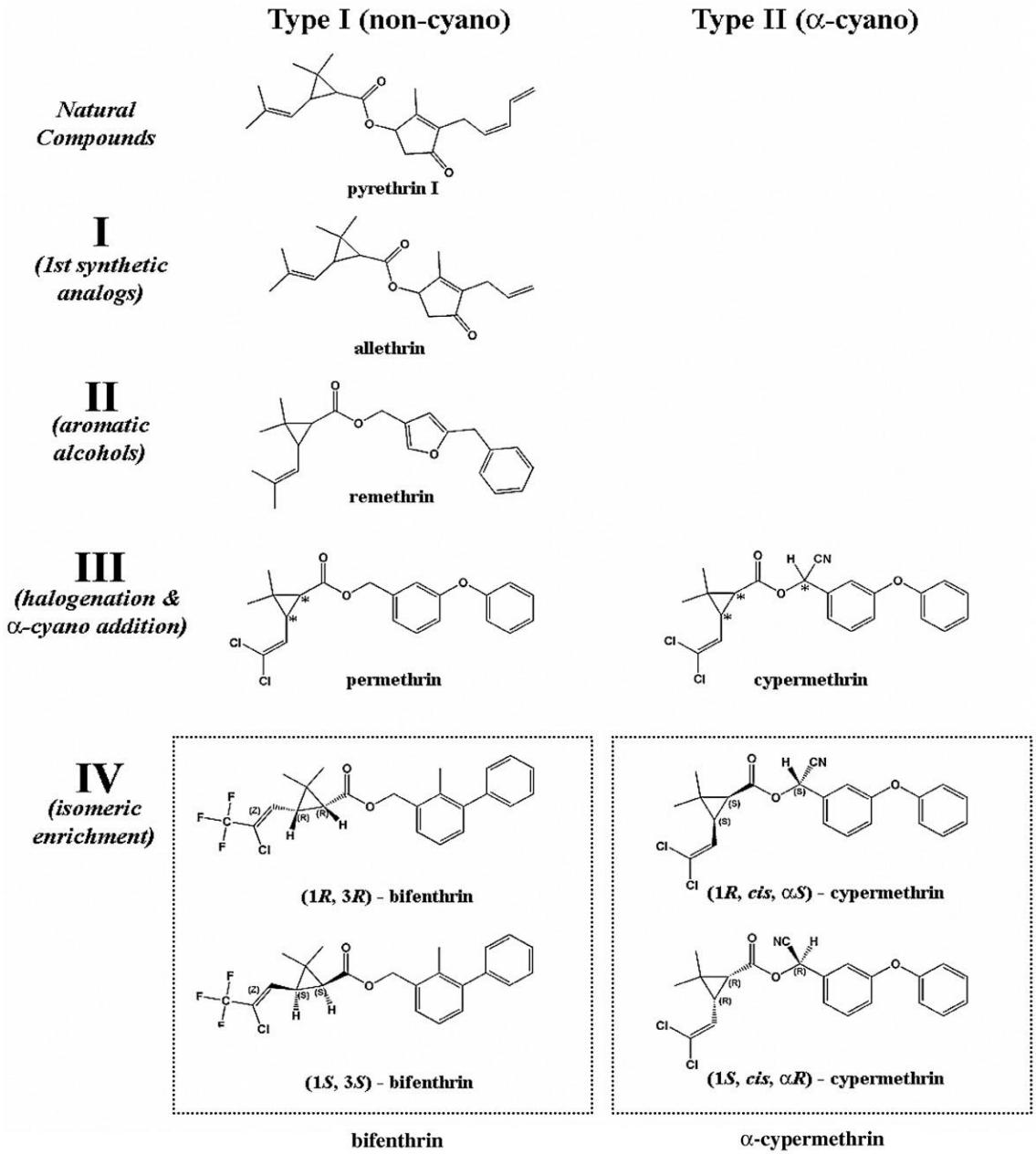


Figure 2.4: Structures of some representative pyrethroids (Adapted from Wolansky and Harrill, 2007).

2.5.2 Clinical signs

The symptoms produced by pyrethroid insecticides intoxication are of three types: Type I (T syndrome) pyrethroids produce abnormal sensitivity and coarse tremors leading to prostration. Type II (CS syndrome) pyrethroids produce ptyalism and coarse tremors progressing to twisting movement of the neck and tail, while type I/II or TS pyrethroids produce signs of both whole-body tremors and salivation (Shafer *et al*., 2005).

Type I pyrethroids, which include allethrin, bifenthrin, d-phenothrin, permethrin, resmethrin and tetramethrin, cause restlessness, hyperexcitation, prostration, and body tremors; Type I pyrethroids produce repetitive nerve discharges. Conversely, exposure to Type II pyrethroids, such as cyhalothrin, cypermethrin, cyfluthrin, deltamethrin, esfenvalerate, fenvalerate, and fluvalinate, and lambda-cyhalothrin, results in hyperactivity, incoordination, convulsions and writhing; Type II pyrethroids produce stimulus-dependent nerve depolarisation and blockage (Ecobichon, 1996; Soderlund and Bloomquist, 1989).

This distinction was primarily derived using mammalian studies, and although exposed insects also exhibit different symptoms between the two types, these are not so distinct (Palmquist *et al*., 2012). In contrast to OP insecticides, pyrethroids exhibit low toxicity to mammals and birds, while demonstrating strong selectivity for insects and invertebrates (Ecobichon, 1996). Chronic animal feeding studies have produced high no-effect levels, indicating both a low potential to bioaccumulate and proficient detoxicification in mammalian receptors. In addition, although these compounds are categorised as highly toxic to very highly toxic to non-target fish and invertebrate species, they are reportedly practically nontoxic to birds (Thatheyus and Seivam, 2013). In addition to low avian and mammalian toxicity, physical properties of pyrethroids also are an improvement over their predecessors. The low vapour pressures and high octanol-water coefficients of pyrethroids indicate a low propensity to volatilise and a high affinity for organic matter, soils and clay (Palmquist *et al*., 2012).

2.5.3 Historical perspective of pyrethroids

Pyrethroids are synthetic analogues of pyrethrins, insecticidal substances obtained from the flowers of a species of chrysanthemum (*Chrysanthemum cinerariaefolium*). The valuable insecticidal properties of pyrethrum (a mixture of pyrethrins, cinerins, and jasmolins) became known in the 19th century, and their properties stimulated detailed examination of the chemical constitution of the active esters in the first quarter of the 20th century (Ecobichon, 1996). Chemical research was conducted initially by British, Japanese and French scientists to find more stable and effective compounds with a mammalian toxicity as low as that of the model pyrethrin I (Pap *et al*., 1996). Although the acid moieties of the esters were correctly identified earlier, only in 1947 were the structures of the alcohols settled. The first synthetic pyrethroid, allethrin, was synthesised in 1949, and marketed in 1952 for the control of household insects (Oberemok *et al*., 2015). The first set of synthetic compounds containing chrysanthemic acid showed only moderately higher insecticidal potency than that of pyrethrin I, but they proved to be potent knock-down agents (Soderlund and Bloomquist, 1989; Palmquist *et al*., 2012).

Pyrethroids and pyrethrins affect nerve impulse transmission in insects mainly through their action on voltage-sensitive sodium channels (Soderlund and Bloomquist, 1989). Efficacy was increased by changing the alcoholic component to the 5-benzy-3-furylmethyl moiety, resulting in tetramethrin, which was first synthesized in 1964 and marketed in 1965 for indoor pest control; and resmethrin, first produced in 1967 and marketed in 1969 for control of household and public health insects (WHO, 1989a). These were the first synthetic compounds with good knockdown activity, greater insecticidal activity and lower mammalian toxicity than the natural esters (Palmquist *et*

al., 2012). Esterifying (1RS, *cis, trans*) - 2, 2 – dimethyl – 3 - (2, 2 - dimethylvinyl) cyclopropanecarboxylic acid (chrysanthemic acid) with 3-phenoxybenzyl alcohol resulted in a relatively cheap molecule, with better insecticidal activity, leading to phenothrin in 1969; which has been in commercial use since 1977 to control insects in the home and in public areas, and to protect stored grain (WHO, 1990; Pap *et al*., 1996). Halogenation of the vinyl group on the cyclopropane ring led to permethrin, which was synthesized in 1973 gave the photostability required for field use. Marketing of permethrin began in 1977 mostly for agricultural purposes, but also for body lice, mosquito nets and other household uses (WHO, 1990a; Pap *et al*., 1996). Fenvalerate and deltamethrin came on the market in 1976 and 1977, respectively, for use on cotton and other crops, and also for control of cattle insects. In 1974, the introduction of the alpha-cyano group resulted in cypermethrin, which was initially marketed in 1977 as having exceptionally high efficacy against pests in the household, in agriculture, public health, and animal husbandry (WHO, 1989b**;** Pap *et al*, 1996). Many second generation pyrethroids (foe example, bifenthrin, cyfluthrin, lambda cyhalothrin) became available in the 1980s (Weston and Lydy, 2010). Original patents expired in the early 1990s, allowing many other manufacturers to offer new active ingredients by the mid-1990s (Palmquist *et al*., 2012). The synthetic pyrethroid insecticides were developed at Rothamsted Research, which was funded by grant from Biotechnology and Biological Sciences Research Council (BBSRC), in the 1960s and 1970s. Today they account for around one sixth of global insecticide sales (Davies *et al*., 2007), and an annual global sales of pyrethorids that exceeds US$1.4 Billions.

2.5.4 Mechanism of action

Pyrethroids are known nerve poisons, like the organophosphate group of insecticides. Pyrethroids are known to alter the normal function of insect nerves by modifying the kinetics of voltage-sensitive sodium channels (VSSCs), which mediate the transient increase in the sodium permeability of the nerve membrane that underlines the nerve action potential (Soderlund and Bloomquist, 1989). Perturbations of sodium channel function by pyrethroids is stereospecific (Narahashi, 1996). Those stereoisomers that are the most potent disruptors of sodium channel function also have the most potent insecticidal or toxicological activity (Shafer *et al*., 2005). Pyrethroids slow the activation (opening), inactivation (closing) of voltage sensitive sodium channels (VSSC) and shift the membrane potential to more hyperpolarized potentials at which VSSCs open. Thus smaller depolarizing changes in membrane potential activate VSSCs, which remain open for extended time so that more Na^+ ions cross and depolarize the neuronal membrane. Pyrethroids are also known inducer of oxidative stress through increased free radical generation and reduced antioxidant capacity of the body (Fetoui *et al*., 2010; Prarabdh *et al*., 2015).

2.5.5 Toxicity

Pyrethroids are some 2250 times more toxic to insects than mammals because insects have increased sodium channel sensitivity, smaller body size and lower body temperature. In addition, mammals are protected due to poor dermal absorption and rapid metabolism to non-toxic metabolites (Bradberry *et al.*, 2005). In spite of their long history of use, there are relatively few reports of pyrethroids toxicity and deaths in mammals from their usage. Comparing the oral toxicities to rats, the pyrethroids have acute oral LD_{50} values following administration in vegetable oils, between 50 and 500

mg/kg (except few pyrethroids), and are therefore considered to be moderately toxic (EPA category II) (Soderlund *et al.*, 2002). In contrast to the moderate oral toxicity of most pyrethroids, the pyrethroids as a class exhibit very low levels of systemic toxicity following dermal exposure (Klainbart *et al*., 2014). Acute regulatory neurotoxicity studies for the pyrethroids show that the effects of pyrethroids were transient with peak effects observed within hours of exposure and full recovery within 1-14 days after treatment (WHO, 2005; Bardullas *et al*., 2015).

2.5.6 Toxicokinetics

Absorption of pyrethroids is poor through the skin and not much more effective through the gastrointestinal tract. Following oral administration of these compounds to rodents, the lipophilic pyrethroids are rapidly absorbed from the gastrointestinal tract and distributed throughout the body. However, absorption through the skin is not an efficient route of internal exposure to pyrethroids (Hughes and Edwards, 2010; Javed *et al*., 2015). In contrast, the lung can efficiently absorb inhaled doses of pyrethroids. Thus, the oral and inhalation exposure routes are important modes of pyrethroid entry into an organism (Kim *et al*., 2017). Synthetic pyrethroids undergo biotransformation in mammals both oxidatively (by P-450 monooxygenases) and hydrolytically (by esterases); and depending on the type of compound either of the pathways may predominate (Soderlund *et al.*, 2002).

Pyrethroids are rapidly hydrolysed in the liver to their inactive acid and alcohol components. Then, further degradation and hydroxylation at the fourth position occurs and oxidation produces a wide range of metabolites (Hutson, 1979; Anadon *et al.*, 2009). Pyrethroids are rapidly metabolized through ester cleavage and hydroxylation.

Pyrethroids accumulate in adipose tissue, which has a varying affinity for the different isomeric forms of cypermethrin. The detoxified metabolites are eliminated by the kidneys for several days after exposure. Pyrethroids are metabolized so rapidly, therefore the concentrations of intact pyrethroids in serum or plasma are much lower than those of urinary metabolites; which are considered to reflect short-term exposures in low-exposure, with the potential for misclassification (Bradman *et al*., 2005; Koureas *et al*., 2012). The major metabolites of pyrethroid insecticides detected in the urine are 3-PBA (a common metabolite of up to 20 synthetic pyrethroid insecticides), 4-F-3-PBA (a metabolite of the fluorine-substituted pyrethroid insecticide cyfluthrin), cis-DCCA and trans-DCCA (geometric isomeric metabolites of the chlorinated pyrethroid insecticides permethrin, cypermethrin, and cyfluthrin), and cis-DBCA (a selective metabolite of deltamethrin) (Viel *et al*., 2015). Faecal excretion can also be significant depending on the animal species and isomeric form (Anadon *et al*, 2009). Since humans possess enzymes that quickly break down pyrethroid insecticides, the pyrethroids are only toxic to humans in large quantities or over long periods of time (Mikata *et al*., 2012).

2.5.7 Uses of pyrethroids

Pyrethroid insecticides are one of the most widely used domestic and agricultural pesticides, comprising one-quarter of the world market as early as 1995 (Casida and Quistad, 1998); and still rising in popularity because they are generally more toxic to insects than non-target species (Soderlund *et al*., 2002; Wolansky and Harrill, 2007). Pyrethroids have also been used extensively for mosquito control following out-breaks of the West Nile virus, and most recently, pyrethroids have become the pesticide of choice to combat bed-bug infestations (DeMicco *et al*., 2010). Different pyrethroids are

extensively and widely used against ectoparasites in domestic animals. Although, spraying the walls of poultry house or stud/livestock farm is considered sufficient, application directly to the back of grazing animals is also necessary (Ahmad *et al*., 2011). Following the removal of the OP pesticide, CPF, for household use, pyrethroids have become a top choice for household pest control (DeMicco *et al*., 2010). Indeed, a recent study demonstrated that biomarkers of pyrethroid pesticide exposure significantly increased, while biomarkers of chlorpyrifos exposure decreased, as its household uses were in the process of being phased out (Williams *et al*., 2008).

Pyrethroids are used effectively against a wide variety of economically important pests, because they have a low oral toxicity to mammals. Their insect to mammal toxicity ratio is much higher than that of the other major classes of insecticides. However, they are lipophilic in nature, they bioaccumulate and affect animals indirectly (Kumar *et al*., 2006; Suman *et al*., 2006). They are reported to elicit reproductive and neuro-developmental toxicity, endocrine disruption and adverse immune system effects (Wang *et al*., 2009b).

2.5.8 Health effects

Synthetic pyrethroids are among the newest pesticides to enter the market place. About 30% of the pesticides used world wide are synthetic pyrethroids (Barr *et al*., 2010). Despite their extensive use, few poisonings in humans and animals have been reported **(**Chandra *et al*., 2013**).** Although pyrethroids are often considered to be ‘‘safer’’ pesticides because of their low to moderate acute toxicity to non-target species, their increased use raises concerns of potential adverse effects, particularly in sensitive populations such as children (Quiros-Alcala *et al*., 2014; de Joode *et al*., 2016). Use of

pyrethroids, particularly in residential settings, has increased dramatically over the past decade and are on the increase further as they are replacing other pesticides (for example, OP pesticides) that are considered to have higher mammalian toxicity and are linked to adverse health effects in children (Williams *et al*. 2008; Barr *et al*., 2010; Horton *et al*., 2011). This concern is intensified by recent studies, indicating that children are exposed to pyrethroids during foetal development. For example, pyrethroid metabolites have been found in the urine of pregnant women (Ding *et al*., 2015). A recent study also found that immigrant mothers and children living in poor housing conditions had detectable levels of the pyrethroid, metabolite 3-phenoxybenzoic acid (3BPA) in their urine (Morgan *et al*., 2007; Trunnelle *et al*., 2014). Lu *et al*. (2006, 2009) have also reported pyrethroid metabolites in the urine of primary school-aged children, that appear to be primarily the result of residential exposure.

The findings that the developing foetus and children are exposed to measurable levels of pyrethroid pesticides raises concern over the potential for developmental neurotoxicity because animal studies have demonstrated that some pyrethroids are more acutely toxic to developing animals than adults (Sheets, 2000). Furthermore, recent data demonstrate that developmentally expressed isoforms of voltage-gated sodium channels, the molecular target of pyrethroids, are uniquely susceptible to some pyrethroids (Meacham *et al*., 2008). Taken together, these suggest that developing animals are uniquely susceptible to pyrethroid toxicity. However, there are few published studies that have evaluated the developmental neurotoxicity of pyrethroid pesticides (Shafer *et al*., 2005).

When acute pyrethroid intoxication occurs in rats, two patterns of symptoms are observed, depending on the chemical configuration of the modified pyrethrins. The type

I pyrethroids, lacking a cyano group, produce the T syndrome (tremors, aggressive sparring, and enhanced startle response). The type II variants, containing a cyano group, produce the choreoathetosis and salivation (CS) syndrome that includes choreoathetosis, salivation, and seizures. Both types interact with the sodium channel on neuronal cell membranes, delaying closure of these channels. Type II pyrethroids also block the effect of the inhibitory neurotransmitter, GABA.

The type II pyrethroids postpone the closure of VSSCs for a considerably extended period, and membrane potential is depolarized extensively preventing action potential generation (Shafer *et al*., 2005). Pyrethroids increase peripheral natural killer and antibody-dependent cytotoxicity/immunotoxicity (Corsini *et al*., 2013), embryonic resorption and foetal mortality (Giri *et al*., 2003), foetotoxicity (Ahmad *et al*., 2009; 2011), inhibition of Na^+, K^+ and Mg^{2+} dependent ATPase activity in liver (Khan *et al*., 2009), and neurotoxicity (Sharaf *et al*., 2010).

2.6 Cypermethrin

Cypermethrin (CYP) is a member of the family of synthetic pyrethroids, belonging to type II class pyrethroids and is widely used in agricultural and other nonagricultural settings (Wielgomas and Krechniak, 2007a). It is one of the most commonly used synthetic pyrethroids.

2.6.1 Uses

Cypermethrin is a synthetic pyrethroid widely used to control many pests viz., moth pests of cotton, fruits, and vegetables crops (Meister, 1992); pests (Lepidoptera, cockroaches, and termites) in stores, ware-houses, industrial buildings, houses and

apartments, greenhouses, laboratories, ships, railcars, buses, trucks, aircrafts and non-food areas such as schools, nursing homes, hospitals, restaurants, hotels and food processing plants (United State Environmental Protection Agency, 1989); and ectoparasites in veterinary practice (Oheme and Mannala, 2001).

2.6.2 Toxicity

Accidental exposure with pyrethroids in human and animals result from advertent use. Populations at highest risk of high dose exposure are producers, hygiene and pesticide workers, and farmers that apply CYP for plant protection (Danis *et al*., 2011; Al-Shaikh, 2013). Low-dose exposure originates mainly from the household application of insecticides, and contaminated food and water (Gorell *et al*., 1998). The CYP can be found in trace amounts or at higher concentrations in soil and air. In mammals, CYP accumulates in body fat, skin, liver, kidneys, adrenal glands, ovaries, lung, blood, and heart (Hall *et al*., 1980; Manna *et al*., 2004). It is highly hydrophobic and this suggests that their action in biological membranes may be related to association with integral proteins and with phospholipids (Michelangeli *et al*., 1990).

2.6.3 Mechanism of action

Cypermethrin, like all synthetic pyrethroids, kills insects by disrupting normal functioning of the nervous system. In insects, as well as all other animals including humans, nerve impulses travel along nerves when the nerves become momentarily permeable to sodium ions, allowing it to flow into the nerve. Cypermethrin delay the closing of the gated channels that allows the sodium flow (Soderlund and Bloomquist, 1989; Soderlund, 2012). This results in multiple nerve impulses instead of the usual single one. In turn, these impulses cause the nerve to release the neurotransmitter ACh

and stimulate other nerves (Neelima *et al*., 2015). Cypermethrin also affects integral protein ATPase in the neuronal membrane (Kakko *et al*., 2003). Cypermethrin also has other effects on the nervous system. It inhibits the GABA receptor, causing excitability and convulsions (Ramadan *et al*., 1988). In addition, it inhibits calcium uptake by nerves (Meijer *et al*., 2014), and also inhibits monoamine oxidase (Hussien *et al*., 2013), an enzyme that breaks down neurotransmitters. It also affects an enzyme not directly involved with the nervous system, adenosine triphosphatase, that is involved in cellular energy production, transport of metal atoms, and muscle contraction (Kakko *et al*., 2003). A recent mechanism described for CYP toxicity is the induction of oxidative stress, a phenomenon that has gained wide acceptance in the science world. The toxicant has been shown to cause free radical-mediated tissue damage in liver, brain, kidney and erythrocytes (Kale *et al*., 1999; Giray *et al*., 2001; Sankar *et al*., 2012; Mossa *et al*., 2015). The mechanism behind the induction may not be far from the increased generation of ROS in tissues, decreased tissue antioxidant defence potentials, as well as decreased ability to rapidly excrete the offending agents and/or their metabolite from the body system.

2.6.4 General properties

2.6.4.1 Chemical structure

Cypermethrin belongs to type-II pyrethroids cyanopyrethroids and has the chemical or IUPAC name of: -cyano- (3 - phenoxyphenyl) methyl (±) - cis/trans - 3- (2, 2 - dichloro-vinyl) 2, 2- dimethylcyclo – propanecarboxylate (Figure 2.5). It has a molecular formula of $C_{22}H_{19}Cl_2NO_3$ with a molecular weight of 416.29716 (Burr, 2014).

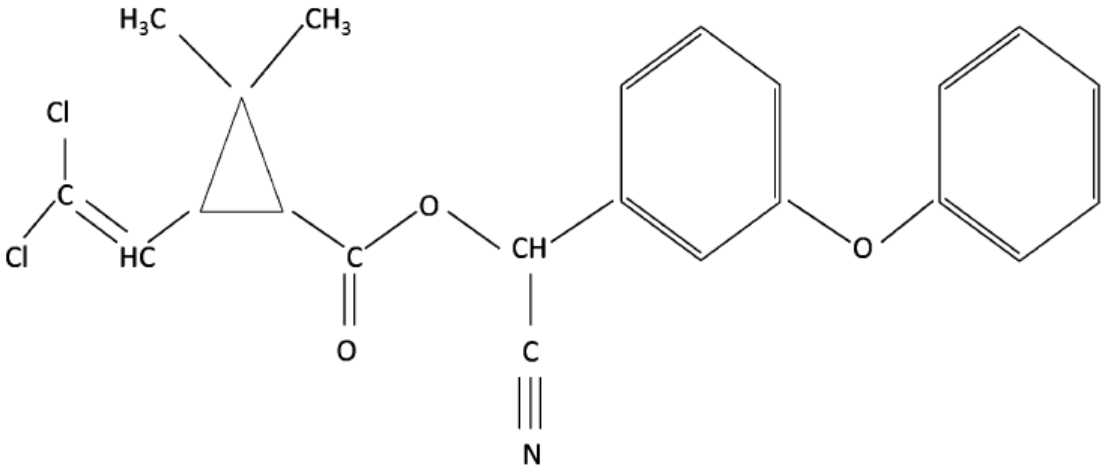


Figure 2.5: Structural formula of cypermethrin (Adapted from Prusty *et al*., 2015).

2.6.4.2 Toxicokinetics

The metabolism of CYP involves a wide range of pathways, with the primary pathway being cleavage of the ester bond. In mammals, hydrolysis of pyrethroid (PYRs) including CYP is a major pathway of detoxification. Cypermethrin, like other pyrethroids is metabolized by carboxylesterase (Nakamura *et al*., 2007), resulting in 3-phenoxybenzyl alcohol (3PBAlc) production. 3PBAlc is metabolized to 3-phenoxybenzaldehyde (3PBAld) by alcohol-dehydrogenase (ADH) and further metabolized to 3-phenoxybenzoic acid (3PBAcid) by aldehyde-dehydrogenase (ALDH) (Hodgson, 2003). Nakamura *et al*. (2007) reported that cytochrome P450s play a metabolic role of these alcohol and aldehyde oxidations (Ueyama *et al*., 2010). Pyrethroids accumulate in adipose tissue, which has a varying affinity for the different isomeric forms of CYP. Although CYP has a high lipoaffinity, it is not significantly stored in the fatty tissues and is excreted primarily intact (Leahey, 1985). In rat, elimination half-lives of 3.4 and 18 days have been measured for *trans* and *cis* isomers of CYP, respectively. Urinary excretion is the primary route of elimination. Faecal excretion can also be significant depending on the animal species and isomeric form

(Burr, 2014). Cypermethrin is readily excreted by rats and mice, leaving low residues after 8 days (Leahey, 1985).

2.6.5 Neurodevelopmental effects

The introduction of new, more toxic and rapidly disseminating pesticides into the environment has necessitated accurate identification of their potential hazards to human and animal health (Assayed *et al*., 2010a), with many of them being extremely toxic to mammals and other non-target creatures (Abdollahi *et al*., 2004a) . Pesticides may affect human reproduction by direct toxicity to the reproductive organs or by interference with hormonal function (Garcia, 1998). The developing foetus and infants are excessively susceptible to the health effects of pesticides. Developmental toxicity of pesticides may result in spontaneous abortion, growth retardation, structural birth defects, or functional deficits (National Research Council, 1993).

Pyrethroid insecticides interact with a variety of neurochemical processes, but not all of these actions are likely to be involved in the disruption of nerve function. Several lines of evidence suggest that the voltage-sensitive sodium channel is the single principal molecular target site for all pyrethroids and DDT analogues in both insects and mammals. The alterations of sodium channel functions, identified in both biophysical and biochemical studies are directly related to the effects of these compounds on intact nerves. The pyrethroid recognition site of the sodium channel exhibits the stringent stereospecificity predicted by *in vivo* estimates of intrinsic neurotoxicity in both insects and mammals (Soderlund and Bloomuist, 1989). Few reports have been documented about the developmental toxicity of many pyrethroids, with no exception to CYP. Although concentration data suggest that age dependency to toxicity is due to lower

metabolic capabilities in young rats (Sheets *et al*., 1994). Similarly, CYP was 17-fold more lethal in PND8 rats compared with adults; metabolic inhibitors were used to demonstrate that toxicokinetic factors were responsible for this age-dependent susceptibility (Cantalamessa, 1993). Malaviya *et al*. (1993) reported that binding of ^{3}H-spiroperidol to striatal membranes from PND 21 rats was increased after lactational exposure to a commercial product containing CYP (Shafer *et al*., 2005). In their work, Assayed *et al*. (2010b) showed that CYP treatment induced significant increase in the percentages of post-implantation deaths, dwarf foeti and subcutaneous oedema beside significant decrease in percentages of live born foetus and uterine implants. Cypermethrin was also noted to cause many developmental characteristics including nasal, ophthalmic, cerebral, pulmonary, cardiac and renal malformations. Developmental toxicity in the embryo-larval stages of zebrafish exposed to CYP, results in increased malformations and apoptosis signal in the nervous system. The underlying mechanism of this developmental toxicity appears to be related to the generation of oxidative stress, repressed DNA repair capacity as well as increased p53 and caspase pathway (Shi *et al*., 2011).

2.6.6 Oxidative stress in cypermethrin toxicty

Cypermethrin is a widely used insecticide and, at high doses, induces oxidative stress in mammals (Hongsibsong *et al*., 2014). Cypermethrin and other pyrethroids are metabolized in the liver via hydrolytic ester cleavage and oxidative pathways by the CYP-450 enzymes that yield ROS, which may be responsible for its oxidative stress-induced toxicity in mammals (Floodstrom *et al*., 1988; Klimek, 1990). The ROS directly react with cellular biomolecules; damage lipids, proteins and DNA in cells and ultimately lead to cell death (Filomeni *et al*., 2015). Pyrethroids are rapidly metabolised

in mammals, and several studies have shown that CYP damages the brain, liver, kidney, and erythrocytes through oxidative stress (Kale *et al*., 1999; Giray *et al*., 2001; Zegura *et al*., 2004). Sankar *et al*. (2012) reported CYP-induced oxidative damage in various organs of rats, which was attributed to decreased antioxidant enzymes in rats. Elevated CYP-induced oxidative stress and lipid peroxidation was also reported in an acute toxicity study in mice (Ince *et al*., 2012).

2.7 Oxidative Stress

The term ‘‘oxidative stress’’ began to be used frequently in the 1970s, but its origins can be traced back to the 1950s when researchers were pondering about the toxic effects of ionising radiation, free radicals, and similar toxic effects of molecular oxygen and the potential contribution of such processes to the phenomenon of ageing. The acceptance of free radical biology was remarkably slow, probably due to the largely theoretical and hypothetical nature of its beginnings, the effervescent nature of free radicals, and the lack of experimental tools to study them (Hybertson *et al*., 2011). The recognition in 1968 that biological systems could produce substantial quantities of the superoxide free radical, O_2^-, through normal metabolic pathways (McCord and Fridovich, 1968) and that enzymes (including superoxide dismutases: SOD) had evolved with the apparent sole purpose of protecting aerobic organisms from the presumed toxicity of this free radical (McCord *et al*., 1971) spurred much interest. For several decades, free radical biology has been ‘‘superoxide-centric’’, owing largely, perhaps, to the fact that superoxide is quantitatively the predominant free radical produced by biological systems (Hybertson *et al*., 2011). An example of a biologically-important free radical process that does not necessarily involve superoxide is lipid peroxidation, propagated by the characteristic ‘‘free radical chain reaction’’ (Hybertson *et al*., 2011). Oxidative stress is an inbalance between oxidants and antioxidants in

favour of the oxidants, leading to a disruption of redox signalling and control and / or molecular damage (Pisoschi and Pop, 2015; Sies, 2015).

2.8 Free Radicals

A free radical can be defined as any molecular species capable of independent existence that contains an unpaired electron in an atomic orbital (Lobo *et al*., 2010). The presence of an unpaired electron results in certain common properties that are shared by most radicals (Lobo *et al*., 2010). Many radicals are unstable and highly reactive. They can either donate an electron or accept an electron from other molecules, therefore behaving as oxidants or reductants (Cheeseman and Slater, 1993). The ROS play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body (Pham-Huy *et al*., 2008). The most important ROS in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, singlet oxygen, hypochlorite, nitric oxide radical, and peroxynitrite radical. These are highly reactive species, capable in the nucleus, and in the membranes of cells, damaging biologically important molecules such as DNA, proteins, carbohydrates, and lipids (Young and Woodside, 2001); leading to cell damage and homeostatic disruption (Lobo *et al*., 2010). Tissue lipid peroxidation (LPO), a sequelae of degradative action, resulting from increase free-radical chain production and propagation, affects polyunsaturated fatty acids; hence their involvement in the pathogenesis of several degenerative diseases, such as arteriosclerosis, diabetes, cancer and rheumathoid arthritis, as well as toxicity associated to drugs and other pharmaceuticals, pesticides and aging (Ayala *et al*., 2014; Sultan, 2014).

The ROS are derived either from normal essential metabolic processes in the human and animal's body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals (Pham-Huy *et al*., 2008; Lobo *et al*., 2010; Sultan, 2014). The formation of ROS occurs continuously in the cells as a consequence of both enzymatic and non-enzymatic reactions (Lobo *et al*., 2010). Enzymatic reactions, which serve as source of free radicals, include those involved in the respiratory chain, phagocytosis, prostaglandin synthesis, and the cytochrome P-450 system (Liu *et al*., 1999). Other sources of internally generated ROS include processes involving xanthine oxidase, peroxisomes, inflammation, arachidonate pathways, exercise, ischaemia/reperfusion injury, while some of the externally generated sources of ROS are: cigarette smoke, environmental pollutants, radiation, certain drugs, pesticides, industrial solvents and ozone among others. The ROS can also be formed in non-enzymatic reactions of oxygen with organic compounds (Pham-Huy *et al*., 2008).

2.8.1 Types of oxygen-based free radicals

The oxygen-based free radicals include (Figure 2.6):

i. *Hydroperoxyl radical***:** The hydroperoxyl radical, also known as the perhydroxyl radical, is the protonated form of superoxide with the chemical formula HO_2. Hydroperoxyl is formed through the transfer of a proton to an oxygen atom. HO_2 can act as an oxidant in a number of biologically important reactions, such as the abstraction of hydrogen atoms from tocopherol and polyunstaturated fatty acids in the lipid bilayer. As such, it may be an important initiator of lipid peroxidation (Tegeli *et al*., 2014).

ii. *Superoxide radical***:** Superoxide can act either as oxidant or reductant. It can oxidize sulphur, ascorbic acid or NADPH, and it can reduce Cytochrome C and metal ions. A

dismutation reaction leading to the formation of hydrogen peroxide and oxygen can occur spontaneously or is catalyzed by the enzyme superoxide dismutase. In its protonated form, superoxide forms a perhydroxyl radical, which is a powerful oxidant (Kumar, 2011; Phaniendra *et al*., 2015), but its biological relevance is probably minor because of its low concentration at physiological pH (Tegeli *et al*., 2014).

iii. *Hydrogen peroxide***:** The univalent reduction of superoxide produces hydrogen peroxide, which is not a free radical because all its electrons are paired. It readily permeates through the membranes and is therefore not compartmentalized in the cell (Kumar, 2011). The main damage caused by this radical is breaking up of DNA, resulting in single strand breaks and formation of DNA protein cross link (Gebicki and Gebicki, 1999). Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecule. This is an oxidizing agent but not specially reactive, and its main significance lies in it being a source of hydroxyl radical in the presence of reactive transition metal ions (Gribas *et al*., 2016; Hu *et al*., 2017).

iv. *Hydroxyl radical*: In living organisms, there are two major reactive oxygen species, superoxide radical and hydroxyl radical that are continuously formed in a process of reduction of oxygen to water (Lipinski, 2011). In the Haber-Weiss reaction hydroxyl radicals are generated in the presence of hydrogen peroxide and in ions pairs. The first step involves reduction of ferric into ferrous ion (Equation 1):

$$Fe^{3+} + {}^{\bullet}O_2^{-} \rightarrow Fe^{2+} + O_2 \quad (1)$$

The second step is the Fenton reaction (Equation 2):

$$Fe^{2+} + H_2O_2^{-} \rightarrow Fe^{3+} + OH^{-} + {}^{\bullet}OH \quad (2)$$

The requirement of hydrogen peroxide in the Fenton reaction led to the misleading concept of oxidative stress that ignores the fact that hydroxyl radical (˙OH), known to be the most biologically active free radical, is formed *in vivo* under hypoxic conditions (Michiels, 2004). It is considered one of the most active and the most harmful among reactive structures. It has been proven that reactivity of hydroxyl radical is limited by coming across other molecules, which can be attacked by the reactive structure (Wojtunik-Kulesza *et al*., 2016). It is able to damage almost all organelle and cell structure, leading to mutation and development of numerous diseases (Sen *et al*., 2010).

v. *Singlet oxygen***:** It is not a free radical per se but it can be formed in some radical reactions and can trigger off others. This arises from hydrogen peroxide molecules. Singlet oxygen on decomposition generates superoxide and hydroxyl radicals (Tegeli *et al*., 2014).

vi. *Triplet oxygen*: Triplet oxygen can react with elements and ions to form oxides, but usually not with organic compounds, which are in singlet state. However, it reacts easily with free radical molecules produced by the action of other active radicals, radiations, ultra violet light, and heat or by complex formation with oxygen and transition metal to produce active peroxide radicals and trigger auto-oxidation of unsaturated fatty acids and others (Tegeli *et al*., 2014).

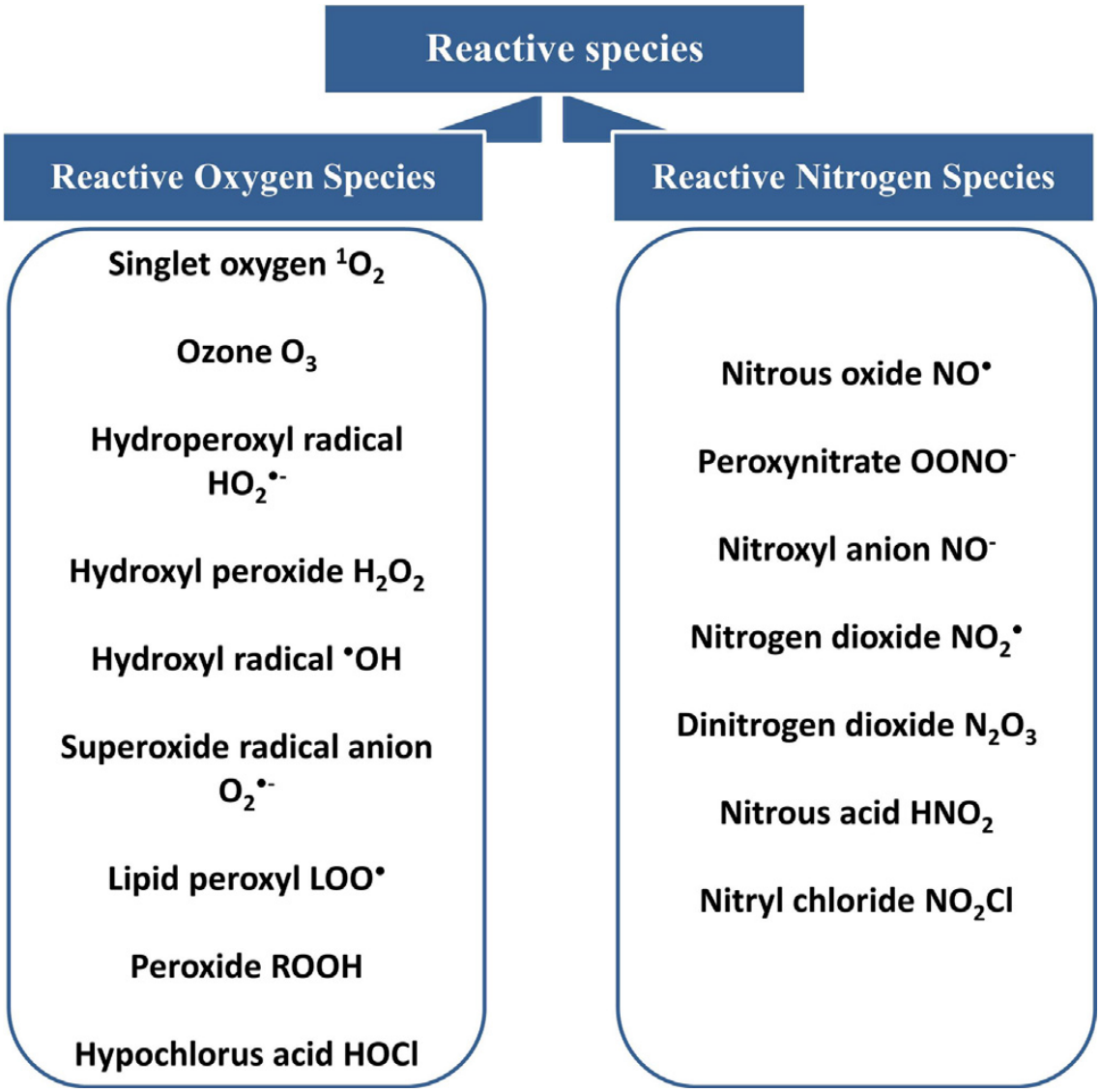


Figure 2.6: Examples of reactive forms of reactive species, divided into reactive oxygen and nitrogen species (Adapted from Wojtunik-Kulesza *et al*., 2016).

2.8.2 General formation of free radicals

Formation of free radicals involving ROS and RNS can occur in the cells by two ways: enzymatic and non-enzymatic reactions. Enzymatic reactions generating ROS and RNS include those involved in the respiratory chain, the phagocytosis, the prostaglandin synthesis and the cytochrome P_{450} system (Bahorun *et al*., 2006; Halliwell and Gutteridge, 2007). For example, the superoxide anion radical (O_2^{o-}) is generated via several cellular oxidase systems such as NADPH oxidase, xanthine oxidase,

peroxidases. Once formed, it participates in several reactions yielding various ROS and RNS such as hydrogen peroxide, hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^{-}$), hypochlorous acid (HOCl), etc. H_2O_2 (a non-radical) is produced by the action of several oxidase enzymes, including amino acid oxidase and xanthine oxidase. The latter catalyses the oxidation of hypoxanthine to xanthine, and subsequently to uric acid. Hydroxyl radical ($OH^{\bullet}$), the most reactive free radical *in vivo*, is formed by the reaction of $O2^{\bullet-}$ with H_2O_2 in the presence of Fe^{2+} or Cu^{+} (catalyst). This is exemplified by Fenton and Haber-Weiss reactions (Figure 2.7). Hypochlorous acid (HOCl) is produced by the neutrophil-derived enzyme, myeloperoxidase, which oxidizes chloride ions in the presence of H_2O_2. Nitric oxide radical (NO•) is formed in biological tissues from the oxidation of L-arginine to citrulline by nitric oxide synthase (Valko *et al*., 2004, 2007; Genestra, 2007).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + {}^{-}OH \text{ (Fenton reaction)}$$
$$OH + H_2O_2 \rightarrow O_2\text{-} + H+ + H_2O$$

$$Cu^{+} + O_2\ Cu^{2+} + O_2^{-}$$
$$O_2^{-} + O_2^{-} + 2H+\ H_2O_2$$
$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-} \text{ (Fenton reaction)}$$

$$O_2^{-} + H_2O_2 \xrightarrow{Fe^{3+}/Fe^{2+}} OH^{\bullet} + OH^{-} + O_2 \text{ (Haber-Weiss reaction)}$$

Figure 2.7: Formation of Fenton and Haber-Weiss reaction (Kanti Das *et al*., 2014)

Free radicals can be produced from non-enzymatic reactions of oxygen with organic compounds as well as those initiated by ionising radiations. The non-enzymatic process can also occur during oxidative phosphorylation (i.e. aerobic respiration) in the mitochondria (Droge, 2002; Genestra, 2007; Valko *et al*., 2007).

The ROS and RNS are generated from either endogenous or exogenous sources. Endogenous free radicals are generated from immune cell activation, inflammation, mental stress, excessive exercise, ischemia, infection, cancer, aging. Exogenous

ROS/RNS result from air and water pollution, cigarette smoke, alcohol, heavy or transition metals (Cd, Hg, Pb, Fe, As), certain drugs (cyclosporine, tacrolimus, gentamycin, bleomycin), industrial solvents, cooking (smoked meat, used oil, fat) and radiation. (Valko *et al*., 2007). After penetration into the body by different routes, these exogenous compounds are decomposed or metabolised into free radicals (Phaniendra *et al*., 2015).

2.8.3 Reactive oxygen species

The superoxide anion is formed by the univalent reduction of triplet-state molecular oxygen ($3O_2$). This process is mediated by enzymes such as NAD(P)H oxidases and xanthine oxidase or non-enzymically by redox reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. Superoxide dismutases (SODs) convert superoxide, enzymically into molecular oxygen and hydrogen peroxide in biological tissues (Perry *et al*., 2010). In the presence of reduced transition metals (for example, ferrous or cuprous ions), hydrogen peroxide can be converted into the highly reactive hydroxyl radical ($^{\cdot}OH$) (Perry *et al*., 2010). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase (Weydert and Cullen, 2010). In the glutathione peroxidase reactions, glutathione is oxidised to glutathione disulfide, which can be converted back to glutathione by glutathione reductase in an NADPH-consuming process. Superoxide and NO are readily converted by enzymes or non-enzymatic chemical reactions into reactive nonradical species such as singlet oxygen ($^1O_2$), hydrogen peroxide, or peroxynitrite ($ONOO_2$); that is, species which can in turn give rise to new radicals; therefore, the regulatory effects of these non-radical species have also been included. Most of the regulatory effects are indeed not directly mediated by superoxide, but rather

by its ROS derivatives. Frequently, different reactive species coexist in the reactive environment and make it difficult to identify unequivocally which agent is responsible for a given biological effect (Suresh *et al*., 2014). .

2.8.4 Reactive nitrogen species

The NO radical (NO_2) is produced in higher organisms by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine (Gautam and Jain, 2007). This process is catalyzed by the enzyme nitric oxide synthase (NOS). Depending on the microenvironment, NO can be converted to various other RNS such as nitrosonium cation (NO_1), nitroxyl anion (NO_2) or peroxynitrite ($ONOO_2$) (Shoman and Aly, 2016). Some of the physiological effects may be mediated through the intermediate formation of *S*-nitroso-cysteine or *S*-nitroso-glutathione (Broniowska *et al*., 2013).

2.9 Mechanism of Oxidative Damage

Depending upon their nature, ROS (for example, $^{\bullet}OH$ radicals) reactions with biomolecules such as lipid, protein and deoxyribonucleic acid (DNA) produce different types of secondary radicals like lipid radicals, sugar and base derived radicals, amino acid radicals and thiyl radicals (sulphur centered). These radicals in the presence of oxygen are converted to peroxyl radicals (Kunwar and Priyadarsini, 2011). Peroxyl radicals are critical in biosystems, as they often induce chain reactions (Kunwar and Priyadarsini, 2011). The biological implications of such reactions depends on several factors like site of generation, nature of the substrate, activation of repair mechanisms, redox status, among many others. For example, cellular membranes are vulnerable to oxidation by ROS due to the presence of high concentration of unsaturated fatty acids in their lipid components (Lobo *et al*., 2010; Sharma *et al*., 2012). ROS reactions with

membrane lipids cause lipid peroxidation, resulting in formation of lipid hydroperoxide (LOOH) which can further decompose to an aldehyde such as malondialdehyde, hydroxy nonenal (4-HNE) or form cyclic endoperoxide, isoprostanes, and hydrocarbons (Devasagayam *et al*., 2003; Ayala *et al*., 2014). The consequences of lipid peroxidation are cross linking of membrane proteins, change in membrane fluidity and formation of lipid-protein and lipid-DNA adducts, which may be detrimental to the functioning of the cell (Sharma *et al*., 2012; Ayala *et al*., 2014).

Proteins can undergo direct and indirect damage following interaction with ROS resulting in peroxidation, changes in their tertiary structure, proteolytic degradation, protein-protein cross linkages and fragmentation. The side-chains of all amino acid residues of proteins, in particular tryptophan, cysteine and methionine residues are susceptible to oxidation by ROS (Rinalducci *et al*., 2008). Protein oxidation products are usually carbonyls such as aldehydes and ketones.

Although DNA is a stable and well-protected molecule, ROS can, however, interact with it and cause several types of damage such as modification of DNA bases, single and double strand DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair systems (Kunwar and Priyadarsini, 2011). A variety of adducts are formed on reaction of ˙OH radical with DNA. The ˙OH radical can attack purine and pyrimidine bases to form ˙OH radical adducts, which are both oxidizing and reducing in nature (Bauer *et al*., 2015). This induces base modifications and sometimes release of bases (Kunwar and Priyadarsini, 2011). Some of the important base modifications include 8-hydroxydeoxyguanosine (8-OHdG), 8 (or 4-, 5-) -hydroxyadenine, thymine peroxide,

thymine glycols and 5-(hydroxymethyl) uracyl (Kalam *et al*., 2015). Free radicals can also attack the sugar moiety, which can produce sugar peroxyl radicals and subsequently induce strand breakage. The consequence of DNA damage is the modification of genetic material resulting in cell death, mutagenesis, carcinogenesis and ageing (Behrens *et al*., 2014).

2.10 Antioxidants

Antioxidants are substances that neutralize free radicals or their actions (Hamid *et al*., 2010). Their importance and essentiality in human, animals' and plants sustenance can not be overemphasize. They inhibit or quench free radical reactions and delay or inhibit cellular damage (Young and Woodside, 2001). Though the antioxidant defenses are different from species to species, the presence of the antioxidant defense is universal (Rahal *et al*., 2014; Nimse and Pal, 2015). Antioxidants exist both in enzymatic and non-enzymatic forms in the intracellular and extracellular environment (Nimse and Pal, 2015). They are substances that protect cells from the damage caused by unstable molecules known as free radicals (Hamid *et al*., 2010).

2.10.1 Classification

Antioxidants are grouped into two, namely;

(1) Primary or natural antioxidants.

(2) Secondary or synthetic antioxidants.

2.10.1.1 Natural antioxidants

They are the endogenous chain-breaking antioxidants which react with lipid radicals and convert them into more stable products, involving conversion of dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water, in a multi-step process in

presence of co-factors such as copper, zinc, manganese, and iron (Shahidi and Zhong, 2010). Antioxidants of this group are mainly phenolic in structure (Hurrell, 2003). They are made up of naturally occurring antioxidants of high or low molecular weight that differ in composition, physical and chemical properties, mechanisms and site of action. They can be further divided into the following categories:

a) *Enzymes***:** Enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase attenuate the generation of reactive oxygen species by removing potential oxidants or by transferring ROS/RNS into relatively stable compounds (Kumar, 2011).

i) *Superoxide dismutase* (SOD): This enzyme was discovered in late 60s. It catalyses the transformation of the superoxide radical into hydrogen peroxide, which can then be transformed by the enzyme catalase into water and molecular oxygen (Tegeli *et al*., 2014). Though superoxide anion in itself is not that particularly reactive, it can reduce transition metal ions, such as iron and gets converted to a more reactive radical - the hydroxyl radical (Uttara *et al*., 2009). Thus, elimination of superoxide radical can attenuate the formation of hydroxyl radical (Tegeli *et al*., 2014).

ii) *Glutathione peroxidase* (GPx): reduces lipid peroxides (ROOH), formed by the oxidation of polyunsaturated fatty acids (PUFA), to a stable, non-toxic molecule - hydroxyl fatty acid (ROH) (Kumar, 2011). Together with phosholipase, GPx can also convert phospholipids hydro-peroxide (PL-OOH) into phospholipids hydroxide (PL-OH) (Ursini *et al*., 1982).

iii) Catalase (CAT): It is a haeme enzyme, which catalyses decomposition of hydrogen peroxide to water and molecular oxygen. It is one of the main enzymes of the

antioxidant defence system of the cell (Krych-Madej and Gebicka 2017). Catalase is one of the most important antioxidant enzymes, present in almost all aerobically respiring organisms. Its main function is decomposition of hydrogen peroxide (H_2O_2) to water and molecular oxygen (the catalatic pathway). Catalase is a four subunit haeme enzyme containing a haeme group with ferric iron within each of identical subunits. The catalytic reaction proceeds as follows:

$$\text{Catalase} + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (1)$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{Catalase} + H_2O + O_2 \quad (2)$$

Where compound I is an oxoferryl porphyrin (por) π-oxidation product of a haeme group ($por^{\bullet+}Fe^{IV} = O$) (Nicholls *et al*., 2001). The rate constants of both reactions, in the case of mammalian catalases, are of the order of 10^7 M^{-1} s^{-1} (Chance *et al*., 1979). Under conditions of low concentration of H_2O_2 catalase is able to act as a peroxidase, i.e. it uses H_2O_2 (or other relatively small peroxides) to oxidize secondary two-electron and one electron donors (the peroxidative pathway) (Krych-Madej and Gebicka, 2017).

b) *Low molecular weight antioxidants***:** These are sub-divided into lipid-soluble antioxidants (tocopherol, carotenoids, quinones, bilirubin and some polyphenols) and water soluble antioxidants (ascorbic acid, uric acid and polyphenols). These delay or inhibit cellular damage mainly through free radical scavenging property.

The lipid-soluble low molecular weight antioxidants tend to accumulate in lipid plasma lipoprotein (for example, low density lipoprotein), where they act as highly efficient scavengers against lipid peroxyl radical, which are formed within the lipoprotein as a consequence of free-radical chain reaction of lipid peroxidation (Lobo *et al*., 2010).

The water-soluble low molecular weight antioxidants cannot enter the lipid moiety of low density lipoprotein (LDL); thus, are less efficient in lipid milleu and are principally unable to counter most of the lipophilic radicals (Bano *et al*., 2012). However, such compounds may act in a synergistic manner with lipophilic antioxidants by regenerating them (Tegeli *et al*., 2014).

Ascorbate has also been demonstrated to be an effective antioxidant. It can act both directly, by reaction with aqueous peroxyl radicals, and indirectly, by restoring the antioxidant properties of fat-soluble vitamin E (Traber and Stevens, 2011). The overall consequence of these antioxidant activities is the beneficial control of lipid peroxidation of cellular membranes including those surrounding as well as within intracellular organelles.

2.10.1.2 Synthetic antioxidants

These are synthetic phenolic compounds that perform the function of capturing free radicals and stopping the chain reactions (Hurrell, 2003). They are the most effective antioxidants and are synthetic in nature. They include;

i. Butylated hydroxyl anisole (BHA).

ii. Butylated hydroxyrotoluene (BHT).

iii. Propyl gallate (PG) and metal-chelating agent (EDTA).

iv. Tertiary butyl hydroquinone (TBHQ).

v. Nordihydroguaretic acid (NDGA).

They are approved by Food and Drug administration agency for addition to foods, e.g., Butylated hydroxyl anisole (BHA), butylated hydroxy toluene (BHT), tertiary butylated hydroxy quinone (TVHQ) (Hamid *et al*., 2010).

Antioxidants can be categorized as water-soluble and lipid-soluble antioxidants. The water-soluble antioxidants (for example, vitamin C) are present in the cellular fluids such as cytosol, or cytoplasmic matrix. The lipid-soluble antioxidants (for example, vitamin E, carotenoids, and lipoic acid) are predominantly located in cell membranes. The antioxidants can also be categorized according to their size, the small-molecule antioxidants and large-molecule antioxidants. The small-molecule antioxidants neutralize the ROS in a process called radical scavenging and carry them away. The main antioxidants in this category are vitamin C, vitamin E, carotenoids, and glutathione (GSH). The large-molecule antioxidants enzymes are SOD, CAT and GPx; and sacrificial proteins (albumin) that absorb ROS and prevent them from attacking other essential proteins (Nimse and Pal, 2015).

2.10.2 Mechanisms of action

In general, the antioxidants act in the following manner:

i. Chain breaking reaction eg. α-tocopherol, which act in lipid phase to trap free radical by acting in the membranes and lipoprotein dormain. The chain-breaking mechanism involves the primary antioxidants donating electrons to the free radicals present in the system, such as example lipid radicals. Vitamin E is the collective name for a set of eight-related tocopherols and tocotrienols, which are fat-soluble vitamins with antioxidant properties. Of these, α-tocopherol has been most studied as it has the highest bioavailability, with the body preferentially absorbing and metabolising this form (Herrera and Barbas, 2001; Hamid *et al*., 2010; Lobo *et al*., 2010). It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. This removes the free radical intermediates and prevents

the propagation reaction from continuing. This reaction produces oxidised α-tocopheroxyl radicals that can be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate and retinol. This is in line with findings that α-tocopherol, but not water-soluble antioxidants, efficiently protects glutathione peroxidase (GPx)-deficient cells from death. The GPx is the only known enzyme that efficiently reduces lipid-hydroperoxides within biological membranes (Herrera and Barbas, 2001; Packer *et al*., 2001; Hamid *et al*., 2010).

ii. By reducing concentration of ROS, for example, glutathione. Glutathione is a cysteine-containing peptide found in most forms of aerobic life. It is not required in the diet and is instead synthesised in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems, such as ascorbate in the glutathione-ascorbate cycle, glutathione peroxidases and glutaredoxins, as well as reacting directly with oxidants (Meister and Anderson, 1983). Due to its high concentration and its central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants. In some organisms glutathione is replaced by other thiols, such as mycothiol in the actinomycetes, or by trypanothione in the kinetoplastids (Fahey, 2001; Hamid *et al*., 2010).

iii. By scavenging initiating radicals such as those performed by superoxide dismutase which act in a lipid phase to trap superoxide free radicals (Tegeli *et al*., 2014) .

iv. By chelating transition metal catalyst: some antioxidants act by sequestrating transition metals (Fe^{2+}, Cu^{2+}, Mn^{2+}) that are well established pro-oxidants. Antioxidants such as transferrin, lactoferrin and ferritin function to keep iron induced oxidant stress in check, while ceruloplasmin and albumin act as copper sequestrants (Kumar, 2011).

2.11 Antioxidants and Nervous System

Oxidative stress refers to the pathological state in which the production of ROS and RNS is increased above the body's antioxidant defence and repair capacity (Park *et al*., 2016). Functional damage, with subsequent cell death, may occur as a consequence of the oxidization of cellular components, such as, proteins, lipids, and nuclear material. Several features of the brain suggest that it is particularly vulnerable to oxidative stress. The brain possesses the highest oxygen metabolic rate and is continually exposed to excitatory amino acids and neurotransmitters. The brain also contains a high concentration of oxidisable polyunsaturated fatty acids, but has comparatively limited endogenous antioxidant defence mechanisms (Guest and Grant, 2012).

There is an increasing evidence that antioxidants could be prophylactic against central nervous system (CNS) disease (Kamat *et al*., 2008). The importance of the cerebellum is well known in controlling various motor activities in the body, and the developing brain is susceptible to the detrimental effects of ROS. It has been reported that antioxidants prevent oxidative damage in cerebellar development and play an important role in general wellness as well as maintenance of wellness (Imosemi, 2013). Few antioxidants have been reported as therapeutic agents for acute CNS injury (Cheremisinoff, 1989). Several factors in the CNS contributes to the brain's increased vulnerability to oxidative stress (Guest and Grant, 2012). These include:

(i) The brain is one of the most energy-consuming organs and is entirely dependent on aerobic metabolism, utilizing oxygen and glucose to generate adenosine-5-triphosphate (ATP). In order to fulfill its high energy needs the brain utilizes approximately 20% of basal oxygen (O_2) consumption, despite accounting for only 2% of the total body mass (Molina and DiMaio, 2012). Consequently, a large amount of oxygen is processed per unit of tissue, producing favourable conditions for the production of ROS.

(ii) Glutamate is the principal CNS neurotransmitter. Under normal physiological conditions, the concentration of glutamate in the synaptic cleft can reach 1 mM (Ankarcrona *et al*., 1995). Higher extracellular levels have been shown to be neurodestructive, through an excitotoxic process (Cotman *et al*., 1987).

(iii) Excluding glutamate and glycine, a number of neurotransmitters autooxidises. For example, serotonin, noradrenaline, dopamine, and its precursor L-3, 4-dihydroxyphenylalanine react with O_2, resulting in the formation of both $O_2^{\bullet-}$, quinones and semiquinones. This can deplete GSH levels in the surrounding tissue, increasing the vulnerability to an oxidative attack (Valencia-Olvera *et al*., 2014; Diaz-Vivancos *et al*., 2015). The autoxidation of dopamine may explain the loss of dopaminergic neurones in senescence and in Parkinson's disease (Fornstedt *et al*., 1989).

(iv) The average adult brain also contains approximately 60 mg of non-haeme iron (Burdo and Connor, 2003). Within a healthy brain, the majority of iron is bound to ferritin and some to hemosiderin (Friedman *et al*., 2006). However, damage to the brain results in the liberation of ferric ions in forms that are able to catalyze free radical reactions via Fenton chemistry (Burdo and Connor, 2003).

(v) Neuronal membranes also contain high concentrations of PUFA side chains that are vulnerable to oxidation (Montine *et al*., 2004). Docosahexaenoic acid (DHA), the principal long-chain PUFA in the brain, is particularity vulnerable to oxidation due to the presence and location of its six double bonds (Gawrisch *et al*., 2003).

(vi) Activation of the brain's resident immunocompetent cells, the microglia, may also contribute to neurodegeneration. Microglia represent 10 to 20% of the total population of glial cells in the adult CNS (Napoli *et al*., 2009). They are located in the parenchyma, behind the blood-brain barrier and assist in the removal of cellular debris and foreign neuronal threats. Their activation is controlled by neuronal soluble mediators and cell-to-cell contact (Vilhardt, 2005). However, once activated, microglia produce a range of ROS, including superoxide and hydrogen peroxide. They also excrete inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interlukin-1 (IL-1), and interlukin-6 (IL-6). These cytokines can magnify ROS production by activating additional microglia and other CNS cells to produce more ROS as well as nitric oxide synthase (NOS), and subsequently peroxynitrite; thus, increasing the potential for oxidative stress within the brain (Guest and Grant, 2012).

Some reports have shown that low doses of organophosphates like malathion, in a repeated treatment regimen, are unable to reduce AChE activity in the rat brain, in contrast to the inhibitory effect in acute treatment. This latter study also indicates that as an alternative to AChE inhibition, interference with the antioxidant defence system may be another important target for such toxicity (Trevisan *et al*., 2008). It has been reported that acute exposure to pesticides elicits a reduction in some antioxidant enzymes, especially in the cerebral cortex (Brocardo *et al*., 2005); which was also

reported for repeated low-dose exposure (Brocardo *et al*., 2007). Antioxidant enzyme activity has also been pointed out as a potential target for OPs toxicity by other authors (Akhgari *et al*., 2003; Hazarika *et al*., 2003).

2.12 Antioxidants in Reproduction

Oxidative stress results from the imbalance between production of the ROS and the protective effect of the antioxidant system responsible for their neutralization and removal. An excess of ROS causes a pathological reaction resulting in damage to cells and tissues. Spermatozoa are particularly vulnerable to the harmful effects of ROS. Oxidative stress affects their activity, damages DNA structure, and accelerates apoptosis, all of which consequently decrease their numbers, hinders motility and development of normal morphology, and impairs function (Walczak-jedrzejowska *et al*., 2013; Archibong *et al*., 2018). Defective sperm function is the most common cause of infertility, and until recently, was difficult to evaluate and treat. Mammalian spermatozoa membranes are rich in polyunsaturated fatty acids and are sensitive to oxygen-induced damage mediated by lipid peroxidation. Hence, free radicals and ROS, associated with oxidative stress are likely to play a number of significant and diverse roles in reproduction (Sheweita *et al*., 2005). Limited endogenous mechanisms exist to reverse these damages, in a normal situation, the seminal plasma contains antioxidant mechanisms which are likely to quench these ROS and protect against any likely damage to spermatozoa. However, in increased ROS generation, these antioxidant mechanisms may be downplayed and create a situation called oxidative stress, which perturb and cause deficits in the reproductive system. Therefore, numerous protective antioxidants system in the seminal plasma and semen which is composed of enzymes including SOD, CAT and GPx, as well as nonenzymatic substances, may closely

interact with each other to ensure optimal protection against ROS. Non–enzymatic antioxidants include vitamins A, E, C, and B complex, glutathione, pantothenic acid, coenzyme Q10 and carnitine, and micronutrients such as zinc, selenium, and copper (Sheweita *et al*., 2005). Therefore, deficit in any of these antioxidants can cause decrease in total antioxidant status.

2.13 Melatonin

Melatonin (MEL) (Figure 2.8), a structurally identified tryptophan derivative, was first isolated from bovine pineal gland about six decades ago by Lerner (Chowdhury *et al*., 2008). It was then, subsequently, found to be a sleep promoter, a chemical signal of light and darkness as well as a regulator of photoperiod-dependent seasonal reproduction in some vertebrates (Tan *et al*., 2010; Acuna-Castroviejo *et al*., 2014; Anwar *et al*., 2015). Using the fluctuating endogenous MEL signals, vertebrates synchronise both their circadian rhythms and their circannual reproductive activities (Reiter *et al*., 2009). Thus, the daily and seasonally changing MEL rhythms are involved in signaling time of day and time of year and, thus, they serve as a bioclock and a bio-calendar in vertebrates (Reiter, 1993). Although melatonin is mainly produced by the pineal gland, it is also found in several extra-pineal organs including the retina, cerebellum, skin, ovary, liver, pancreas, kidneys (Reiter, 1991; Tan *et al*., 2002). There has been increasing evidence showing its involvement in many other functions. For example, it is now accepted that it has immune enhancing (Yaprak *et al*., 2003) and anti-inflammatory (Vollrath and Huesgen, 1988; Hardeland *et al*., 1993) properties; exerts homeostatic roles in the mitochondrion (Karolczak *et al*., 2005); and inhibits cancer progression (Ceinos *et al*., 2004; Johnston *et al*., 2004; Liu and Borjigin, 2005). Accordingly, it is known that melatonin is a versatile and ubiquitous molecule, and it is

not surprising that it has drawn the attention of the scientific community since its discovery. The antioxidant role of MEL has been profusely reported in different studies (Umosen *et al*., 2012; Mondal *et al*., 2017; Sharif *et al*., 2017).

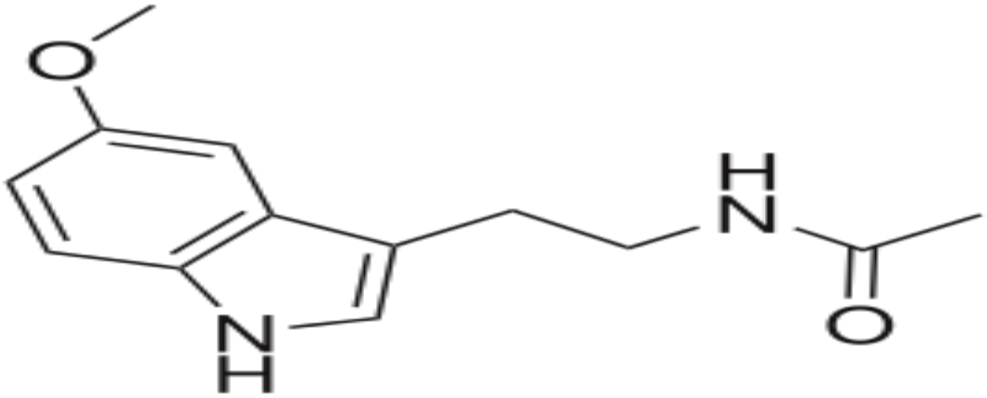


Figure 2.8: Structure of melatonin (Chowdhury *et al*., 2008).

2.13.1 Sources

It has been established that pineal gland is not the only source of melatonin; extrapineal melatonin is widespread in animal and man (Terry *et al*., 2009). Furthermore, active synthesis of melatonin has also been demonstrated in the non-endocrine cells such as mast cells, natural killer cells, eosinophilic leucocytes, platelets, endothelial cells etc. Thus, explaining the role of melatonin as a neuroendocrine regulator and coordinator of many complex and interrelated biological processes in the body (Kvetnoy *et al*., 2002).

Dietary sources of melatonin has been established, while MEL has been reported in foods, including cherries (Burkhardt *et al*., 2001), bananas and grapes, rice and cereals, herbs, olive oil, wine and beer (Lamont *et al*., 2011). When avian species ingest MEL-rich plant feed, such as rice, the MEL binds to its receptors in their brains (Hattori *et al*., 1995). When humans consume foods rich in MEL such as banana, pineapple and orange, the blood levels of MEL significantly increase (Sae-Teaw *et al*., 2012).

Beverages containing MEL are sold in grocery stores, convenience stores, and clubs in the developed world especially to enhance relaxation (Pigeon *et al*., 2010).

2.13.2 Physiological mechanisms and effects

Melatonin production, physiology and pathophysiology is derived from the essential amino acid tryptophan (Peuhkuri *et al*., 2012). Melatonin is an indoleamine molecule that is found widely throughout nature. Melatonin is synthesised and immediately secreted into the blood vascular system and cerebro-spinal fluid by the pineal gland during the night, whereas the daytime production of MEL is virtually nil. The surge of MEL during nights represent a biological timing signal that is internally driven by the activity of a central pace-maker in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Kriegsfeld and Silver, 2006; Blask, 2009). The nocturnal melatonin signal provides time of day information to all the cells, tissues and organs of the body and is the most stable and reliable peripheral biomarker of the timing of the central biological clock (Noguti and Ribeiro, 2012). Since the duration of the MEL surge defines the length of the biological night, the pineal gland not only acts as a clock, but also as a calendar by providing an organism with information about seasonal changes in day length (Walton *et al*., 2011). Melatonin is often referred to as the chemical expression or hormone of darkness (Masters *et al*., 2014). At either physiological or pharmacological concentrations, MEL is involved in a variety of physiological and pathophysiological processes, including the control of sleep, circadian rhythms, retinal physiology, seasonal reproductive cycles, cancer development and growth, immune activity, antioxidation and free-radical scavenging, mitochondrial respiration, cardiovascular function, bone metabolism, intermediary metabolism and gastrointestinal physiology (Reiter, 2002; 2005; Arendt, 2006).

The central circadian pacemaker or master biological clock is located in the hypothalamus and is composed of a specialized group of neurons in the suprachiasmatic nuclei (SCN) (Mohawk *et al*., 2012). The endogenous activity of this internal clock drives the complex molecular mechanisms responsible for the production of MEL at night (Kovacic and Somanathan, 2014). In addition to the nocturnal rhythm of MEL production, the central circadian pacemaker regulates other daily rhythms such as the sleep/wake cycle, body temperature, secretory patterns of hormones, food consumption and metabolism (Eckel-Mahan and Sassone-Corsi, 2013). The circadian timing system facilitates an organism's adaptation to changes in its environment via the temporal coordination of these and other physiological functions; the rhythmic output of MEL every night plays a pivotal role in this biological timing mechanism (Brainard and Hanifin, 2005). Photic signals reaching the retina of eye play an essential role in synchronising this dynamic process (Ben-Moshe *et al*., 2014). Light entering the eyes stimulates specialized photoreceptors in the retina from which neural signals are then sent not only to the primary visual system but also to non-visual centres of the brain, most particularly the SCN. During the day, light input to the SCN resets the central circadian pace-maker and thus, synchronises internal near 24-hour rhythms, including the melatonin rhythm, precisely to the environmental 24-hour light/dark cycle (Brainard and Hanifin, 2005).

When an organism experiences a change in the timing of the ambient light/dark cycle, the central circadian pace-maker responds appropriately by advancing or delaying its own timing referred to as a phase shift (Lockley, 2009). Therefore, changes in an individual's exposure to light and darkness can induce changes in the phasing of the MEL rhythm and other circadian rhythms as are experienced during shift work,

transcontinental jet travel, space flight. However, light present during darkness, provided that it is bright enough and of the proper wavelength, is detrimental to the circadian system and immediately turns off MEL production (Gooley *et al*., 2011). Although light is the most potent stimulus to the mechanisms governing internal time-keeping, much more light is actually required for circadian regulation than for vision (Brainard and Hanifin, 2005).

Following its synthesis, MEL is immediately secreted from the pineal directly into both the blood stream and cerebrospinal fluid of the third ventricle (Tan *et al*., 2010). The amount of MEL produced during the night varies considerably from one individual to another and this appears to be genetically determined (Peuhkuri *et al*., 2012). Once it is released into the circulation, MEL has a short half-life and it is primarily metabolised in the liver, where it is converted by cytochrome P450 enzymes to its main metabolite, 6-sulfatoxymelatonin that is excreted in the urine. Melatonin is also metabolized in a number of extrahepatic tissues, most notably the brain, by both enzymatic and non-enzymatic mechanisms (Reiter, 1991; 2002).

Melatonin in other body fluids such as saliva, urine, ovarian follicular fluid, breastmilk and semen also exhibits a nocturnal rhythm. Due to its ability to not only produce but also take-up and store melatonin, the gastro-intestinal tract contains amounts of MEL that far exceed the levels present in the pineal gland (Chen *et al*., 2011). Bile from the liver and gall bladder contains levels of MEL that greatly exceed those in the general blood circulation (Reiter, 1991; 2002).

Physiological or pharmacological concentrations of MEL exert an important regulatory influence over a broad range of physiological and pathophysiological processes in both lower animals and humans. Although current knowledge of the mechanisms by which MEL modulates these diverse processes is incomplete, specific plasma membrane-associated MEL receptors play a major role in mediating several of MEL actions at the cellular level. Melatonin receptors are expressed in diverse areas of the brain including the SCN as well as in several peripheral organs and tissues. Some physiological and pathophysiological situations may involve nuclear MEL receptors whereas in other biological contexts no specific receptors appear to be required for melatonin's actions (Reiter, 2002; Pandi-Perumal *et al*., 2006; Blask, 2007).

Other physiological and pathophysiological responses modulated by MEL via MEL receptor-mediated mechanisms include pituitary hormone release, testosterone production by the testes, cortisol secretion by the adrenal cortex, vascular tone, energy metabolism, fatty acid transport, immune activity, cancer cell proliferation and tumour growth, retinal physiology, seasonal reproductive cycles, cancer development and growth, immune modulation, antioxidation and free-radical scavenging, mitochondrial respiration, cardiovascular function, bone metabolism, intermediary metabolism and gastro-intestinal physiology (Arendt, 2006; Ekmekcioglu, 2006). Actions of MEL that appear to involve nuclear receptors include stimulation of immune cells to produce biologically active substances called ILs, as well as inhibition of the proliferation of certain types of colon cancer cells (Reiter, 2002; Pandi-Perumal *et al*., 2006; Blask, 2007).

2.13.3 Antoxidant effects

Melatonin has amphiphilic features. In contrast to other antioxidants that are either hydrophilic or lipophilic, MEL can cross physiological barriers, thereby reducing oxidative damage in both the lipid and aqueous environments of cells (Reiter *et al*., 2004). Both direct and indirect antioxidant properties of MEL have been reported (Tan *et al*., 2002; Mihandoost *et al*., 2014). Melatonin exhibits indirect antioxidant role by supporting SOD and GPx activities (Reiter *et al*., 2002, 2005), possibly via epigenetic mechanisms (Korkmaz and Reiter, 2008). It also stimulates glutathione production in cells (Winiarska *et al*., 2006; Reiter *et al*., 2008) by stimulating γ-glutamylcysteine synthase. It promotes the activity of glutathione reductase which converts oxidised glutathione (GSSG) back to its reduced form, GSH (Reiter *et al*., 2002).

Direct antioxidant properties of MEL have been demonstrated both *in vitro* (Gulcin *et al*., 2002) and *in vivo* (El-Missiry *et al*., 2007), by inhibiting lipid peroxidation and DNA degradation (Mathes, 2010; Reiter *et al*., 2014). The latter action could be due to a direct scavenging of ROS and/or the activation of DNA reparation enzymes (Vijayalaxmi *et al*., 1995). The protection of DNA-cyclophosphamide-induced damage by MEL has also been reported, which may be due to its hydroxyl radical scavenging capacity (Ferreira *et al*., 2013). By virtue of its amphiphilic capacity, MEL has both lipophilic and hydrophilic properties which make it able to cross physiological barriers and enter cells (Reiter *et al*., 2004). This property confers to MEL an advantage as compared to other antioxidants whose action is limited due to their solubility limiting their partitioning between intra- and extra-cellular compartments. The efficiency of radical scavenging by MEL is therefore highly dependent on its partitioning between the lipidic and aqueous phases. Several studies demonstrated the ability of MEL to

scavenge oxygen-derived free radicals, such as hydroxyl (Poeggeler *et al*., 2002) and peroxyl (Reiter *et al*., 2003) radicals. However, MEL seems poorly able to react with superoxide radicals (Walters-Laporte *et al*., 1998; Zang *et al*., 1998).

Melatonin is also able to neutralize singlet oxygen, peroxynitrite anion, and nitric oxide (Reiter *et al*., 2004; Topal *et al*., 2005; Ucar *et al*., 2007). The first reports on this subject placed MEL among the very best scavengers in terms of its ability to neutralise the $LOO^{\cdot}$; thus, supporting the claim that MEL is twice as effective as vitamin E, the primary chain breaking antioxidant, in interfering with the propagation of lipid peroxidation (Pieri *et al*., 1994; 1996). Moreover, protective effects of MEL have been reported in case of *in vitro* oxidation of LDL by copper or by macrophages (Bonnefont-Rousselot and Collin, 2010). This protection has been explained by the ability of MEL to directly scavenge the free radicals involved in these oxidation processes (Allegra *et al*., 2003). Similarly, models of MEL-leaded nanoparticles have shown their efficiency to protect liposomes or microsomes against lipid peroxidation (Schaffazick *et al*., 2005). Apart from directly scavenging oxygen and chloride-based free radicals, MEL has been shown to scavenge nitrogen-based free radicals (Reiter *et al*., 2010). Melatonin was shown to react with $ONOO^{-}$ with first-order kinetics; however, the rate constant for the reaction of MEL with ONOOH was considerably higher (a reaction unimportant at physiological pH). Blanchard *et al.* (2000) also reported that MEL interacted with $ONOO^{-}$. According to Gitto *et al.* (2001), under *in vitro* conditions and using end products of lipid peroxidation as an index of free radical damage, MEL augments the protective actions of vitamin E, vitamin C and GSH against free radical-mediated oxidation of polyunsaturated fatty fats. Thus, indicating that combinations of MEL with other antioxidants increase their efficacy. The mechanism of the synergy remains

unknown *in vivo*. When compared under conditions of high oxidative stress *in vivo*, MEL has proven superior to vitamins C and E in reducing oxidative damage (Tan *et al*., 2002).

2.13.4 Actions of melatonin at the level of the mitochondria

Mitochondria are a major source of free radicals and as a consequence these subcellular organelles are exposed to extensive oxidative abuse. The inner mitochondrial membrane is the site of the electron transport chain (ETC), a system of oxido-reductant protein complexes (complexes I, II, III and IV). In aerobic cells, mitochondrial oxidative phosphorylation is responsible for an estimated 90-95% of the total ATP generated by cells. Deficiencies in the ETC can lead to the leakage of electrons which thereafter form free radicals and other toxic reactants which results in molecular damage in mitochondria; this damage culminates in and contributes to what are referred to as mitochondria-related diseases (Chen *et al*., 2010). That MEL has important actions at the level of mitochondria is suggested by a number of observations: a), MEL is an efficient scavenger of ROS/RNS which are abundantly produced in mitochondria; b), although, mitochondria are incapable of GSH synthesis (they take it up from the cytosol), they do possess GPx and glutathione reductase (GRD) for GSH cycling, both enzymes of which are stimulated by melatonin; c), MEL has been shown to have antiapoptotic effects, with the apoptotic signals originating in mitochondria; d), MEL may be found in higher concentration in mitochondria than elsewhere in the cell, and even higher in the serum (Acuna-Castroviejo *et al*., 2002). Long term melatonin administration has been reported to increase the number of mitochondria in cells (Escames *et al*., 2010). Additionally, MEL was shown to inhibit NADPH-dependent lipid peroxidation in human placental mitochondria (Milczarek *et al*., 2010), to protect

the foetal rat brain against oxidant-mediated mitochondrial damage (Alonso-Alconada *et al*., 2013) and to stimulate mitochondrial respiration in the brain and liver of senescence-accelerated mice (Okatani *et al*., 2002).

2.14 Oxidative Stress - A Common Mechanism in Chlorpyrifos- and Cypermethrin-induced Reproductive and Neurodevelopmental Toxicity

Oxidative stress caused by increased production of ROS has been implicated in the toxicity of many chemical agents including pesticides (El-Demerdash, 2011a,b). Humans and animals are potentially exposed to pesticides either directly, as workers in green-houses and in agriculture, or indirectly, via food consumption (Damalas and Eleftherohorinos, 2011). In addition, it is likely that a significant amount of these pesticides and their metabolites get into rivers and estuaries via run-off from farmland. These are potentially toxic to wildlife and other animals (El-Shenawy, 2010). In recent times, oxidative stress has been implicated as a common mechanism of action in toxicities associated with OPs, PYRs and their combination. OPs affect mammalian brain development through a variety of mechanisms beyond their shared property of cholinesterase inhibition (Slotkin and Seidler, 2007a). Although it was originally thought that these agents act solely through inhibition of cholinesterase and consequent cholinergic hyperstimulation, it is now evident that there are multiple mechanisms that contribute to neurodevelopmental abnormalities (Barone *et al*., 2000; Qiao *et al*., 2002; Yanai *et al*., 2002; Gupta, 2004).

Synthetic pyrethroids are one of the most commonly used insecticides. In recent years, the use of these insecticides have increased over organochlorines, organophosphates and carbamates because of their high effectiveness against a wide range of insects, rapid biodegradation, low mammalian toxicity and target oriented mechanism of action. Thus,

exposure to them is often. CYP is a common synthetic pyrethroid used in agriculture, forestry as well as in public and animal health programmes. Although considered nontoxic to mammals, recent studies have shown adverse effects of CYP on the nervous system (Singh *et al*., 2012a), hepatic and renal systems (Sushma and Devasena, 2010) and male reproductive system (Wang *et al*., 2009a; Hu *et al*., 2013) in laboratory animals. Another study also incriminated CYP in oxidative stress (Singh *et al*., 2012b).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Animals

A total of ninety six (96) healthy adult nulliparous female and seventy two (72) male Wistar rats weighing between 140-160 grams used for this experiment were obtained from the Animal House of the Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria. Twenty four (24) adult female nulliparous Wistar rats were used for the median lethal dose determination while seventy two (72) adult nulliparous female and seventy two (72) male Wistar rats were used for the reproductive toxicity study. The animals were housed in the Department of Veterinary Anatomy Research Laboratory, Ahmadu Bello University, Zaria, Nigeria. They were fed pellets made from grower's mash (Vital Feeds Ltd., Jos, Nigeria). They were given access to water *ad libitum*. The rats were acclimatised to the laboratory environment for two weeks prior to use and identified by coloured tail markings.

3.2 Chemicals

Commercial grade Chlorpyrifos (20% E.C. TERMICOT®, Sabero Organics, Gujarat, India), Cypermethrin (10% EC, GLOBATHRIN®, Heranba Industries Limited, Vapi, India) was obtained from a reputable agrochemical outlet in Kaduna. Nigeria. Melatonin (MEL, 3 mg/Tablet, Nature Made Nutritional Product, USA) was obtained from a reputable Pharmaceutical outlet in Ilorin, Nigeria. Soya oil (Grand Pure Soya Oil, Grand Cereals Limited, Jos, Nigeria) was obtained from Samaru Market, Samaru, Nigeria. Other chemicals, including thiobarbituric acid, trichloroacetic acid and thiocholine

iodide used for this study were of analytical grade, and obtained from Sigma-Aldrich (Germany).

3.3 Experimental Protocols

3.3.1 Sample preparation

Chlorpyrifos (CPF) was dissolved in S/oil to make a 1% working solution, while cypermethrin (CYP) and melatonin (MEL) were dissolved in distilled water to make a 1% and 1.5 mg/2 ml concentrations, respectively.

3.3.2 Determination of Median Lethal Dose of CPF

The median lethal dose (LD_{50}) of CPF was determined using modified method of Lorke (1983). Briefly, phase I consisted of nine adult female Wistar rats, divided into three groups of three animals each. Groups I, II, III were dosed orally with 50, 150 and 450 mg/kg of the reconstituted CPF, respectively. The result obtained from phase I was used to determine the dose for the phase II study. In phase II, three groups of one animal per group were dosed at 80 mg/kg, 90 mg/kg and 100 mg/kg, respectively. The LD_{50} was thereafter determined based on dose-response as described by Lorke (1983).

3.3.3 Determination of Median Lethal Dose of CYP

The median lethal dose (LD_{50}) of CYP was determined using modified method of Lorke (1983). Briefly, phase I consisted of nine adult female Wistar rats divided into three groups of three animals each. Groups I, II, III were dosed orally with 100, 300 and 500 mg/kg of the reconstituted CYP, respectively. The result obtained from phase I was used to determine doses used in the phase II study. In phase II, three groups of one animal per group were dosed at 300, 350 and 400 mg/kg, respectively. The LD_{50} was thereafter determined based on dose-response as described by Lorke (1983).

3.3.4 Animal breeding and dosing protocol

Seventy-two sexually matured female nulliparous Wistar rats weighing between 140-160 grams were bred with 72 adult males to obtain 60 pregnant animals after being mated overnight in a 1 : 1 mating scheme, based on Organisation for Economic Co-operation and Development (OECD) (1995) guidelines on reproductive toxicity study. Evidence of mating was assessed in each female animal by the presence of spermatozoa deposit in the vagina or the presence of vaginal plug. Day 1 of gestation was regarded as the day copulation plug/spermatozoa was found in the vagina. The sixty pregnant dams were thereafter divided at random into 6 groups of 10 animals each. Group I (DW) was given distilled water (2 ml/kg), while group II (S/oil) was given Soya oil only (2 ml/kg). Group III (MEL) was dosed MEL [0.5 mg/kg (Gultekin *et al*., 2006; Umosen *et al*., 2012)] only, while group IV (CC) was co-administered with CPF (1.9 mg/kg, $1/50^{th}$ of the LD_{50}) and CYP (7.5 mg/kg, $1/50^{th}$ of the LD_{50}). Group V (MCC) was pretreated with MEL (0.5 mg/kg) and then co-exposed to CPF (1.9 mg/kg, $1/50^{th}$ of the LD_{50}) and CYP (7.5 mg/kg, $1/50^{th}$ of the LD_{50}), respectively, 30 minutes later. Group VI (CCM) was co-exposed to CPF (1.9 mg/kg, $1/50^{th}$ of the LD_{50}) and CYP (7.5 mg/kg, $1/50^{th}$ of the LD_{50}), respectively, and post-treated with MEL (0.5 mg/kg), 30 minutes later. The regimens were given to the dam by gavage once daily from gestation day 1 (GD 1) to postnatal day (PND) 21. Weekly monitoring of body weight and clinical signs on the dam were carried out and recorded. The dams were allowed to deliver normally and some behavioural, reproductive and biochemical parameters were thereafter evaluated on the pups.

3.4 Evaluation of Treatment on Developmental Parameters of Pups

3.4.1 Litter size on day of parturition (PND 0)

Litter size was determined on PND 0 by physical counting and recording the number of pups, delivered by each dam.

3.4.2 Number of live and dead pups

Number of live and dead pups in each litter was evaluated on the day of birth (PND 0). The number of pups delivered per individual dam was counted based on the number alive versus the number dead at parturition.

3.4.3 Evaluation of the litter weight and sex

Evaluation of the litter weight of each pup was done on PND 1 and subsequently on PND 4 (that is, before and after culling), 7, 14 and 21 (Breslin *et al*., 1996) using precision weighing balance (Mettler® P161, Switzerland). Each pup was weighed and the sum of weight of all the pups per dam was averaged and recorded. The sex of the animal was determined by physical examination of the sex organs.

3.4.4 Determination of anogenital distance

Anogenital distance was measured using digital Vernier caliper (Tresna® China). Each pup was carefully held at the scruff, then digital Vernier caliper was used to measure the distance between the anterior end of the anal orifice to the posterior end of the preputial opening in millimetre (mm) at PND 1 as described by Schwarz *et al*. (2009).

3.4.5 Determination of Crown-rump length

Crown-rump length was evaluated by placing a measuring tape from the centre of the head to the posterior end of the pups at the base of the tail at PND 4 as described by Archibong *et al*. (2002).

3.4.6 Time of ear and eye opening

The time of ear and eye openings was recorded between PNDs 7-21 as described by Robinson and Wallace (2001) and Cole *et al*. (2012). Briefly, evaluation of ear opening was done by clapping of the hands follow by lateral deviation of the head to the sound, while that of the eye opening was by visual and blinking of the eye when approached with a sensor.

3.4.7 Time of testicular descent

Time of testicular descent in all the groups was recorded as the time the testes descended into the scrotal sac according to the method of Schwarz *et al*. (2009). This was conducted starting from PND 16.

3.5 Evaluation of Treatment on Reflexogenic Parameters

The pups were also subjected to the following reflexogenic tests:

3.5.1 Surface righting reflex

Each pup was placed in its supine position on PND 1. the time that it took the pup to turn over on its four feets was recorded in seconds, with a limit of 60s (Chanda and Pope, 1996; Laviola *et al*., 2006).

3.5.2 Ability to walk and time pivoting

On PND 8, all the pups were examined on whether they walked or not in a one-minute observation period and timed for the number of seconds they spent pivoting. This test was repeated on PND 15 (Alfano *et al*., 2014).

3.5.3 Negative geotaxis test

The pups were placed on an inclined (15^{o}) board with the head pointing downwards on PND 10. The time required for each pup to rotate its body axis (180^{o}) was recorded as described by Moser (1999) and Cole *et al*. (2012).

3.5.4 Aerial righting reflex

Each pup was dropped in the supine position from 45 cm height on a cotton wool pad on two consecutive trials on PND 14. A pup was given a score of 1, if it landed on its four feet; otherwise it received a score of 0 (Ghandhi *et al*., 2012).

3.5.5 Neuromuscular coordination

Neuromuscular co-ordination of each rat was evaluated on an inclined plane based on the method described by Ambali and Aliyu (2012). Briefly, an inclined plane was placed on an apparatus made with an angled rough wooden plank with thick foam pad at its bottom end. The plank was first raised to an inclination of 35°, and thereafter gradually increased step-wise by 5° until the subject could no longer stay and be stable horizontally on the plank for 3 seconds, without sliding down. Angles were measured and marked on the apparatus beforehand, and were obtained by propping the plank on a vertical bar with several notches. The test was performed with the head of the rat first facing left, and then right hand side of the experimenter. The highest angle at which

each rat stayed and stood horizontally, and facing each direction was recorded. Two trials were performed for each testing period. This procedure was carried out on each rat in all the groups on PNDs 30, 60 and 90.

3.5.6 Efficiency of locomotion

Ladder-walk test was used to assess the effect of treatment on efficiency of locomotion as described by Ambali and Aliyu (2012). Briefly rat from each group was encouraged to walk across a black wooden ladder of 106 cm × 17 cm, with 0.8-cm diameter rungs, and with 2.5-cm spaces between them. The number of times each rat missed a rung was counted by one rater on each side. This was evaluated on PNDs 30, 60 and 90.

3.5.7 Motor coordination

The effect of treatment on motor coordination was performed using the beam-walk performance task as described by Ambali *et al*. (2010d). Briefly, the beam walk performance was evaluated by allowing individual rat from each group to walk across a wooden black beam of 106-cm length, beginning at 17.2-cm width and ending at 1.0-cm width. Periodic widths were marked on the side of the apparatus. On each side of the narrowing beam, there was a 1.8-cm step-down to a 3.0-cm area, where subjects may step if necessary. As the subject walked across, the width of the beam at which it stepped down was recorded by one rater on each side, and this was repeated twice during each trial session and the average width recorded. This was done on PNDs 30, 60 and 90.

3.5.8 Excitability scores

The excitability score is a neurobehavioural parameter and was evaluated as described by Ambali and Ayo (2012). Briefly, each rat was held by the tail upside down and held in that position for 30 seconds. Response of each rat was then scored using an ordinal scale of 0 - 5 as follows:

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

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

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

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

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

Grade 5- In addition to observations in grade 3 above, rat successfully climbs the tip of its tail. The excitability scores was assessed on PNDs 40 and 80.

3.5.9 Motor strength

The fore-paw grip time was used to evaluate the motor strength of the rats, as described by Abou-Donia *et al*. (2001). This was conducted by having rats hung down from a 5-mm diameter wood dowel gripped with both forepaws. The time spent by each rat before releasing its grips was recorded in seconds. This parameter was evaluated on PND 80.

3.5.10 Forced swimming test

The forced swimming test (FST), used to evaluate the effect of treatment on depression, was carried out on the rats according to the method of Porsolt *et al*. (1978). Rats were individually forced to swim in an open plastic cylinder (height 40 cm, diameter 30 cm),

containing 25 cm of water, and maintained at 25 ± 1^{o}C. The pre-test session was carried out 24 hours prior to the test day, which was performed on PND 80. In the pre-test session, rats were allowed to swim for 15 min and then returned to their home cages. In the test session, 24 hours later, rats were again subjected to the forced swimming test, with immobility time measured during a 5 min period. Each rat was then judged to be immobile when it remained floating motionless in the water, making only those movements necessary to keep its head above water.

3.5.11 Cognition test

The effect of various groups on cognitive ability of each rat was assessed via learning and short-term memory tests as follows:

3.5.11.1 Evaluation of treatment on learning

The effect of treatment on learning task in pups was assessed 48 h to the termination of the test (PND 89), using the step-down inhibitory avoidance learning task as described by Zhu *et al*. (2001) and modified by Ambali (2009). The apparatus used for the learning test was an acrylic chamber 40 × 25 × 25 cm, consisting of a floor made of parallel 2-mm-calibre stainless steel bars spaced 1 cm apart. An electric shock was delivered through the floor bars. A 2.5-cm-high, 8 cm × 25 cm wooden platform was placed on the left extreme of the chamber. Each animal was gently placed on the platform. Upon stepping down, the rat immediately received a single 80-volt foot shock. If the animal did not returned to the platform, the foot shock was repeated every 5 sec. A rat was considered to have learned the avoidance task, if it remained on the platform for more than 2 min. The number of foot shocks was recorded as an index of learning acquisition.

3.5.11.2 Evaluation of treatment on short-term memory

Short-term memory was evaluated in individual rat from each group using the step-down avoidance inhibitory task as described by Zhu *et al*. (2001) 24 hours after the assessment of learning. The apparatus used for the memory test was the same as that used earlier for the assessment of learning (PND 90). In this test, the rat was again placed gently on the platform 24 h after performing the learning task. The time an animal remained on the platform was recorded as an index of memory retention. Staying on the platform for 2 min was counted as maximum memory retention (ceiling response). This was assessed on PND 90.

3.6 Evaluation of Reproductive Toxicity

Reproductive toxicity was evaluated on sexually matured F1 generation males from different groups, the following endpoints of reproductive toxicity were evaluated as described by USEPA (1996).

3.6.1 Mating index

On PND 80, F1 generation males, that have been previously separated from the females counterparts were co-habited with a sexually matured female in a cage overnight. The mating index was calculated as follows:

$$\frac{\text{Number of males mated}}{\text{Number of females co-habited}} \times 100$$

3.6.2 Live birth index

Live birth index was determined at the end of gestation period of the co-habited females with the male animals in the F1 generation. This was done by visual counting of the

number of live offspring littered by individual dam versus the total number of offsprings delivered and then averaged per group and the live birth index calculated as follows:

$$\frac{\text{Number of live offspring}}{\text{Number of offspring delivered}} \times 100$$

3.6.3 Sex ratio

Sex ratio was determined at the end of gestation period of the cohabited F1 male generation and sexually mature female. This index was determined by taking note of the anogenital distance by use of a digital Vernier caliper per litter of individual dam and then calculated as below:

$$\frac{\text{Number of male offspring}}{\text{Number of female offspring}} \times 100$$

3.6.4 Viability index

Viability index was determined by taking note of the number of litter that were alive at PND 4 relative to those that were delivered at the parturition of the co-habited F1 male and sexually mature females and then calculated as below:

$$\frac{\text{Number of live offspring at lactation day 4}}{\text{Number of live offspring delivered}} \times 100$$

3.6.5 Gestational length

Gestational length was based on the number of days in between gestation day 1 and day of parturition. This was determined by noting the day vaginal plug was observed in the female reproductive tract as gestation day 1 and the day of parturition.

3.6.6 Fertility index

Fertility index was assessed by taking note of the dams that were apparently pregnant at gestation day 14 in order to be sure of state of pregnancy after an overnight co-habiting with males:

$$\frac{\text{Number of co-habited females pregnant}}{\text{Number of non-pregnant couples co-habited}} \times 100$$

3.6.7 Gestation (pregnancy) index

Gestation or pregnancy index was assessed by counting the number of females that delivered live pups at parturition and the number of females with evidence of pregnancy after co-habitation.

$$\frac{\text{Number of females that delivered live young}}{\text{Number of females with evidence of pregnancy co-habited}} \times 100$$

3.7 Evaluation of Treatment on Sperm Characteristics

On PND 91, five male rats in each group were sacrificed and the epididymides were harvested for the determination of sperm characteristics while pituitary glands, testes, prostate, seminal vesicles, brain and thyroid glands were obtained and weighed and then kept at 4^{o}C in a refrigerator until assayed for total proteins and sialic acid concentrations.

3.7.1 Sperm motility

Sperm motility was evaluated as described by Sonmez *et al*. (2005). Briefly, the cauda epididymis from each F1 male rat was cut into small pieces and transferred into the Petri dishes, containing prewarmed 0.5 ml Tris buffer solution. The sperm cells were allowed to swim out within 5 min, at 37^{o}C. An aliquot of this solution was observed under the light microscope examined at × 40. The percentage sperm motility was calculated using

the number of live sperm cells over the total number of sperm cells, both motile and non-motile. The sperm cells that were not moving at all were considered to be non-motile, while the rest; which displayed some movement were considered to be motile.

3.7.2 Spermatozoa concentration

Spermatozoa concentration was evaluated based on the method described by Yokoi and Mayi (2004). Briefly, the epididymis was minced with anatomic scissors in 5 ml physiological saline; it was then placed in a rocker for 10 min and allowed to incubate at room temperature for 2 min. After incubation, the supernatant fluid was diluted 1:100 with solution containing 5 g sodium bicarbonate and 1 ml formalin (35%). Total sperm number was determined by using the new improved Neuber's counting chamber (haemocytometer). Approximately, 10 µl of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and was allowed to stand for 5 min. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was then focused and the number of spermatozoa counted in five 16-celled squares was recorded. The sperm concentration was then calculated, multiplied by 5 and expressed as $[X] \times 10^6$, where [X] is the number of spermatozoa in a 16-celled square.

3.7.3 Spermatozoa live-dead ratio

Evaluation of live-dead ratio was carried out using the method of Kvist and Bjokndahl (2002). Briefly, 50 µl of semen was mixed with 50 µl of eosin-nigrosin mixture. It was then allowed to stand, and after 30 min, smears were made and allowed to air-dry. Smears were examined with light microscope using oil immersion (× 100 objective). A total of 100 sperm cells were counted, from five different fields and the stained and

unstained cells were counted. Stained sperm cells were designated as dead sperm cells and appeared pinkish, while the unstained ones were said to be alive.

3.7.4 Sperm morphology

Sperm morphology was evaluated by mixing a 1:10 dilution of Giemsa solution using distilled water. Exactly 10 µl of semen was spread onto a glass slide and allowed to air dry at room temperature (25-26 ^{o}C). Air-dried smear was stained with the diluted Giemsa solution for 40 min. Stained smear was briefly rinsed in distilled water and allowed to air-dry. Stained smear was examined under bright field microscope using × 100 objectives. According to WHO criteria, a morphologically normal spermatozoon have an oval head and an acrosome covering 40–70% of the head area. A normal spermatozoon have no neck, mid-piece, tail abnormalities or cytoplasmic droplets larger than 50% of the sperm head (WHO, 1992).

3.8 Evaluation of Treatment on Serum Sex Hormone Profiles

At the end of the dosing period, each rats was sacrificed by severing the jugular vein, after light chloroform anaesthesia. Thereafter, 5 ml of blood was collected from each animal into a test tube and incubated at room temperature (25-26 ^{o}C) for 30 min on the shelf before being centrifuged at 1000 × *g* for 10 min. Thereafter, the serum was collected from the test tube into a clean sample bottle, which was subsequently used for the analysis of serum concentrations of FSH, LH and testosterone and the thyroid hormones, using standard ELISA methods as summarized below:

3.8.1 Determination of serum follicle-stimulating hormone concentration

The concentration of FSH was determined using the ALPCO®EIA kit. It is an enzyme immunoassay procedure. The principle of the enzyme immunoassay test followed a typical two-step capture or " sandwich" type assay. The assay made use of two highly specific monoclonal antibodies: A monoclonal antibody specific for FSH is immobilized onto the microwell plate and another monoclonal antibody specific for a different region of FSH was conjugated to horse radish peroxidase (HRP). The FSH from the sample and standards were allowed to bind to the plate, washed, and subsequently incubated with the HRP conjugate. After a second washing step, the enzyme substrate was added. The enzymatic reaction was terminated by addition of the stop solution. The absorbance was measured on a microtiter plate reader. The intensity of the colour formed by the enzymatic reaction was directly proportional to the concentration of FSH in the sample. A set of standards were used to plot a standard curve from which the amount of FSH in test samples and controls was directly read. After stopping the reaction, the resulting colour was measured using a spectrophotometer (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China) at 450 nm. Briefly, the working solutions of the anti-FSH-HRP conjugate and wash buffer were prepared. The required number of microwell strips were removed, the bag resealed and the unused were returned to the refrigerator. Twenty five micromole of each calibrator, control and specimen sample was pipetted into correspondingly labeled wells in duplicate. Exactly 100 µl of assay buffer was then added into each well using a multichannel pipette. This was incubated on a plate shaker at approximately 1000 × *g* for 30 min at room temperature (25-26 ^{o}C).

The wells were washed 3 times with 300 µl of diluted wash buffer per well followed by tapping of the plate firmly against absorbent paper to ensure that it was dry. Exactly 100 µl of the conjugate working solution was then pipetted into each well using a multichannel pipette. These were then incubated on a plate shaker at approximately 200 × g for 30 min at room temperature. The wells were washed again in the same manner as above. Exactly 100 µl of tetramethylbenzidine substrate were pipetted into each well at timed intervals. These was incubated on a plate shaker for 15-20 min at room temperature until it attained a dark blue colour for optical density (OD) reading. Exactly 50 µl of stop solution was added into each well at the same timed intervals. The plate was finally read on a microwell plate reader (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China**)** at 450 nm within 20 min after addition of the stop solution.

3.8.2 Determination of serum luteinizing hormone concentration

The concentration of LH was determined using the ALPCO®EIA kit. The principle of the ELISA test followed a typical two-step capture or 'sandwich' type assay. The assay made use of two highly specific monoclonal antibodies: A monoclonal antibody specific for LH was immobilized onto the microwell plate and another monoclonal antibody specific for a different region of LH was conjugated to horse radish peroxidase (HRP). The LH from the sample and standards are allowed to bind to the plate, washed, and subsequently incubated with the HRP conjugate. After a second washing step, the enzyme substrate was added. The enzymatic reaction was terminated by addition of a stop solution. The absorbance was measured on a microtitre plate reader.

The intensity of the colour formed by the enzymatic reaction was directly proportional to the concentration of LH in the sample. A set of standards were used to plot a standard curve from which the amount of LH in the test samples and controls was directly read. Briefly, the working solutions of the anti-hLH-HRP conjugate and wash buffer were prepared. The required number of microwell strips were removed, the bag resealed and the unused were returned to the refrigerator. Twenty five microlitre of each calibrator, control and specimen sample was pipetted into correspondingly labeled wells in duplicate. Exactly 100 µl of assay buffer were then added into each well using a multichannel pipette. This was incubated on a plate shaker at approximately 200 × *g* for 30 min at room temperature. The wells were washed 3 times with 300 µl of diluted wash buffer per well, followed by tapping of the plate firmly against absorbent paper to ensure that it was dry. One hundred microlitre of the conjugate working solution was then pipetted into each well using a multi-channel pipette. These were then incubated on a plate shaker at approximately 200 × *g* for 30 min at room temperature. The wells were washed again in the same manner as above. Exactly 100 µl of tetramethylbenzidine substrate was pipetted into each well at timed intervals. These was incubated on a plate shaker for 15-20 min at room temperature until it attained a dark blue color for optical density (OD) reading. Then a 50 µl of stop solution was added into each well at the same timed intervals. The plate was finally read on a microwell plate reader (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China**)** at 450 nm within 20 min, after addition of the stop solution.

3.8.3 Determination of serum testosterone concentration

Testosterone was assayed using enzyme linked immunosorbent assay (ELISA) method (Syntron Bioresearch, Inc. Carlsbad, U.S.A.). The principle of the ELISA test followed

the typical competitive binding procedure. Competition occured between an unlabeled antigen (present in standards, control and test samples) and an enzyme-labeled antigen (conjugate) for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedure remove unbound materials. After the washing step, the enzyme substrate was added. The enzymatic reaction was terminated by addition of the stop solution. The absorbance was measured on a microtitre plate reader. The intensity of the colour formed was inversely proportional to the concentration of testosterone in the sample.

A set of standards was used to plot a standard curve from which the amount of testosterone in test samples and controls can be directly read. Briefly, the working solutions of the testosterone-HRP conjugate and wash buffer were prepared. The required number of microwell strips were removed, the bag resealed and the unused were returned to the refrigerator. Exactly 50 µl of each calibrator, control and specimen sample were pipetted into correspondingly labeled wells in duplicate. One hundred microlitre of conjugate working solution was then added into each well using a multichannel pipette. This was incubated on a plate shaker at approximately 200 × g for 1 hour at room temperature.

The wells were washed three times with 300 µl of diluted wash buffer per well followed by tapping of the plate firmly against absorbent paper to ensure that it was dry. Exactly 150 µl of tetramethylbenzidine substrate was pipetted into each well at 10 min intervals. These were incubated on a plate shaker for 10-15 min at room temperature until dark blue colouration appears on calibrator for desired optical density (OD) reading. Then a 50 µl of stop solution was added into each well at the same timed interval as mentioned

above. After stopping the reaction, the resulting colour was measured using a microplate reader (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China**)** at 450 nm within 20 min after adding the stop solution.

3.8.4 Evaluation of treatment on thyroid hormones concentration

3.8.4.1 Triiodothyronine concentration

A commercially-prepared microwell competitive ELISA kit (ALPCO^{TM} Diagnostics, U.S.A) was used for the determination of triiodothyronine (T_3). The same principle underlied the function of both T_4 and T_3. Briefly, certain amount of anti-T_3 antibodies was coated on microtitre well of the T_3 plates. A measured amount of sample serum and constant amount of T_3 conjugated with horse-radish peroxidase was added to the microtitre wells. This was incubated at room temperature for 60 min. During this period, the T_3 in the test samples and conjugated T_3, competed for the limited binding site on the anti-T_3 antibodies, respectively. The wells were then washed to remove the unbound T_3 conjugates. A solution of tetramethylbenzidine was then added and incubated for 10 min at room temperature, resulting in the development of blue colour. The colour development was stopped by the addition of 2N HCl and the absorbance taken with a spectrophotometer (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China) at 450 nm. The intensity of the colour formed was proportional to the amount of enzyme present, but inversely related to the amount of unlabelled T_3 in the sample. By making reference to the series of T_3 standards assayed in the same way, the concentration of T_3 in the unknown samples was determined.

3.8.4.2 Thyroxine concentration

A commercially prepared microwell ELISA kit (ALPCO^{TM} Diagnostics, U.S.A) was used for the determination of serum thyroxine (T_4). The principle for the T_4 ELISA was that certain amount of anti-T_4 antibody was coated on microtitre wells. Then, a measured amount of the sample serum and a constant amount of T_4 conjugated with horse radish peroxidase were added to the microtitre wells. During incubation, T_4 and conjugated T_4 competed for the binding sites on the anti- T_4 antibody. After 60 min of incubation at room temperature, the wells were washed five times with water to remove the unbound T_4 conjugate. A solution of tetramethylbenzidine was then added and incubated for 20 min, resulting in development of blue colour. The colour development was stopped with addition of 2N HCl, and the absorbance was measured using a spectrophotometer (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China) at 450 nm. The intensity of the colour formed was proportional to the amount of enzyme present, and it was inversely related to the amount of unlabelled T_4 in the sample.

3.8.4.3 Thyroid-stimulating hormone concentration

The test kit is a solid phase enzyme immunometric assay (ELISA) (ALPCO^{TM} Diagnostics, U.S.A) in the microplate format, designed for the quantitative measurement of thyroid-stimulating hormone (TSH) in rat serum. The microplate is coated with a monoclonal antibody specific for TSH. Calibrators and samples are pipetted into the antibody coated microplate. A polyclonal horseradish peroxidase-labeled antibody was then added. During an 18-20 hours incubation at 4°C, sandwich complexes consisting of the two antibodies and the rat TSH was formed. Non-reactive components were removed by a washing step. A chromogenic substrate, TMB (3, 3', 5,

5’-Tetra-Methyl-Benzidine), was added to all wells. During a 30 min incubation, the substrate was converted to a coloured end product (blue) by the fixed enzyme. The enzyme reaction was stopped by adding hydrochloric acid as the stop solution (change from blue to yellow). The colour intensity was directly proportional to the concentration of rat TSH present in the sample. The optical density of the colour solution was measured with a microplate reader at 450 nm.

Briefly, a sufficient number of microplate wells to accommodate calibrators and samples in duplicates were prepared. This was followed by labeling five tubes: F (40 ng/ml), E (20 ng/mL), D (10 ng/ml), C (5 ng/ml) and B (2.5 ng/ml). 0.1 mL of the Calibrator/Sample Diluent were pipetted into all tubes. This was then followed by 0.1 mL of the reconstituted Rat TSH Master Calibrator into tube F (40 ng/ml), and mixed thoroughly. This was done in two fold dilution. The reconstituted Rat TSH Calibrator served as highest calibrator G (80 ng/ml). Exactly 25 µl of each calibrator, control and sample were pipetted into the wells prepared. This was followed by addition of 200 µl of Enzyme-Labeled Anti-Rat TSH antibody to all wells. It was then incubated for 18-20 hours at 4°C. The content of the wells were discarded and washed 4 times with 300 µl diluted wash solution. Absorbent paper was used to remove as much wash solution as possible. Two hundred microlitre of tetramethylbenzidine-substrate solution was added to all wells. Incubated for 30 min in the dark and followed by adding 50 µl of Stop Solution to each well and mixed carefully. The optical density was read at 450 nm using a spectrophotometer (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China).

3.9 Evaluation of Treatment on Oxidative Stress Parameters

The effect of treatment on the level of malondialdehyde in the brain, pituitary gland, thyroid gland, testes, prostate, epididymis and seminal vesicles were evaluated using a commercially prepared kits, while activities of antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase were analysed using standard ELISA methods on PND 91. Slices of the organs were homogenized in ice-cold phosphate buffered-solution (PBS) to make 10% homogenates. Then the homogenates were centrifuged at 10,000 × *g* for 15 min at 4 °C and the aliquots were separated and kept refrigerated until required for assay.

3.9.1 Evaluation of brain, pituitary gland, thyroid gland, testicular, prostate, epididymal and seminal vesicles malondialdehyde concentration

The malondialdehyde (MDA) concentrations in the brain, pituitary gland, thyroid gland, testicular, prostate, epididymis and seminal vesicles were evaluated using MDA assay kit (NWLSS^{TM} Malondialdehyde Assay, K-MDA01, Northwest Life Science Specialities, LLC, 5131 NE 94th Avenue, Suite 201Vancouver, WA 98662). The assay principle was based on the reaction of MDA with thiobarbituric acid (TBA); forming an MDA-TBA2 adduct that absorbs strongly at 532 nm. Briefly, after cutting tissue samples, they were weighed and 10% Phosphate buffered solution (PBS) (PH-7.4) was added, homogenized by hand using pestle and mortar, and the mixture centrifuged for 20 min at the speed of 2000-3000 × *g*. The supernatant was then decanted and used for the assay. A 10 µl butylated hydroxytoluene (BHT) reagent was then added to microcentrifuge vial; it was then followed by addition of 250 µl sample. Afterward, 250 µl of acid reagent was added and then 250 µl thiobarbituric acid (TBA) reagent. This content was vortex vigorously (5-count) and then incubated for 60 minutes at 60°C. It

was then centrifuged at 10,000 × *g* for 2-3 min. The reaction mixtures were then transferred to a cuvette and the absorbance recorded (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China) at 450 nm. The protein concentrations were evaluated using the method of Lowry *et al*., (1952).

3.9.2 Evaluation of brain, pituitary gland, thyroid gland, testicular, prostate, epididymal and seminal vesicles superoxide dismutase activity

Superoxide dismutase activities in the tissues listed above were analysed using NWLSS^{TM} SOD activity assay kit (Northwest Life Science Specialities, LLC, 5131 NE 94th Avenue, Suite 201, Vancouver, WA 98662). The principle of the test was based on the monitoring of the auto-oxidation rate of hematoxylin as originally described by Martin *et al*. (1987), with modifications to increase robustness and reliability. Briefly, in the presence of SOD enzyme at specific assay pH, the rate of auto-oxidation was inhibited and the percentage of inhibition is linearly proportional to the amount of SOD present within a specific range. Sample SOD activity was determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample and expressed as traditional McCord Fridovich "cytochrome c" units. The basic principal of the assay is shown by the following equation:

$$O_2 + HTH_2 \xrightarrow{SOD} H_2O_2 + HT\ (Abs560\uparrow)$$

Briefly, to each well used for testing, 230 µl of assay buffer was added. It was then followed by adding 10 µl of assay buffer (for blank) or 10 µl Sample. The contents were then shaken to mix and then incubated for 2 min. After incubation, 10 µl of hematoxylin reagent was added to begin the reaction. This was mixed quickly using the instrument's shaker function, and immediately, recording of the absorbance (Rayto Microplate

Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China) at 560 nm every 10 seconds was started for at least 5 minutes.

3.9.3 Evaluation of brain, pituitary gland, thyroid gland, testicular, prostate, epididymal and seminal vesicles catalase activity

Catalase activity in the tissues listed above was analysed using Catalase (CAT) assay kit (NWLSS^{TM} Catalase Activity Assay, NWK-CAT01, Northwest Life Science Specialities, LLC, 5131 NE 94th Avenue, Suite 201, Vancouver, WA 98662). The principle of the test was based on measuring the consumption of H_2O_2 substrate at 240 nm which was based on the method described by Beers and Sizer (1952), with the following modifications to increase robustness and convenience. Catalase calibrator of known activity was provided in order to negate the need for tedious H_2O_2 substrate calibrations. Reagents were also formulated to provide better stability of diluted H_2O_2 substrate and diluted catalase standards allowing for more convenient test condition. Briefly, to each clean UV microplate well, 15 µl of diluted standard or sample was pipetted. This was followed by adding 290 µl of Assay Cocktail to each well. This was mixed as quickly as possible using reader's shaker function. Recording of absorbance (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China) was started immediately at 240 nm, every 2 seconds for 15 seconds.

3.9.4 Evaluation of brain, pituitary gland, thyroid gland, testicular, prostate, epididymal and seminal vesicles glutathione peroxidase activity

The activity of GPx in the brain, pituitary gland, thyroid gland, testes, prostate, epididymis and seminal vesicles were assayed using the Glutathione Peroxidase Assay Kit (NWLSS^{TM} Glutathione peroxidas assay, NWK-GPx01, Northwest Life Science Specialities, LLC, 5131 NE 94th Avenue, Suite 201Vancouver, WA 98662). The

NWLSS™ Glutathione peroxidase assay was an adaptation of the method of Paglia and Valentine (1967). Glutathione peroxidase catalyses the reduction of hydrogen peroxide (H_2O_2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). The GSSG was then reduced by glutathione reductase (GR) and nicotinamide adenine dinucleotide phosphate (NADPH) forming $NADP^+$ (resulting in decreased absorbance at 340 nm) and recycling the GSH. Because GPx was limiting, the decrease in absorbance at 340 nm was directly proportional to the GPx concentration. GPx activity was reported as units based on the definition:

One unit of GPx-1 = the amount of enzyme necessary to catalyze the oxidation (by H_2O_2) of 1.0 µmole GSH to GSSG, per min at 25 ºC, pH 7.0. Briefly, all reagents were brought to room temperature. The microplate was removed from plastic bag. Fifty microlitre of diluted sample was added to the wells. This was then followed by adding of 50 µl of working NADPH to each well. In addition, 50 µl of working H_2O_2 was also added to each well. A 1 min waiting period was observed; absorbance was monitored at 340nm (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China) for 5 minutes with a recording interval of every 30 seconds. The GPx activity was then calculated from the net rate.

3.10 Evaluation of Treatment on Acetylcholinesterase Activity

The acetylcholinesterase activity was evaluated in the brain, pituitary gland, thyroid gland, testes, prostate and seminal vesicles on PND 91.

3.10.1 Evaluation of organs acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined by the method of Ellman *et al*. (1961), using acetylthiocholine iodide as a substrate. Briefly, the brain, pituitary gland,

thyroid gland, testes, prostate and seminal vesicles of each animal were homogenised in cold (0-4 °C) 20 mM PBS, incubated with 0.01 M 5,5-dithio-bis(2-nitrobenzoic acid) in 0.1 M PBS, pH 7.0. Incubations were allowed to proceed at room temperature for 10 min. Then, acetylthiocholine iodide (0.075 M in 0.1 M PBS, pH 8.0) was added to each tube, and absorbance at 412 nm was measured continuously for 30 min using ELISA reader (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China). The assay of the organs were done in triplicate and AChE activity calculated based on the rate of colour change per min, using the extinction coefficient of 1.36×10^4 which was expressed as nanomoles/min/mg protein. The protein content of each of the tissue was determined using the method of Lowry *et al*. (1952).

3.11 Evaluation of Testicular and Epididymal Total and Free Sialic Acid Concentration

The sialic acid content was assayed using QuantiChrome™ Sialic Acid Assay Kit (BioAssay Systems, 3191 Corporate Place, Hayward, CA 94545, USA) as described by the manufacturer using the calometric method of Warren (1959).

Standards were prepared and equilibrated at room temperature. One thousand micromols sialic acid standard was premixed by mixing 25 µl of the 10 mM Standard and 225 µl distilled water (dH_2O). The standard was then diluted as follows:

No	Premix + dH_2O	Vol (µL)	Sialic Acid (µM)
1	100 µL + 0 µL	100	1000
2	60 µL + 40 µL	100	600
3	30 µL + 70 µL	100	300
4	0 µL + 100 µL	100	0

Exactly 20 µl standards was transfered into four labeled Eppendorf tubes, from where 5 µl 10% trichloro acetic acid (TCA) was added. Total sialic acid in the samples was then hydrolyzed to release bound sialic acid as follows. In an Eppendorf tube, 20 µl sample, 40 µl dH_2O and 40 µl hydrolysis reagent were mixed at 80°C for 60 min and then cooled briefly and centrifuged. A 25 µl 10% TCA was added, vortexed and then centrifuged at 14,000 × g for 10 min. A 25 µl of the supernatant was then transferred into a clean tube and then labeled as total sialic acid. The free sialic acid (FSA) was determined by directly precipitating protein in the mixture by mixing 40 µl sample and 10 µl 10% TCA. This was vortexed and centrifuged at 14,000 × g for 10 min. A 25 µl of the supernatant was then transfered into a clean tube and labeled as free sialic acid (FSA). The mixtures were then auto-oxidated by preparing a working reagent for each tube, by mixing 15 µl hydrolysis reagent, 50 µl dH_2O and 65 µl oxidation reagent. Exactly 125 µl working reagent was then added to each tube and allowed to stand for 60 min at room temperature.

Colour reaction was achieved by adding 50 µl dye reagent to each tube, mixed and heated for 10 min at 100°C. The mixture was then allowed to cool for another 5-10 min. A 100 µl dimethyl sulphoxide was added to each tube, mixed and centrifuged for 5 min at 14,000 × g. Finally, 250 µl of the supernatant was transfered into separate wells of a clear flat-bottom 96-well plate and the optical density read at 549 nm (540-555 nm) (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China).

3.12 Evaluation of Epididymal Fructose Concentration

Fructose levels were determined in the epididymis as described by Anderson *et al*. (1979). The method was based on reduction of fructose by a commercially available preparation of sorbitol dehydrogenase with the concomitant oxidation of nicotinamide adenine dinucleotide (NADH). The initial rate of NADH oxidation, which was proportional to the fructose content of the sample, was measured using a recording spectrophotometer. Briefly, after centrifuging the epididymal sample at 1200 × *g* for 25 min, 25-100 µl of epididymal supernatant was added to water to give a total volume of 0.5 ml, The tube was placed in a boiling water bath for 7 minutes, and then centrifuged for 20 min to remove precipitated material. Then 0.1 to 0.3 ml of the supernatant was added to give a reaction mixture containing 1.5 mmol of NADH per liter and sorbitol dehydrogenase preparation (10 µg of protein) in sodium phosphate (0.1 mol/l, pH 6.8), to make a final concentration and total volume of 1 ml. The concentration of fructose was determined by comparing the initial rate of decrease in absorbance at 340 nm (25°C) to that of fructose standards treated identically using ELISA reader (Rayto Microplate Reader, RT-6000, Rayto Life and Analytical Sciences Co. Ltd., China).

3.13 Post-mortem Gross and Histopathological Examinations

Post-mortem gross examination of the brain, pituitary glands, thyroid gland, liver, testes, epididymides, prostate and seminal vesicles were conducted on PND 91. For histopathological evaluation, sections of these organs were fixed in Bouin's fluid, and paraffin sections were made and then stained with haematoxylin and eosin using the method described by Luna (1960).

3.14 Data Analyses

Values were recorded as mean ± standard error of mean (±SEM) and subjected to one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test to determine differences in the parameters evaluated between various experimental groups. The non-parametric ANOVA followed by Tukey's *post-hoc* was used to analysed excitability score. Values of $P < 0.05$ was considered significant. Graphpad prism version 4.0 for windows (2003) from Graphpad prism software, San Diego, Califonia (www.grapphpad.com) was used. Values of $P < 0.05$ were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Median Lethal Dose for Chlorpyrifos

There was no death recorded in the three rats dosed with 50 mg/g of CPF during the phase I LD_{50} evaluation, while two rats died in the group dosed 150 mg/kg. All the three animals died within 24-72 hours in the group dosed with CPF at 450 mg/kg (Table 4.1). The result of phase I of the LD_{50} determination was then used as a guide in selecting the dose for the phase II as shown in Table 4.2. In the phase II, there was no death recorded in the animals dosed at 80 mg/kg and 90 mg/kg, respectively, with CPF. However, the animal, dosed at 100 mg/kg died. The LD_{50} was calculated to be 95 mg/kg. Clinical signs observed included, back-arching, huddling, convulsion, tachypnoea, paresis, hind-limb and-quarters paralysis and death.

4.2 Median Lethal Dose for Cypermethrin

There was no mortality seen in the rats (n=3/group) treated in groups 1 and 2 at 100 mg/kg and 300 mg/kg, respectively, while all the rats in group 3 dosed at 500 mg/kg died within 24 - 72 hours post-exposure in the phase I LD_{50} evaluation (Table 4.3). In the phase II, no death was recorded in groups 1 and 2 rats dosed at 300 mg/kg and 350 mg/kg, respectively while the rats in group III dosed with CYP at 400 mg/kg died (Table 4.4). The LD_{50} was determined to be 375 mg/kg. Clinical signs observed included, spastic limb movement, ataxia, paresis, hind-limb and-quarters paralysis and death.

Table 4.1: Summary of Phase I LD_{50} determination for CPF

Group	Dose (mg/kg)	Number of Death
1	**50**	**0/3**
2	**150**	**2/3**
3	**450**	**3/3**

Table 4.2: Summary of Phase II LD_{50} determination for CPF

Group	Dose (mg/kg)	Number of Death
1	**80**	**0/1**
2	**90**	**0/1**
3	**100**	**1/1**

Based on the modified method of Lorke (1983), the LD_{50} for chlorpyrifos =

$\frac{90 \text{ mg/kg} + 100 \text{ mg/kg}}{2} = 95 \text{ mg/kg}$

Table 4.3: Summary of Phase I LD_{50} determination for CYP

Group	Dose (mg/kg)	Number of Death
1	**100**	**0/3**
2	**300**	**0/3**
3	**500**	**3/3**

Table 4.4: Summary of Phase II LD_{50} determination for CYP

Group	Dose (mg/kg)	Number of Death
1	**300**	**0/1**
2	**350**	**0/1**
3	**400**	**1/1**

Based on the modified method of Lorke (1983), the LD_{50} for cypermethrin =

$\frac{400\ mg/kg+350mg/kg}{2}$ = 375 mg/kg

4.3 Neurodevelopmental and Reproductive Toxicity Study

4.3.1 Clinical signs

No clinical signs of toxicity were observed in dams of groups I (DW), II (S/oil) and III (MEL), respectively. Group IV (CC) dams showed toxicity signs, including muscle tremors, wet faecal mass, huddling, reduced feed intake, nasal discharges, dystocia, dysgalatia, and death. Most of the pups delivered by dams in CC group were born weak and were smaller in weight compared to the control and the treated groups. In dams that developed dysgalactia, the pups were fostered to a dam with fewer pups in the same group. Groups V (MCC) and VI (CCM) showed milder signs of toxicity. The signs observed included huddling and reduced feed intake, which disappeared a week after commencement of treatment. Two dams died of dystocia at GD 20 of pregnancy in the CC group.

4.4 Effects of Treatment on Developmental Parameters of Pups

4.4.1 Effect of treatment on litter size in F1 generation male Wistar rats

Figure 4.1 shows the effect of treatment on F1 generation litter size. There was a significant ($P < 0.05$) decrease in the litter size in the CC group, compared to the DW ($P < 0.01$), MEL ($P < 0.01$), MCC ($P < 0.001$)and CCM ($P < 0.05$) groups, respectively. Although not significant ($P > 0.05$), the litter size of S/oil group was relatively higher (16.5%) compared to that of the CC group.

4.4.2 Effect of treatment on foetal live/dead ratio of F1 generation male Wistar rats

Foetal live/dead ratio in the CC group was significantly lower, compared to the DW ($P < 0.05$), S/oil ($P < 0.05$), MEL ($P < 0.001$), MCC ($P < 0.001$) and CCM ($P < 0.01$) groups, respectively. There was a significant increase ($P < 0.05$) in the foetal live/dead ratio in the MEL group when compared to that of the DW and S/oil groups. Although not significant, the foetal live/dead ratio in the MCC (19%) group was relatively higher when compared to that of CCM (17%) group (Figure 4.2).

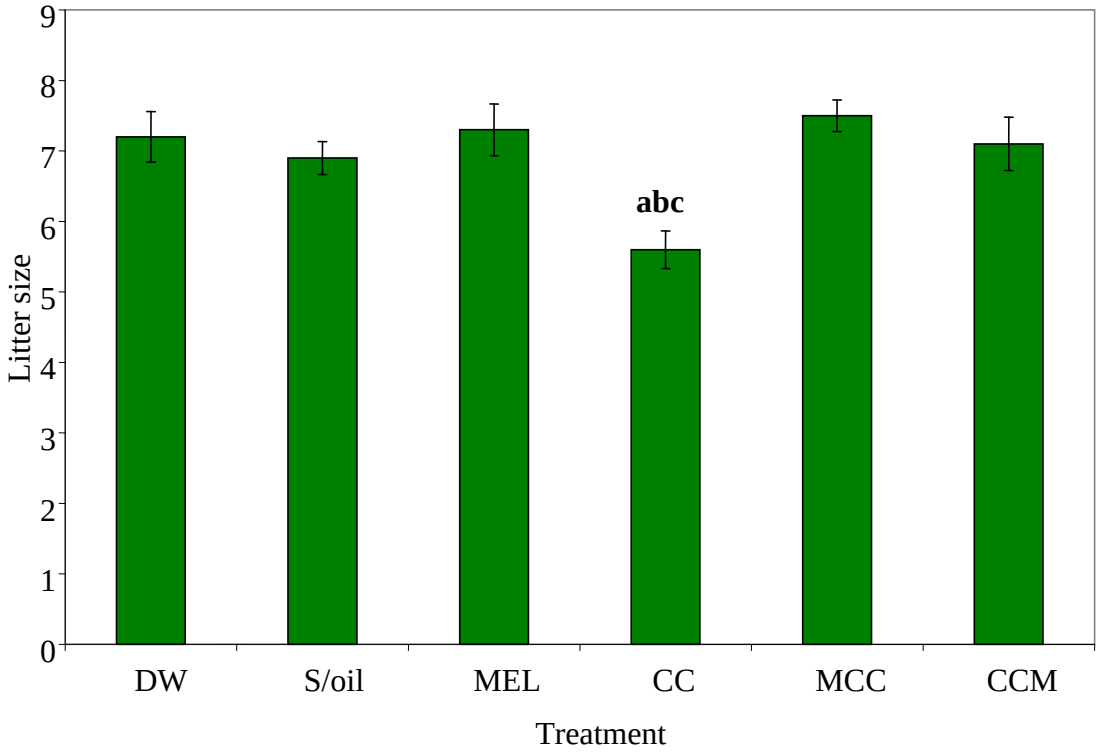


Figure 4.1: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on litter size. $^{a}P < 0.01$ versus DW, MEL groups; $^{b}P < 0.001$ versus MCC; $^{c}P < 0.05$ versus CCM.

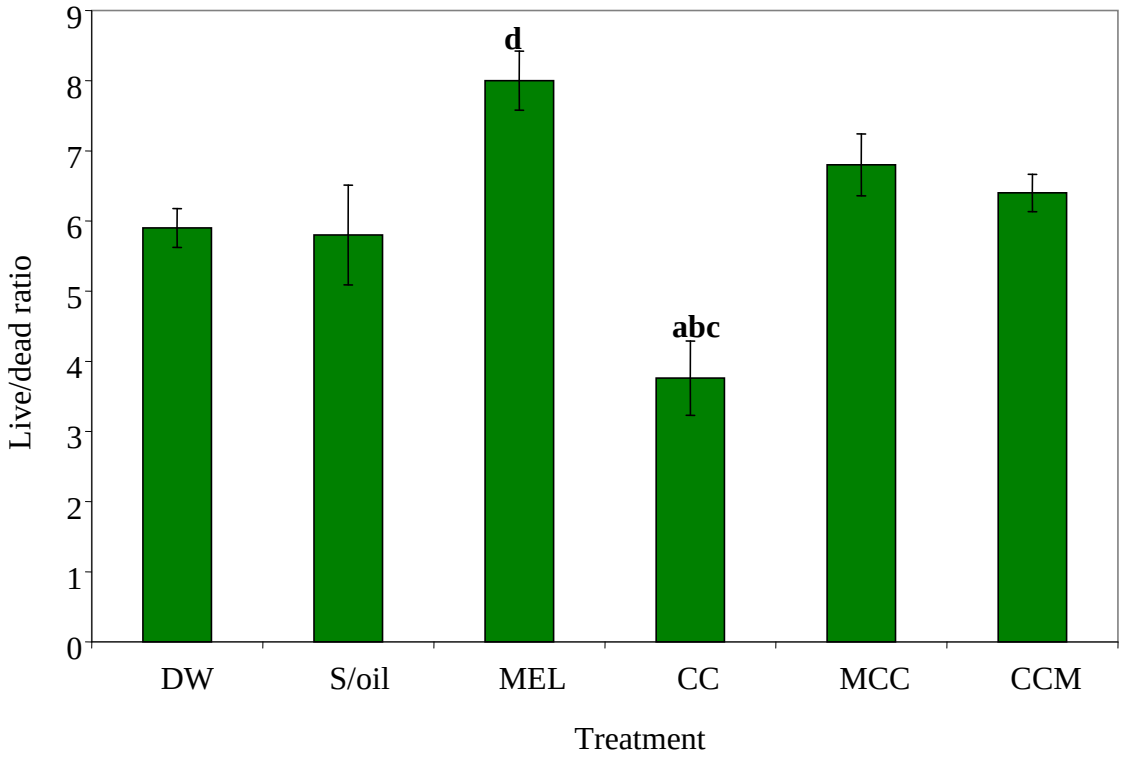


Figure 4.2: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on foetal live/dead ratio. $^{a}P < 0.05$ versus DW and S/oil groups; $^{b}P < 0.001$ versus MEL and MCC groups; $^{c}P < 0.01$ versus CCM group; $^{d}P < 0.05$ versus DW and S/oil groups.

4.4.3 Effect of treatment on dynamics of litter weight of F1 generation male Wistar rats

At PND 0, there was a significant decrease in the litter weight of the CC group relative to that of the DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.01$) and MCC ($P < 0.01$) groups, respectively. There was a significant ($P < 0.05$) increase in the litter weight in the S/oil group compared to that of the CCM group. There was a marginal increase ($P > 0.05$) in the litter weight of S/oil group when compared to that of MCC group. In addition, there was a non-significant ($P > 0.05$) increase in the litter weight of CCM group relative to that of CC group. There was no significant change ($P > 0.05$) in the litter weight of MCC group when compared to that of CCM group.

At PND 4, there was a significant decrease in the litter weight in the CC group relative to that of the DW ($P < 0.05$), S/oil ($P < 0.001$), MEL ($P < 0.01$) and MCC ($P < 0.05$) groups, respectively. There was a non-significant increase ($P > 0.05$) in the litter weight of S/oil group when compared to that of MCC group. Similarly, there was no significant ($P > 0.05$) increase in the litter weight of CCM group relative to that of CC group. There was no significant change ($P > 0.05$) in litter weight in the MCC group compared to that of the CCM group.

At PND 7, there was a significant decrease in the litter weight of CC group when compared to that of the DW ($P < 0.01$), S/oil ($P < 0.001$), MEL ($P < 0.05$) and MCC ($P < 0.05$) groups, respectively. In addition, there was a significant decrease in the litter weight in the CCM group when compared to that of the DW ($P < 0.01$), S/oil ($P < 0.001$) and MEL ($P < 0.01$) groups. There was a non-significant ($P > 0.05$) increase in the litter weight of CC group relative to that of CCM group. Furthermore, there was a significant ($P < 0.01$) increase in the litter weight of the MCC group relative to that of the CCM group.

At PND 14, there was a significant decrease in the litter weight of the CC group relative to that of the DW ($P < 0.001$), S/oil ($P < 0.001$) and MCC ($P < 0.05$) groups, respectively. There was a significant decrease in the litter weight of the CCM group relative to that of the DW ($P < 0.01$) and S/oil ($P < 0.001$) groups, respectively. There was a non-significant ($P > 0.05$) increase in the litter weight of CCM group relative to that of CC group. In addition, there was a significant ($P < 0.05$) increase in the litter weight of the S/oil group when compared to that of the MCC group. There was no change in the litter weight of MCC group relative to that of CCM group.

At PND 21, there was a significant decrease in the litter weight of CC group relative to that of the DW ($P < 0.001$), S/oil ($P < 0.001$), MCC ($P < 0.001$) and CCM ($P < 0.05$) groups, respectively. There was a significant decrease in the litter weight of MEL group relative to that of the DW ($P < 0.05$) and S/oil ($P < 0.001$) group, respectively. Furthermore, there was significant increase in the litter weight of S/oil group relative to that of MCC ($P < 0.05$) and CCM ($P < 0.001$) groups, respectively. Although not significant ($P > 0.05$), the litter weight of the MCC group was relatively higher compared to that of the CCM group (Figure 4.3).

Within the DW group, there was a significant increase ($P < 0.001$) in the litter weights of the F1 rats at PND 7, 14 and 21 when respectively compared to that of the PND 0. In addition, data showed a significant increase ($P < 0.001$) in the litter weights at PND 14 and 21, respectively relative to that of PND 4; while significant increases ($P < 0.001$) in the litter weight were recorded in the F1 rats at PND 7, 14 and 21, when respectively compared to that of PND 7

The S/oil group showed a progressive increase ($P < 0.001$) in the litter weight of F1 rats at PNDs 7, 14 and 21 when respectively compared to PND 0; there were significant

increases in litter weight at PND 14 and 21, when respectively compared to that of PND 4 or PND 7. In addition, there was a significant increase ($P < 0.001$) in the litter weight of F1 rats at PND 14 relative to that of PND 21.

In the MEL group, there was a significant increase in the litter weights of the F1 rats at PNDs 4, 7, 14 and 21, respectively when compared to that of the PND 0. Further more, a significant increase ($P < 0.001$) in the litter weights at PND 14 and 21, respectively relative to that of PND 4 were observed; while significant increases ($P < 0.001$) in the litter weight were recorded in the F1 male offsprings at PND 14 and 21, when compared to that of PND 7. In addition, there was a significant increase ($P < 0.001$) in the litter weight of F1 rats at PND 21 relative to that of PND 14.

In the CC group, the litter weights of the F1 rats were significantly higher at PNDs 4 ($P < 0.05$), 7 ($P < 0.001$), 14 ($P < 0.001$) and 21 ($P < 0.001$), compared to that of PND 0, respectively. In addition, the litter weights were higher ($P < 0.001$) in the F1 male offsprings at PND 14 and 21, compared to that of PND 4. There were significant increases in the litter weights of the rats at PND 14 ($P < 0.01$) and 21 ($P < 0.001$), compared to that of PND 7. There was a significant ($P < 0.001$) increase in the litter weight of F1 rats at PND 21 relative to that of PND 14.

In the MCC group, the litter weights significantly ($P < 0.001$) increased at PNDs 4, 7, 14 and 21 relative to that of PND 0, respectively; then at PNDs 7, 14 and 21 relative to that of PND 4, respectively ($P < 0.001$); and at PNDs 14 and 21 relative to that of PND 7 ($P < 0.001$),respectively. There was a significant ($P < 0.001$) increase in the litter weight of F1 rats at PND 21, relative to that of PND 14.

In the CCM group, the litter weights significantly ($P < 0.001$) increased at PNDs 4, 7, 14 and 21, relative to that of PND 0, respectively; then at PNDs 7, 14 and 21 relative to

that of PND 4, respectively ($P < 0.001$); and at PNDs 14 and 21 relative to that of PND 7 ($P < 0.001$), respectively. There was a significant increase ($P < 0.001$) in the litter weight of F1 rats at PND 21 relative to that of PND 14.

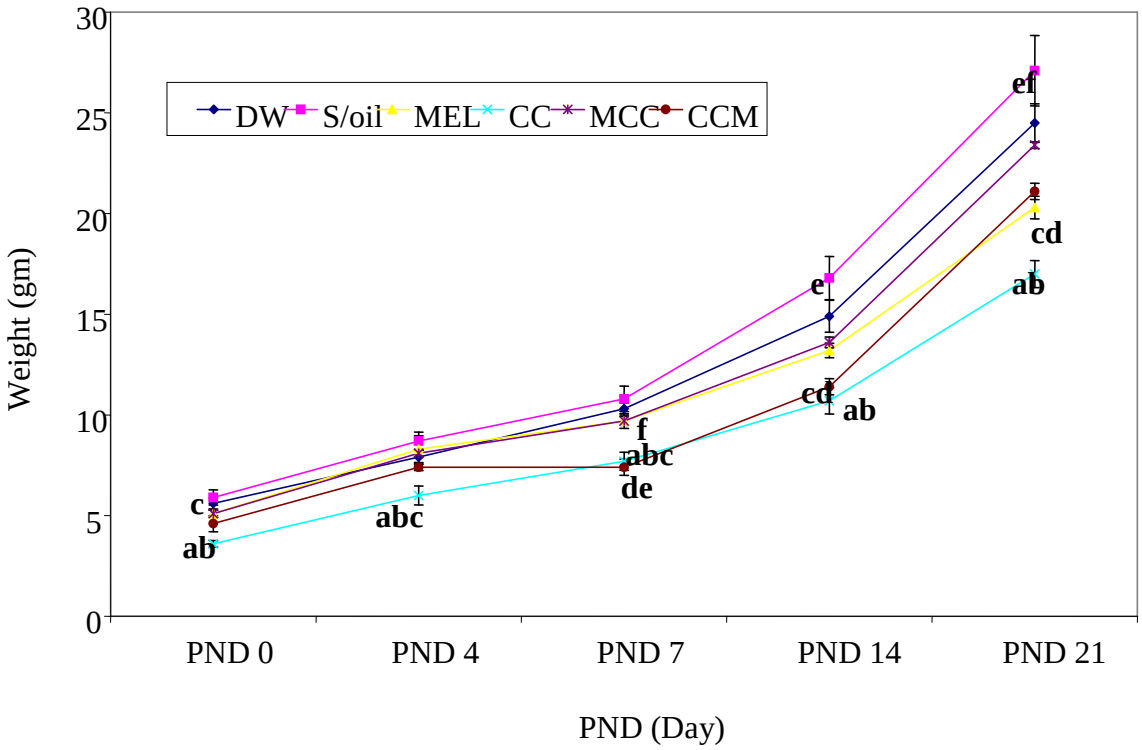


Figure 4.3: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on dynamics of Litter weight. $^{a}P < 0.001$ versus DW and S/oil groups; $^{b}P < 0.01$ versus MEL and MCC groups; $^{c}P < 0.05$ versus CCM at PND 0. $^{a}P < 0.05$ versus DW and MEL groups; $^{b}P < 0.01$ versus MEL group; $^{c}P < 0.001$ versus S/oil group at PND 4. $^{a}P < 0.01$ versus DW group; $^{b}P < 0.001$ versus S/oil; $^{c}P < 0.05$ versus MEL and MCC groups; $^{d}P < 0.01$ versus DW group; $^{e}P < 0.001$ versus S/oil group; $^{f}P < 0.01$ versus CCM group at PND 7. $^{a}P < 0.001$ versus DW and S/oil groups; $^{b}P < 0.05$ versus MEL; $^{c}P < 0.01$ versus DW group; $^{d}P < 0.001$ versus S/oil group; $^{e}P < 0.05$ versus MCC group at PND 14. $^{a}P < 0.001$ versus DW, S/oil and MCC groups; $^{b}P < 0.05$ versus CCM group, $^{c}P < 0.05$ versus DW group; $^{d}P < 0.05$ versus S/oil group; $^{e}P < 0.05$ versus MCC; $^{f}P < 0.001$ versus CCM group at PND 21.

4.4.4 Effect of treatment on crown-rump length and anogenital distance in F1 generation male Wistar rats

The crown-rump length in the CC group was significantly ($P < 0.001$) lower, compared to those obtained in the DW, S/oil, MEL, MCC and CCM treated groups. There was no significant ($P > 0.05$) change in the crown rump length in the MCC group when compared to that of the CCM group (Figure 4.4).

There was no significant ($P > 0.05$) change in the anogenital distance in between the groups. The anogenital distance in the CC group however decreased by 17%, 19%, 15%, 17% and 18%, respectively relative to that of the DW, S/oil, MEL, MCC and CCM groups (Figure 4.5).

4.4.5 Effect of treatment on time of ear opening of F1 generation male Wistar rats

The time of opening of the ear increased significantly ($P < 0.001$) in the CC group, when compared to that of DW, S/oil, MEL, CCM and MCC treated groups. There was no significant ($P > 0.05$) change in time of ear opening in the MCC, when compared to that of the CCM group (Figure 4.6).

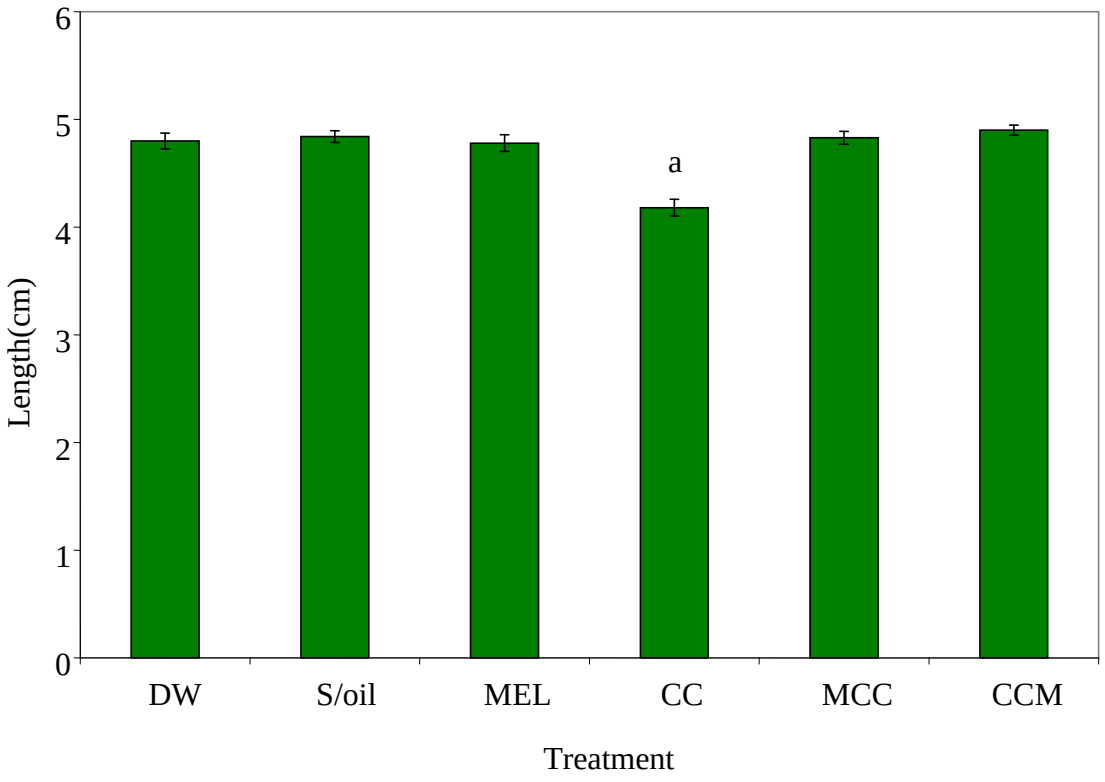


Figure 4.4: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on crown rump lenghth. $^{a}P < 0.001$ versus DW, S/oil, MEL, MCC and CCM groups.

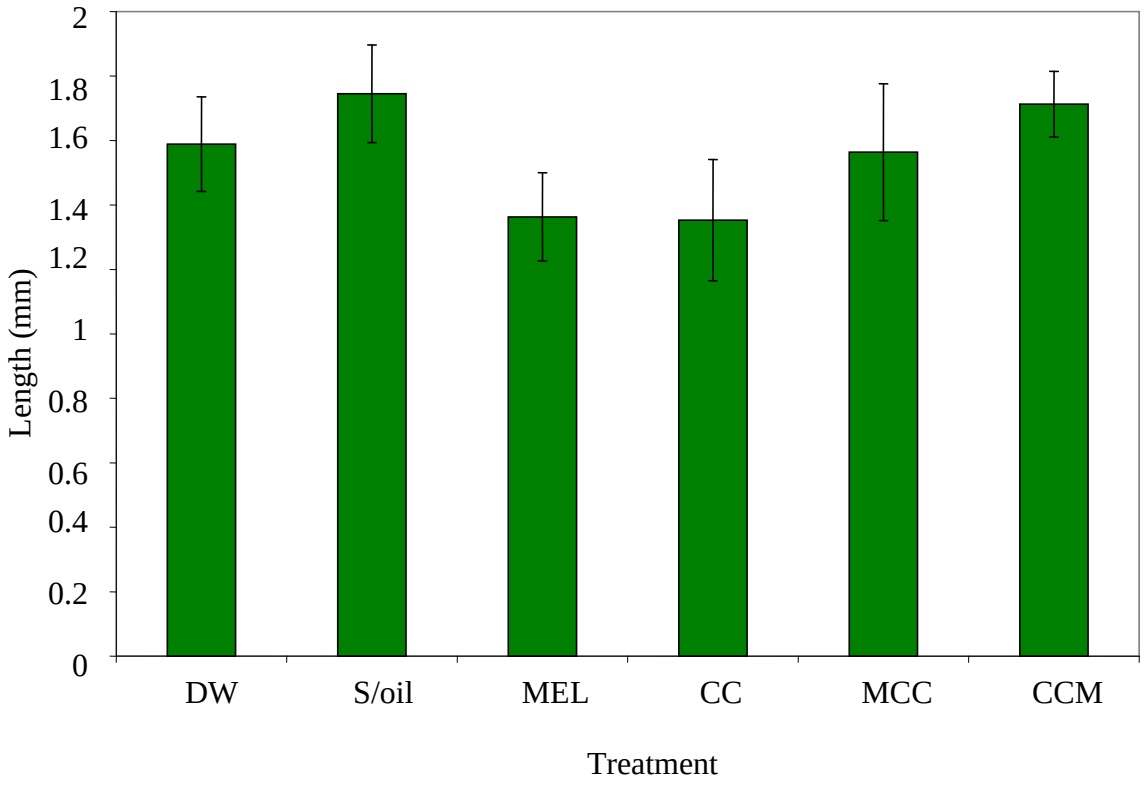


Figure 4.5: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on anogenital distance.

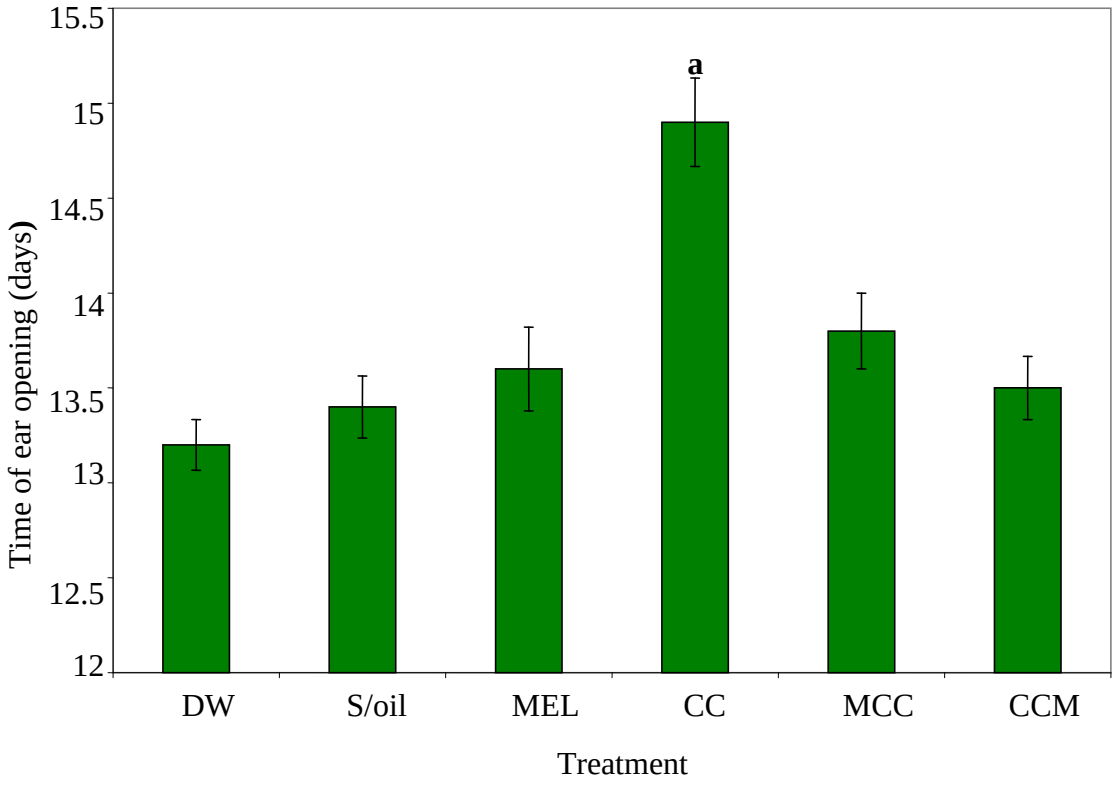


Figure 4.6: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on Time of ear opening. $^{a}P < 0.001$ versus DW, S/oil, MEL, MCC and CCM groups.

4.4.6 Effect of treatment on time of eye opening of F1 generation male Wistar rats

There was a significant ($P < 0.001$) increase in the time of eye opening in the CC group compared to that of DW, S/oil, MEL, MCC and CCM treated groups. Time of eye opening was significantly shorter in the DW group, relative to that the MCC ($P < 0.01$) and CCM ($P < 0.001$) groups. A shorter time of eye opening was observed in the S/oil group relative to that of the MEL ($P < 0.01$), MCC ($P < 0.001$) and CCM ($P < 0.001$) groups, respectively. In addition, the time of eye opening in the MEL group significantly ($P < 0.01$) decreased, relative to that of the CCM group. There was no significant difference in the time of eye opening in the MCC group, when compared to that of the CCM group (Figure 4.7).

4.4.7 Effect of treatment on time of testicular descent of F1 generation male Wistar rats

Figure 4.8 shows the effect of treatment on the time of testicular descent. There was a significant ($P < 0.05$) increase in time of testicular descent in the CC group compared to that of the DW group. Although not significant ($P > 0.05$), the time of testicular descent in the CC group increased by 16%, 17%, 17% and 16%, respectively when compared to that of the S/oil, MEL, MCC and CCM groups.

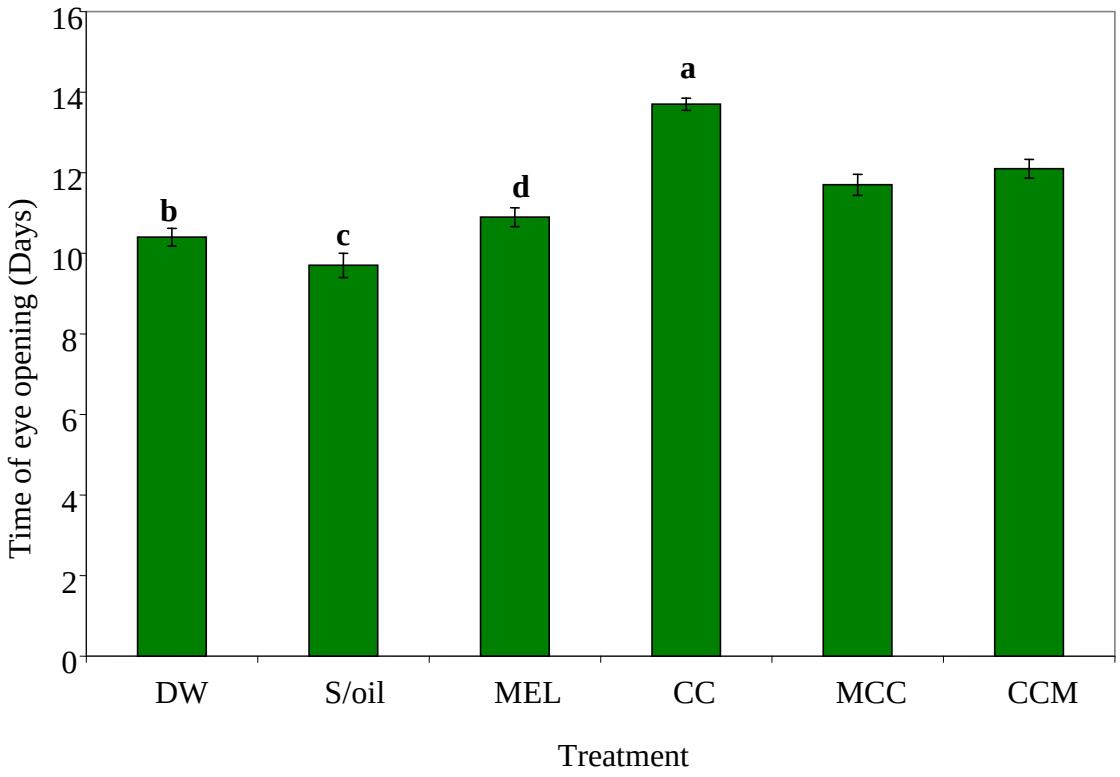


Figure 4.7: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on time of eye opening. $^{a}P < 0.01$ versus DW, S/oil, MEL, MCC and CCM groups. $^{b}P < 0.01$ versus MCC and CCM groups, respectively; $^{c}P < 0.01$ versus MCC and CCM groups. $^{d}P < 0.01$ versus CCM groups.

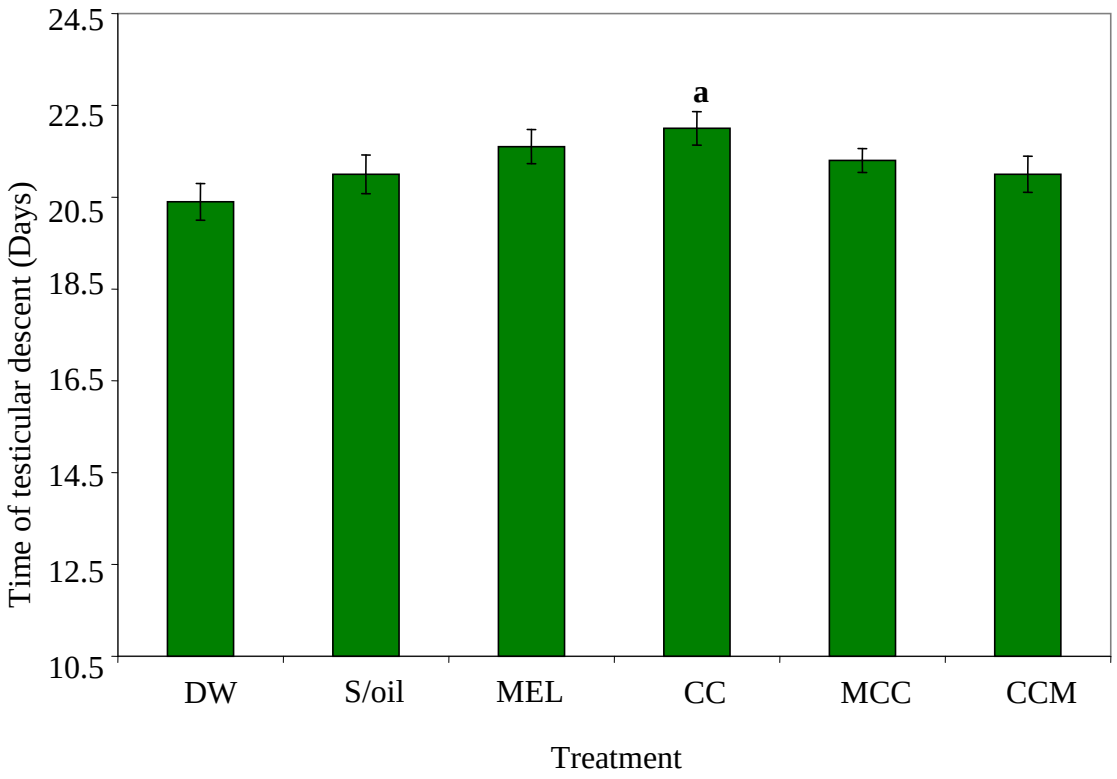


Figure 4.8: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on time of testicular descent. $^{a}P < 0.05$ versus DW group.

4.5 Effects of Treatment on Neurobehavioural Parameters in F1 Generation Pup

4.5.1 Effect of treatment on surface righting reflex of F1 generation male Wistar rats

There was a significant ($P < 0.001$) increase in the surface righting reflex time in the CC group when compared to that of the DW, S/oil, MEL and MCC treated groups. Furthermore, there was a significant increase in the time of surface righting reflex in the CCM group when compared to that of S/oil ($P < 0.01$) and MEL ($P < 0.05$) treated groups. Reflex righting time in the CC group was relatively higher by 23% than that of CCM treated group (Figure 4. 9).

4.5.2 Effect of treatment on negative geotaxis in F1 generation male Wistar rats.

Figure 4.10 shows the effect of treatment on negative geotaxis of F1 rats. There was a significant ($P < 0.001$) increase in the time it took for pups in the CC group to rotate their body, when compared to that of the DW, S/oil, MEL, MCC and CCM treated groups.

4.5.3 Effect of treatment on Aerial righting reflex of F1 generation male Wistar rats

There were no significant differences ($P > 0.05$) in the aerial righting reflex between the groups. However, the time of aerial righting reflex recorded for rats in the CC group was not significantly different when compared to the DW (14%), S/oil (17%), MEL (23%), MCC (20%) and CCM (14%) treated groups (Figure 4.11).

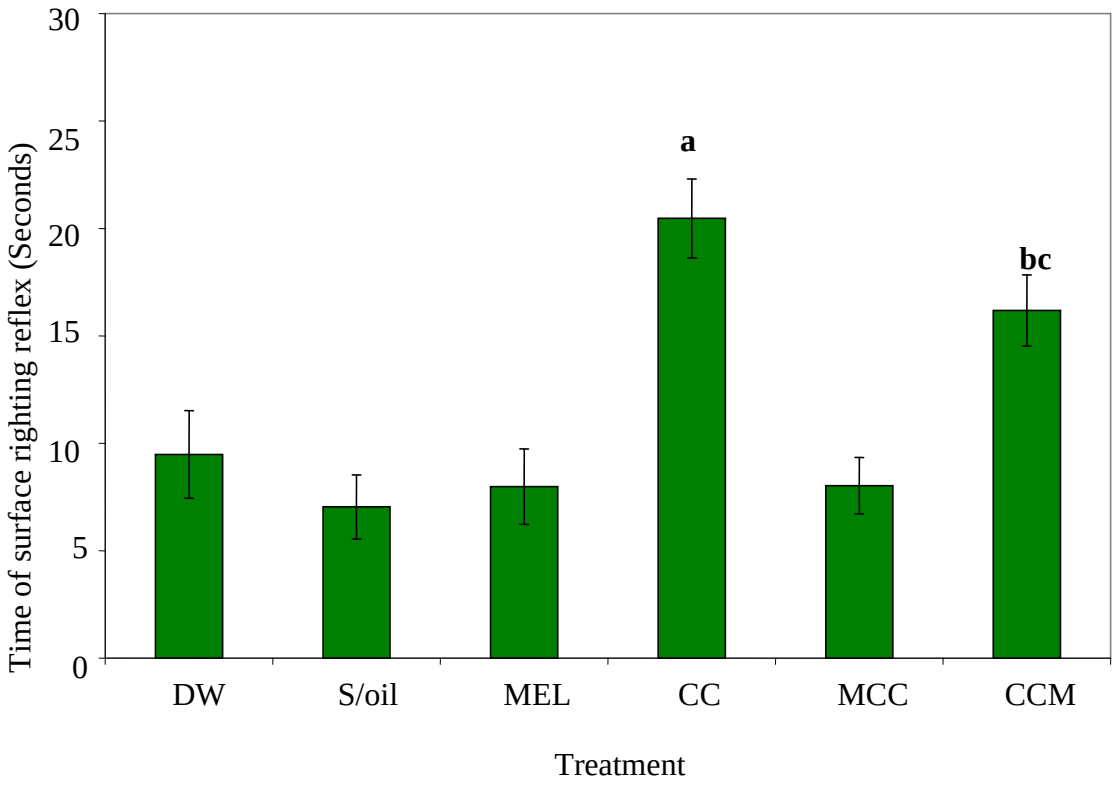


Figure 4.9: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on time of surface righting reflex. $^{a}P < 0.001$ versus DW, S/oil, MEL and MCC groups. $^{b}P < 0.01$ versus Soil group; $^{c}P < 0.05$ versus MEL group.

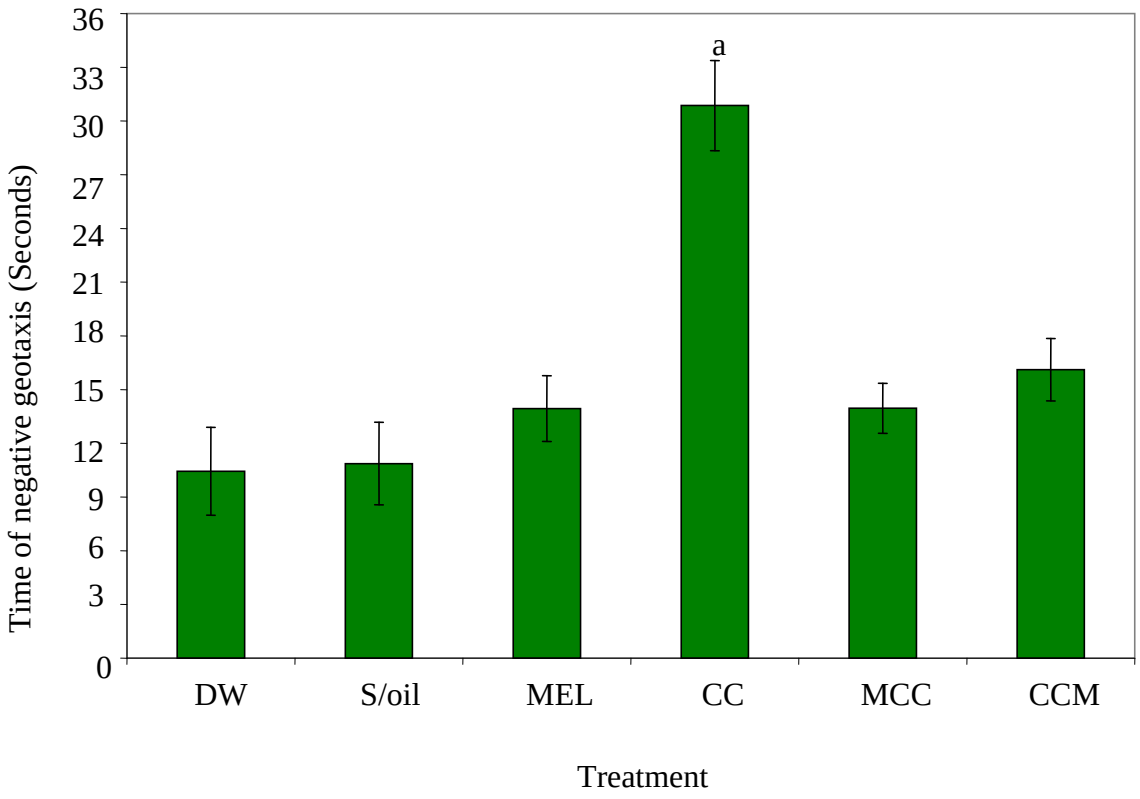


Figure 4.10: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on time of negative geotaxis. $^{a}P < 0.001$ versus DW, S/oil, MEL, MCC and CCM groups.

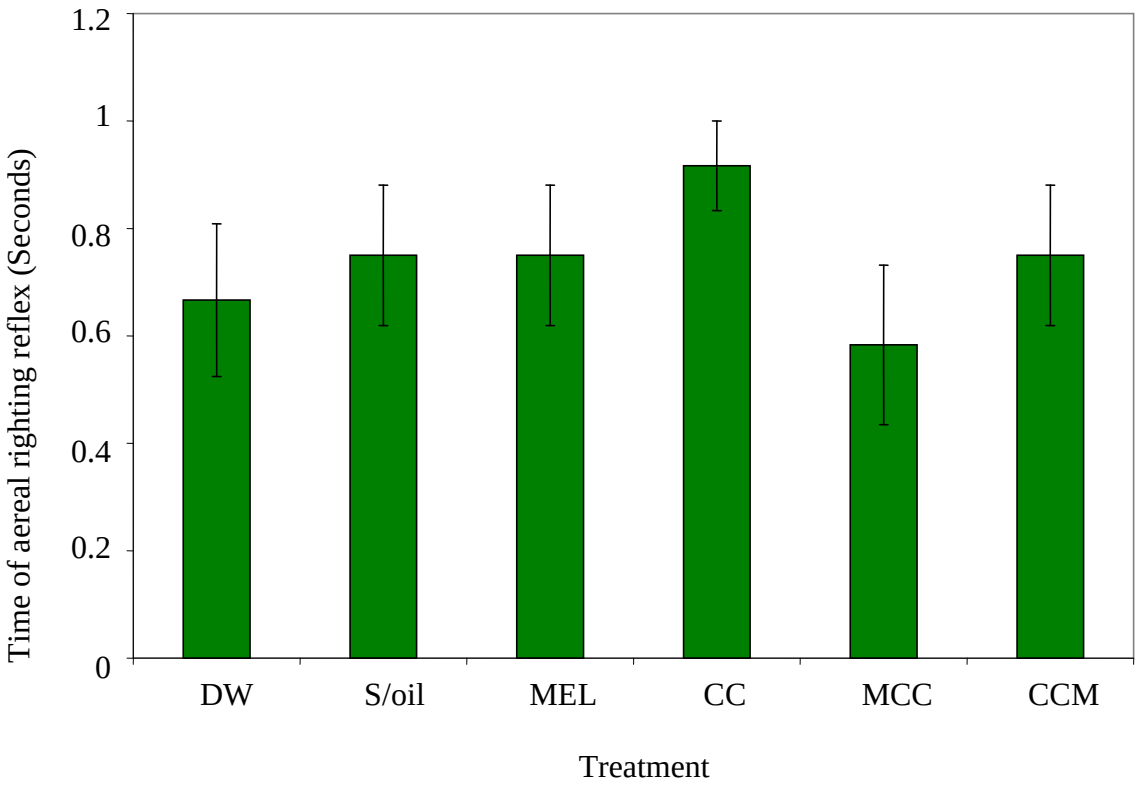


Figure 4.11: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on time of aerial righting reflex.

4.5.4 Effect of treatment on one-minute ability to walk in F1 generation male Wistar rats

Figure 4.12 shows the dynamics of one-minute pup's ability to walk. There was a significant decrease in the □ time to walk□ in the CC group when compared to S/oil ($P < 0.05$), MEL ($P < 0.05$), MCC ($P < 0.001$) and CCM ($P < 0.05$) treated groups at PND 8. However, there was no significant ($P > 0.05$) difference in the one-minute □ time to walk□ of the pups in the CC group as compared to pups of the DW group. In addition, there was a significant ($P < 0.01$) decrease in the time the pups in the DW group took to start walk when compared to that of the MCC group at PND 8.

At PND 15, there was a significant ($P < 0.001$) decrease in the time to walk of pups in the CC group compared to that of the DW, S/oil, MEL, MCC; and CCM ($P < 0.01$) treated groups. Although not significant ($P > 0.05$), there was a relative increase in the time the pups in the S/oil and MEL treated groups walked relative to that of the MCC and CCM groups (Figure 4.12).

Within the group, there was a significant increase ($P < 0.05$) in the time of walk of pups in the DW, S/oil, MEL, CC and CCM groups at PND 8 relative to PND 15.

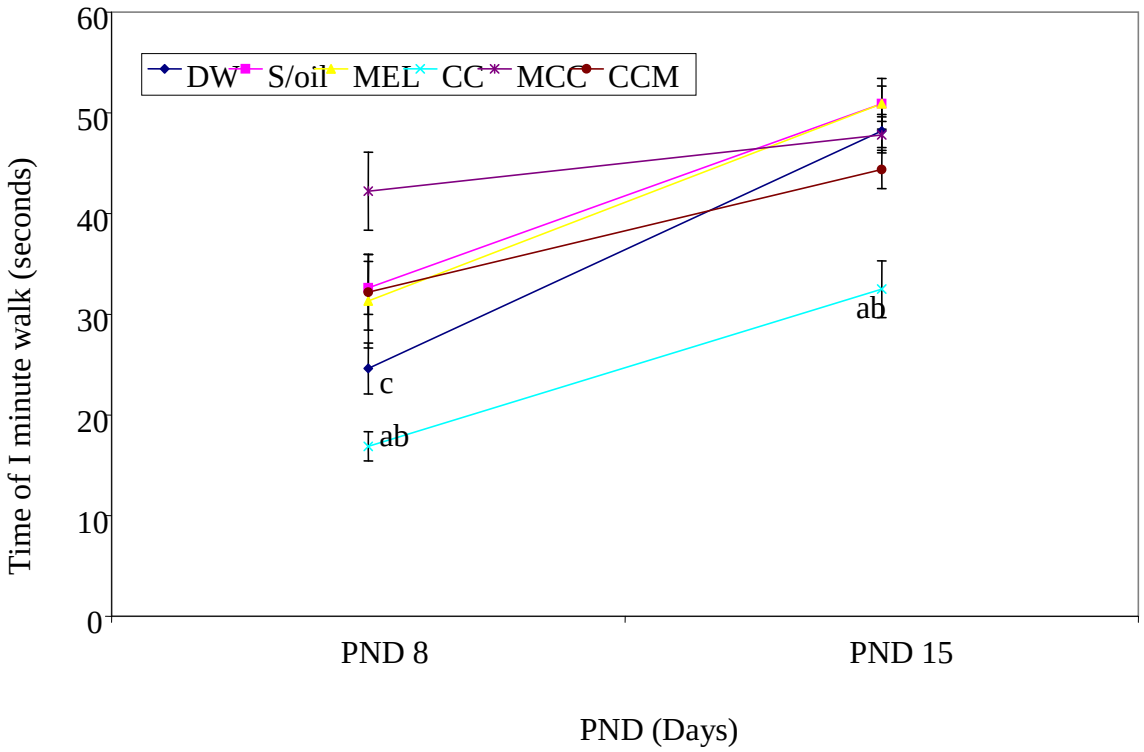


Figure 4.12: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on one-minute ability to walk. [a]P < 0.05 versus S/oil, MEL and CCM groups, respectively; [b]P<0.001 versus MCC; [c]P < 0.01 versus MCC at PND 8. [a]P < 0.001 versus DW, S/oil, MEL and MCC groups, [b]P < 0.001 versus CCM group at PND 15.

4.5.5 Effect of treatment on time of pivoting in F1 generation male Wistar rats

Effects of treatment on time of pivoting is shown in Figure 4.13. The increase in the time of pivoting in the CC group was higher when compared with that of S/oil ($P < 0.05$), MEL ($P < 0.05$), MCC ($P < 0.001$) and CCM ($P < 0.05$) treated groups at PND 8. Although there was no signifcant ($P > 0.05$) difference, time of pivoting in the CC group increased marginally relative to that of DW. Furthermore, time of pivoting in the DW group increased ($P < 0.01$) relative to that of the MCC treated group at PND 8.

At PND 15, a significant increase was recorded in the time of pivoting in the CC group, compared to that of the DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$), MCC ($P < 0.001$) and CCM ($P < 0.01$) treated groups (Fig. 4.13).

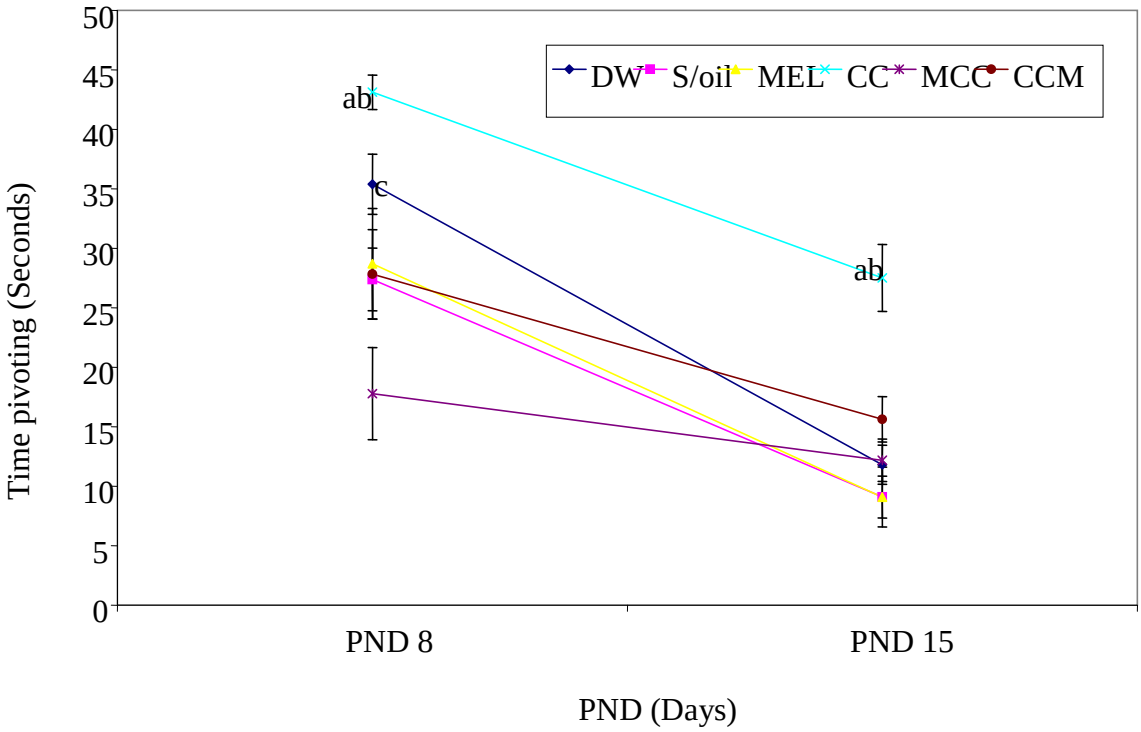


Figure 4.13: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on Time of pivoting. $^{a}P < 0.05$ versus S/oil, MEL and CCM groups, respectively; $^{b}P < 0.001$ versus MCC group; $^{c}P < 0.01$ versus MCC group at PND 8. $^{a}P<0.001$ versus DW, S/oil, MEL, MCC groups, $^{b}P < 0.01$ versus CCM group at PND 15.

4.5.6 Effect of treatment on inclined plane performance in F1 generation male Wistar rats

Effect of treatment on dynamics of neuromuscular coordination as assessed by inclined plane performance is shown in Figure 4.14. At PND 30, there was a significant decrease in the angle of slip in the inclined plane in the CC group compared to the DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.01$) and MCC ($P < 0.01$) treated groups. The angle of slip in the inclined plane in the S/oil group increased significantly ($P < 0.05$) relative to the CCM group. There was no significant change ($P > 0.05$) in the angle of slip in the inclined plane of MCC treated group, when compared to that of CCM group.

At PND 60, there was a significant ($P < 0.001$) decrease in the angle of slip in the inclined plane in the CC group compared to that of DW, S/oil, MEL, MCC and CCM groups, respectively. At PND 90, the angle of slip on the incline plane performance in the CC group was significantly ($P < 0.001$) lower compared to that of the DW, S/oil, MEL, MCC and CCM treated groups.

In the DW group, there was a significant increase ($P < 0.01$) in the angle of slip in the inclined plane at PND 60 and 90, respectively relative to PND 30. There was no significant difference ($P > 0.05$) in the angle of slip in the inclined plane at PND 90 compared to PND 60. In the S/oil group, the angle of slip was significantly higher ($P < 0.001$) at PND 90 compared to PND 30. There was no significant difference ($P > 0.05$) in the angle of slip in the inclined plane at PND 60 compared to PND 90. In the MEL group, there was a significant increase in the angle of slip in the inclined plane at PND 60 ($P < 0.05$) and 90 ($P < 0.01$) relative to that obtained at PND 30. There was no significant difference ($P > 0.05$) in the angle of slip in the inclined plane at PND 90 compared to PND 60. In the CC group, there was no significant change ($P > 0.05$) in the angle of slip of the inclined plane performance between PND 30, 60 and 90. In the

MCC group, there was a significant increase ($P < 0.001$) in the angle of slip in the inclined plane at PND 60 and 90, respectively relative to that of PND 30. There was no significant difference ($P > 0.05$) in the angle of slip in the inclined plane at PND 90 compared to PND 60. Significant increases ($P < 0.01$) were recorded in the angle of slip on the inclined plane at PND 60 and 90, respectively, when compared to that of PND 30 in the CCM group.

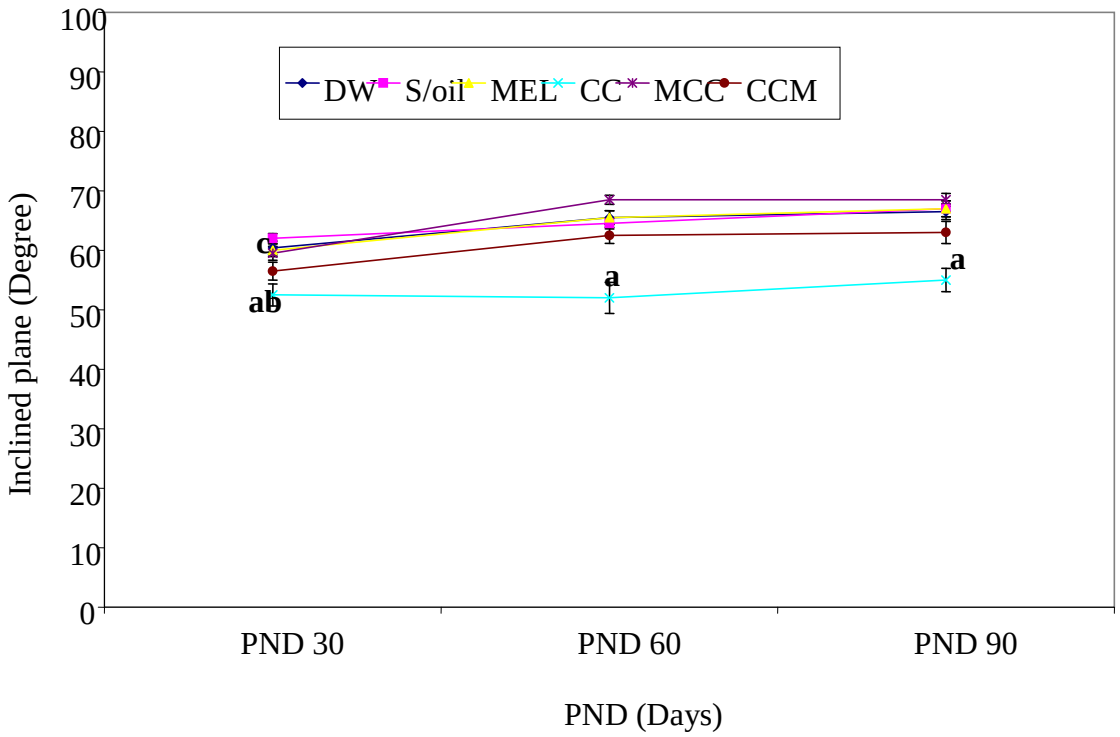


Figure 4.14: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on inclined plane performance. $^{a}P < 0.001$ versus DW and S/oil groups; $^{b}P < 0.01$ versus MEL and MCC groups; $^{c}P < 0.05$ versus CCM group at PND 30. $^{a}P < 0.001$ versus DW, S/oil, MEL, MCC and CCM groups, respectively at PND 60. $^{a}P < 0.001$ versus DW, S/oil, MEL, MCC and CCM groups, respectively at PND 90.

4.5.7 Effect of treatment on ladder walk in F1 generation male Wistar rats

The effect of treatment on efficiency of locomotion as measured by ladder walk test is shown in Figure 4.15. At PND 30, there was a significant decrease in the number of rungs missed in the CC group compared to that of the DW ($P < 0.001$), S/oil ($P < 0.01$) MEL ($P < 0.001$) or MCC ($P < 0.001$) group. Also there was a significant increase in the number of rungs missed in MEL group when compared to that of S/oil ($P < 0.05$) and CCM ($P < 0.01$) groups, respectively. In addition, the number of rungs missed in the MCC group increased significantly ($P < 0.01$) when compared to the CCM group.

At PND 60, there was a significant ($P < 0.001$) decrease in the number of rungs missed in the CC group when compared to DW, S/oil, and MEL groups. There was no significant ($P > 0.05$) difference in the number of rungs missed in the MCC group when compared to the CCM group. There was a significant increase in the number of rungs missed in the DW group when compared to that of the MCC ($P < 0.05$) and CCM ($P < 0.001$) groups. In addition, there was a significant increase in the number of rungs missed in the S/oil group when compared to that of the MCC ($P < 0.05$) and CCM ($P < 0.001$) groups. Furthermore, there was a significant increase in the number of rungs missed in the MEL group when compared to that of the MCC ($P < 0.05$) and CCM ($P < 0.001$) groups.

At PND 90, there was a significant decrease in the number of rungs missed in the CC group compared to that of the DW ($P < 0.05$), S/oil ($P < 0.001$), MEL ($P < 0.001$) and MCC ($P < 0.01$) groups. There was a significant increase in the number of rungs missed in the DW group when compared to that of the MEL ($P < 0.05$) and CCM ($P < 0.05$) groups. In addition, there was a significant ($P < 0.001$) increase in the number of rungs missed in the MEL group when compared to that of the CCM groups. Although not

significant ($P > 0.05$), there are comparable decrease in the number of rungs missed in the CC group relative to that of the MCC (17%) and CCM (13%) groups.

In the DW group, there was a significant increase ($P < 0.001$) in the number of rungs missed at PND 60 when compared to that of PND 30. There was a significant ($P < 0.001$) decrease in the number of rungs missed at PND 90 compared to that of PND 60. There were significant increases in the number of rungs missed at PND 60 ($P < 0.001$) and 90 ($P < 0.01$), respectively compared to PND 30. There was a significant ($P < 0.05$) decrease in the number of rungs missed at PND 90 compared to that of PND 60 in the S/oil group. In the MEL group, the number of rungs missed was significantly higher ($P < 0.01$) at PND 60 compared to PND 30. There was no significant change ($P > 0.05$) in the number of rungs missed at PND 90 compared to that of PND 30 and PND 60, respectively. In the CC group, there was a significant increase ($P < 0.01$) in the number of rungs missed at PND 90 when compared to that of PND 30. Although not significant ($P > 0.05$), there was a relative increase in the number of rungs missed at PND 90 (39%) compaired to that of PND 30 (25%) and PND 60 (34%), respectively. In the MCC group, there were no significant change ($P > 0.05$) in the number of rungs missed within the groups between PNDs 30 (31%), 60 (35%) and 90 (34%). Although not significant ($P > 0.05$), there were progressive increase in the number of rungs missed at PNDs 30 (33%), 60 (30%) and 90 (37%) when compaired to each other in the CCM group.

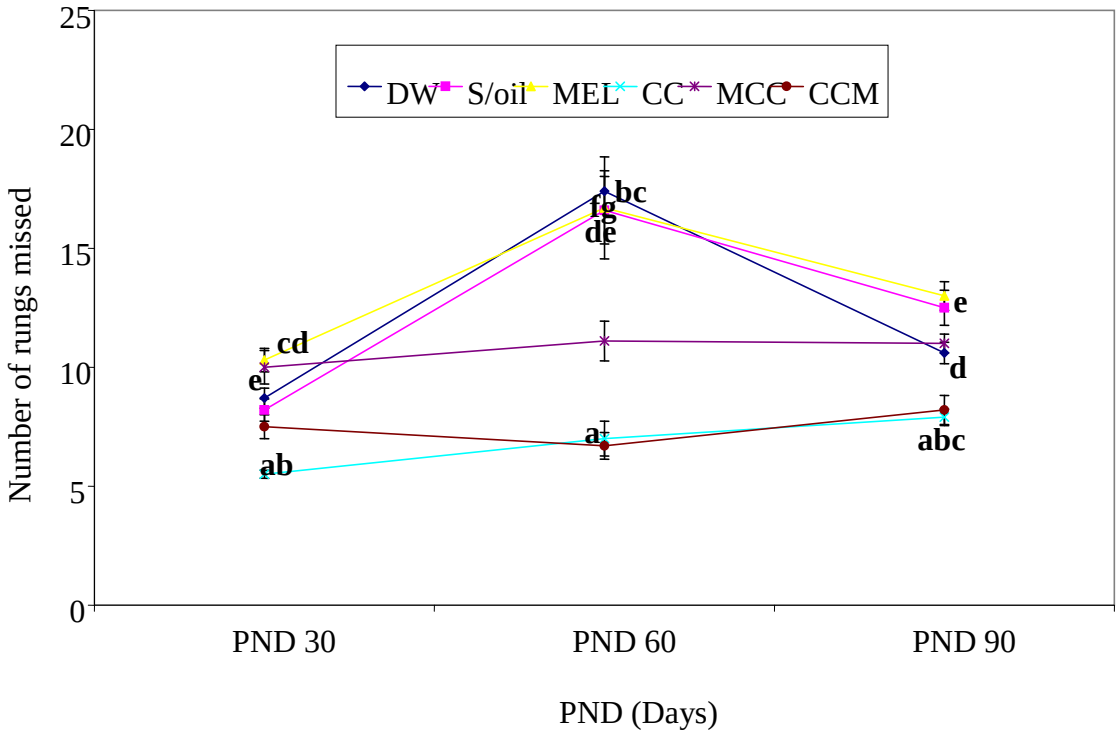


Figure 4.15: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on Ladder walk. $^{a}P<0.001$ versus DW, MEL and MCC groups; $^{b}P < 0.01$ versus S/oil; $^{c}P < .0$ versus S/oil and CCM groups; $^{d}P < 0.01$ versus CC+ MEL group; $^{e}P < 0.01$ versus CCM group at PND 30. $^{a}P < 0.001$ versus DW, S/oil and MEL groups; $^{b}P < 0.05$ versus MCC; $^{c}P < 0.001$ versus CCM group; $^{d}P < 0.05$ versus MCC; $^{e}P < 0.001$ versus CCM; $^{f}P < 0.05$ versus MCC group and $^{g}P < 0.001$ versus CCM group, respectively at PND 60. $^{a}P < 0.05$ versus DW group; $^{b}P < 0.00$ versus Soil group; $^{c}P < 0.01$ versus MCC group; $^{d}P < 0.05$ versus MCC and CCM groups, $^{e}P < 0.001$ versus CCM at PND 90.

4.5.8 Effect of treatment on beam walk performance in F1 generation male Wistar rats

Effect of treatment on motor coordination as demonstrated by cumulative beam walk is illustrated in Figure 4.16. At PND 30, there was a significant increase in the width of slip in the CC group when compared to that of DW ($P < 0.01$), S/oil ($P < 0.01$), MEL ($P < 0.001$) and MCC ($P < 0.05$) groups, respectively. There was a significant ($P < 0.05$) increase in the width of slip in the CCM ($P < 0.01$) group relative to that of S/oil and MEL treated groups.

At PND 60, there was a significant increase in the width of slip in the CC group when compared to that of DW ($P < 0.001$), S/oil ($P < 0.01$), MEL ($P < 0.01$) and MCC ($P < 0.05$) groups, respectively. There was no significant increase in the width of slip of CC group, when compared to that of CCM (18%) group. In addition, the width of slip in the MCC did not differ relative to that of CCM group.

At PND 90, there was a significant increase in the width of slip in the CC group when compared to that of DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$) and MCC ($P < 0.01$) groups, respectively. The width of slip in the CCM group increased relative to that of the DW ($P < 0.05$), S/oil ($P < 0.01$) and MEL ($P < 0.05$) groups, respectively. There was a non-significant increase in the width of slip in the CC group relative to that of CCM (22%) group. The width of slip in the MCC (14%) relative to the CCM (22%) group increased insignificantly.

Although not significant ($P > 0.05$), the width of slip off the beam was higher in day 30 when compared to that of days 60 (15%) and 90 (9%), respectively in the DW group. Although not significant ($P > 0.05$), the width of slip off the beam was higher in day 60 when compared to that of days 30 (11%) and 90 (21%), respectively in the S/oil group. Although not significant ($P > 0.05$), the width of slip off the beam was higher in day 60

relative to that of days 30 (20%) and 90 (12%), respectively in the MEL group. In the CC group, there was no significant change ($P > 0.05$) in the width of slip off the beam in between PNDs with the highest width of slip recorded at PND 60 relative to PND 30 (20%) and PND 90 (20%), respectively. Although not significant ($P > 0.05$), the width of slip off the beam was higher in day 60 when compared to that of days 30 (8%) and 90 (17%), respectively in the MCC group. Although not significant ($P > 0.05$), the width of slip off the beam was higher in day 30 when compared to that of days 60 (10%) and 90 (5%) in the CCM group.

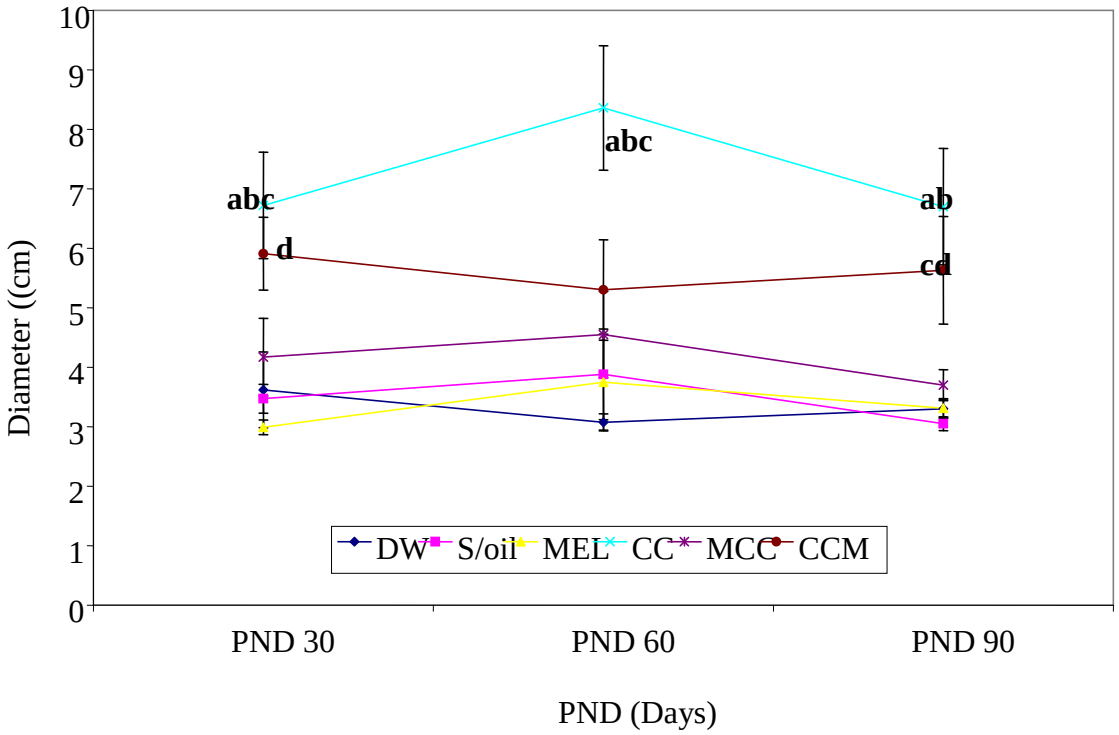


Figure 4.16: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on beam walk. $^{a}P < 0.01$ versus DW and S/oil groups; $^{b}P < 0.001$ versus MEL; $^{c}P < 0.05$ versus MCC; $^{d}P < 0.05$ versus S/oil and MEL group at PND 30. $^{a}P < 0.001$ versus DW group; $^{b}P < 0.01$ versus S/oil and MEL groups; $^{c}P < 0.05$ versus MCC group at PND 60. aP < 001 versus DW, S/oil and MEL groups, respectively; $^{b}P < 0.01$ versus MCC; $^{c}P < 0.05$ versus DW and MEL groups; $^{d}P < 0.01$ versus S/oil group at PND 90.

4.5.9 Effect of treatment on excitability scores in F1 generation male Wistar rats

There was a significant ($P < 0.001$) decline in the excitability score of rats in the CC group in relation to that of the DW, S/oil, MEL and MCC groups, respectively. There was a significant ($P < 0.05$) decline in excitability scores of CCM group when compared to that of the DW and MEL groups, resectively. Although not significant ($P > 0.05$), there was decline in the excitability score of the CC group relative to that of CCM (15%) group. In addition, there was no significant change ($P > 0.05$) in the excitability score in the MCC group relative to the CCM group at PND 40.

At PND 80, the excitability score in the CC group declined relative to that of the DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$) and MCC ($P < 0.01$) groups, respectively. There was a significant ($P < 0.05$) increase in the excitability scores in the DW group relative to that of the CCM group. Although not significant ($P > 0.05$), the excitability score in the CC group was lower relative to that of CCM group. In addition, MCC (17%) group excitability scores increased non-significantly when compared to that of CCM (15%) group (Figure 4.17).

In the DW group, there was a significant increase ($P < 0.05$) in the excitability score at PND 80 when compared to that of PND 40. In the S/oil, MEL, CC, MCC and CCM groups, there was no significant change ($P > 0.05$) in the excitability score between the PNDs 40 and 80 within the groups.

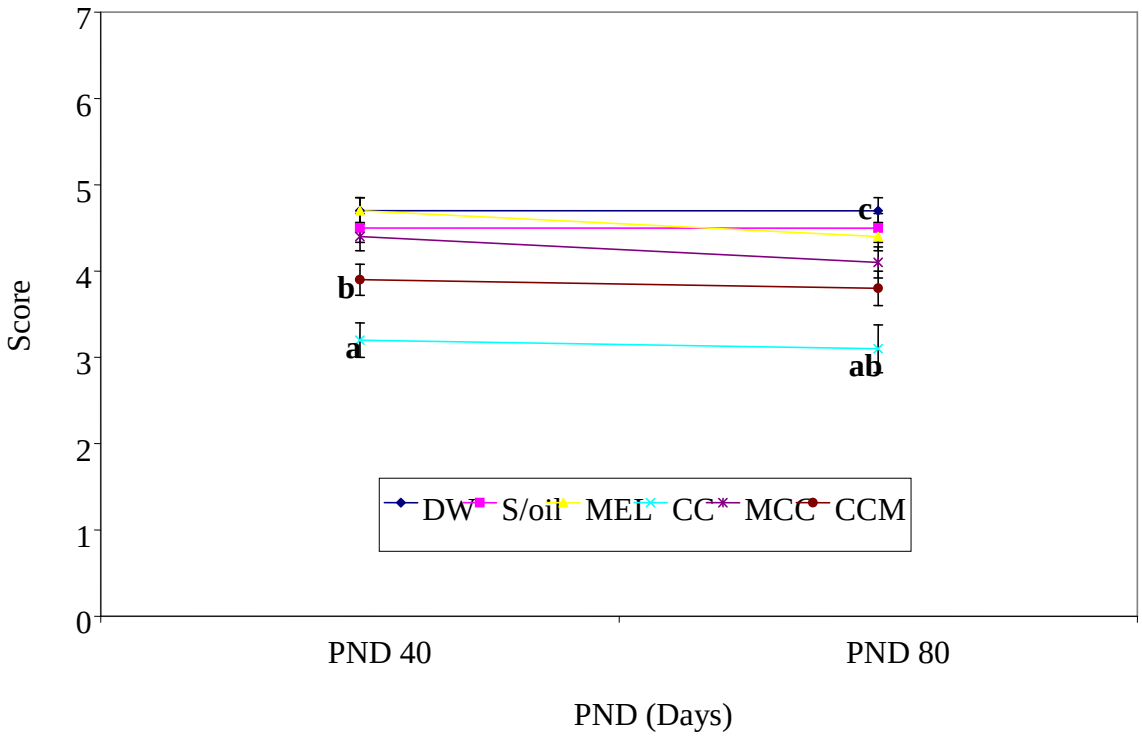


Figure 4.17: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on excitability score. [a]P < 0.001 versus DW, S/oil, MEL and MCC groups, [b]P < 0.05 versus DW and MEL groups at PND 40. [a]P<0.001 versus DW, S/oil, MEL groups, [b]P < 0.01 versus MCC group, [c]P < 0.05 versus CCM group at PND 80.

4.5.10 Effect of treatment on fore-paw grip time in F1 generation male Wistar rats

Effect of treatment on motor strength measured by forepaw grip time is shown in Figure 4.18. The forepaw grip time in the CC group, decreased compared to that of DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$) and MCC ($P < 0.05$) treated groups. There was a significant decline in the fore-paw grip time in the MCC relative to that of the DW ($P < 0.01$) and S/oil ($P < 0.05$) group. In addition, there was a significant decrease in the fore-paw grip time in the CCM group when compared to that of the DW ($P < 0.001$), S/oil ($P < 0.01$) and MEL ($P < 0.05$) treated groups. Furthermore, although there was no significant ($P > 0.05$) change, a relative increase in the fore-paw grip time in the MCC (10%) compared to that of CCM was recorded at PND 80.

4.5.11 Effect of treatment on forced swimming test (FST) in F1 generation male Wistar rats

There was a significant decrease in the mobility time in the CC group compared to DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.01$), MCC ($P < 0.001$) and CCM ($P < 0.001$) groups, respectively. In addition, there was a significant increase ($P < 0.001$) in the mobility time in the DW group relative to the MEL, MCC and CCM groups, respectively. The S/oil group showed a significant increase ($P < 0.001$) in mobility time compared to the MEL, MCC and CCM treated groups. Although not significant ($P > 0.05$), there was a relative increase (5%) in the mobility time in the MCC group relative to that of the CCM group at PND 80 (Figure 4.19).

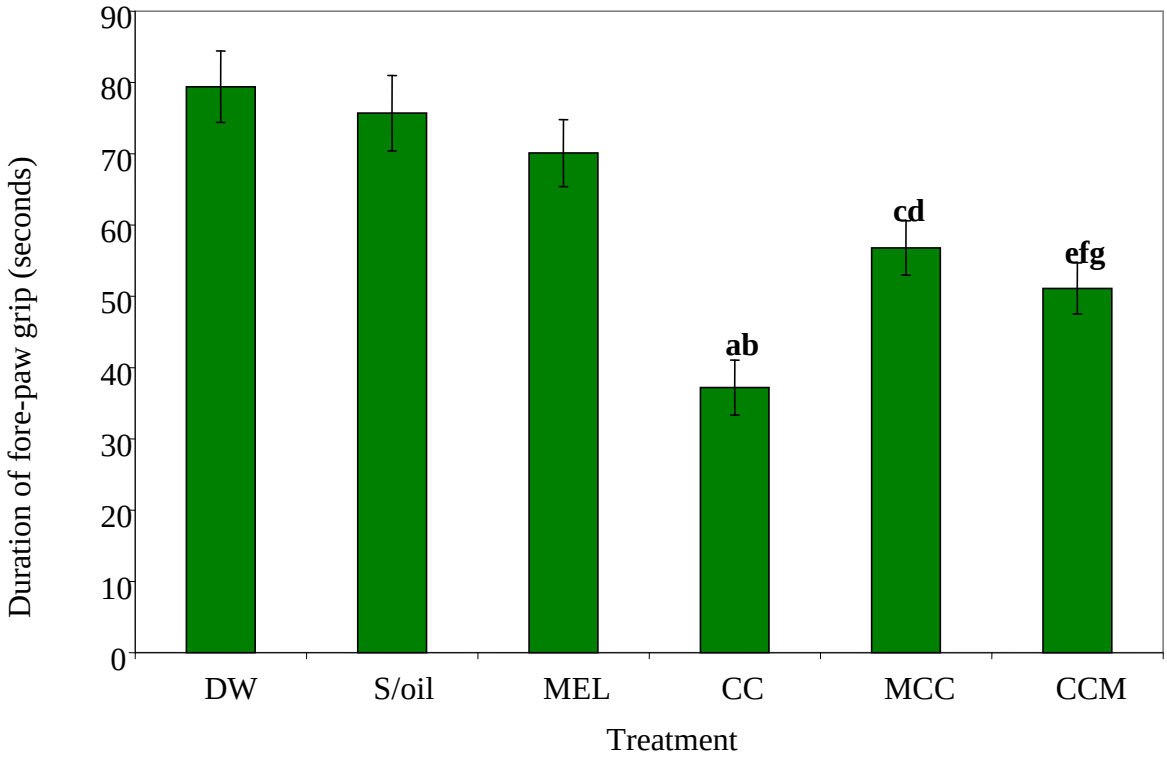


Figure 4.18: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on fore-paw grip test. $^{a}P < 0.001$ versus DW, S/oil and MEL groups; $^{b}P < 0.0$ versus MCC; $^{c}P < 0.01$ versus DW group; $^{d}P < 0.05$ versus S/oil group; $^{e}P < 0.001$ versus DW group; $^{f}P < 0.01$ versus S/oil group; $^{g}P < 0.05$ versus MEL group at PND 80.

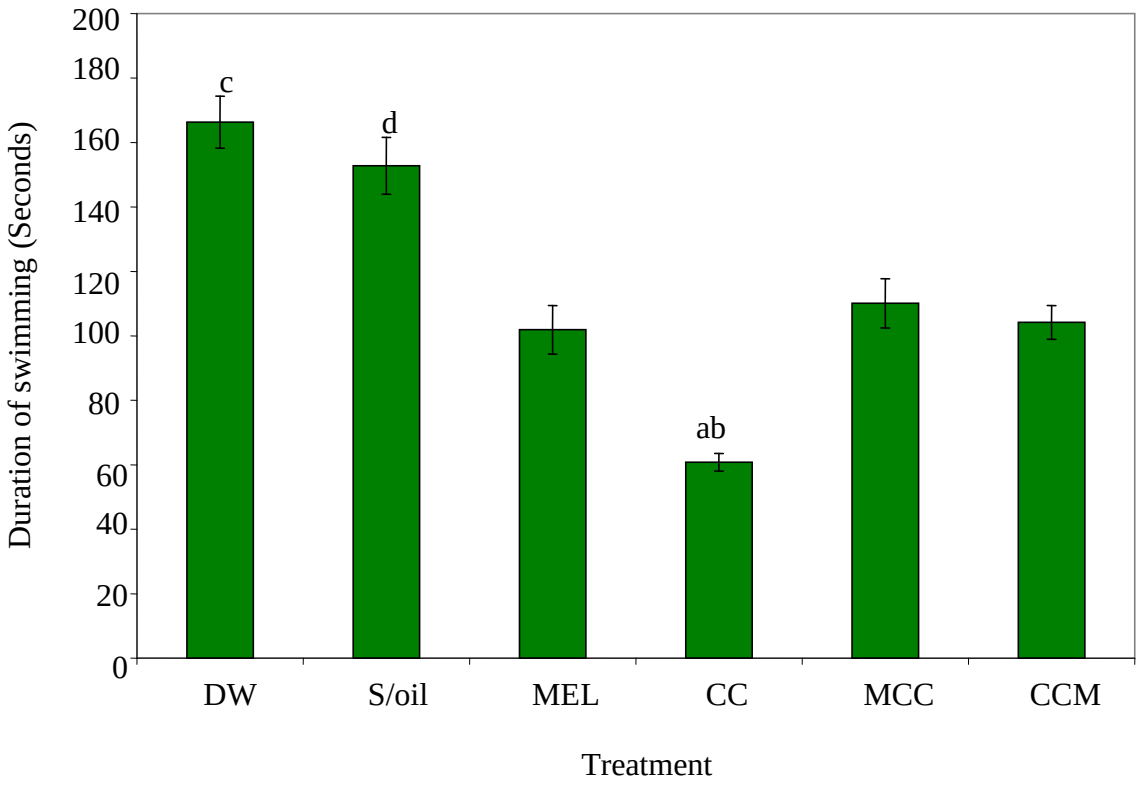


Figure 4.19: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on forced swimming test. $^{a}P < 0.001$ versus DW, S/oil, MCC and CCM groups, respectively; $^{b}P < 0.01$ versus MEL,; $^{c}P < 0.001$ versus MEL, MCC and CCM groups, respectively group and $^{d}P < 0.05$ versus S/oil, MCC and CCM groups, respectively.

4.6 Effect of Treatment on Cognition

4.6.1 Effect of treatment on learning acquisition in F1 generation male Wistar rats

There was a significant increase in the number of foot shocks applied to the CC group compared to that of the DW ($P < 0.001$), S/oil ($P < 0.001$) and MEL ($P < 0.001$), MCC ($P < 0.01$) and CCM ($P < 0.05$) groups, respectively. There was no significant ($P > 0.05$) change in the number of foot-shocks applied to the MCC group (30%), when compared to that of CCM group (Figure 4.20).

4.6.2 Effect of treatment on short-term memory in F1 generation male Wistar rats

The effect of treatment on short-term memory is shown in Figure 4.21. There was a significant decline ($P < 0.001$) in the latency on the platform by the CC group compared to that of the DW, S/oil, MEL, MCC and CCM groups, respectively. There was no significant ($P > 0.05$) change in the latency time on the platform by the MCC group (2%) when compared to that of CCM group.

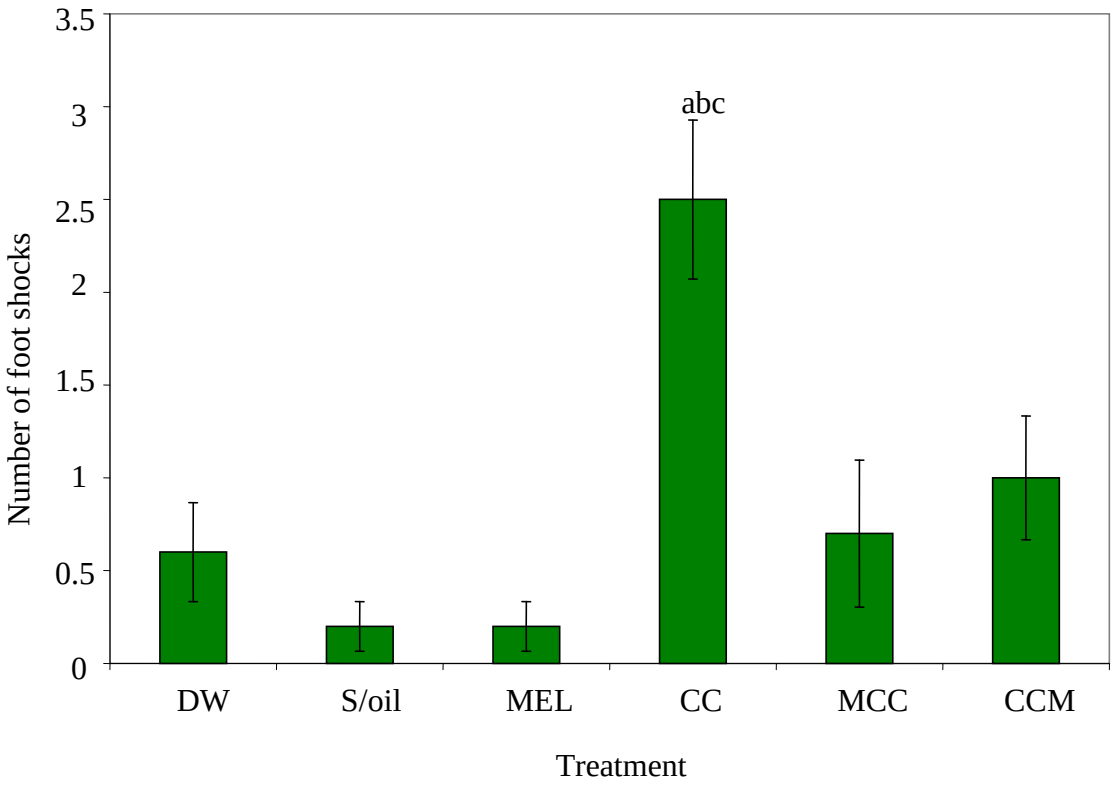


Figure 4.20: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on learning acquisition. $^{a}P < 0.001$ versus DW, S/oil and MEL group, respectively; $^{b}P < 0.01$ versus MCC group and $^{c}P < 0.0$ versus CCM group.

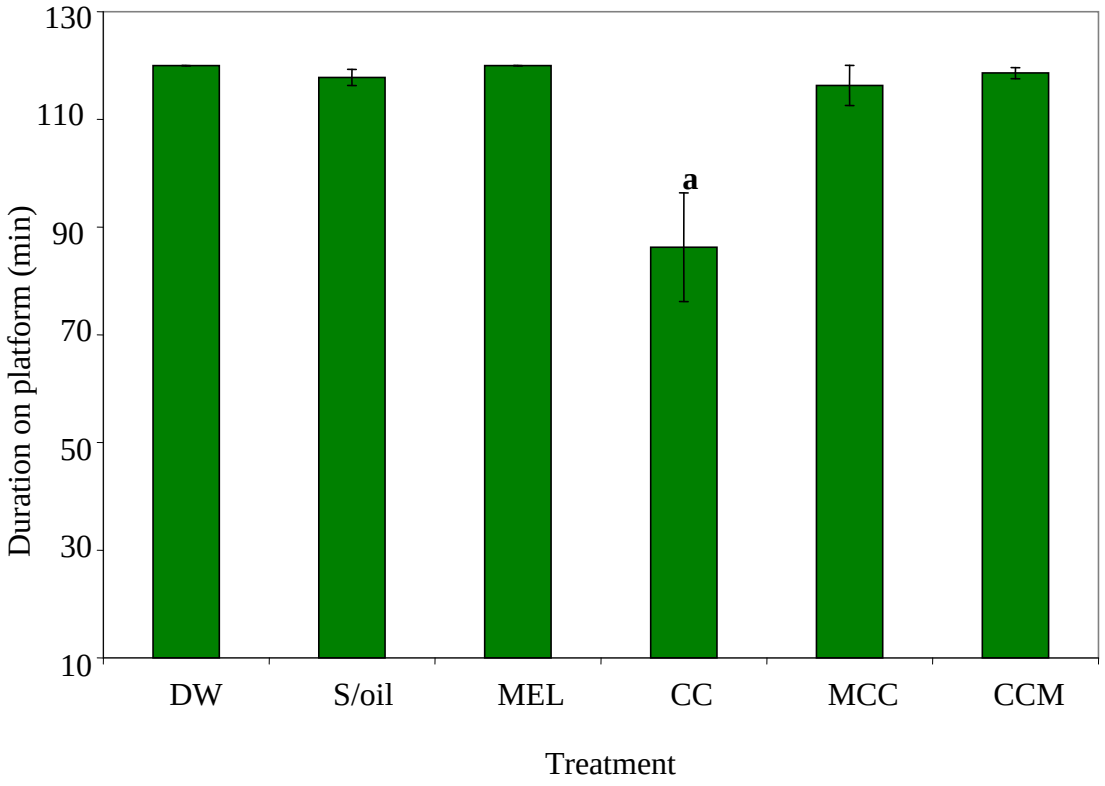


Figure 4.21: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on short-term memory. $^{a}P < 0.001$ versus DW, S/oil, MEL, MCC and CCM groups.

4.7 Effects of Treatment on Reproductive Toxicity End-point

4.7.1 Effect of treatment on gestational length, sex ratio and mating, viability, gestation (pregnancy), fertility and live birth indices.

4.7.1.1 Effect on gestation length

There was a significant reduction in mean gestational length in the CC group relative to that of the DW ($P < 0.01$), S/oil ($P < 0.001$), MEL ($P < 0.01$) and MCC ($P < 0.001$) groups, respectively. There was a significant ($P < 0.001$) increase in the gestation length in the S/oil group when compared to CCM group. The mean gestation length in the CCM group decreased significantly ($P < 0.001$) relative to that of the MCC group (Table 4.5).

4.7.1.2 Effect on mating index

There was no significant ($P > 0.05$) change in the mating index in between the groups. However, the mating index in the CC group decreased by 15%, 18%, 18%, 17% and 18%, when respectively compared to that of the DW, S/oil, MEL, MCC and CCM groups (Table 4.5).

4.7.1.3 Effect on sex ratio

There was no significant ($P > 0.05$) change in the sex ratio in between the groups. The mean male sex ratio in the CC group decreases by 15%, 18%, 18%, 17% and 18% when respectively compared to DW, S/oil, MEL, MCC and CCM groups (Table 4.5).

4.7.1.4 Effect on viability index

There was a decrease in the viability index in the CC group, compared to that of the DW ($P < 0.01$), S/oil ($P < 0.001$), MEL ($P < 0.01$), MCC ($P < 0.01$) and CCM ($P < 0.01$) treated groups (Table 4.5).

4.7.1.5 Effect on gestation (pregnancy) index

Although there was no significant changes ($P > 0.05$) in between the groups. The gestation (pregnancy) index in the CC group was relatively lower, when compared to that of DW (15%), S/oil (19%), MEL (19%), MCC (15%) and CCM (17%) treated groups (Table 4.5).

4.7.1.6 Effect on fertility index

There were no significant differences ($P > 0.05$) in the fertility index in between the groups. However, the mean fetility index in the CC group decreased relative to that of the DW (19%), S/oil (21%), MEL (21%) and CCM (19%) treated groups (Table 4.5).

4.7.1.7 Effect on live birth index

There was no significant ($P > 0.05$) change in the live birth index in between the groups. The mean live birth index in the CC group decrease marginally by 17%,when respectively compared to DW, S/oil, MEL, MCC and CCM groups (Table 4.5).

Table 4.5: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on reproductive indices.

Parameter	Treatment					
	DW	S/Oil	MEL	CC	MCC	CCM
Gestational length (Days)	20.22±0.493	21.33±0.441[c]	20.22±0.2778	18.22±0.3239[ab]	21.33±0.2357[d]	18.89±0.2003
Sex ratio (%)	83.33±20.6	79.81±7.685	72.78±12.98	48.33±9.321	75.56±14.61	57.78±15.19
Mating index (%)	66.67±12.6	80±10.69	80±10.69	60±13.09	73.33±11.82	80±10.69
Viability index (%)	93.22±5.537	98.89±1.111	96.56±2.292	67±10.16 [ab]	94.11±3.914	97.44±1.692
Gestation index (%)	80±10.69	100±0	100±0	73.33±11.82	80±10.69	93.33±6.667
Fertility index (%)	93.33±6.667	100±0	100±0	86.67±9.085	86.67±9.085	93.33±6.667
Live birth index (%)	94.67±2.774	95.11±3.498	93.11±4.569	87.22±5.288	95.56±4.444	95.89±4.111

Gestation length: [a]P < 0.001 versus S/oil, MEL and MCC. [b]P < 0.01 versus DW. [c]P < 0.001 versus CCM. [d]P < 0.001 versus CCM.
Viability index: [a]P < 0.0.01 versus DW, MEL, MCC and CCM. [b]P < 0.001 versus S/oil. Results are presented as mean ± SEM.

4.7.2 Effect of treatment on caudal epididymal sperm count in F1 generation male Wistar rats

Effect of treatment on caudal epididymal sperm concentration is shown in Figure 4.22. The sperm concentration shows a significant ($P < 0.001$) decline in the CC group compared to DW group. Although not significant ($P > 0.05$), the sperm concentration in the CC group decreased compared to S/oil (17%), MEL (14%), MCC (16%) and CCM (14%) groups, respectively. There was a significant increase in the sperm concentration in the DW group relative to that of the S/oil ($P < 0.05$), MEL ($P < 0.01$), MCC ($P < 0.05$) and CCM ($P < 0.01$) treated groups, respectively.

4.7.3 Effect of treatment on active progressive motility of sperm in F1 generation male Wistar rats

Effect of treatment on active progressive sperm movements is shown in Figure 4.23. There was a significant ($P < 0.01$) decrease in the number of active progressive sperm movement in the CC group compared to S/oil or MCC group. Although not significant ($P > 0.05$), there was a marginal decrease in the rate of active sperm movement in the CC group when compared to that of the DW (15.2%), MEL (17.6%) and CCM (16%) treated groups, respectively.

4.7.4 Effect of treatment on non-progressive sperm motility in F1 generation male Wistar rats

There was a significant increase in the non-progressive sperm movement in the CC group relative to that of the DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$) and MCC ($P < 0.001$) groups, respectively. There was a significant increase in the non-progressive sperm movement in the CCM group when compared to that of DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$) and MCC ($P < 0.001$) treated groups (Figure 4.24).

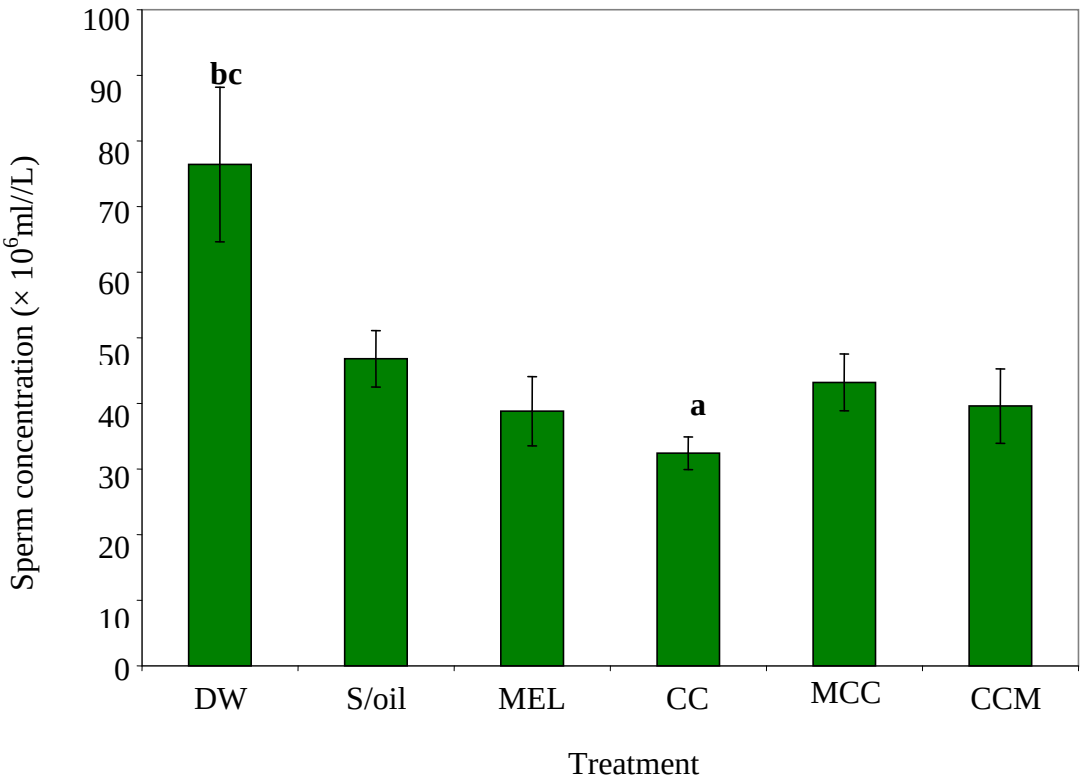


Figure 4.22: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on caudal epididymal sperm concentration. $^{a}P < 0.001$ versus DW group; $^{b}P < 0.05$ versus S/oil and MCC groups, respectively; $^{c}P < 0.01$ versus MEL and CCM treated groups.

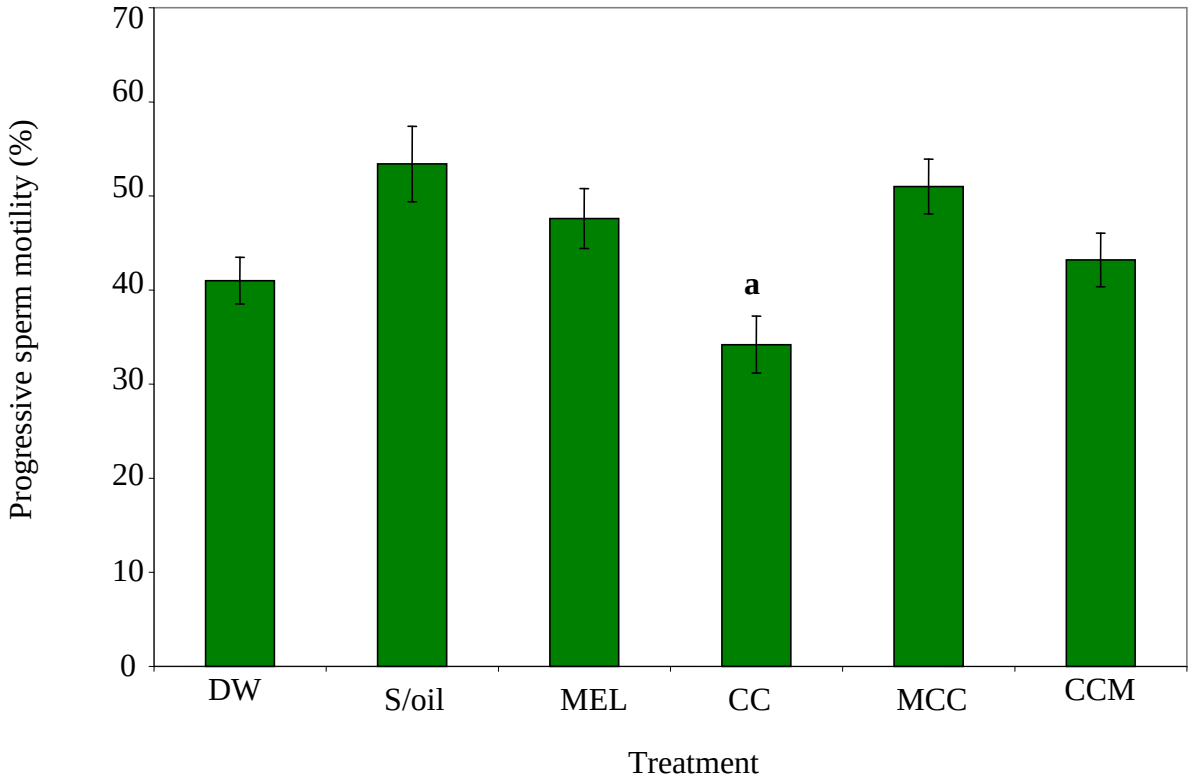


Figure 4.23: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on active progressive sperm motility. $^{a}P < 0.01$ versus S/oil and MCC groups.

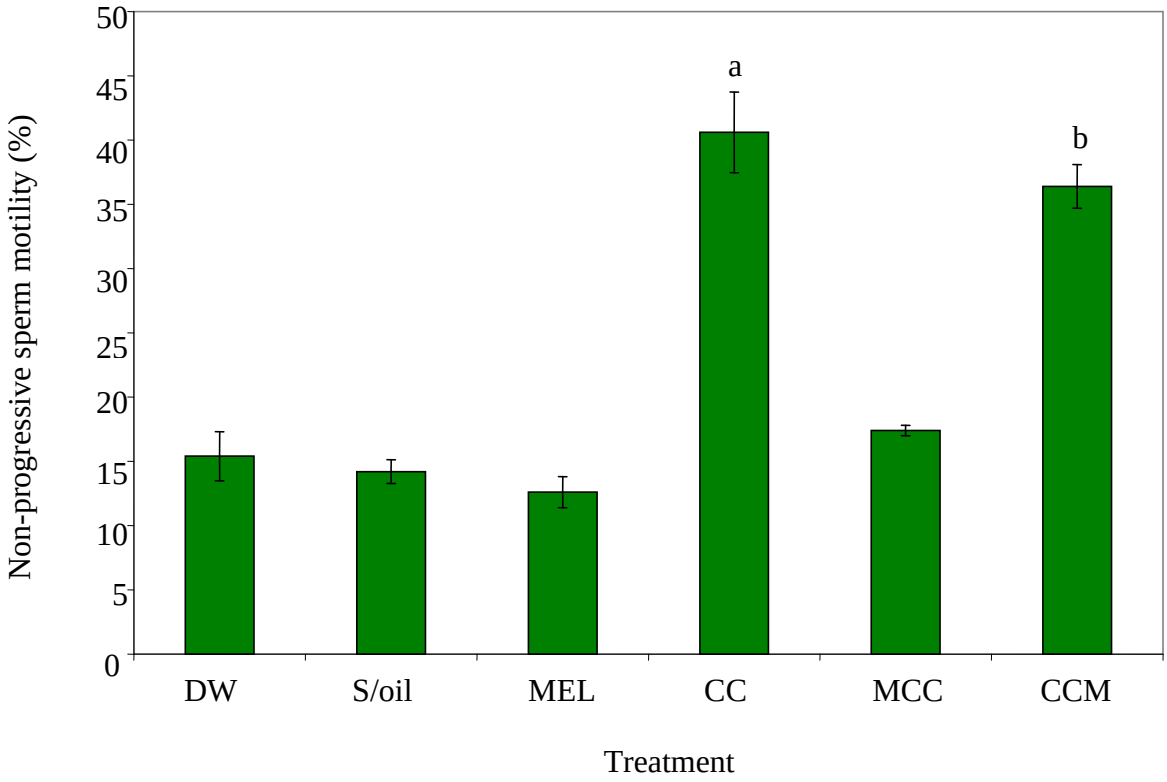


Figure 4.24: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on non-progressive sperm motility. [a]P < 0. 001 versus DW, S/oil, MEL and MCC groups. [b]P < 0.001 versus DW, S/oil, MEL and MCC groups.

4.7.5 Effect of treatment on immotile sperm in F1 generation male Wistar rats

There was a significant ($P < 0.05$) increase in the number of immotile sperm in the CC group compared to the S/oil ($P < 0.05$), MEL ($P < 0.05$) and MCC ($P < 0.01$) groups, respectively. Although not significant ($P > 0.05$), there was a relative increase in the number of immotile sperm in the CC group when compared to that of the DW (18%) and CCM (16%) groups, respectively. There was a relative decrease in the number of immotile sperm in the MCC (10%) compared to CCM (16%) group (Figure 4.25).

4.7.6 Effect of treatment on sperm live/dead ratio in F1 generation male Wistar rats

Figure 4.26 shows the effect of treatment on sperm Live/Dead ratio. There was a significant decrease in the sperm live/dead ratio in the CC compared to that of MCC ($P < 0.01$) and CCM groups, respectively. There was a non-significant ($P > 0.05$) decrease in the sperm live/dead ratio of CC group, compared to that of DW (16%), S/oil (16%) and MEL (17%) treated groups.

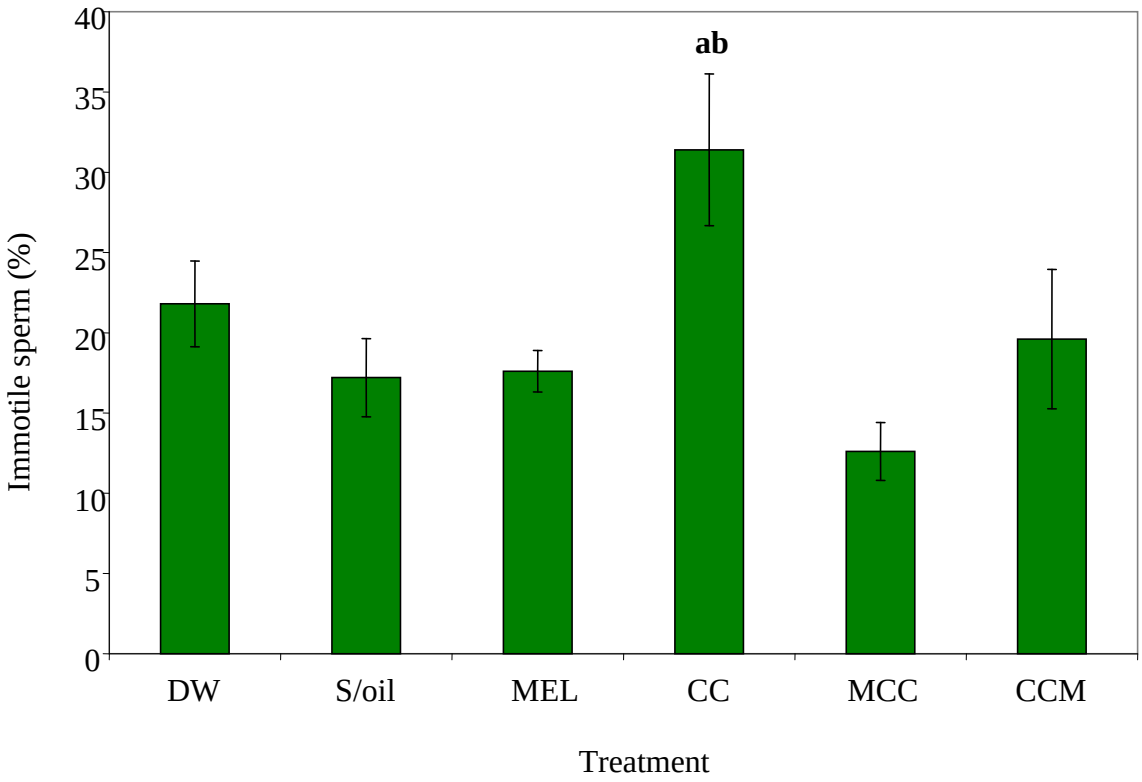


Figure 4.25: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on immotile sperm. $^{a}P < 0.05$ versus DW and MEL groups, respectively. $^{b}P < 0.01$ versus MCC group.

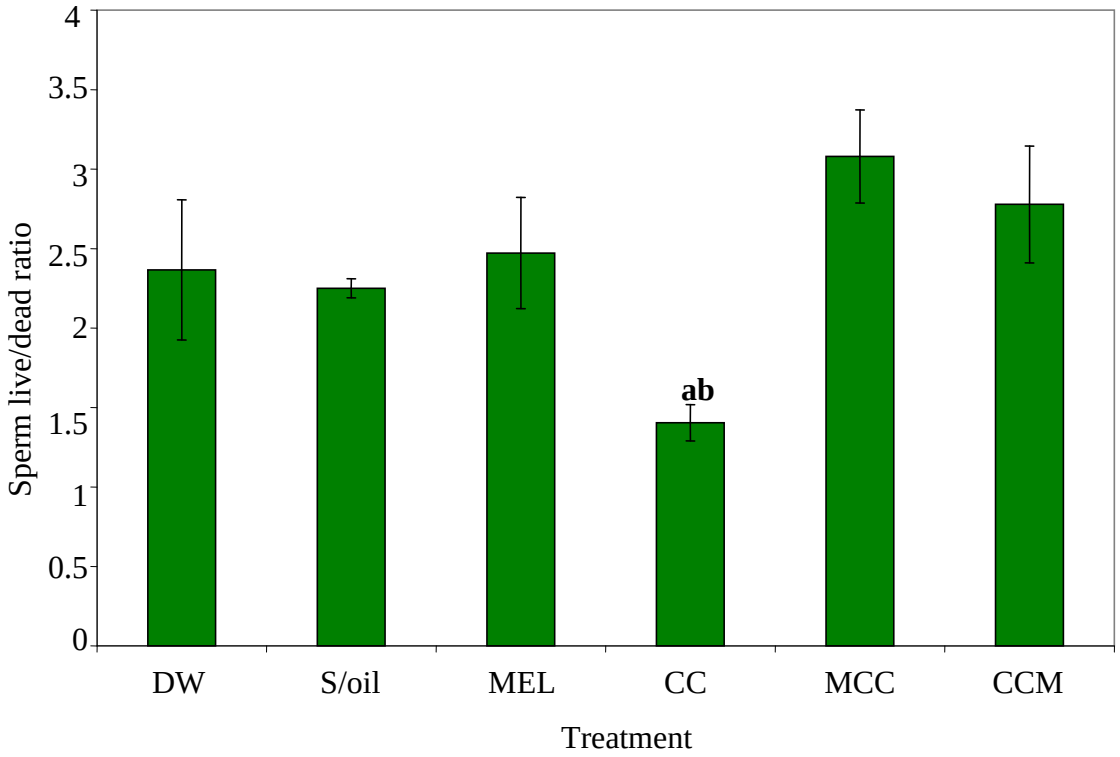


Figure 4.26: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on sperm live/dead ratio. $^{a}P < 0.01$ versus MCC group; $^{b}P < 0.05$ versus CCM group.

4.7.7 Effect of Treatment on Sperm Morphology

4.7.7.1 Effect of treatment on normal sperm cells in F1 generation male Wistar rats

Figure 4.27 shows the effect of treatment on normal sperm cells. There was a significant ($P < 0.001$) decrease in the normal sperm cells in the CC group when compared to that of DW, S/oil, MEL, MCC and CCM groups, respectively. There was a significant decrease in the number of normal sperm cells in the CCM group, compared to that of the DW ($P < 0.001$), S/oil ($P < 0.01$) and MEL ($P < 0.05$) groups, respectively. There was no significant change ($P > 0.05$) in the number of normal sperm cells in the MCC compared to the CCM treated groups.

4.7.7.2 Effect of treatment on sperm head morphology in F1 generation male Wistar rats

Figure 4.28 shows the effect of treatment on sperm head morphology. There was a significant ($P < 0.01$) increase in the number of sperm head defect in the CC group compared to that of the DW, S/oil, MEL and MCC groups, respectively. Although not significant ($P > 0.05$), there was a 20% increase in the number of sperm head defect in the CC group when compared to that of the CCM group. There was a relative increase (26%) in the number of sperm head defect in the CCM compared to MCC group.

4.7.7.3 Effect of treatment on sperm mid-piece defect in F1 generation male Wistar rats

Figure 4.29 shows the effect of treatment on sperm midpiece defect. There were no significant ($P > 0.05$) changes in between the groups. However, there was a relative increase in the number of sperm midpiece defect in the CC group, compared to the DW (14%), S/oil (14%), MEL (10%), MCC (14%) and CCM (24%) treated groups.

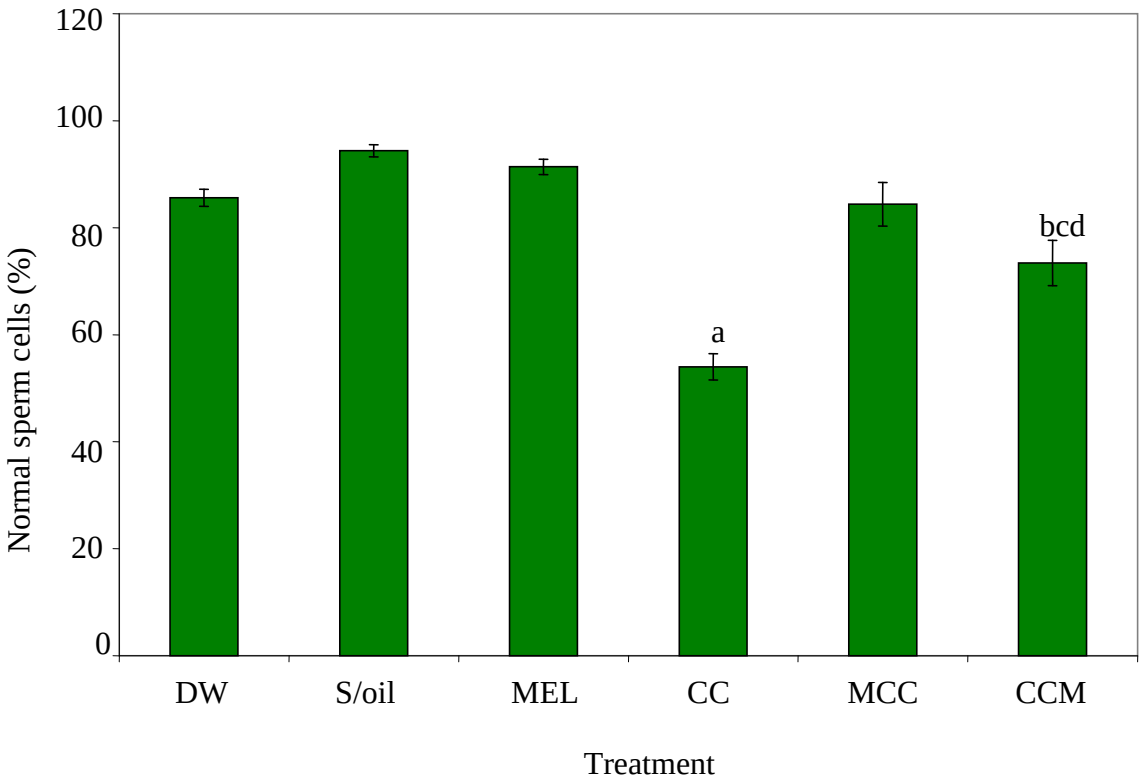


Figure 4.27: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on normal sperm cells. $^{a}P < 0.001$ versus DW, S/oil, MEL, MCC and CCM treated groups, $^{b}P < 0.05$ versus DW group; $^{c}P < 0.001$ versus S/oil group and $^{d}P < 0.01$ versus MEL group.

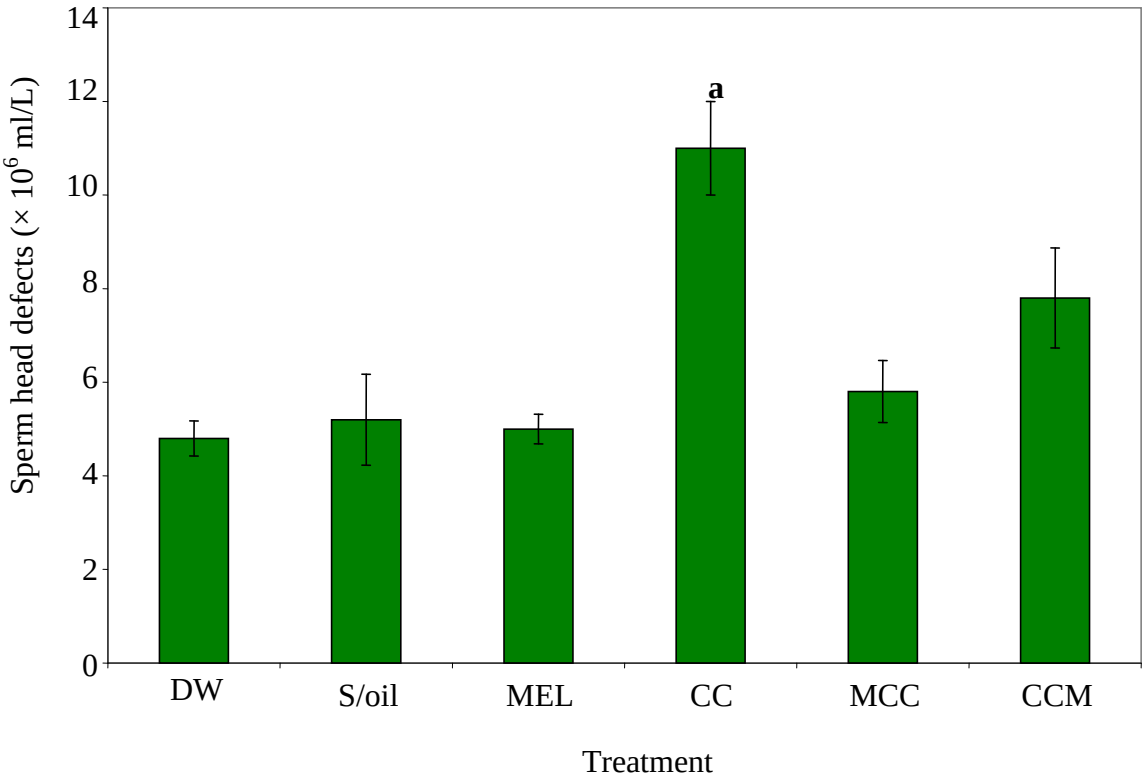


Figure 4.28: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on sperm head defects. $^{a}P < 0.01$ versus DW, S/oil, MEL and MCC treated groups.

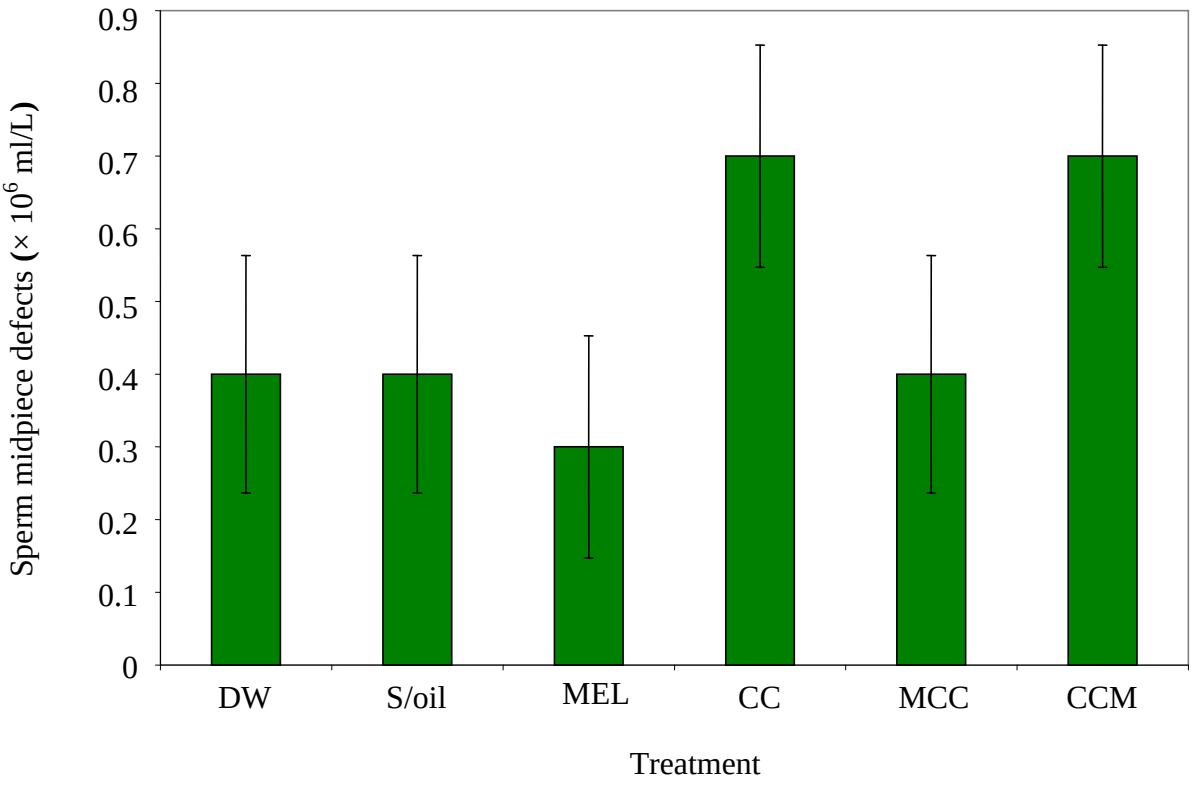


Figure 4.29: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on sperm mid-piece defects.

4.7.7.4 Effect of treatment on sperm tail defect in F1 generation male Wistar rats

Figure 4.30 shows the effect of treatment on sperm tail defect. There was a significant ($P < 0.05$) increase in the sperm tail defect in the CC group, when compared to that of the MEL group. There was no significant ($P > 0.05$) change in the tail defects in the CC group relative to the other groups. However, there was a relative increase in the number of sperm tail defect in the CC group compared to that of the DW (12%), S/oil (12%), MCC (15%) and CCM (19%) treated groups.

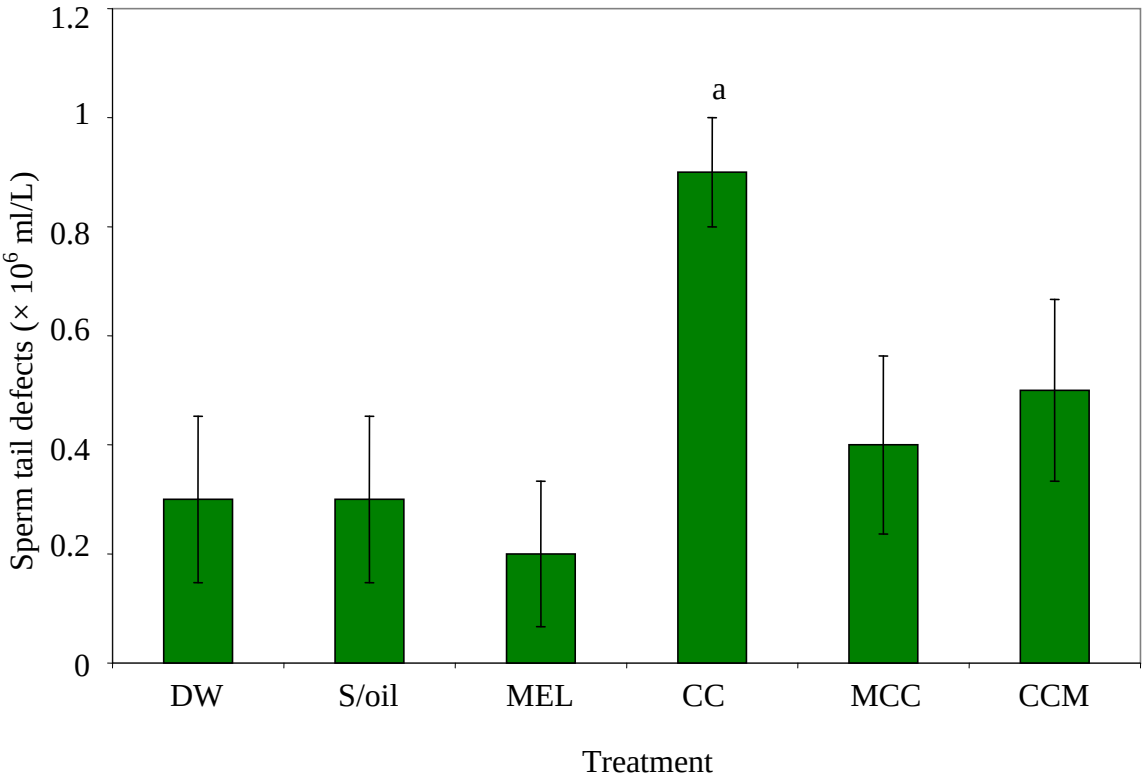


Figure 4.30: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on sperm tail defects. $^{a}P < 0.05$ versus MEL group.

4.8 Effect of Treatment on Sex Hormone Profiles

4.8.1 Effect of treatment on follicle-stimulating hormone concentration

Figure 4.31 shows the effect of treatment on FSH concentration. There was a significant decrease ($P < 0.05$) in the FSH concentration in the CC group compared to that of the S/oil group. However, the CC group showed a relatively but non-significant decrease ($P > 0.05$) in the FSH concentration compared to that of DW (17.6%), MEL (16.8%), MCC (16%) and CCM (16%) treated groups.

4.8.2 Effect of treatment on luteinizing hormone concentration

The effect of treatment on serum LH concentration is shown in Figure 4.32. The LH concentration in the CC group was significantly lower ($P < 0.01$) compared to that of the S/oil group. Similarly, the LH concentration in the DW group was significantly ($P < 0.05$) lower compared to that of S/oil group. In addition, the LH concentration in the S/oil group was higher, compared to that of MEL ($P < 0.05$) and MCC ($P < 0.01$) treated groups.

4.8.3 Effect of treatment on testosterone concentration

Figure 4.33 shows the effect of treatment on serum testosterone concentration. There was no significant change in the testosterone concentration in between the groups. However, the CC group showed a relatively lower testosterone concentration compared to any of the other groups. Testosterone concentration in CC group decreased relative to that of the DW (20%), S/oil (19%), MEL (16.70%), MCC (17%) and CCM (15%) treated groups.

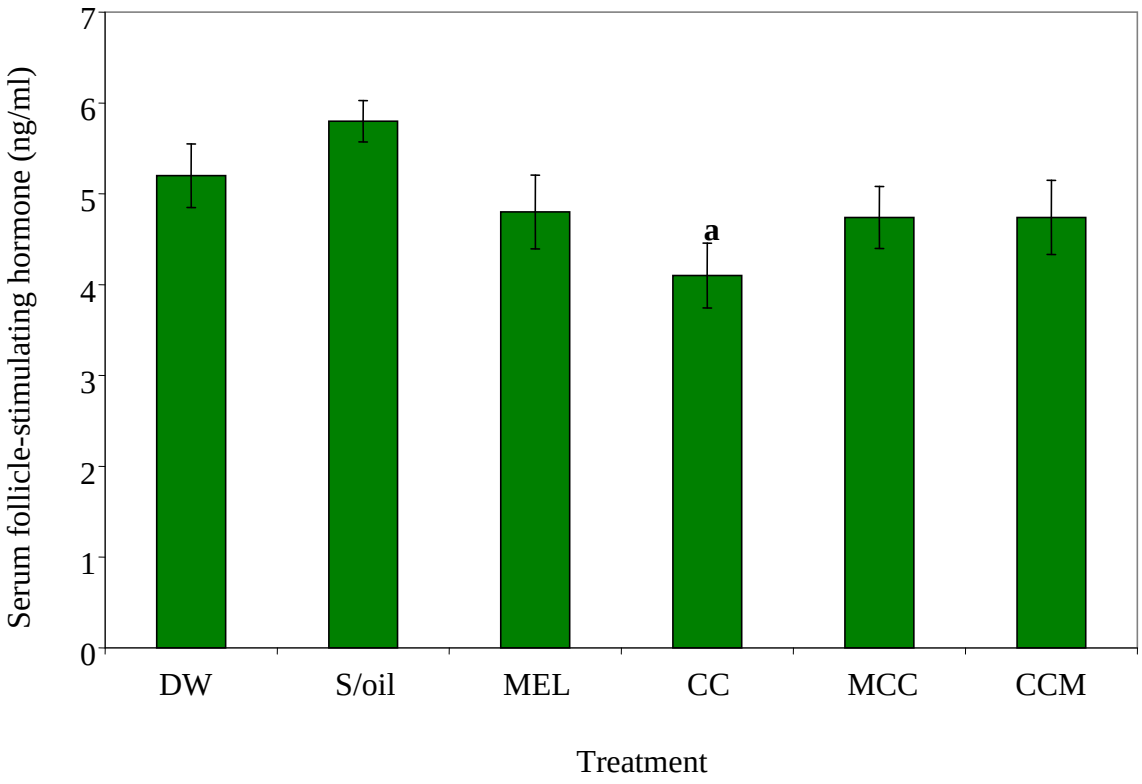


Figure 4.31: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on serum FSH concentration. $^{a}P < 0.05$ versus S/oil group.

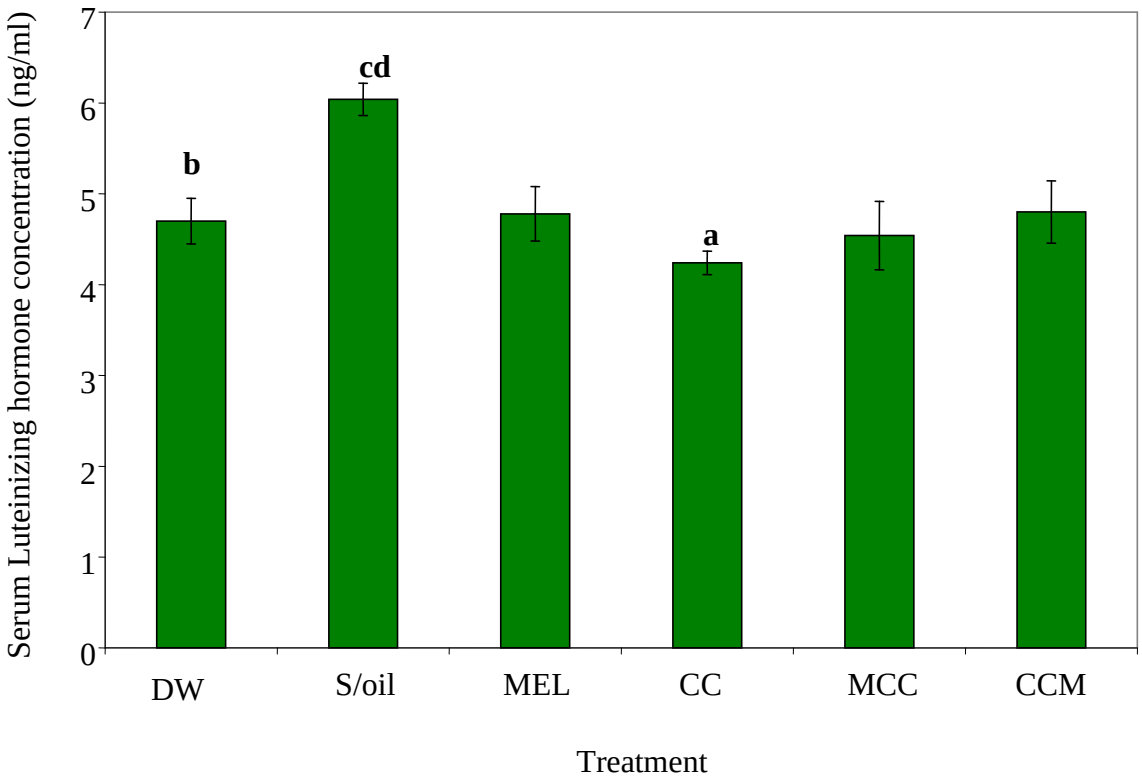


Figure 4.32: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on serum LH concentration. $^{a}P < 0.01$ versus S/oil group; $^{b}P < 0.05$ versus S/oil group; $^{c}P < 0.05$ versus MEL group; $^{d}P < 0.05$ versus MCC group.

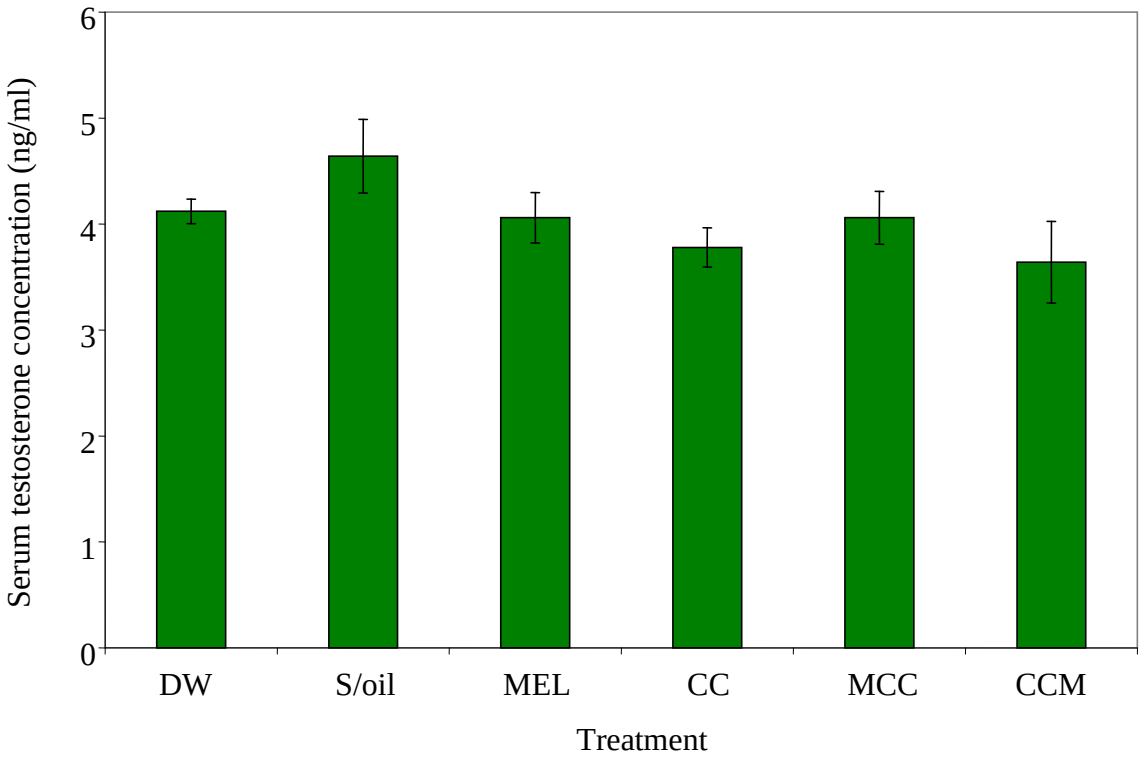


Figure 4.33: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on serum testosterone concentration.

4.8.4 Effect of treatment on serum triiodothyronine concentration

There was a significant decrease in the serum T_3 concentration in the CC group when compared to that of DW ($P < 0.01$), S/oil ($P < 0.05$), MEL ($P < 0.001$) and MCC ($P < 0.05$) groups, respectively. In addition, there was a significant increase ($P < 0.01$) in the serum T_3 concentration in the MEL relative to that of CCM group. Although not significant ($P > 0.05$), serum T_3 concentration in the MCC group was relatively higher (33%) compared to that of CCM group (Figure 4.34).

4.8.5 Effect of treatment on serum thyroxine concentration

Figure 4.35 shows the effects of treatment on serum thyroxine concentration. There was a significant decrease in the serum T_4 concentration in the CC group compared to that of MEL ($P < 0.01$) and MCC ($P < 0.05$) groups, respectively. In addition, there was a significant ($P < 0.01$) increase in the serum T_4 concentration in the MEL group when compared to that of the CCM group. However, the CC group showed a non-significant decrease ($P > 0.05$) in serum T_4 concentration compared to that of DW (17%), S/oil (18%) and CCM (13%) treated groups, respectively. Although not significant ($P > 0.05$), there was an increase of 32% in the serum T_4 concentration in the MCC group relative to that of CCM groups.

4.8.6 Effect of treatment on thyroid-stimulating hormone concentration

Serum TSH concentration in the CC group rose, when compared to that of DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$) and MCC ($P < 0.01$) groups, respectively. In addition, serum TSH concentration in the MEL was higher ($P < 0.01$) than that of CCM group. Although not significant ($P > 0.05$), serum TSH concentration in the CCM group was higher (25%) relative to that of the CCM group (Figure 4.36).

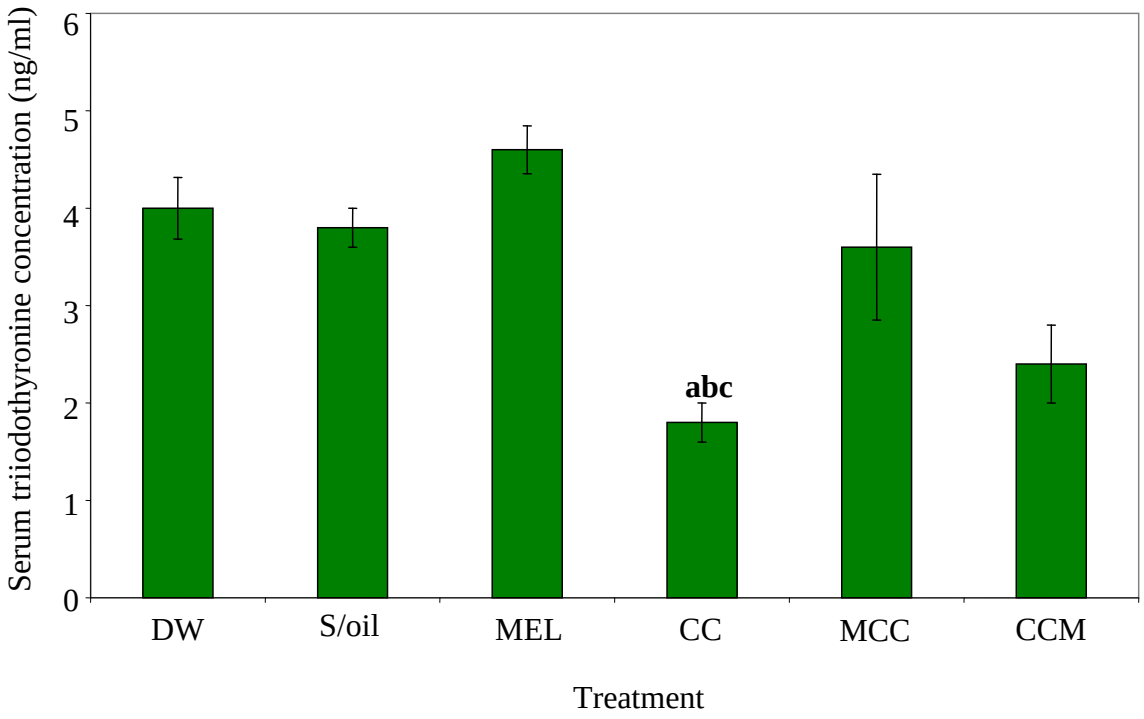


Figure 4.34: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on serum triiodothyronine concentration. $^{a}P < 0.01$ versus DW and CCM groups, $^{b}P < 0.05$ versus S/oiland MCC groups, $^{c}P < 0.001$ versus MEL treated group.

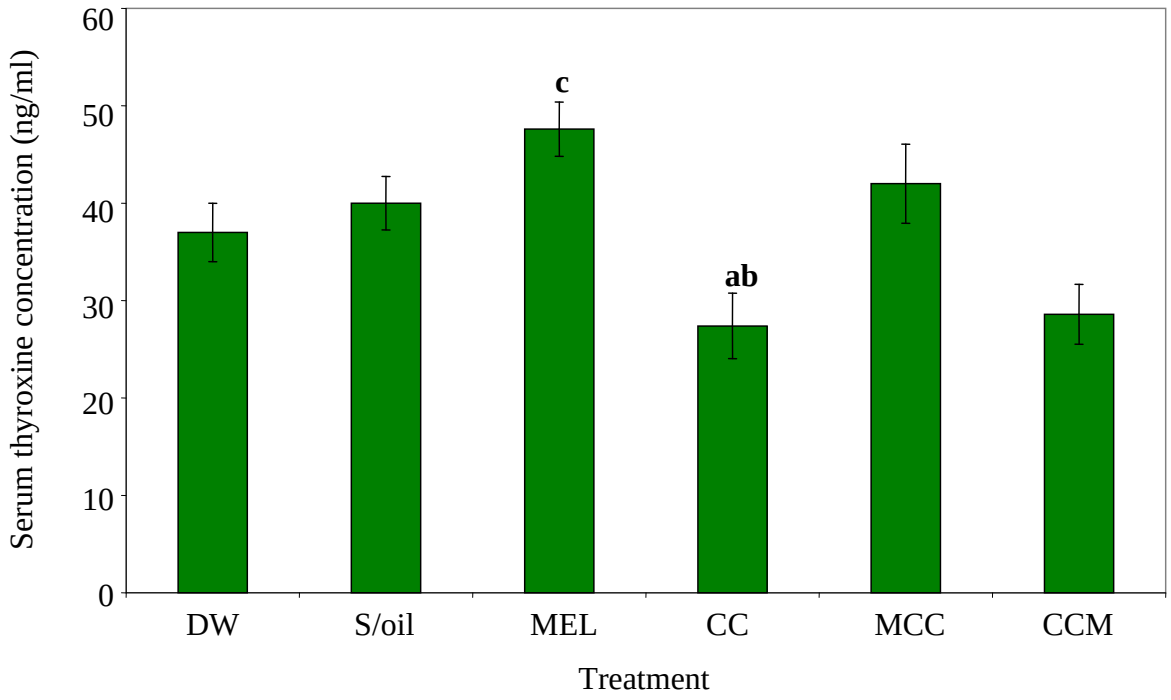


Figure 4.35: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on serum thyroxine concentration. $^{a}P < 0.01$ versus MEL, $^{b}P < 0.05$ versus MCC, $^{c}P < 0.01$ versus CCM treated groups.

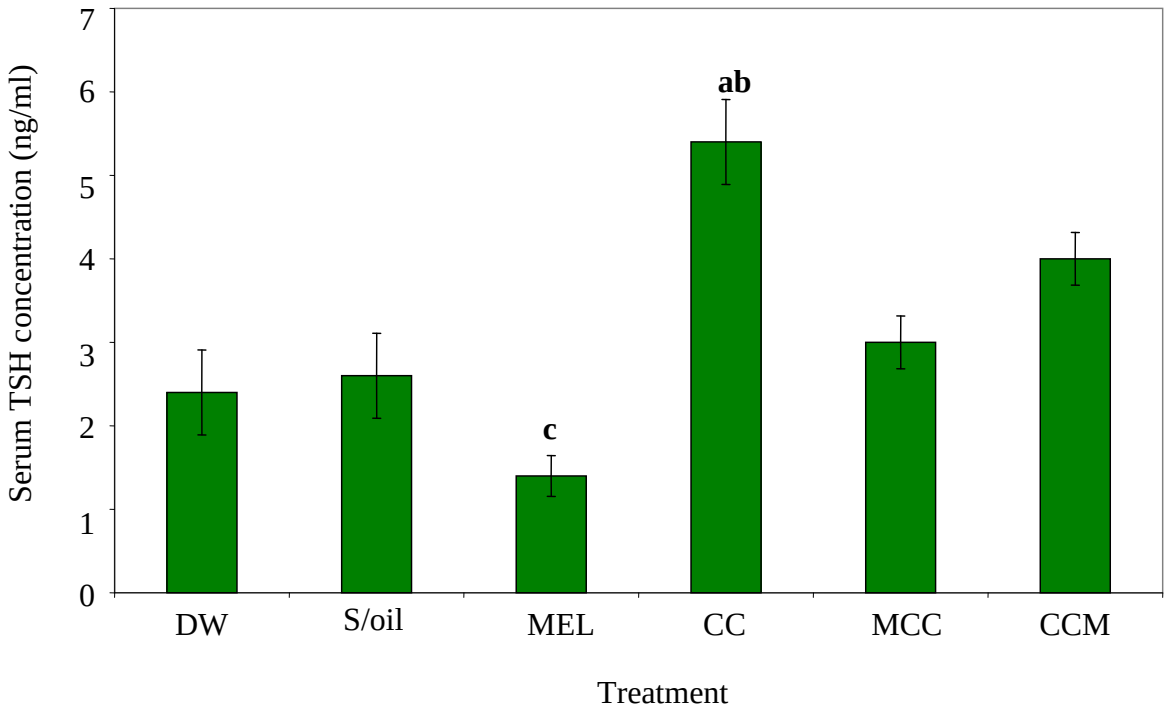


Figure 4.36: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on serum thyroid-stimulating hormone concentration. $^{a}P < 0.001$ versus DW, S/oil and MEL groups, $^{b}P < 0.01$ versus MCC group, $^{c}P < 0.01$versus CCM group.

4.9. Effect of Treatment on Oxidative Stress Biomarkers

4.9.1 Effect of treatment on brain malondialdehyde concentration

The effect of treatment on brain MDA concentration is shown in Figure 4.37. The MDA concentration in the CC group was significantly ($P < 0.05$) higher compared to that of the DW, S/oil, MEL, MCC or CCM group.

4.9.2 Effect of treatment on pituitary gland malondialdehyde concentration

The effect of treatment on pituitary MDA is shown in Figure 4.38. There was a significant ($P < 0.01$) increase in the MDA concentration in the CC group, compared to that of the DW, S/oil, MEL, MCC and CCM groups.

4.9.3 Effect of treatment on thyroid gland malondialdehyde concentration

The effect of treatment on changes in thyroid gland MDA concentration is shown in Figure 4.39. There were no significant changes ($P > 0.05$) in MDA concentrations in between the groups. However, the MDA concentration in the CC group was relatively higher ($P > 0.05$) compared to that of the DW (15%), S/oil (15.3%), MEL (16%), MCC (17%) and CCM (17%) groups.

4.9.4 Effect of treatment on testicular malondialdehyde concentration

The effect of treatment on testicular MDA concentration is shown in Figure 4.40. There was a significant increase in the MDA concentration in the CC group compared to that of the DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$), MCC ($P < 0.01$) and CCM ($P < 0.05$) groups, respectively. there was no significant change ($P > 0.05$) in the testicular MDA concentration in the MCC group when compared to that of CCM group.

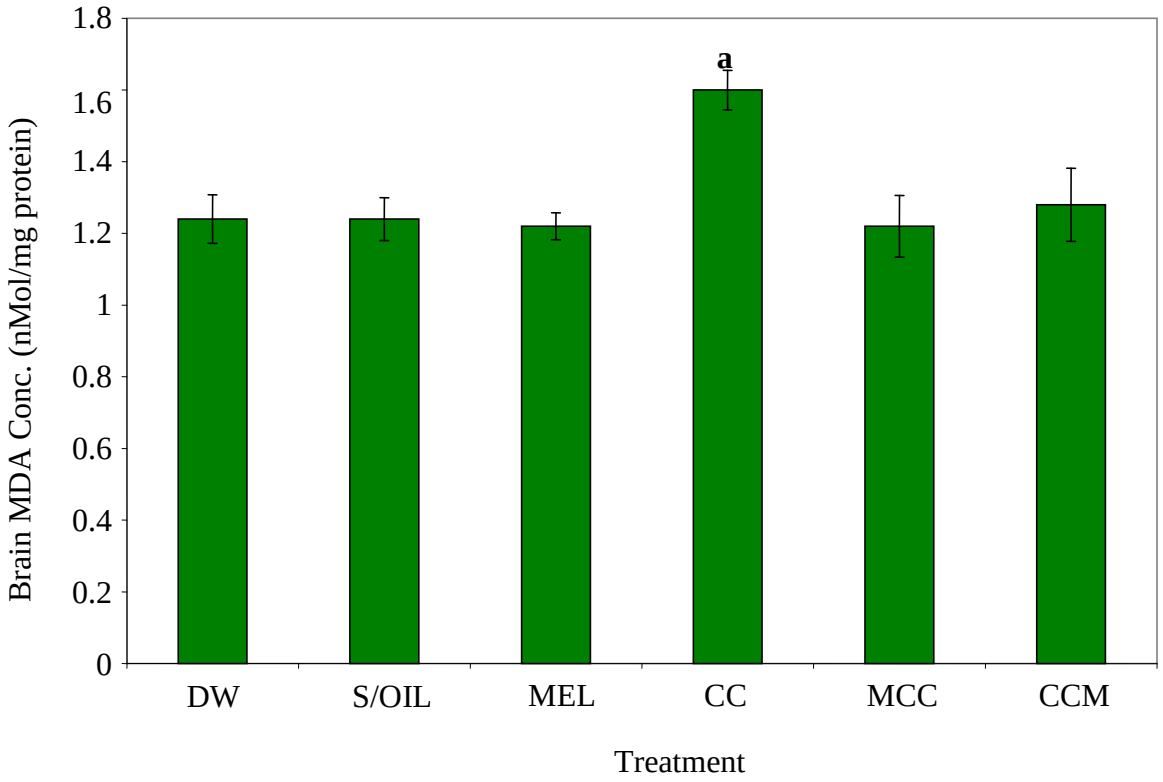


Figure 4.37: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on brain malondialdehyde concentration. $^{a}P < 0.05$ versus DW, S/oil, MEL, MCC and CCM groups.

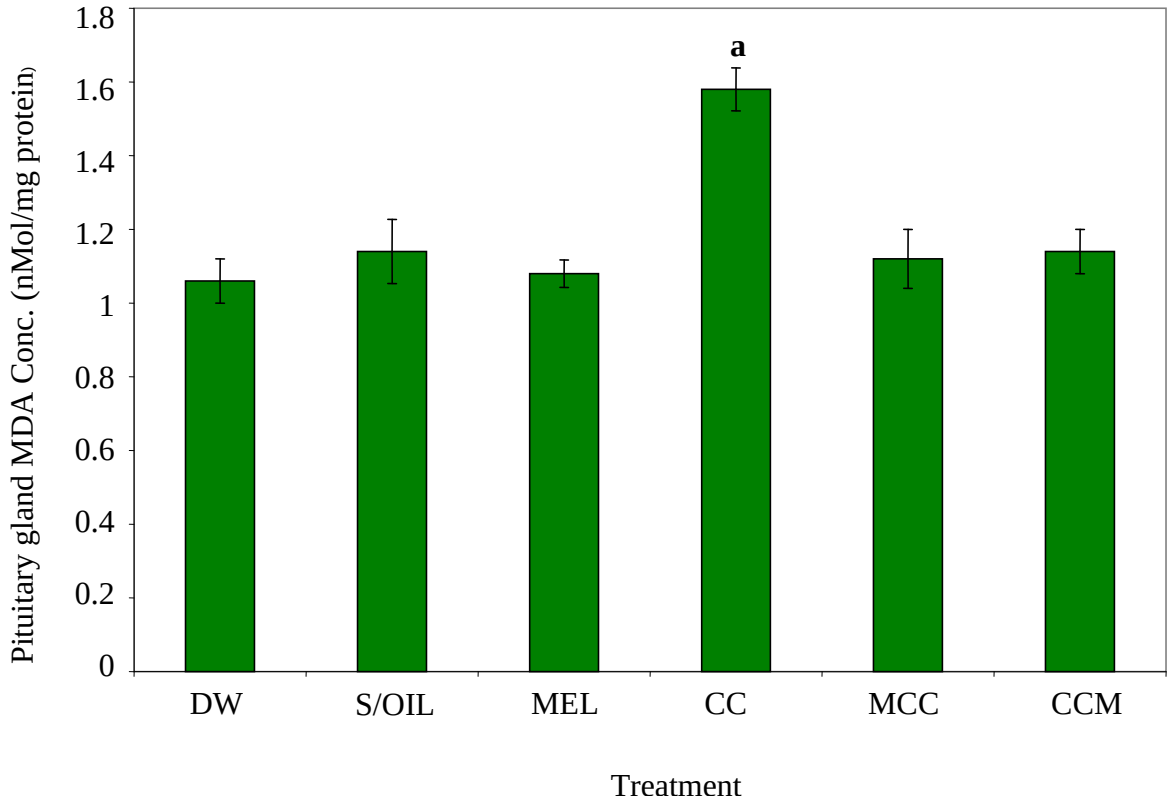


Figure 4.38: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on pituitary glands malondialdehyde concentration. $^{a}P < 0.01$ versus DW, S/oil, MEL, MCC and CCM groups.

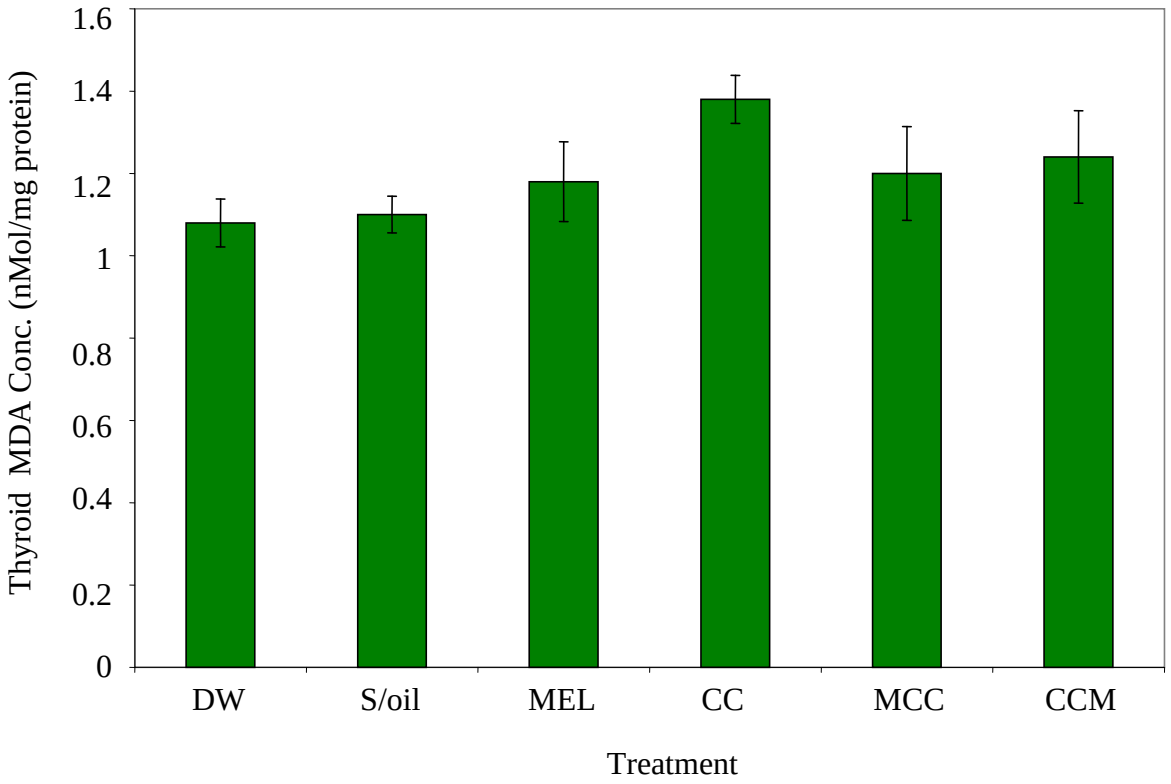


Figure 4.39: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on thyroid gland malondialdehyde concentration.

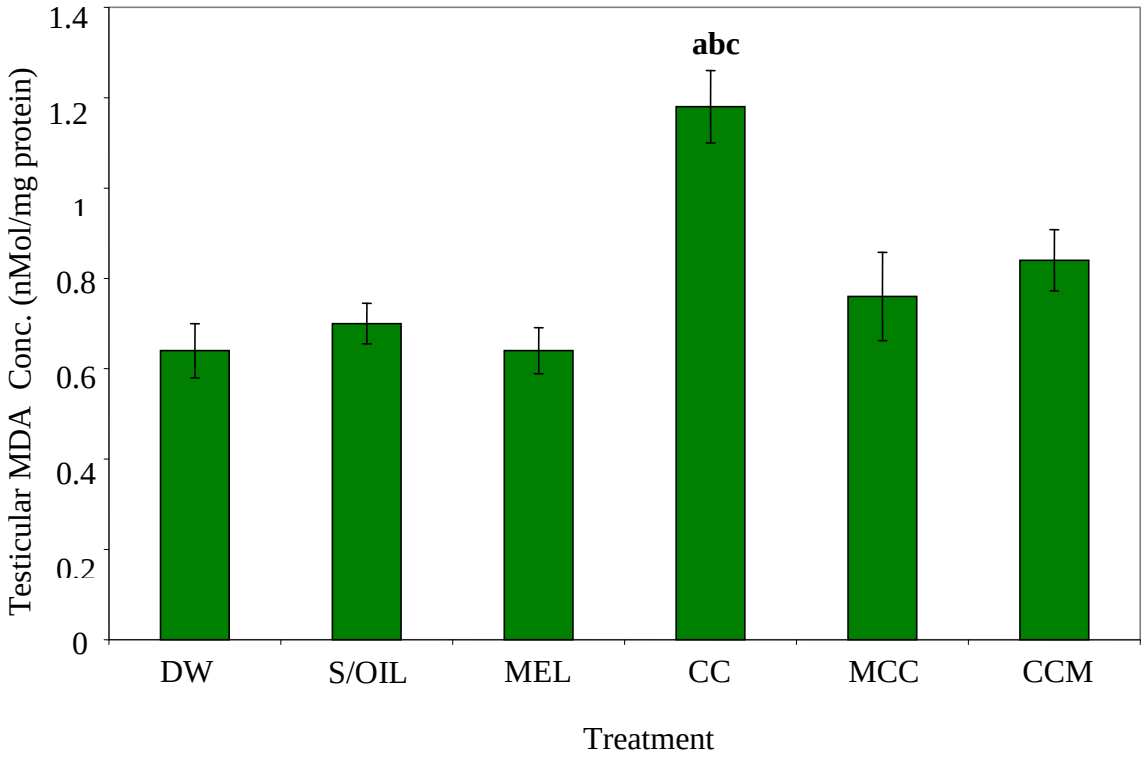


Figure 4.40: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on testicular malondialdehyde concentration. $^{a}P < 0.001$ versus DW, S/oil, MEL groups; $^{b}P < 0.01$ versus MCC; $^{c}P < 0.05$ versus CCM.

4.9.5 Effect of treatment on prostate gland malondialdehyde concentration

The effect of treatment on prostate gland MDA concentration is shown in Figure 4.41. The MDA concentration in the CC group was significantly higher compared to that of the DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$), MCC ($P < 0.001$) and CCM ($P < 0.05$) groups, respectively. There was no significant change ($P > 0.05$) in the prostate MDA concentration in the MCC group, when compared to that of the CCM treated group.

4.9.6 Effect of treatment on epididymal malondialdehyde concentration

The effect of treatment on changes in epididymal MDA concentration is shown in Figure 4.42. There was a significant increase in the epididymal MDA concentration in the CC group relative to that of the DW ($P < 0.01$), MEL ($P < 0.01$), MCC ($P < 0.01$) and S/oil ($P < 0.05$) groups, respectively. Although not significant ($P > 0.05$) however, there was a marginal decrease in the epididymal MDA concentration in the CCM (18%) group when compared to that of the CC group. There was no significant change ($P > 0.05$) in the epididymal MDA concentration in the MCC group when compared to that of the CCM group.

4.9.7 Effect of treatment on seminal vesicle malondialdehyde concentration

The effect of treatment on changes in seminal vesicle MDA concentration is shown in Figure 4.43. There was a significant increase in the seminal vesicle MDA concentration in the CC group when compared to that of the DW ($P < 0.01$), S/oil ($P < 0.05$), MEL ($P < 0.001$) and MCC ($P < 0.05$) groups, respectively. In addition, there was a significant decrease ($P < 0.05$) in the seminal vesicle MDA concentration in the MEL group compared to that of the CCM group. There was no significant change ($P > 0.05$) in the

seminal vesicle MDA concentration in the MCC group when compared to that of the CCM group.

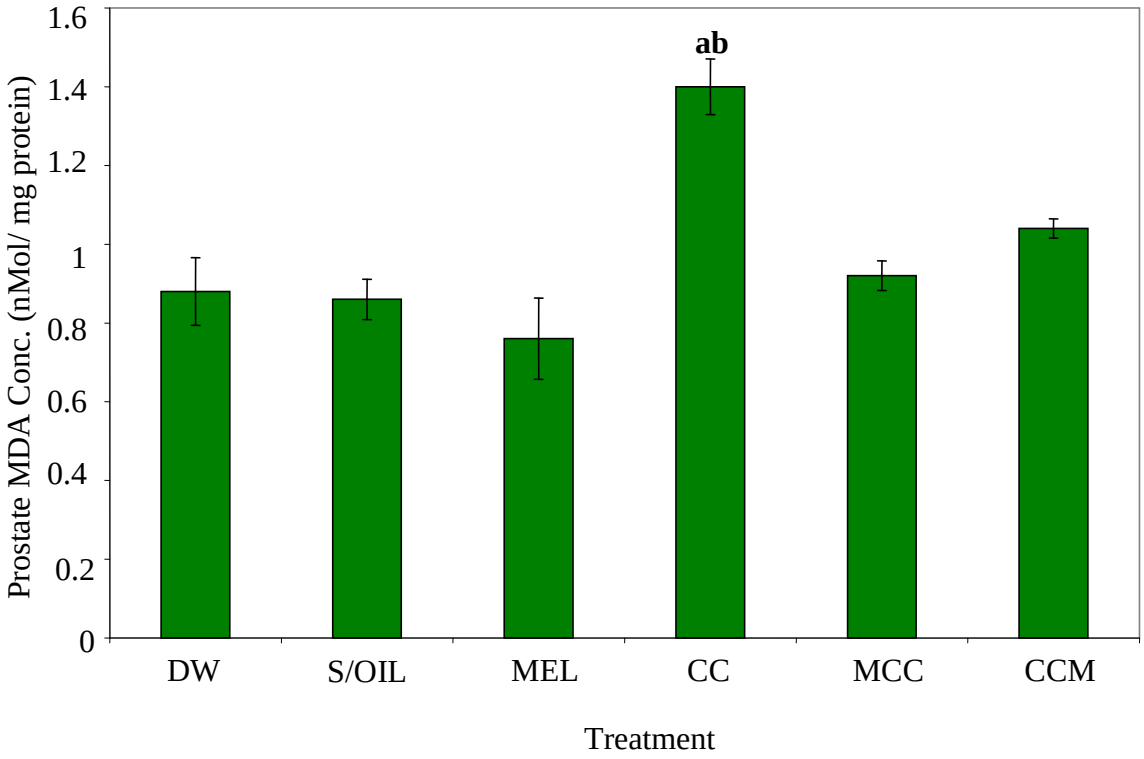


Figure 4.41: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on prostate malondialdehyde concentration. $^{a}P < 0.001$ versus DW, S/oil, MEL and MCC groups, respectively. $^{b}P < 0.05$ versus CCM group.

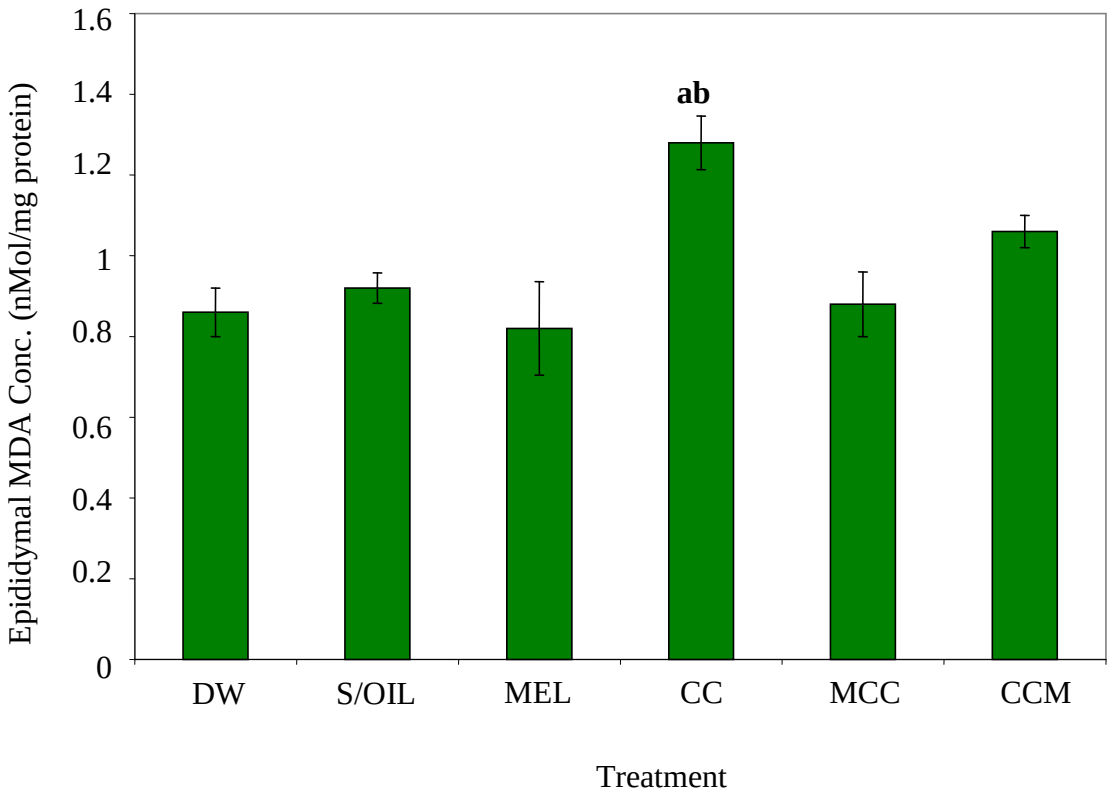


Figure 4.42: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on epididymal malondialdehyde concentration. $^{a}P < 0.01$ versus DW, MEL and MCC groups, $^{b}P < 0.05$ versus S/oil group.

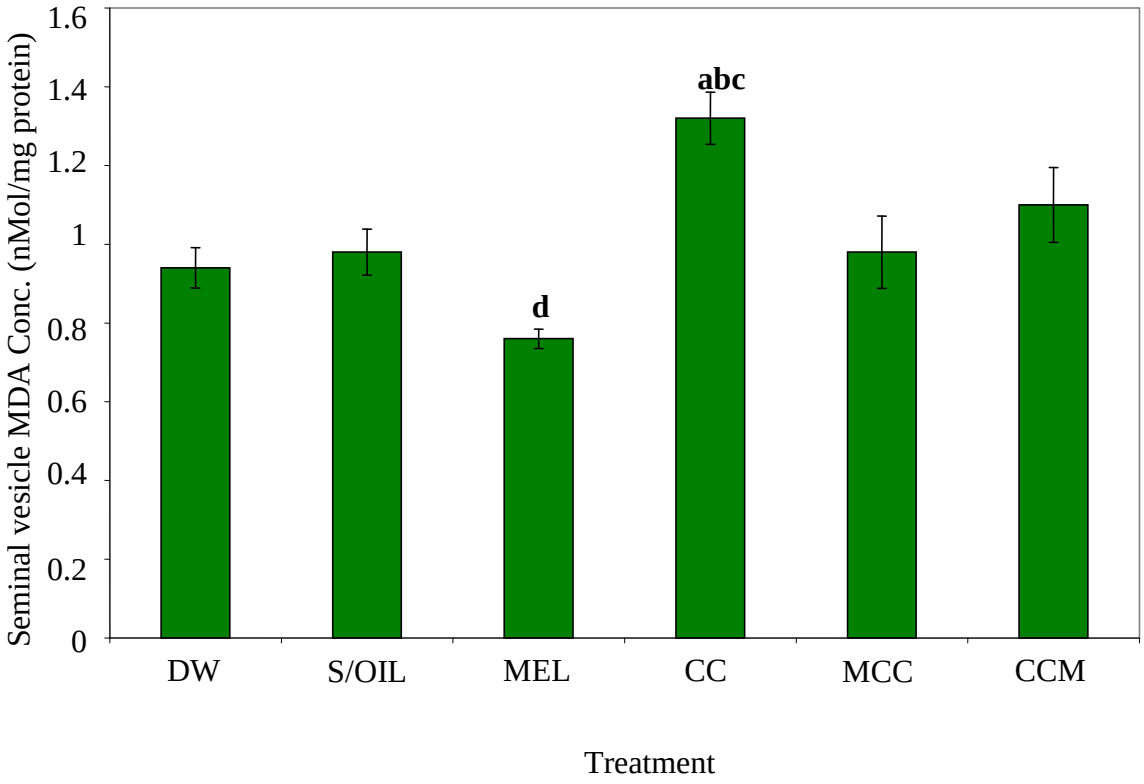


Figure 4.43: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on seminal vesicle malondialdehyde concentration. $^{a}P < 0.01$ versus DW, $^{b}P < 0.001$ versus MEL, $^{c}P < 0.05$ versus S/oil and MCC, $^{d}P < 0.05$ versus CCM groups.

4.9.8 Effect of treatment on brain superoxide dismutase activity

Figure 4.44 illustrates the brain SOD activity. There was a significant increase in the brain SOD activity in the MEL group when compared to that of the CC ($P < 0.05$), DW ($P < 0.01$) and S/oil ($P < 0.01$) groups, respectively. Although not significant ($P > 0.05$), the CC group recorded a relatively lower SOD activity compared to that of the DW (15%), S/oil (13%), MCC (18%) and CCM (18%) treated groups.

4.9.9 Effect of treatment on pituitary gland superoxide dismutase activity

The effect of treatment on pituitary SOD activity is shown in Figure 4.45. There were no significant differences ($P > 0.05$) in SOD activity in between the groups. However, the pituitary SOD activity in the CC group was relatively lower when compared to that of the DW (15%), S/oil (17%), MEL (18%), MCC (18%) and CC+ MEL (17%) groups.

4.9.10 Effect of treatment on thyroid gland superoxide dismutase activity

The effect of treatment on changes in thyroid SOD activity is shown in Figure 4.46. Although not significant ($P > 0.05$), the thyroid SOD activity in the CC group was marginally lower compared to that of the DW (16%), S/oil (17%), MEL (19%), MCC (16%) or CCM (17%) groups.

4.9.11 Effect of treatment on testicular superoxide dismutase activity

The effect of treatment on testicular SOD activity is shown in Figure 4.47. A significant decrease ($P < 0.01$) in the testicular SOD activity was recorded in the CC group when compared to that of the DW group. Although not significant ($P > 0.05$), the SOD activity in the CC group marginally decreased when compared to that of the S/oil (17%), MEL (17%), MCC (16%) and CC+ MEL (17%) groups.

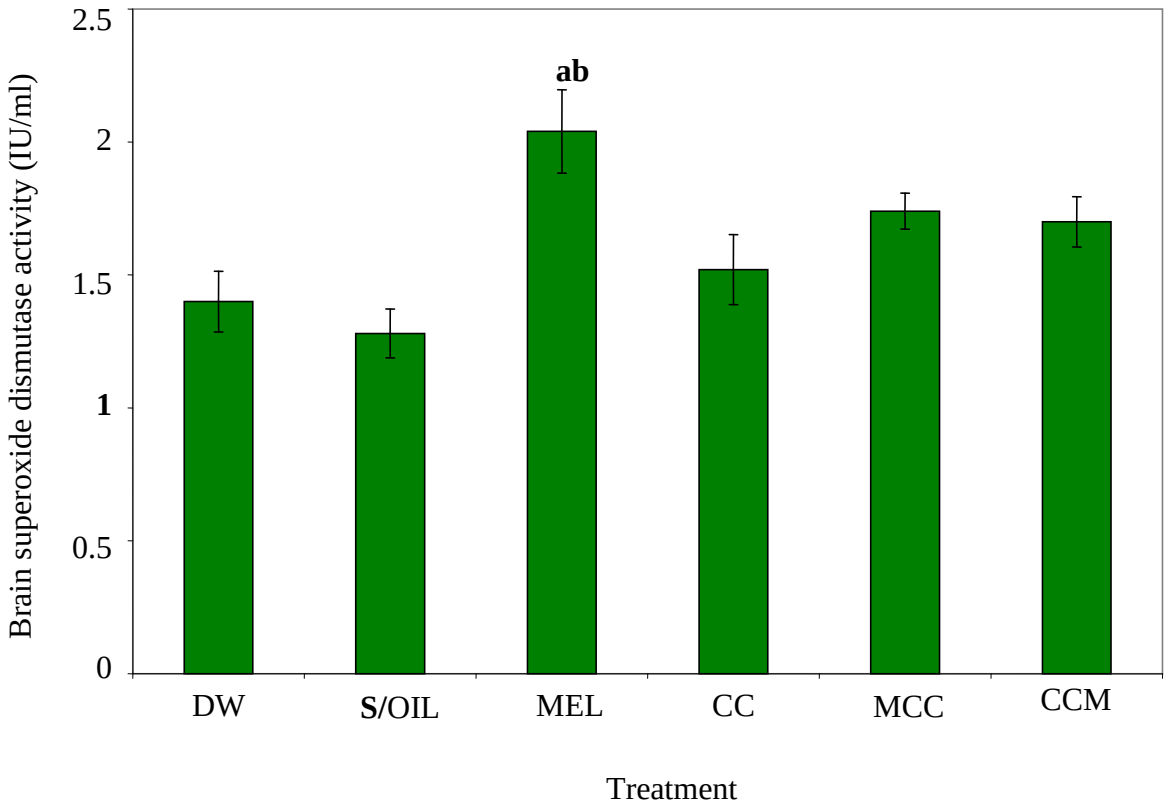


Figure 4.44: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on brain superoxide dismutase activity, $^{a}P < 0.01$ versus DW and S/oil groups, $^{b}P < 0.05$ versus CC group.

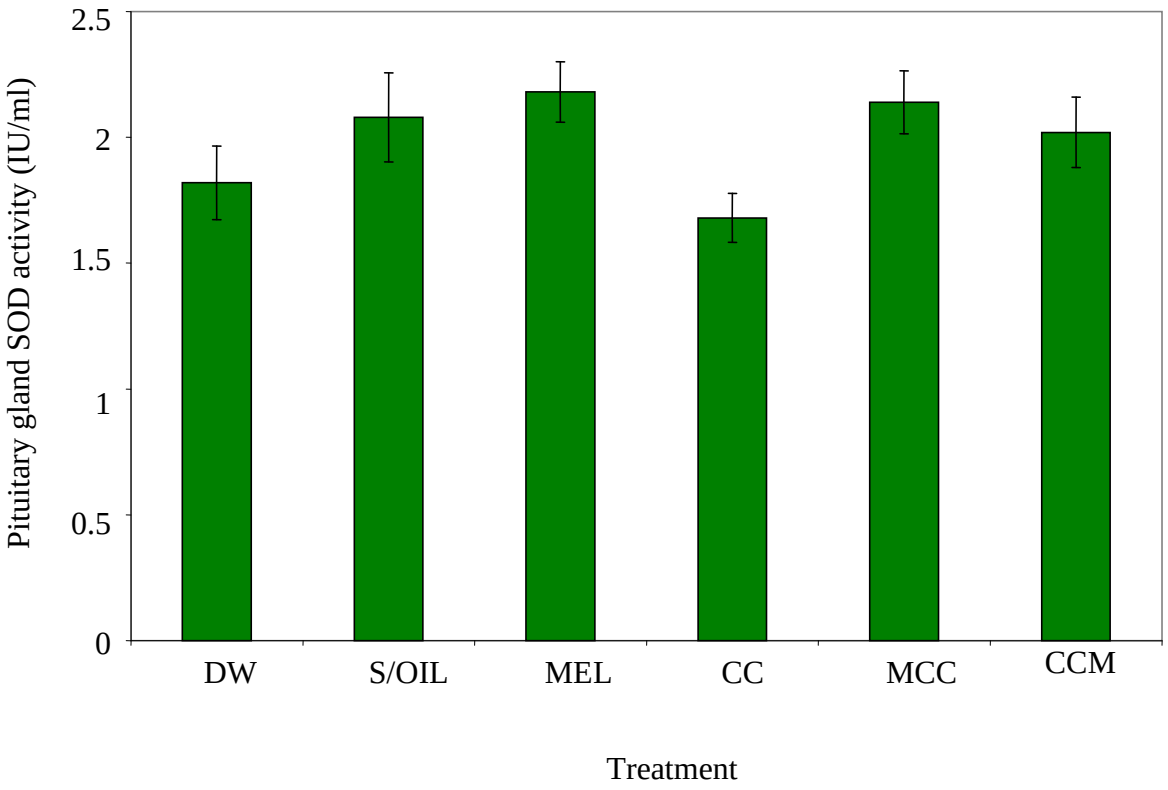


Figure 4.45: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on Pituitary gland SOD activity.

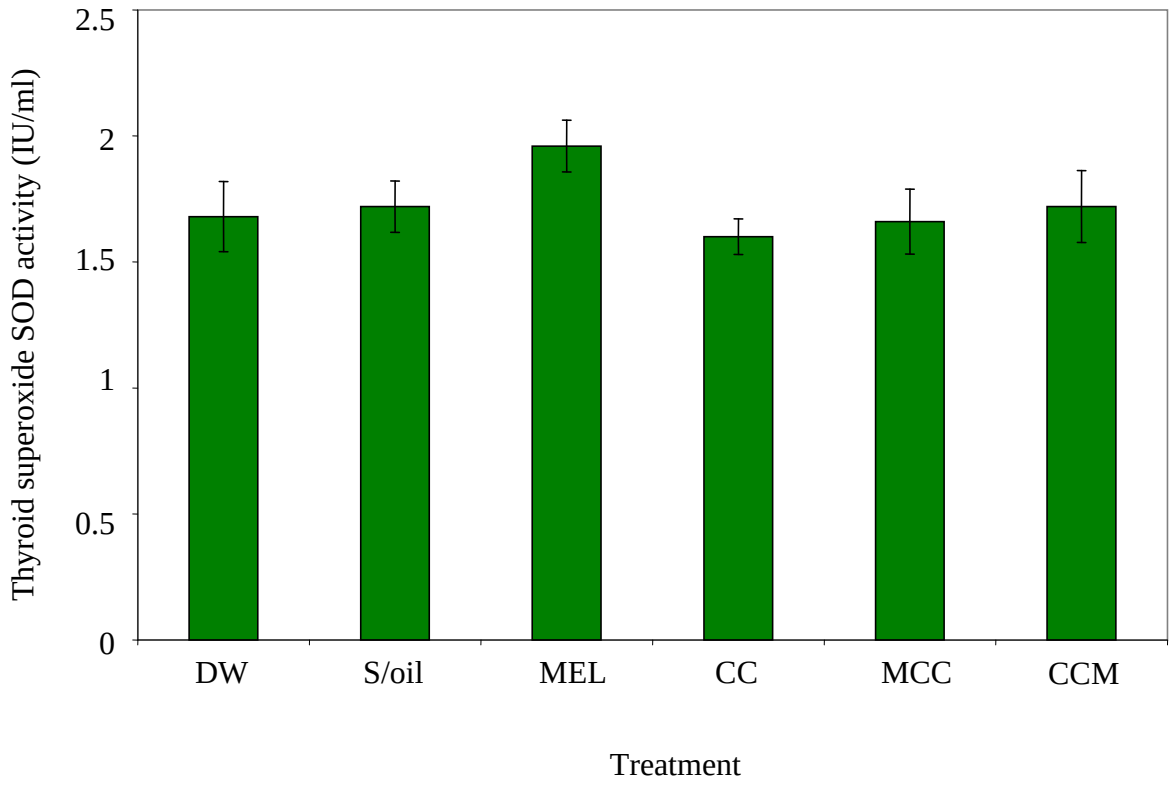


Figure 4.46: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on thyroid glands superoxide dismutase activity.

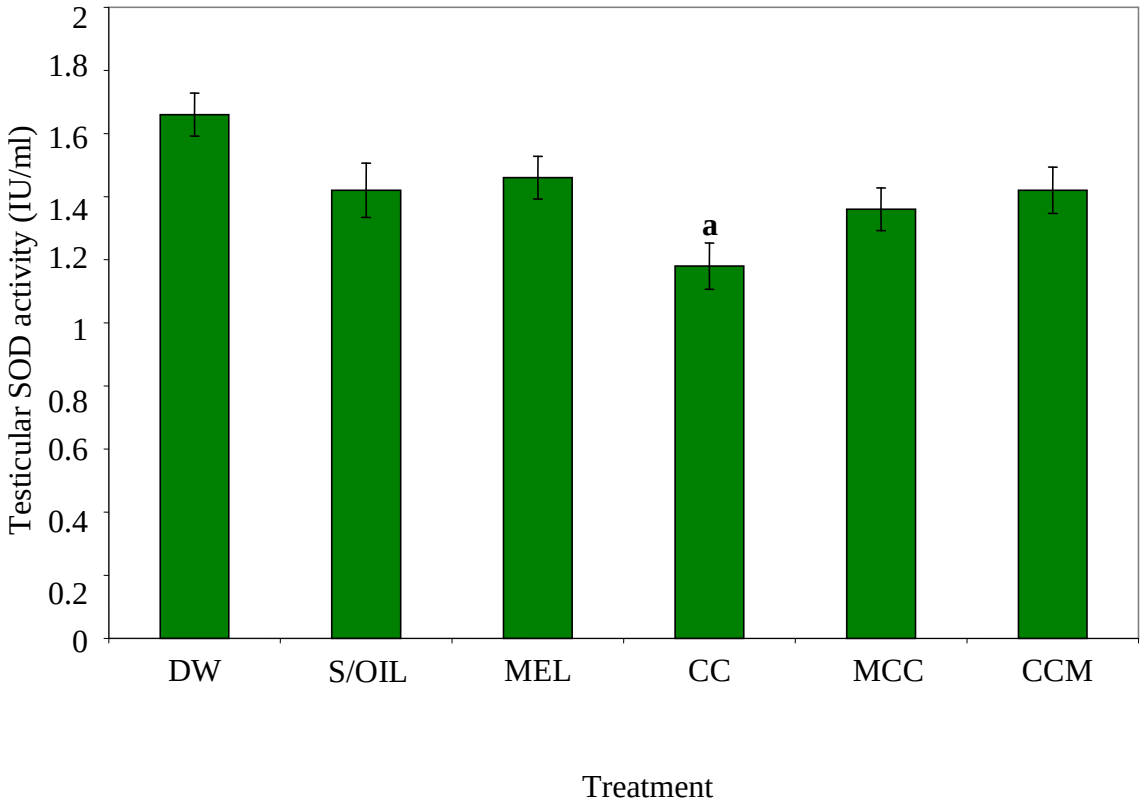


Figure 4.47: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on testicular SOD activity. $^{a}P < 0.01$ versus DW group.

4.9.12 Effect of treatment on prostate gland superoxide dismutase activity

The effect of treatment on changes in prostate SOD activity is shown in Figure 4.48. Although not significant ($P > 0.05$), the prostate gland SOD activity in the CC group was relatively higher compared to that of the DW (16%), S/oil (17%), MEL (16%), MCC (17%) and CCM (17%) treated groups.

4.9.13 Effect of treatment on epididymal superoxide dismutase activity

The effect of treatment on changes in epididymal SOD activity is shown in Figure 4.49. Although not significant ($P > 0.05$), the epididymal SOD activity in the CC group was higher compared to that of the DW (17%), S/oil (17%), MEL (16%), MCC (16%) or CCM (15%) treated groups.

4.9.14 Effect of treatment on seminal vesicle superoxide dismutase activity

The effect of treatment on changes in seminal vesicle SOD activity is shown in Figure 4.50. Although not significant ($P > 0.05$), the seminal vesicle SOD activity in the CC group was lower compared to that of the DW (16%), S/oil (15%), MEL (16%) and CCM (15%) groups, respectively, but was lower when compared to that of the MCC treated groups.

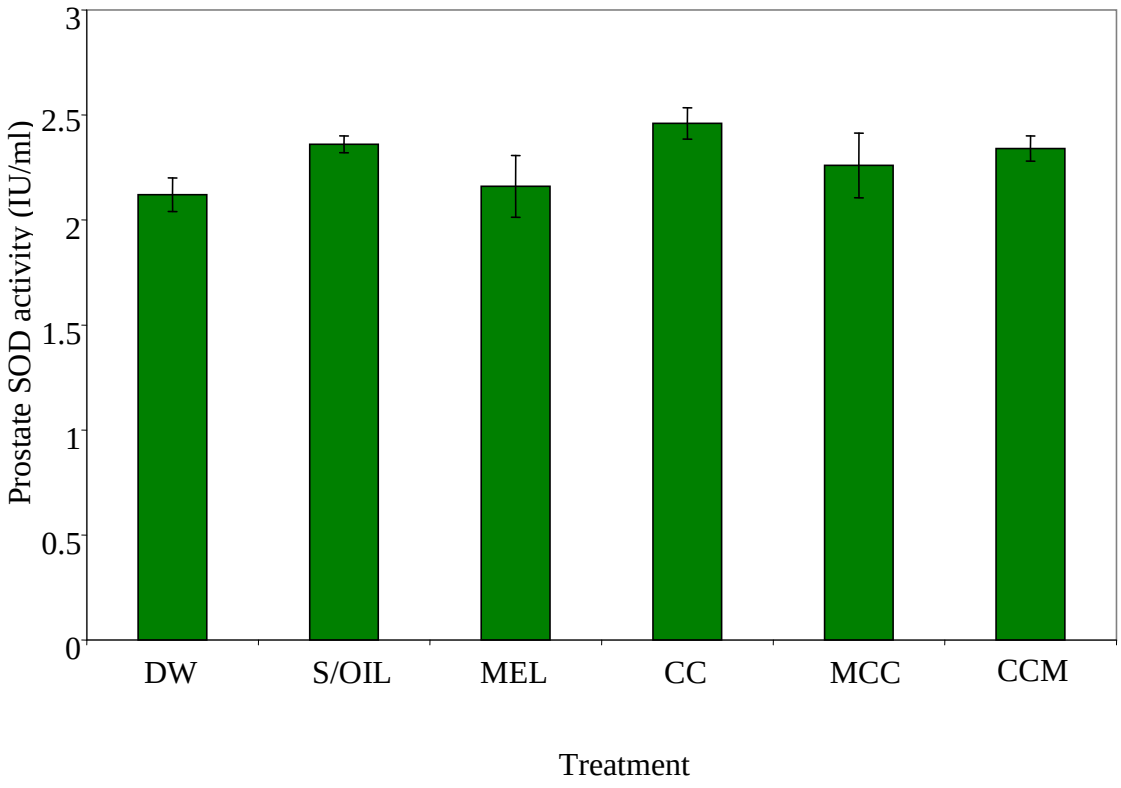


Figure 4.48: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on prostate gland superoxide dismutase activity.

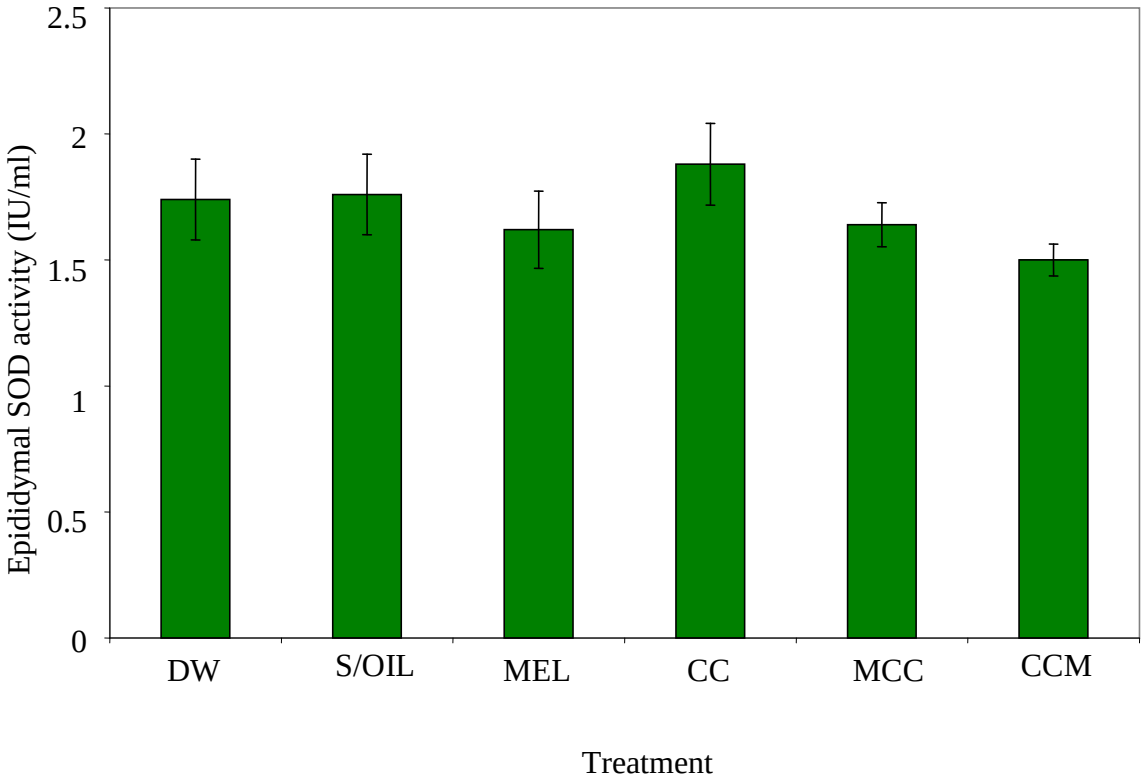


Figure 4.49: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on epididymal superoxide dismutase activity.

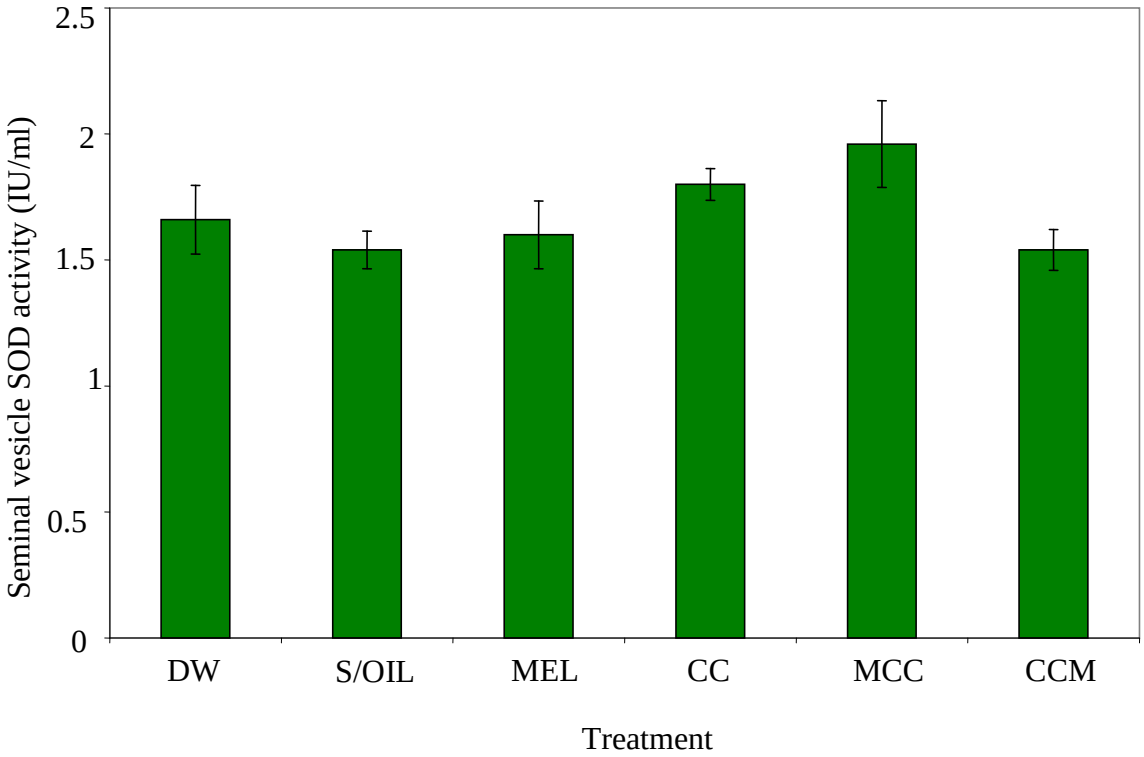


Figure 4.50: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on seminal vesicle superoxide dismutase activity.

4.9.15 Effect of treatment on brain catalase activity

Figure 4.51 shows the effect of treatment on brain CAT activity. There was a significant decrease in the brain CAT activity in the CC group relative to that of the DW ($P < 0.01$), S/oil ($P < 0.05$) and MEL ($P < 0.05$) groups, respectively. The brain CAT activity of CCM group decreased significantly relative to that of the DW ($P < 0.01$), S/oil ($P < 0.05$) and MEL ($P < 0.05$) treated groups.

4.9.16 Effect of treatment on pituitary gland catalase activity

Figure 4.52 shows the effect of treatment on pituitary CAT activity. There was a significant decrease in the pituitary gland CAT activity in the CC group relative to that of the DW ($P < 0.05$), S/oil ($P < 0.05$), MEL ($P < 0.001$) and MCC ($P < 0.05$) groups, respectively. Although not significant ($P > 0.05$), pituitary gland CAT activity in the CC group was relatively lower than that of the CCM group.

4.9.17 Effect of treatment on thyroid gland catalase activity

There was no significant ($P > 0.05$) change in the thyroid CAT activity in between the groups. However, there was marginal decrease in the thyroid gland CAT activity in the CC group relative to that of the DW (16%), S/oil (16%), MEL (18%), MCC (17%) and CC +MEL (17%) groups (Figure 4.53).

4.9.18 Effect of treatment on testicular catalase activity

There was a significant decrease in the testicular CAT activity in the CC group relative to that of the DW ($P < 0.001$), S/oil ($P < 0.01$) and MEL ($P < 0.05$) groups, respectively. There was a significant decrease in testicular CAT activity in the CCM group compared to that of the DW ($P < 0.01$) and S/oil ($P < 0.05$) groups, respectively. Although not

significant ($P > 0.05$), testicular CAT activity in the MCC group was relatively higher (7%) than that of the CCM group (Figure 4.54).

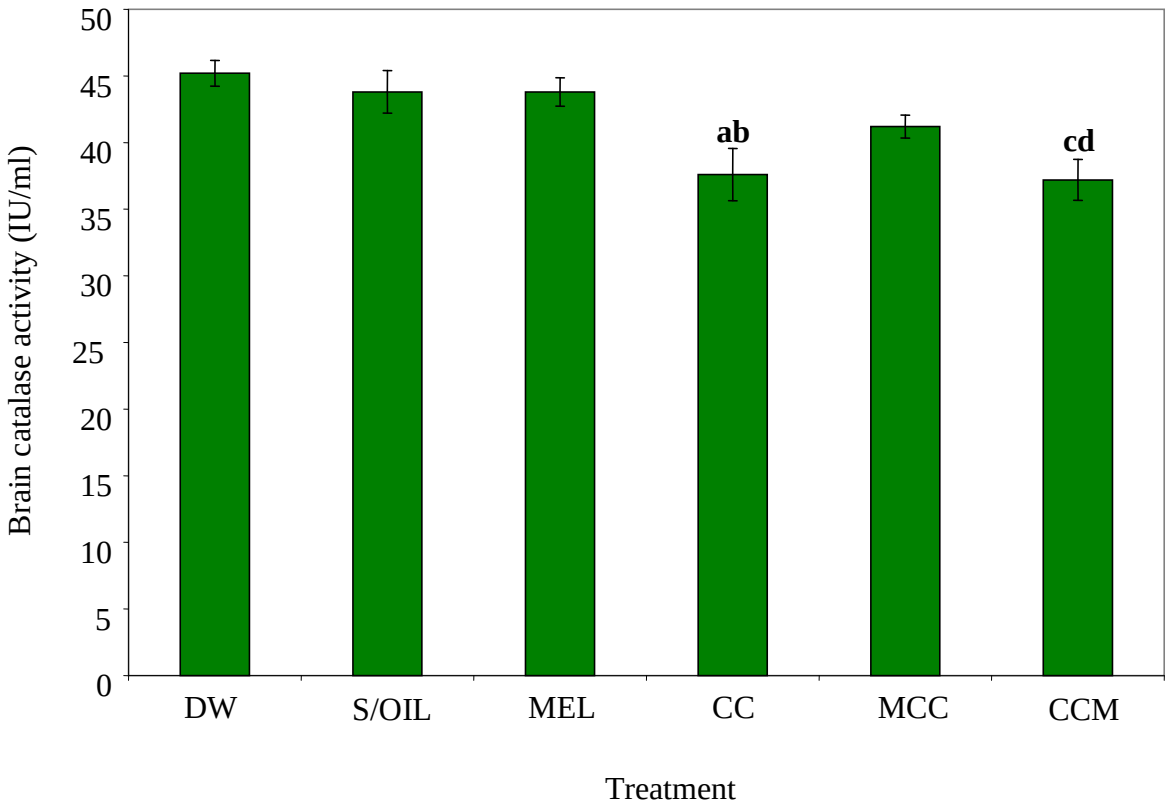


Figure 4.51: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on brain catalase activity. $^{a}P < 0.01$ versus DW; $^{b}P < 0.05$ versus S/oil and MEL. $^{c}P < 0.01$ versus DW; $^{d}P < 0.05$ versus S/oil and MEL groups.

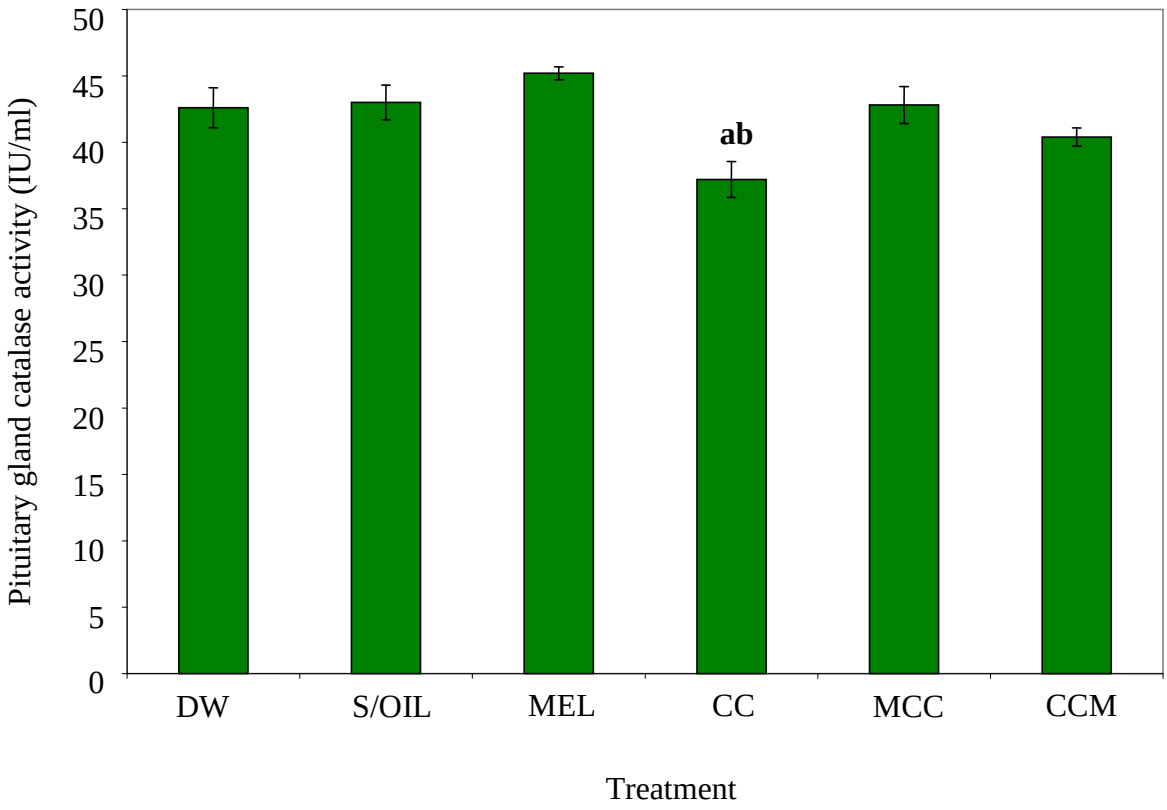


Figure 4.52: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on pituitary gland CAT activity. [a]P < 0.05 versus DW, S/oil and MCC groups, respectively. [b]P < 0.001 versus MEL group.

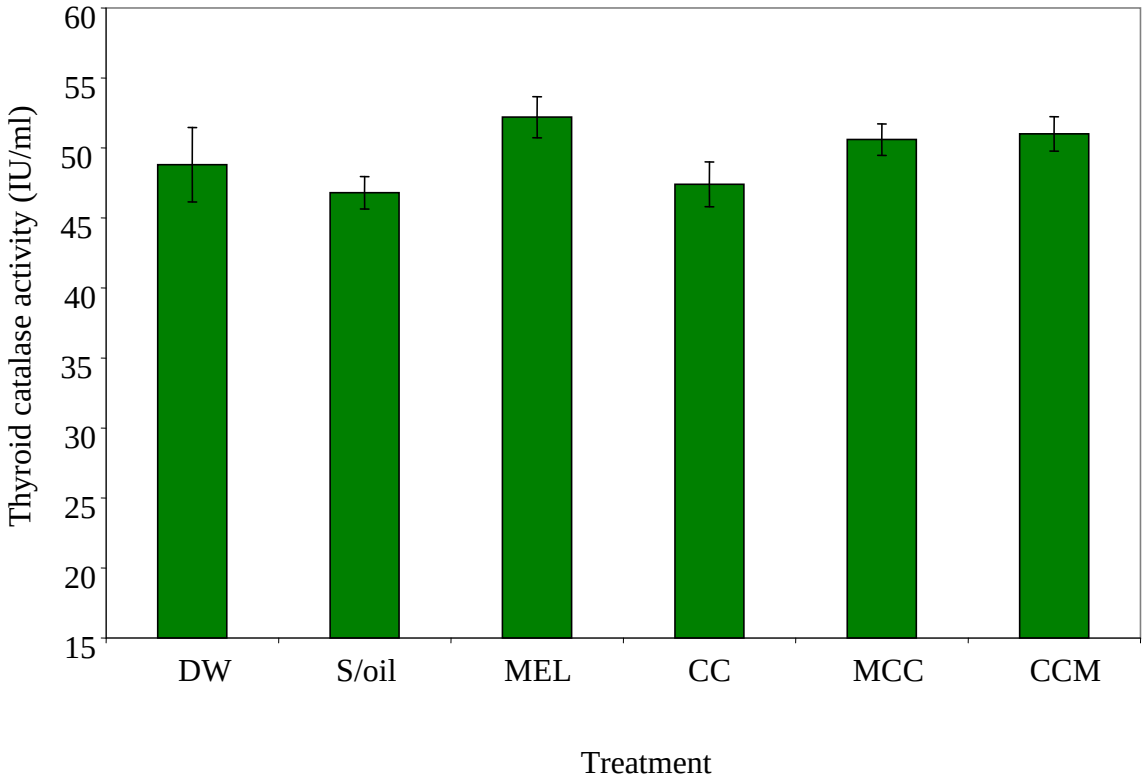


Figure 4.53: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on thyroid glands catalase activity.

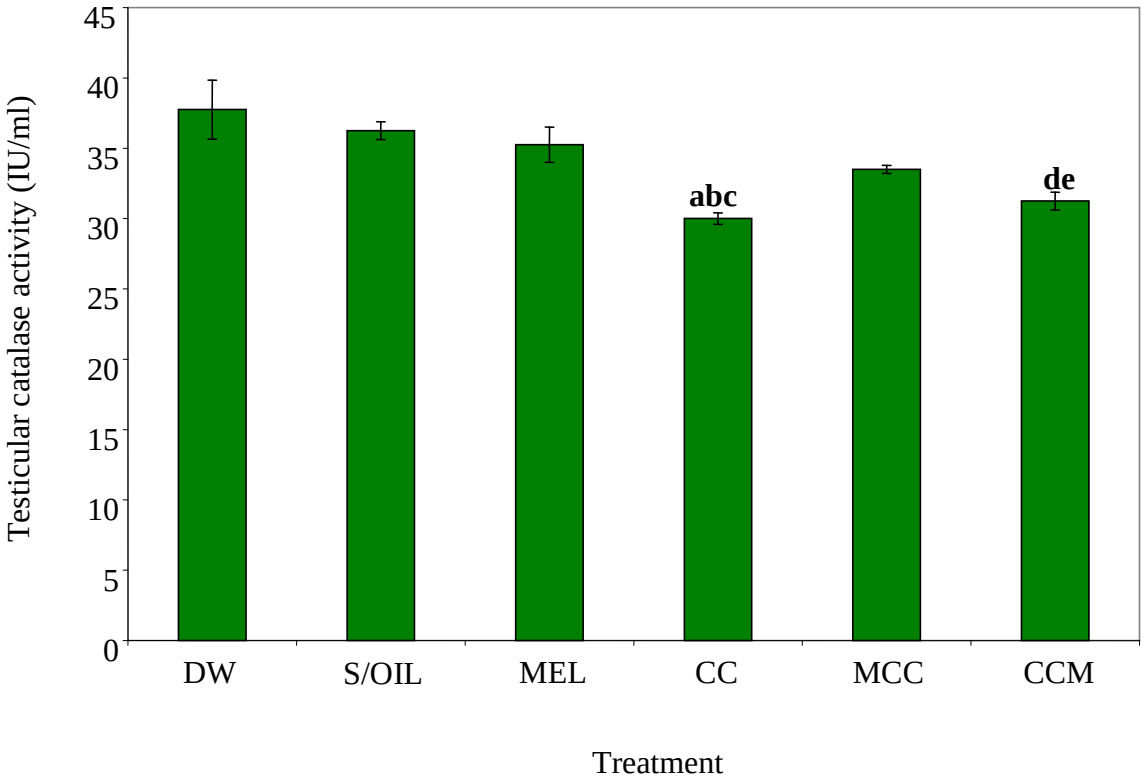


Figure 4.54: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on testicular catalase activity. $^{a}P < 0.001$ versus DW; $^{b}P < 0.01$ versus S/oil; $^{c}P < 0.05$ versus MEL. $^{d}P < 0.01$ versus DW; $^{e}P < 0.05$ versus S/oil groups.

4.9.19 Effect of treatment on prostate catalase activity

Figure 4.55 shows the effect of treatment on prostate CAT activity. There were no significant change ($P > 0.05$) in prostate CAT activity in between the groups. However, there was marginal decrease in the prostate CAT activity in the CC group relative to that of the DW (17%), S/oil (17%), MEL (17%), MCC (17%) and CCM (16%) groups.

4.9.20 Effect of treatment on epididymal catalase activity

Figure 4.56 shows the effect of treatment on epididymal CAT activity. There was a significant ($P < 0.05$) decrease in the epididymal CAT activity in the CC group, relative to that of the MEL group. Although not significant ($P > 0.05$), there was marginal decrease in the epididymal CAT activity in the CC group relative to that of the DW (17%), S/oil (17%), MCC (17%) and CCM (16%) groups.

4.9.21 Effect of treatment on seminal vesicles catalase activity

Figure 4.57 shows the effect of treatment on seminal vesicle CAT activity. There was no significant change ($P > 0.05$) in between the groups. However, there was marginal decrease in the seminal vesicle CAT activity in the CC group relative to that of the DW (17%), S/oil (17%), MEL (17%), MCC (16%) and CCM (17%) groups.

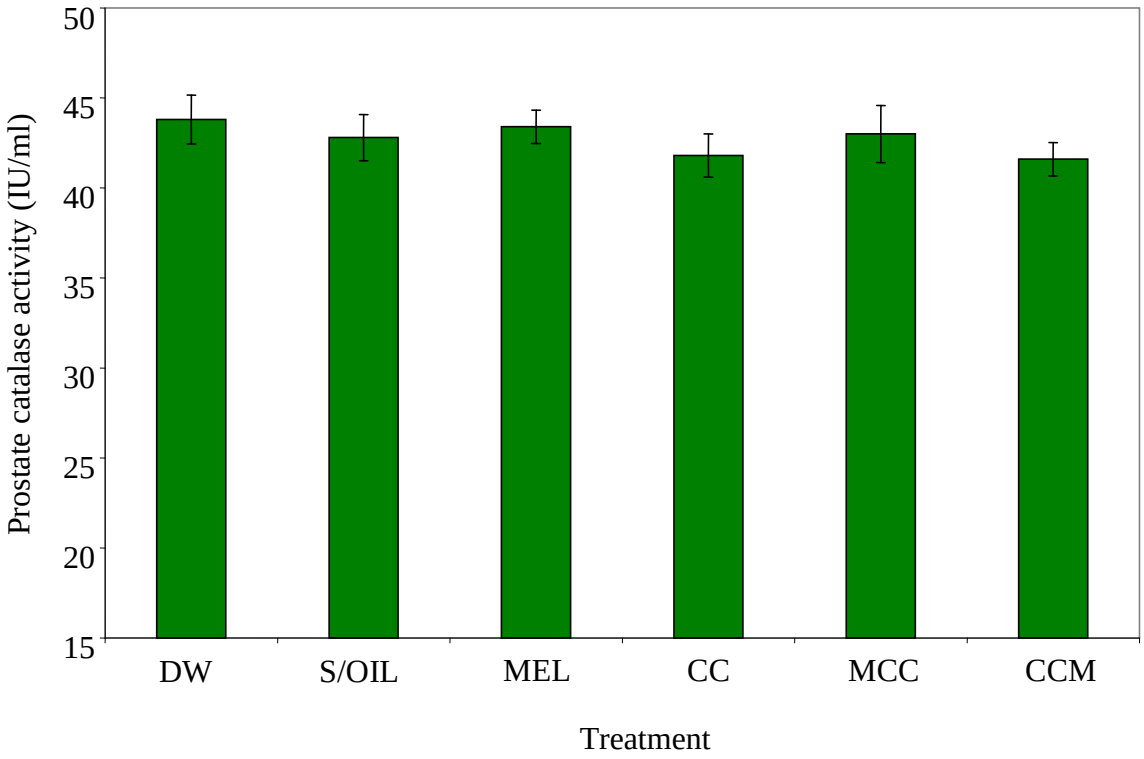


Figure 4.55: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on prostate catalase activity.

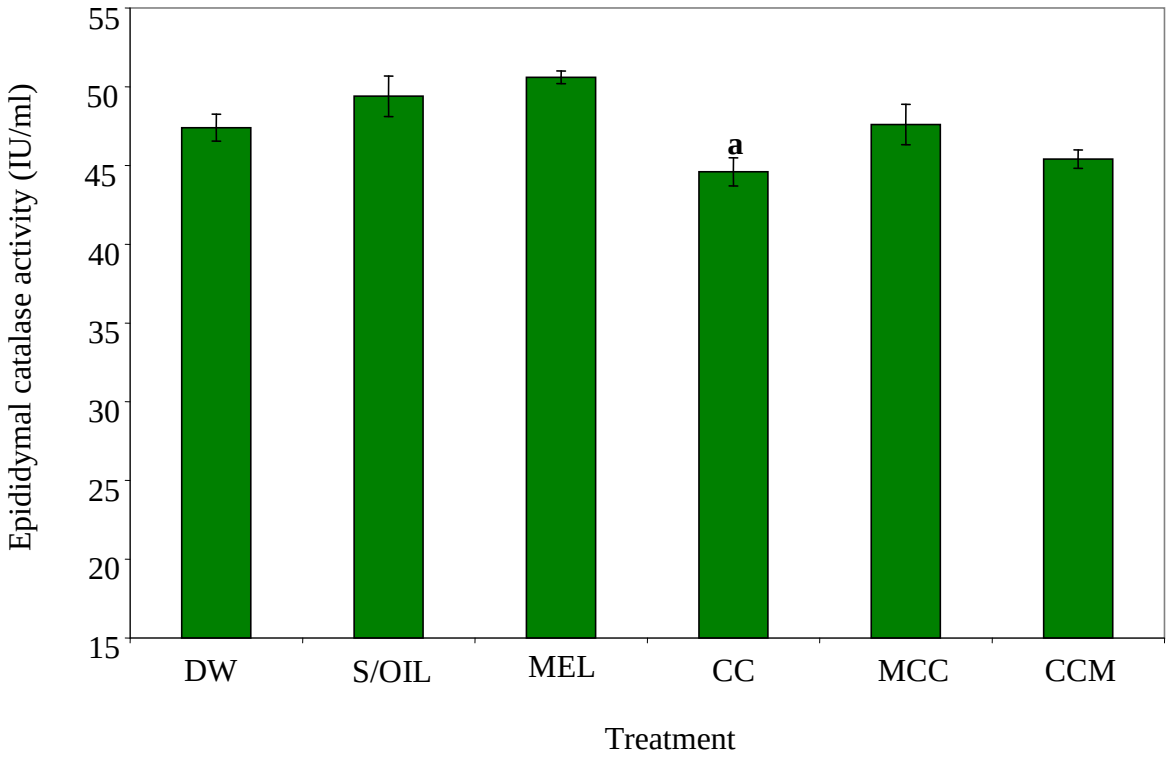


Figure 4.56: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on epididymal catalase activity. $^{a}P < 0.05$ versus MEL group.

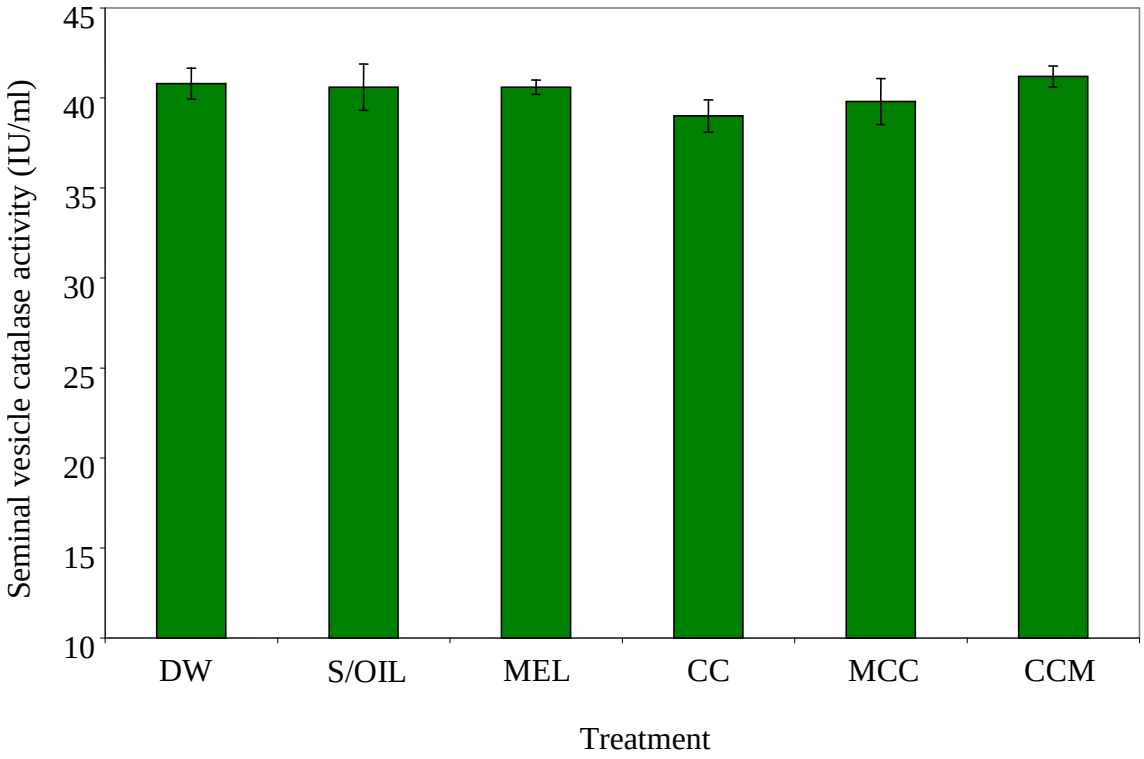


Figure 4.57: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on seminal vesicles catalase activity.

4.9.22 Effect of treatment on brain glutathione peroxidase activity

The effect of treatment on brain GPx activity is shown in Figure 4.58. The brain GPx activity in the CC group was significantly lower compared to that of the DW ($P < 0.05$), MEL ($P < 0.01$) and MCC ($P < 0.001$) groups, respectively. There was a non significant decrease in the brain glutathione activity in the CC group compared to that of the S/oil (17%) and CCM (16%) groups, respectively. There was no significant ($P > 0.05$) change in the testicular GPx activity of MCC group when compared to that of CCM group.

4.9.23 Effect of treatment on pituitary gland glutathione peroxidase activity

There was as a significant decrease in the pituitary GPx activity in CC group relative to that of DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$) and MCC ($P < 0.01$) groups, respectively. There was a significant increase ($P < 0.05$) in the pituitary gland GPx activity in the MCC group compared to that of the CCM group. In addition, there was a significant ($P < 0.05$) decrease in the pituitary gland GPx activity in the CCM group, when compared to that of the DW, S/oil and MEL groups (Figure 4.59).

4.9.24 Effect of treatment on thyroid gland glutathione peroxidase activity

The effect of treatment on thyroid gland GPx activity is shown in Figure 4.60. The thyroid gland GPx activity in the CC group was lower ($P < 0.01$) than that of the MEL group. The thyroid gland GPx activity in the MEL group was significantly ($P < 0.05$) higher, compared to that of the S/oil group. Although not significant ($P > 0.05$), GPx activity in the CC group was relatively lower ($P > 0.05$) compared to that of the DW (16%), S/oil (16%), MCC (17%) or CCM (17%) group.

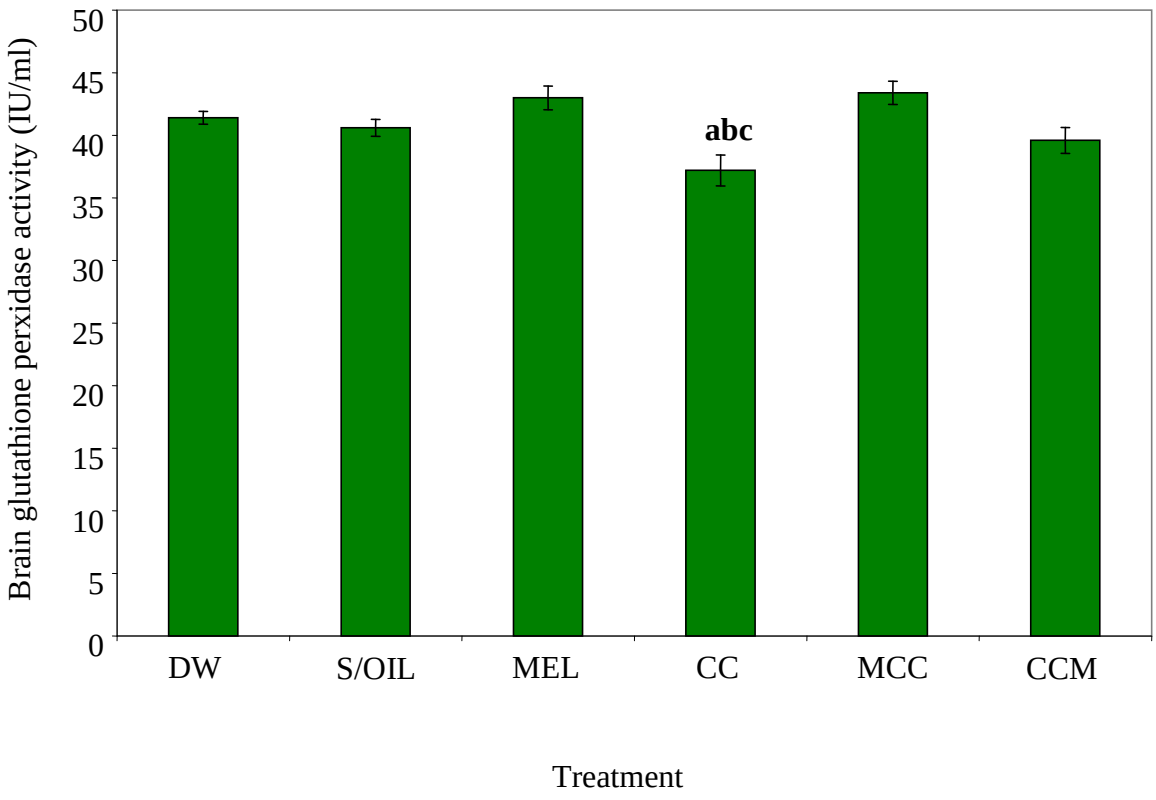


Figure 4.58: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on brain glutathione peroxidase activity. $^{a}P < 0.05$ versus DW; $^{b}P < 0.01$ versus MEL and $^{c}P < 0.001$ versus MCC groups, respectively.

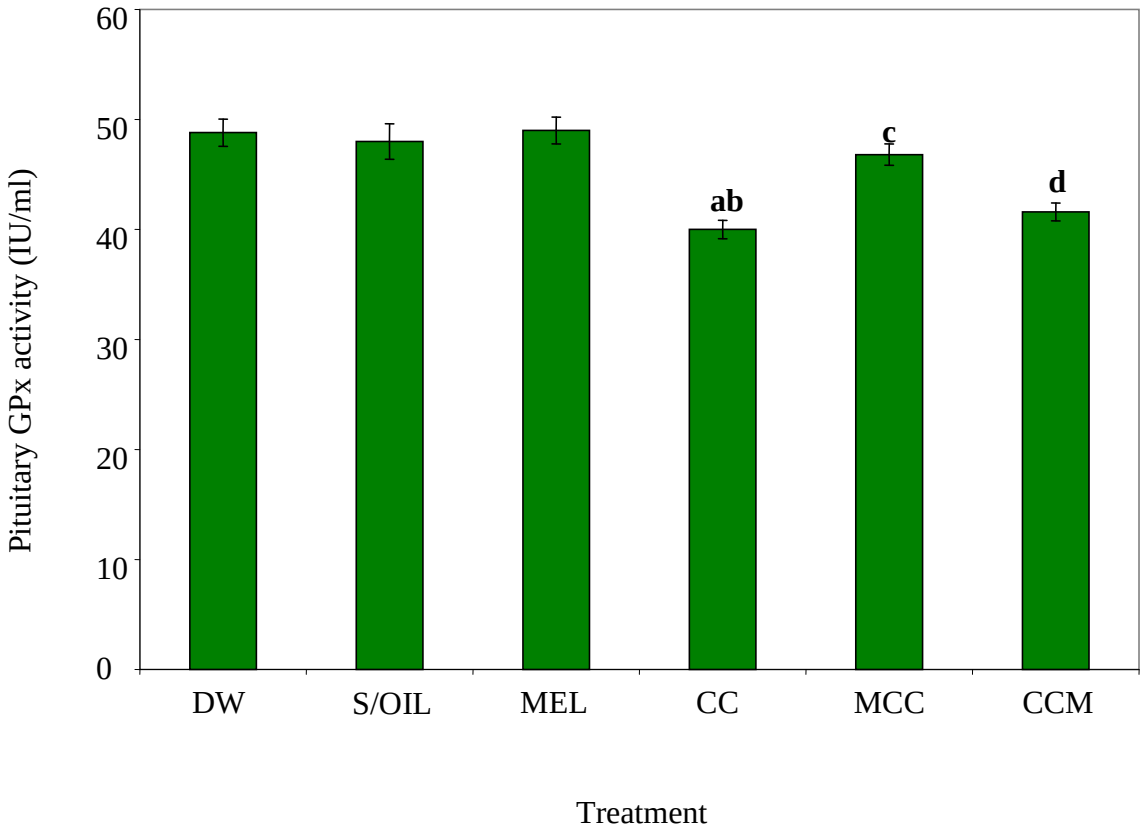


Figure 4.59: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on pituitary gland glutathione peroxidase activity. $^{a}P < 0.001$ versus DW, S/oil and MEL groups, respectively; $^{b}P < 0.01$ versus MCC group; $^{c}P < 0.05$ versus CCM group; $^{d}P < 0.01$ versus DW, S/oil and MEL groups.

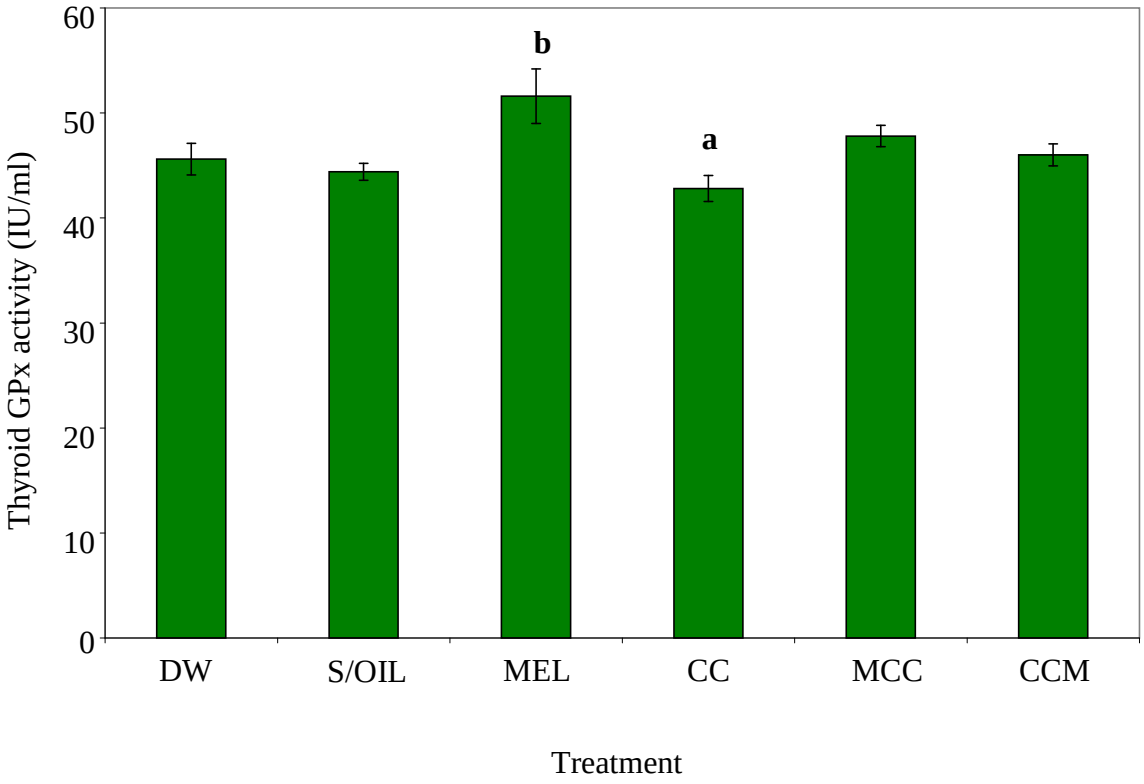


Figure 4.60. Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on thyroid glands glutathione peroxidase activity. $^{a}P < 0.01$ versus MEL group;. $^{b}P < 0.05$ versus S/oil group.

4.9.25 Effect of treatment on testicular glutathione peroxidase activity

The effect of treatment on testicular GPx activity is shown in Figure 4.61. There was a significant decrease in the testicular GPx activity in the CC group relative to that of the DW ($P < 0.001$), S/oil ($P < 0.001$), MEL ($P < 0.001$) group and MCC ($P < 0.05$) groups, respectively. Although not significant ($P > 0.05$), there was a marginal increase (16%) in the testicular GPx activity of CCM (16%) group when compared to that of the CC group. There was no significant ($P > 0.05$) difference in the pituitary gland GPx activity in the MCC group compared to that of the CCM group.

4.9.26 Effect of treatment on prostate glutathione peroxidase activity

Figure 4.62 shows the effect of treatment on prostate glutathione peroxidase activity. There were no significant changes ($P > 0.05$) in the GPx activity in between the groups. However, there was marginal decrease in the prostate GPx activity in the CC group relative to that of DW (18%), S/oil (16%), MEL (16%), MCC (16%) and CCM (17%) groups.

4.9.27 Effect of treatment on epididymal glutathione peroxidase activity

Figure 4.63 shows the effect of treatment on epididymal GPx activity. There was no significant changes ($P > 0.05$) in between the groups. However, there was marginal increase in the epididymal GPx activity in the CC group relative to that of the MEL (17%), while it increased marginally relative to that of the DW (17%), S/oil (17%), MCC (16%) and CCM (16%) groups.

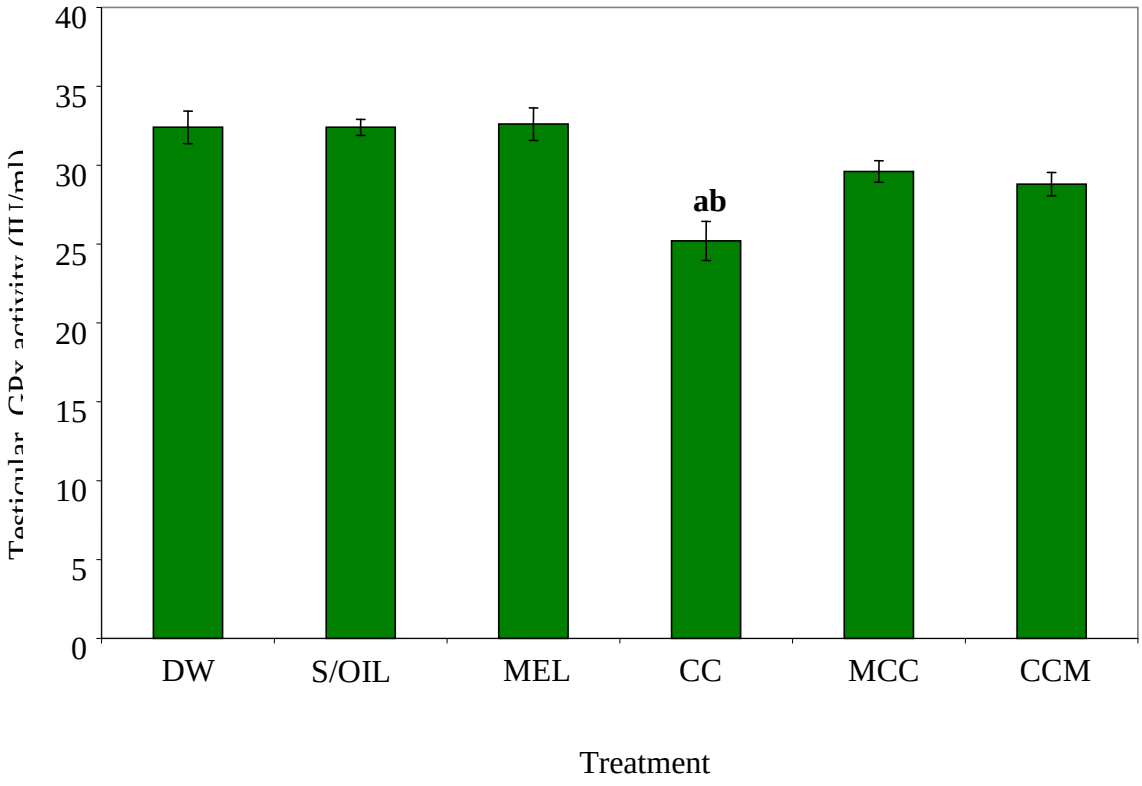


Figure 4.61: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on testicular glutathione peroxidase activity. $^{a}P < 0.001$ versus DW, S/oil and MEL groups, respectively. $^{b}P < 0.05$ versus MCC group.

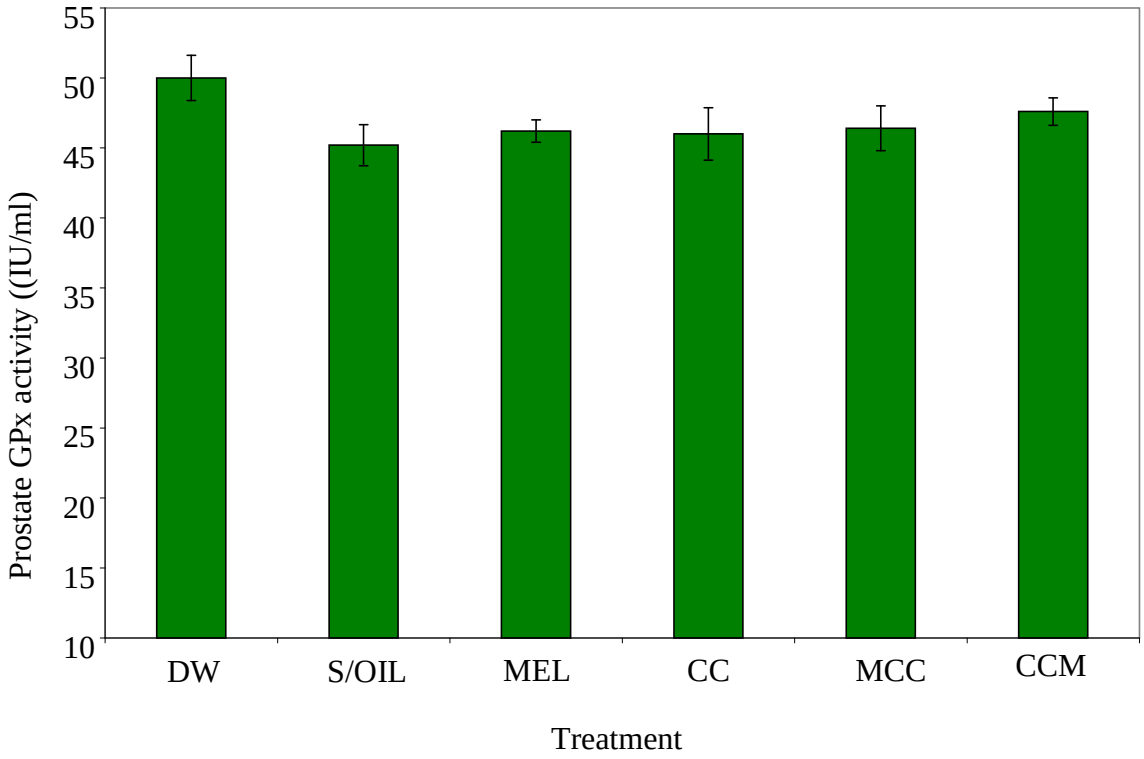


Figure 4.62: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on prostate glutathione peroxidase activity.

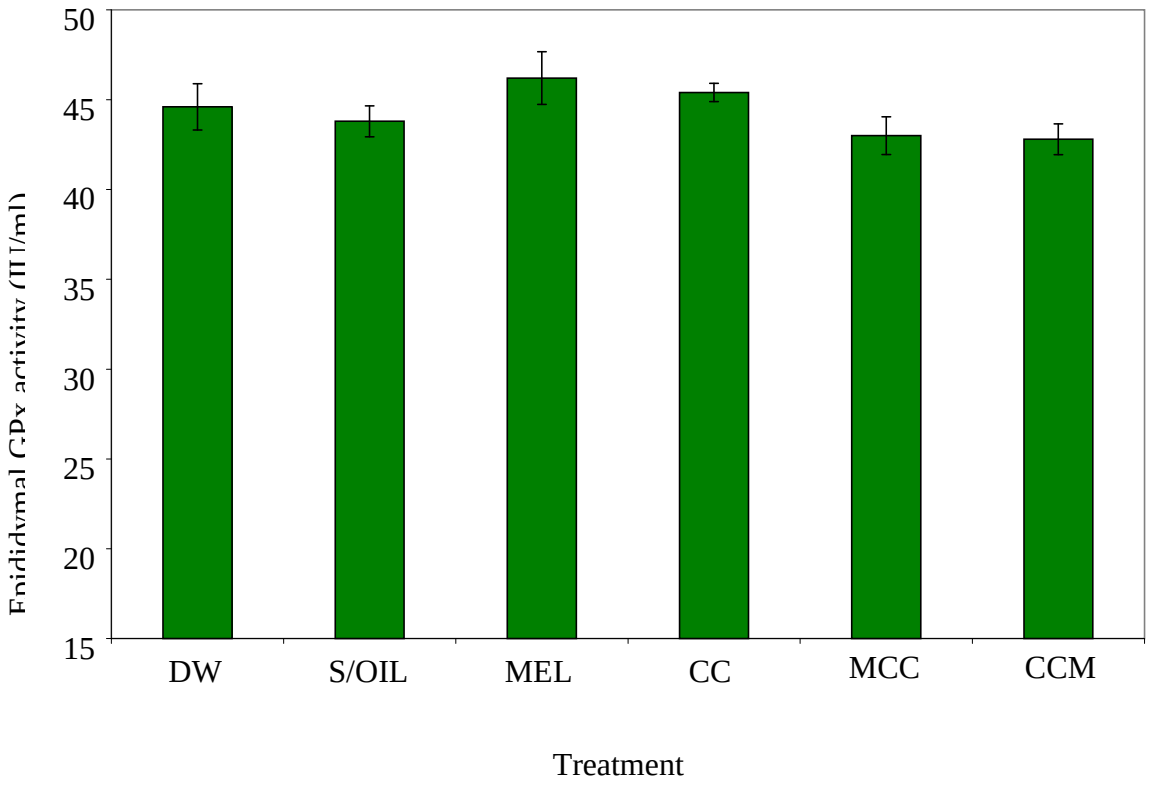


Figure 4.63: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on epididymal glutathione peroxidase activity.

4.9.28 Effect of treatment on seminal vesicles glutathione peroxidase activity

Figure 4.64 shows the effect of treatment on seminal vesicle GPx activity. There were no significant changes ($P > 0.05$) in GPx activities in between the groups. However, there was marginal decrease in the seminal vesicles GPx activity in the CC group when compared to that of the DW (17%), S/oil (17%), MCC (17%), CC +MEL (17%) groups, respectively. Seminal vesicles GPx activity in the CC group increased non-significantly relative to that of the MEL (16%) group.

4.9.29 Effect of treatment on brain acetylcholinesterase activity

Figure 4.65 shows the effect of treatment on brain AChE activity. There was a significant decrease in group exposed to CC, when compared to that of the DW ($P < 0.01$), S/oil($P < 0.05$), MEL ($P < 0.001$) and CCM ($P < 0.05$) groups, respectively. Although not significant ($P > 0.05$), there was decrease in the brain AChE activity in the group exposed to CC relative to that of MCC group.

4.9.30 Effect of treatment on pituitary gland acetylcholinesterase activity

Figure 4.66 illustrates pituitary gland AChE activity, which had a significant decrease ($P < 0.05$) in group exposed to CC, when compared to that of the DW ($P < 0.001$), S/oil($P < 0.01$), MEL ($P < 0.001$), MCC ($P < 0.001$) and CCM ($P < 0.05$) groups, respectively. In addition, there was a significant ($P < 0.05$) increase in the AChE activity in the group, exposed to MEL relative to that of CCM group.

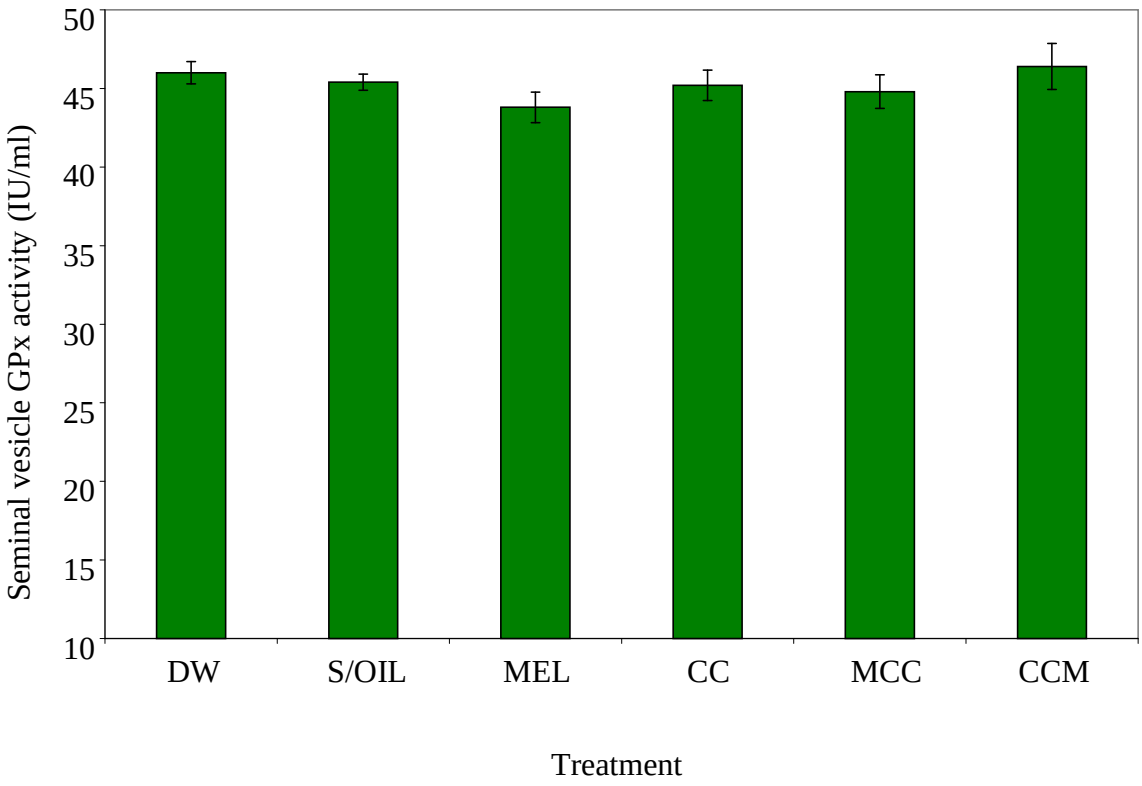


Figure 4.64: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on seminal vesicle glutathione peroxidase activity.

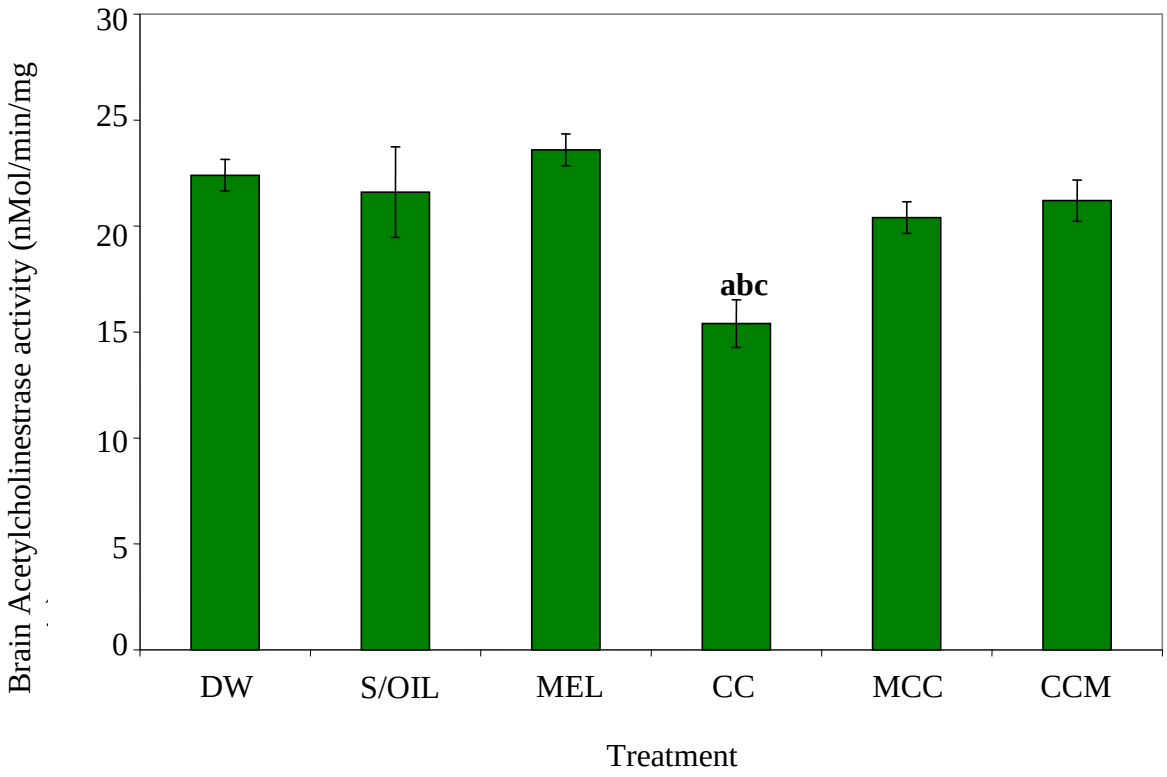


Figure 4.65: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on brain AChE activity. $^{a}P < 0.01$ versus DW; $^{b}P < 0.05$ versus S/oil and CCM groups; $^{c}P < 0.001$ versus MEL group.

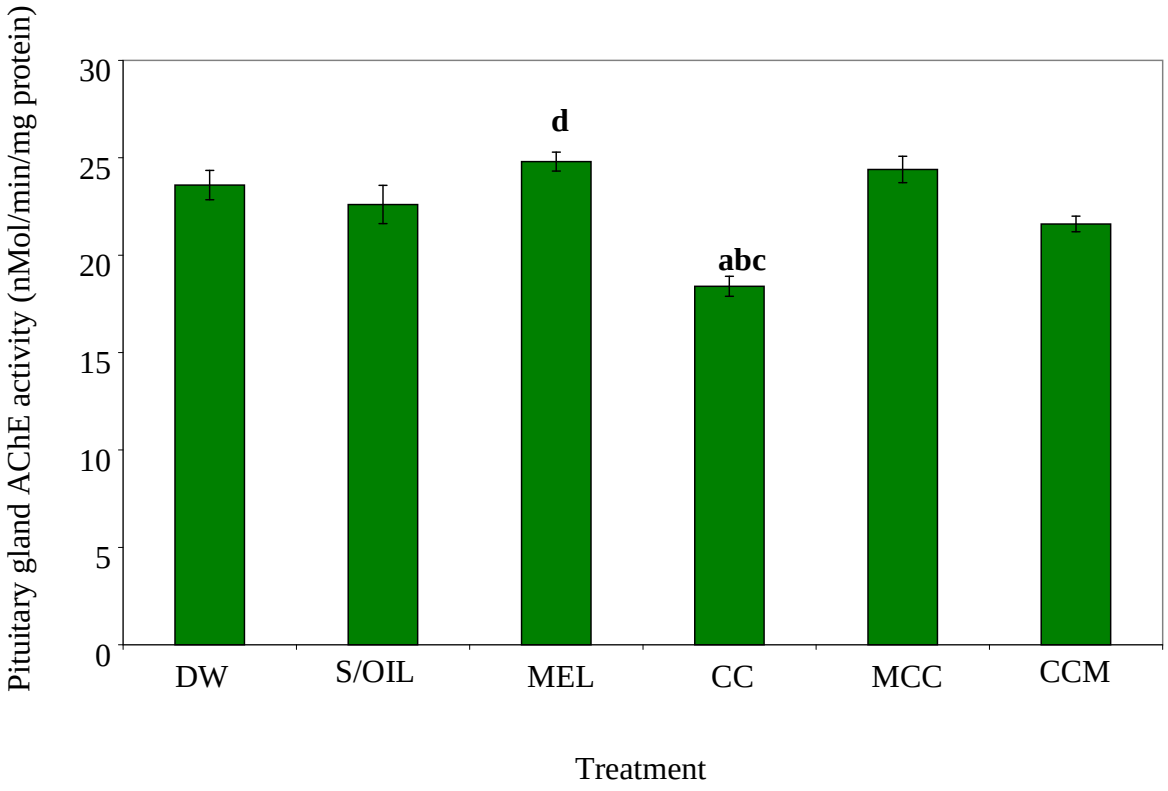


Figure 4.66: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on pituitary AChE activity. $^{a}P < 0.001$ versus DW, MEL and MCC groups, respectively; $^{b}P < 0.01$ versus S/oil group; $^{c}P < 0.05$ versus CCM group; $^{d}P < 0.05$ versus CCM group.

4.9.31 Effect of treatment on thyroid gland acetylcholinesterase activity

Figure 4.67 shows the effect of treatment on thyroid gland AChE activity. There was no significant difference ($P > 0.05$) in between the groups. There was, however, a relative decrease in the thyroid gland AChE activity in CC group relative to that of the DW (19%), S/oil (18%), MEL (17%) and MCC (18%) groups, respectively. However, thyroid gland AChE activity significantly increased ($P < 0.01$) in the S/oil group, compared to that of the CCM group. In addition, there was a significant ($P < 0.05$) increase in the thyroid gland AChE activity of MCC group, relative to that of the CCM group.

4.9.32 Effect of treatment on testicular acetylcholinesterase activity

Figure 4.68 shows the effects of treatment on testicular AChE activity. There was a significant ($P < 0.05$) decrease in the testicular AChE activity in the CC group compared to that of the DW, S/oil, MEL and MCC groups, respectively. There was a significant ($P < 0.05$) change in the testicular AChE activity of MCC group when compared to that of CCM group.

4.9.33 Effect of treatment on prostate acetylcholinesterase activity

Figure 4.69 shows the effect of treatment on prostate AChE activity. There were no significant change ($P > 0.05$) in between the groups. There was, however, a marginal increase in the prostate AChE activity, relative to that of the DW (17%), S/oil (15%), MEL (167%) and MCC (17%) and CCM (17%) groups.

4.9.34 Effect of treatment on seminal vesicles acetylcholinesterase activity

Figure 4.70 shows the effect of treatment on seminal vesicle AChE activity. There were no significant differences ($P > 0.05$) in between the groups. There was a marginal decrease in the seminal vesicle AChE activity of the CC group, relative to that of the DW (16%), S/oil (14%), MEL (18%) and MCC (19%) and CCM (17%) groups.

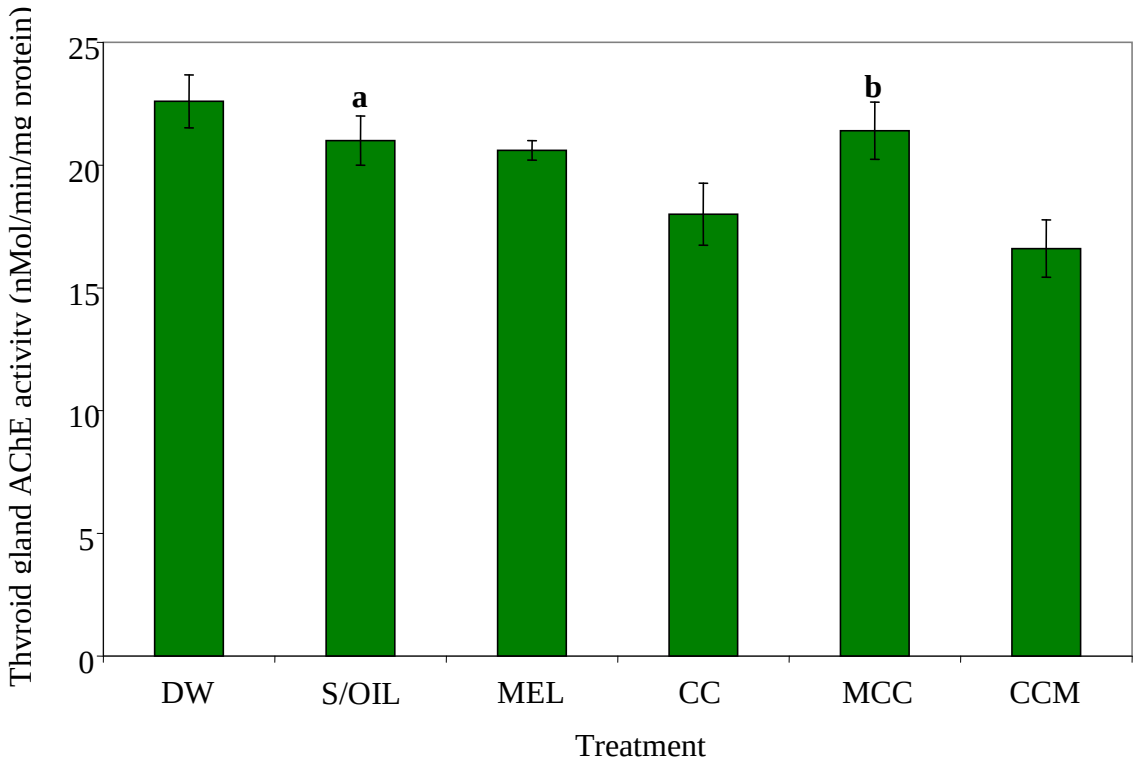


Figure 4.67: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on thyroid gland AChE activity. $^{a}P < 0.01$ versus CCM; $^{b}P < 0.05$ versus CCM group.

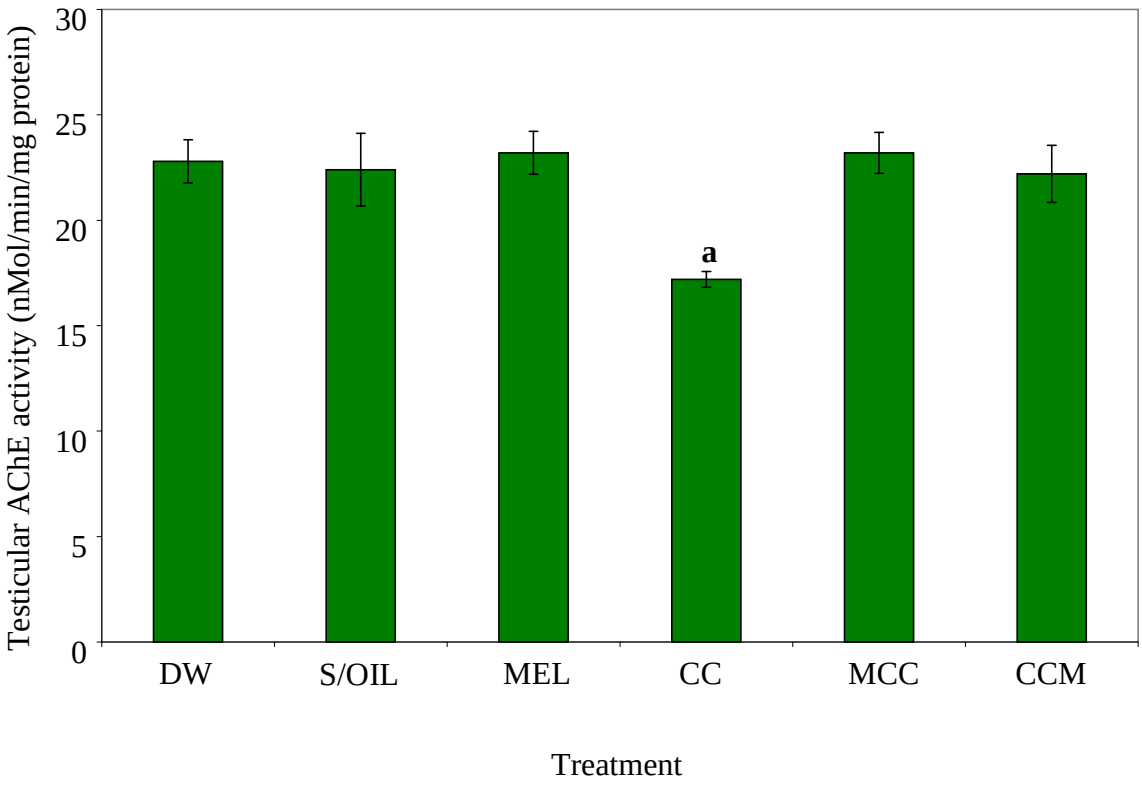


Figure 4.68: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on testicular AChE activity. $^{a}P < 0.05$ versus DW, S/oil, MEL and MCC groups.

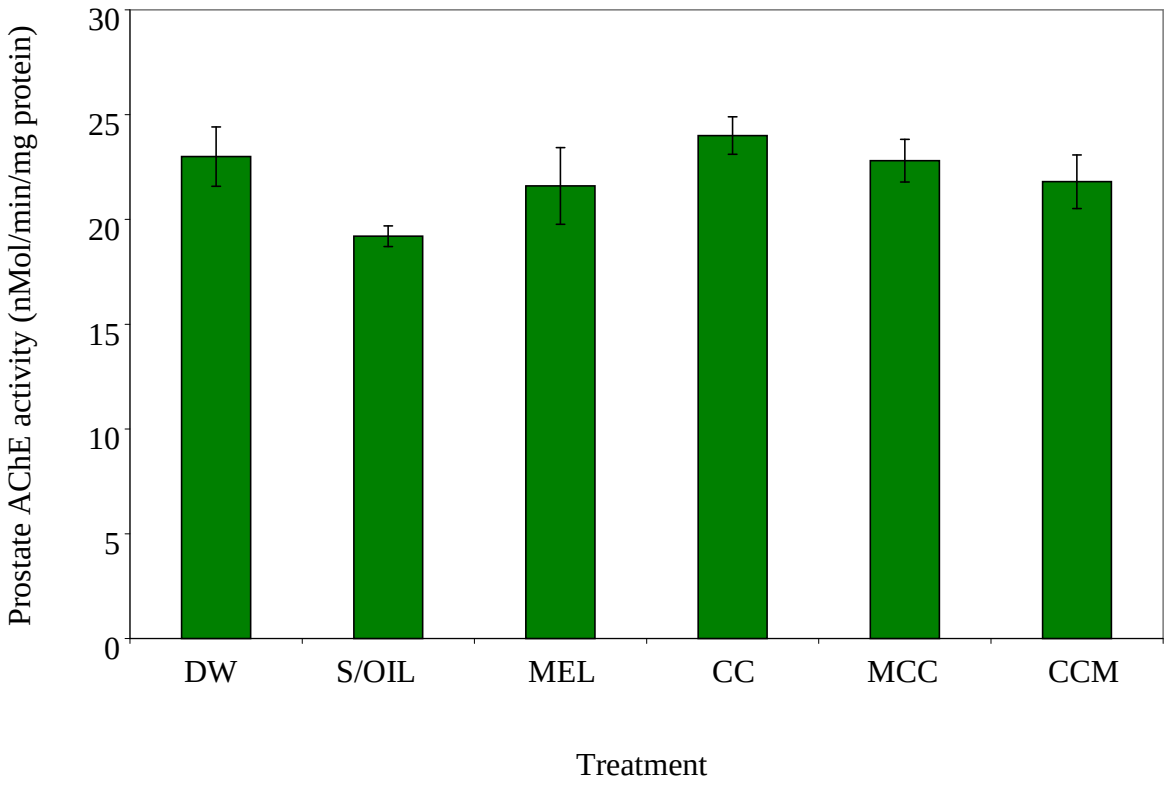


Figure 4.69: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on prostate AChE activity.

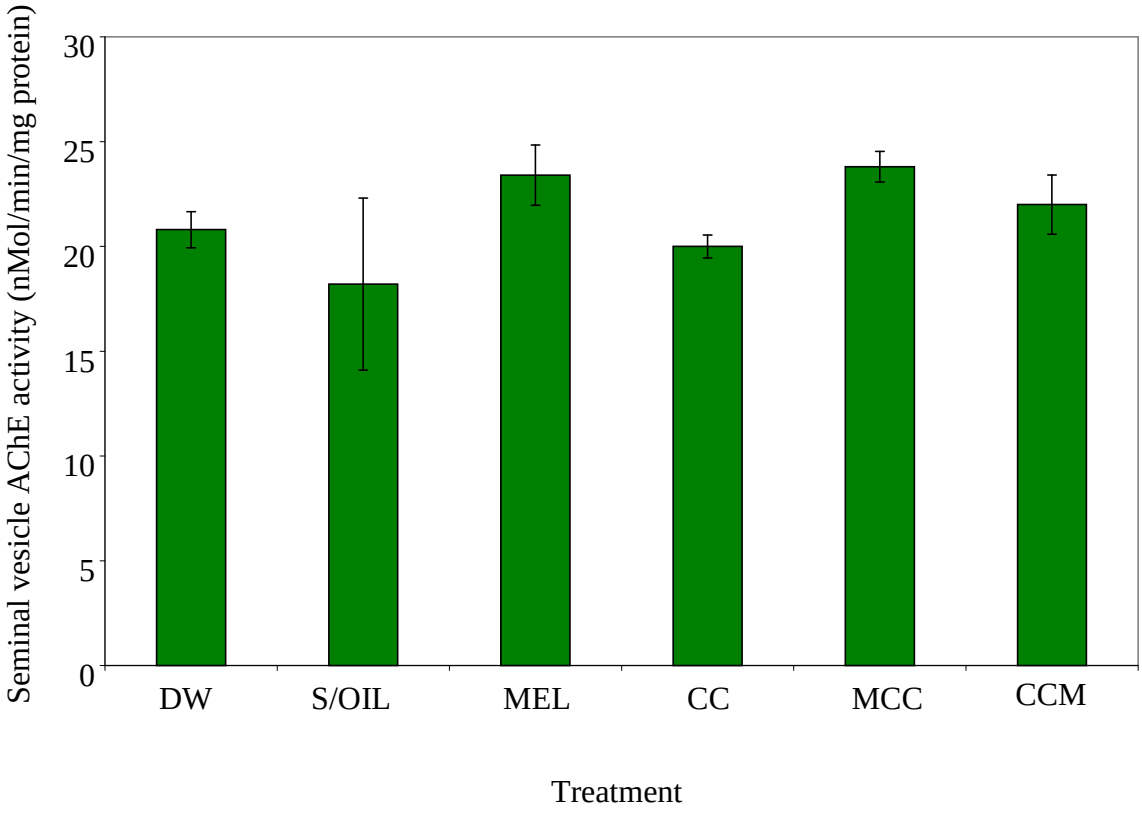


Figure 4.70: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on seminal vesicle AChE activity.

4.9.35 Effect of treatment on testicular total sialic acid concentration

Testicular total sialic acid concentration was decreased in group exposed to CC, when compared to that of DW ($P < 0.05$), S/oil ($P < 0.01$), MEL ($P < 0.05$) and MCC ($P < 0.05$) groups, respectively (Figure 4.71).

4.9.36 Effect of treatment on testicular free sialic acid concentration

There was a significant decrease in the testicular free sialic concentration in the CC group compared to that of the DW ($P < 0.01$), S/oil ($P < 0.001$), MEL ($P < 0.01$) and MCC ($P < 0.05$) groups, respectively. There was a significant increase in the testicular free sialic acid in the S/oil group when compared to that of MCC ($P < 0.05$) or CCM ($P < 0.01$) group. There was no significant change ($P > 0.05$) in the testicular free sialic acid concentration in the MCC group relative to that of the CCM group (Figure 4.72).

4.9.37 Effect of treatment on epididymal total and free sialic acid concentration

Figure 4.73 shows the effect of treatment on epididymal total sialic acid concentration. There were no significant differences ($P > 0.05$) in between the groups. There was however, a relative decrease in the epididymal total sialic acid concentration in CC group compared to that of the DW (16.3%), S/oil (16%), MEL (17.4%), MCC (17.3%) and CCM(17%) groups.

There were no significant difference in between the groups in the epididymal free sialic acid concentrations. However, there was a relative decrease in the epididymal free sialic acid concentration in CC group relative to that of the DW (18%), S/oil (16%), MCC (16%) and CCM (18%) groups, respectively, but also showed an increase concentration relative to that recorded in MEL (15%) group.

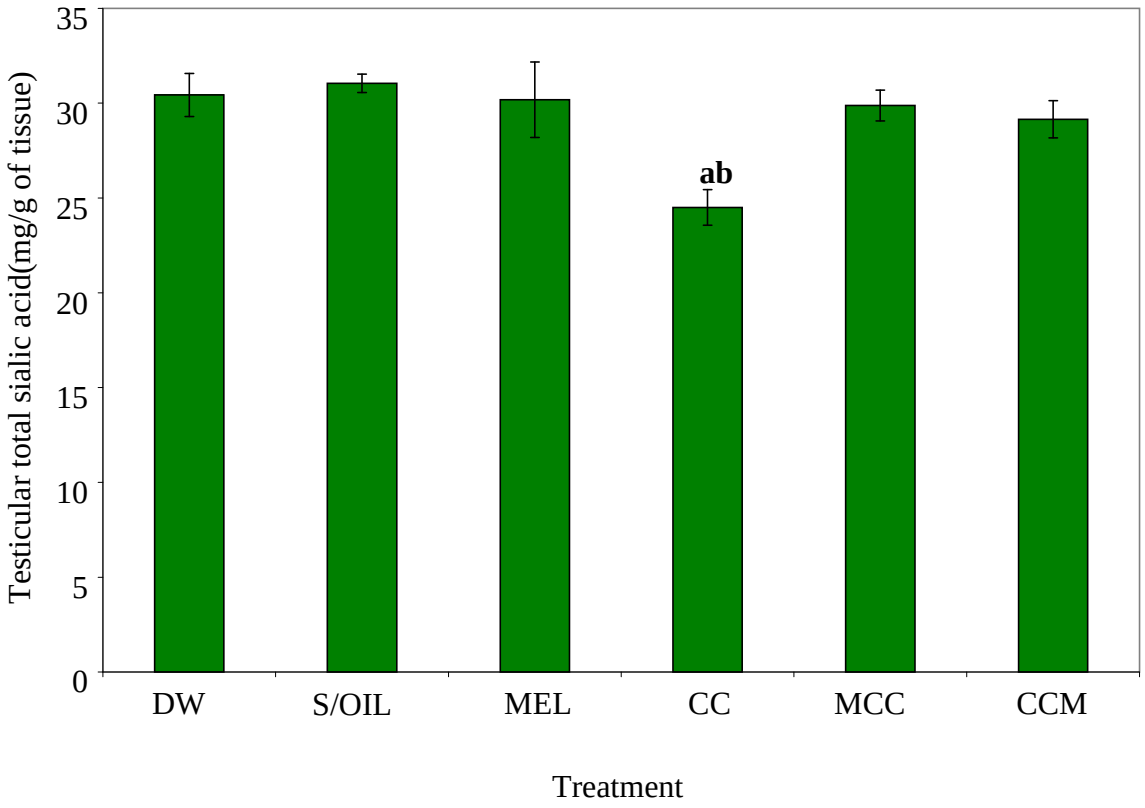


Figure 4.71: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on testicular total sialic acid concentration. $^{a}P < 0.05$ versus DW, MEL and MCC groups, respectively; $^{b}P < 0.01$ versus S/oil group.

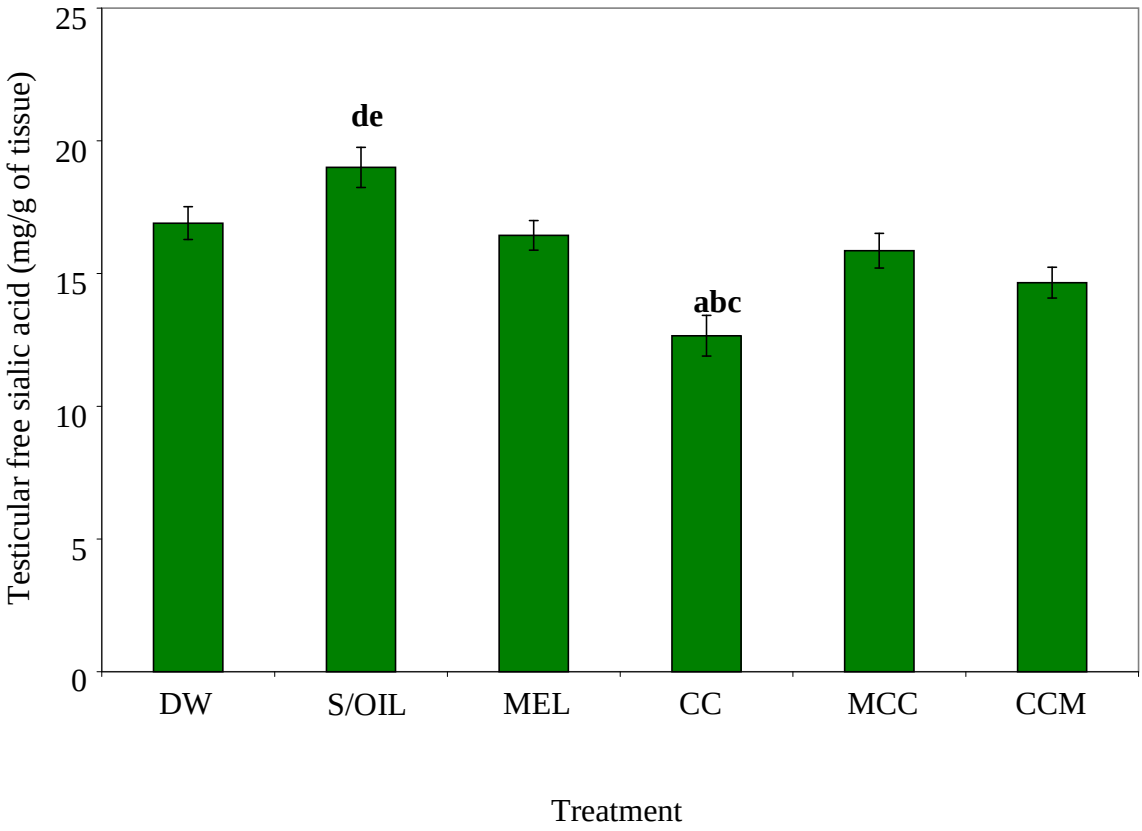


Figure 4.72: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on testicular free sialic acid concentration. [a]P < 0.01 versus DW and MEL groups; [b]P < 0.001 versus S/oil group; [c]P<0.05 versus MCC; [d]P < 0.05 versus MCC group; [e]P < 0.01 versus CCM group.

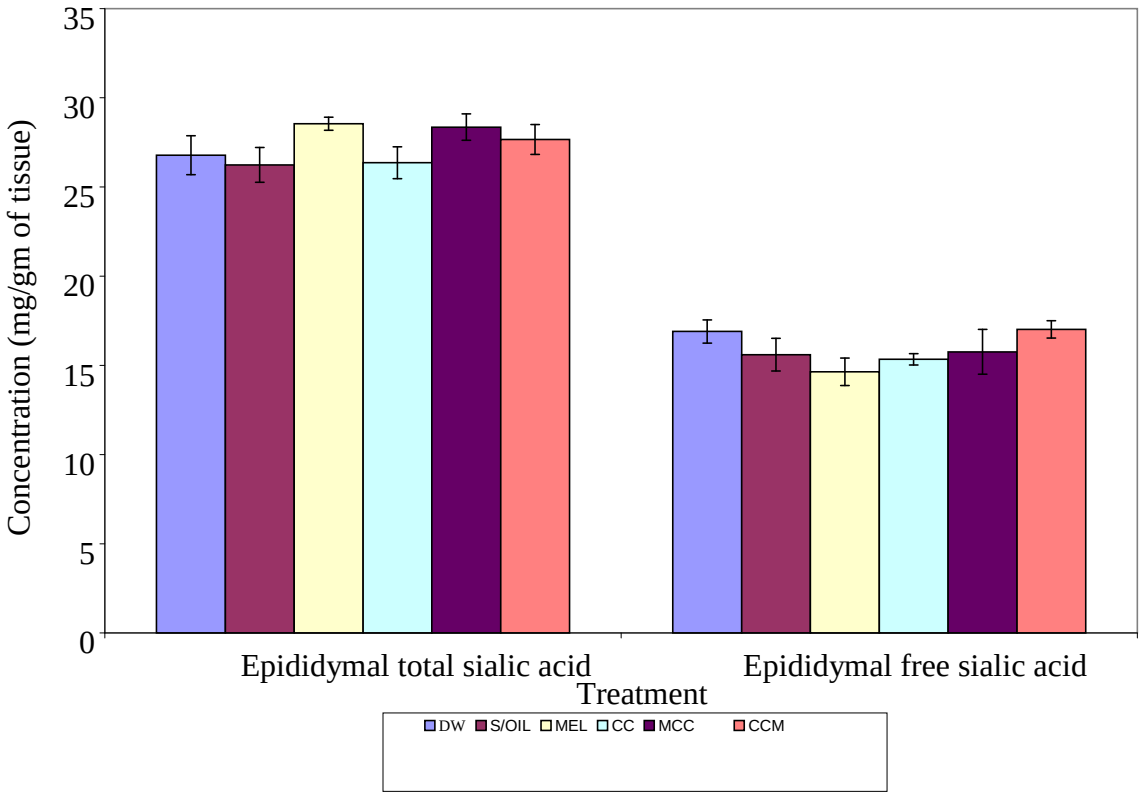


Figure 4.73: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on epididymal sialic acid concentration.

4.9.38 Effect of treatment on epididymal fructose concentration

Figure 4.74 shows the effect of treatment on epididymal fructose concentration. There were no significant differences ($P > 0.05$) in between the groups. However, there was a marginal decrease in the epididymal fructose concentration in the CC group relative to that of the DW (19%), S/oil (15%), MEL (17%) and MCC (17%) and CCM (17%) groups.

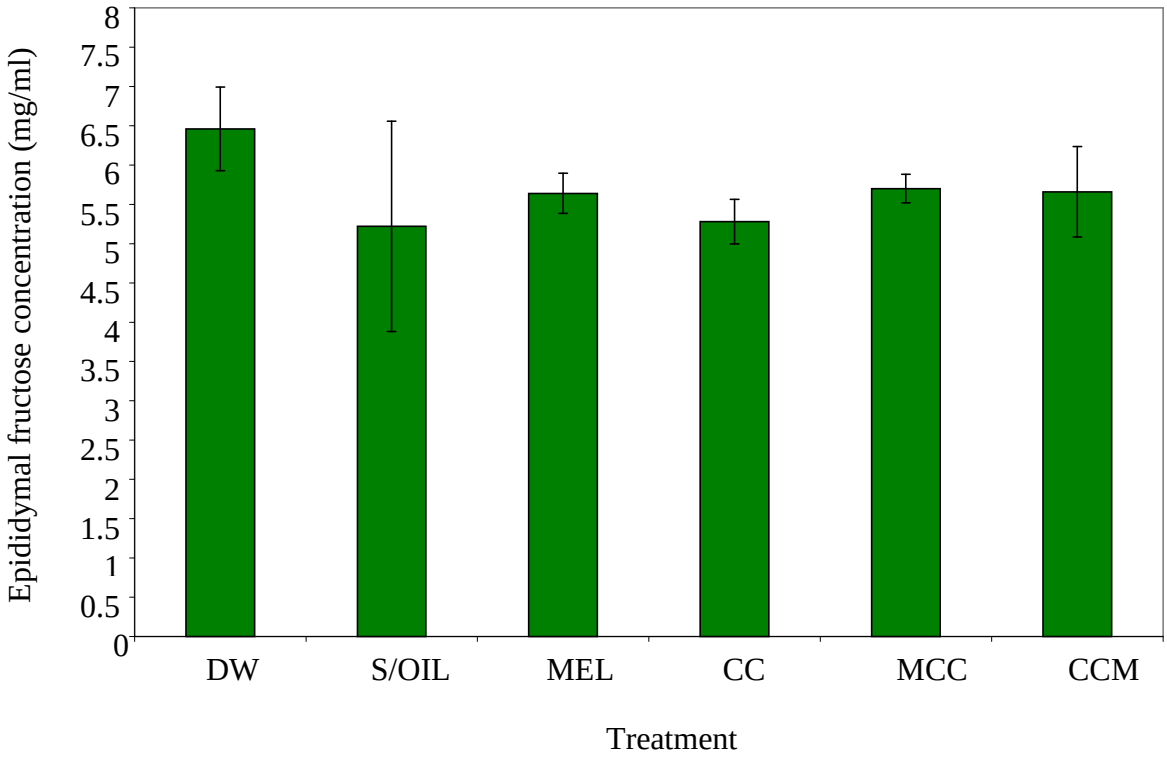


Figure 4.74: Effect of gestational and lactational exposure to distilled water (DW), soya oil (S/oil), melatonin (MEL), chlorpyrifos and cypermethrin (CC), melatonin + chlorpyrifos + cypermethrin (MCC) and chlorpyrifos + cypermethrin + melatonin (CCM) on epididymal fructose concentration.

4.9.39 Effect of treatment on post-mortem gross pathological changes

Post-mortem gross findings in the LD_{50} study include haemorrhagic gastroenteritis, filling of the stomach and intestines with bubbles, petechial haemorrhages of the liver, haemorrhages and congestion, friable and nodular lungs, congested heart, kidneys, testes and brain vessels, flabby thigh muscles and areas of necrosis in the liver.

Post-mortem gross findings in the main studies showed apparently normal findings in all the groups, except for the CC group which showed decreases in the weight of most organs harvested.

4.9.40 Effect of treatment on histopathological changes

Testis: The testicular histoarchitecture of the testes for all the groups is shown in Plates I-V. There was apparently normal testicular histoarchitecture in the DW, S/oil, MEl and MCC groups, respectively. The testicular histoarchitecture of the CC group was characterised by an apparently normal sperm, degenerated spermatocytes, spermatids, and sertoli cells. Degenerated changes in the spermatocytes were recorded in the CCM group.

Epididymis: The cytoarchitecture of the epididymis were apparently normal in each of the treatment groups, when compared to the controls. However, there were degenerative changes in the interstitial (leydig) cells in the CC and CCM groups, in addition to congestion recorded in the former group (Plates VI-X).

Seminal Vesicles: The cytoarchitecture of the seminal vesicles in the DW, S/oil, Mel and MCC groups were apparently normal. However, there were degenerative changes in the epithelial lining of the seminal vesicles in the CC and CCM groups (Plates XI-XV).

Brain: The brain histoarchitecture were apparently normal in the DW, S/oil, MEL, MCC and CCM groups. However, there were degenerative changes in the neurons and glial cells of the CC group (Plates XVI-.XX).

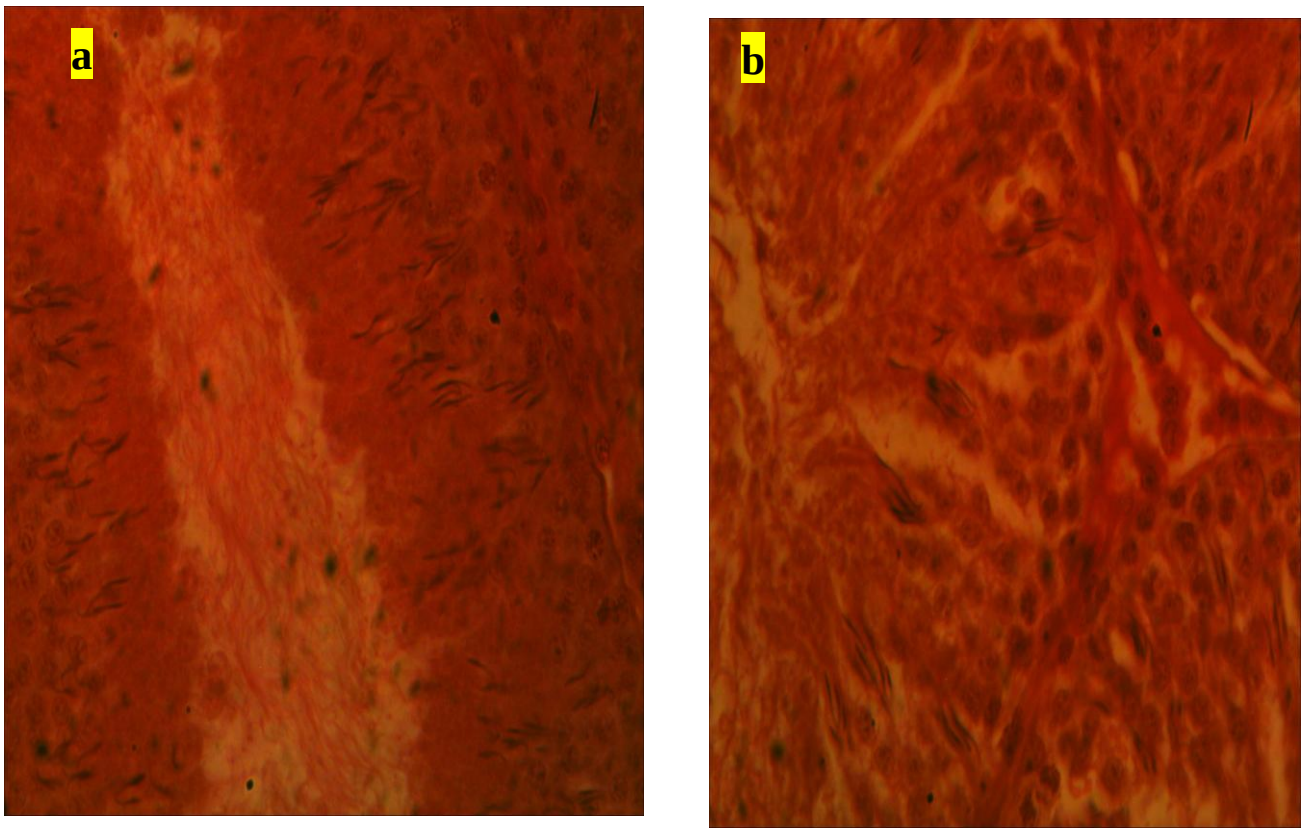


Plate Ia and b: Photomicrograph of the testis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and soya oil (b), respectively showing apparently normal spermatozoa, spermatids, spermatocytes. Haematoxylin and Eosin × 400.

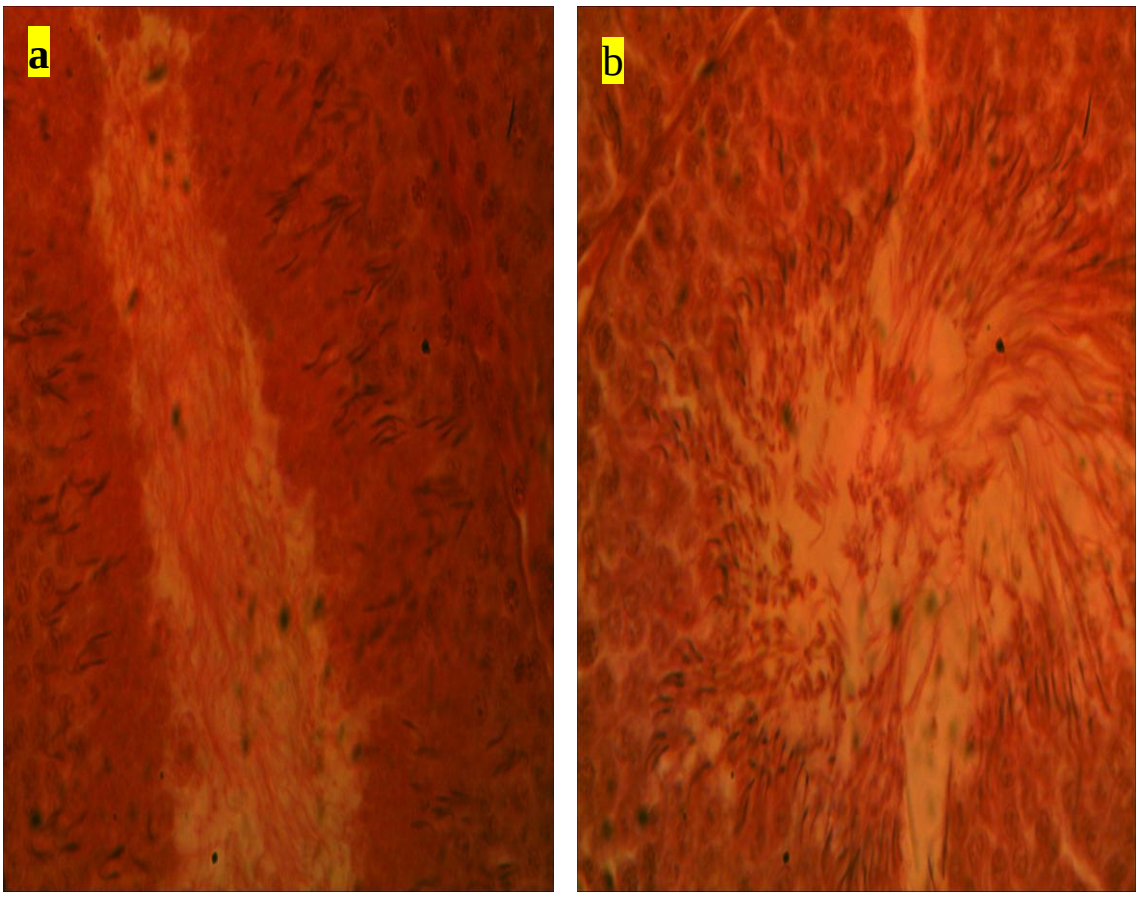


Plate IIa and b: Photomicrograph of the testis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and melatonin only (b) respectively, showing apparently normal testicular architecture, spermatozoa, spermatids, spermatocytes. Haematoxylin and Eosin × 400.

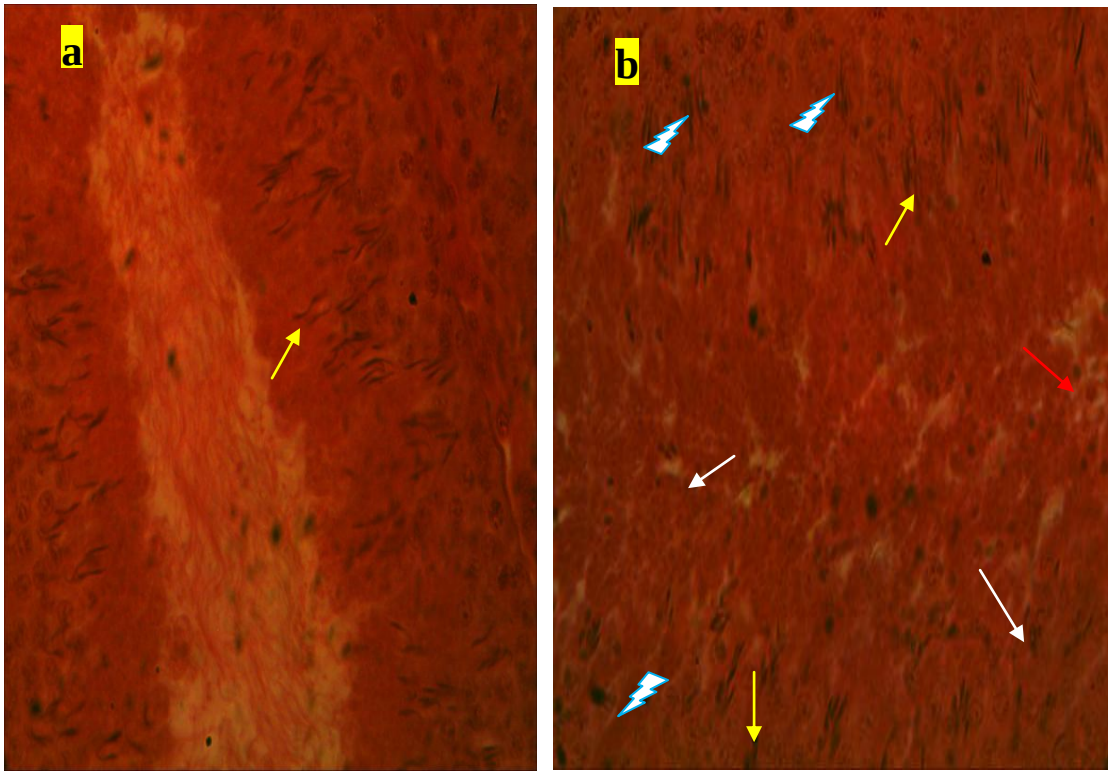


Plate IIIa and b: Photomicrograph of the testis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and chlorpyrifos and cypermethrin (b) showing apparently normal spermatozoa(yellow single arrow), spermatids, spermatocytes; degenerated spermatocytes (lightening bolt), degenerated spermatids (white single arrow) and degenerated Sertoli cells (red single arrow), respevtively. Haematoxylin and Eosin × 400.

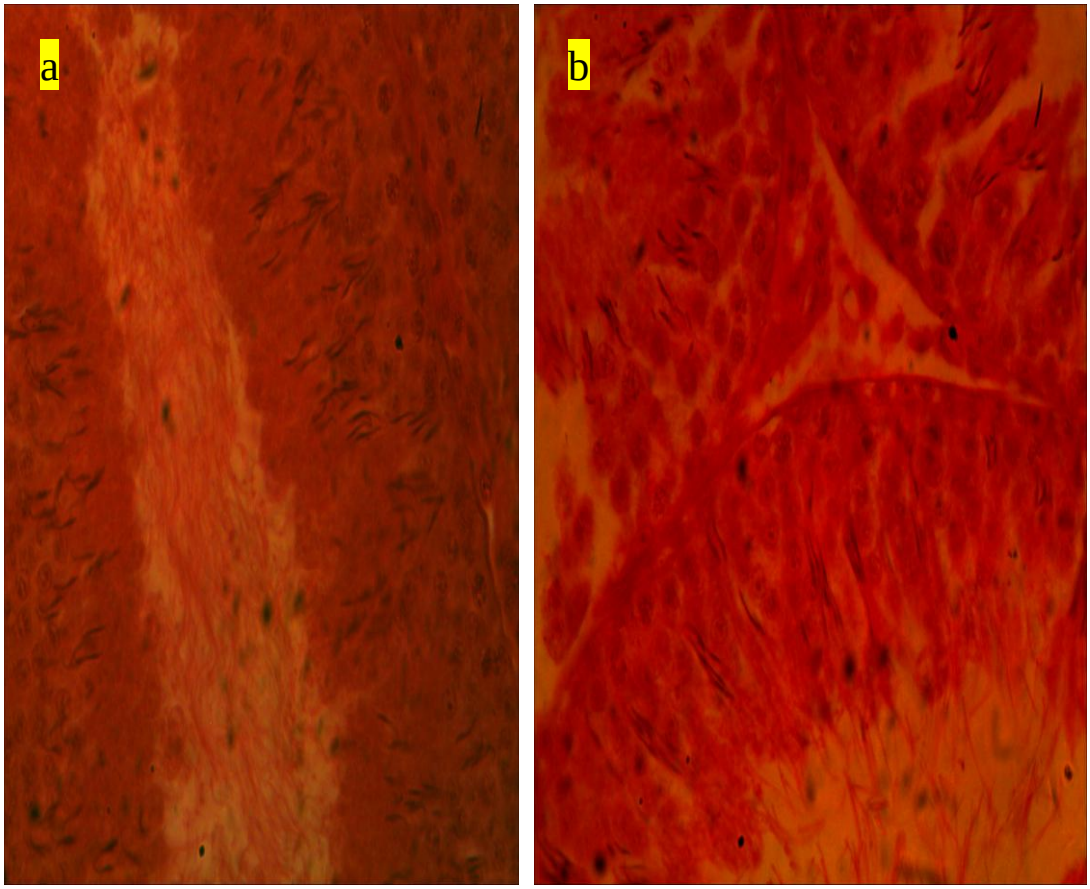


Plate IVa and b: Photomicrograph of the testis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and melatonin pretreatment then followed by chlorpyrifos and cypermethrin (b) respectively showing apparently normal spermatozoa, spermatids, spermatocytes. Haematoxylin and Eosin × 400

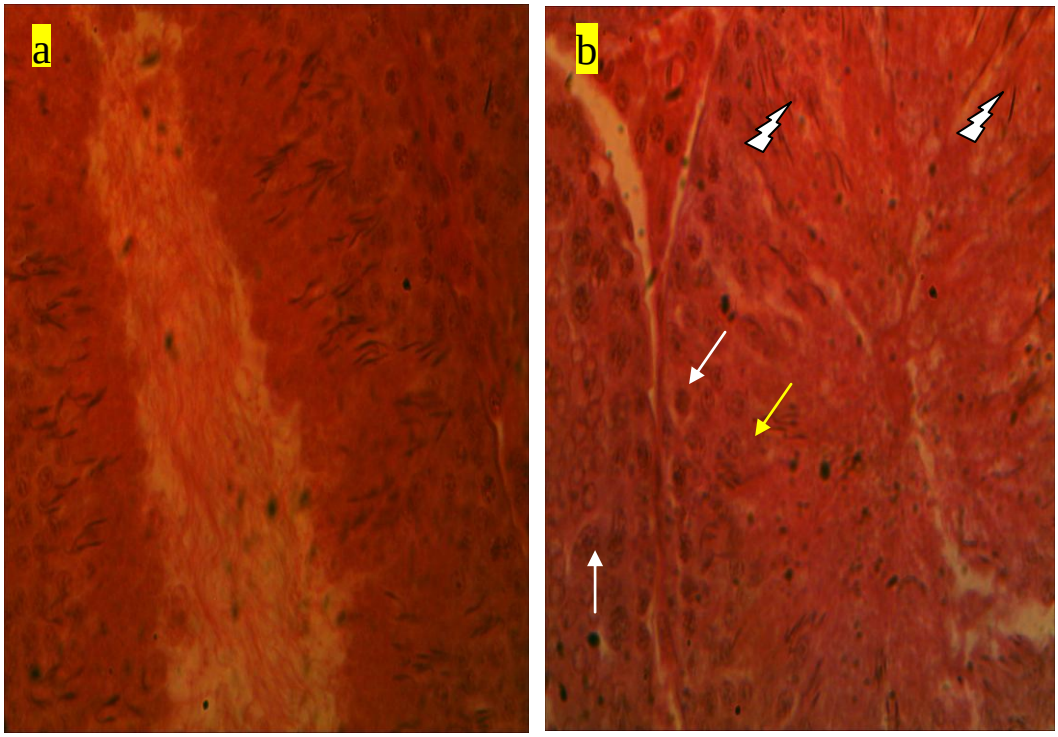


Plate Va and b: Photomicrograph of the testis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and chlorpyrifos and cypermethrin followed by melatonin (b) showing apparently normal spermatozoa, spermatids, spermatocytes; normal spermatozoa (lightening bolt) and spermatid (yellow single arrow); degenerative changes in the spermatocytes (white single arrows) respectively. Haematoxylin and Eosin × 400.

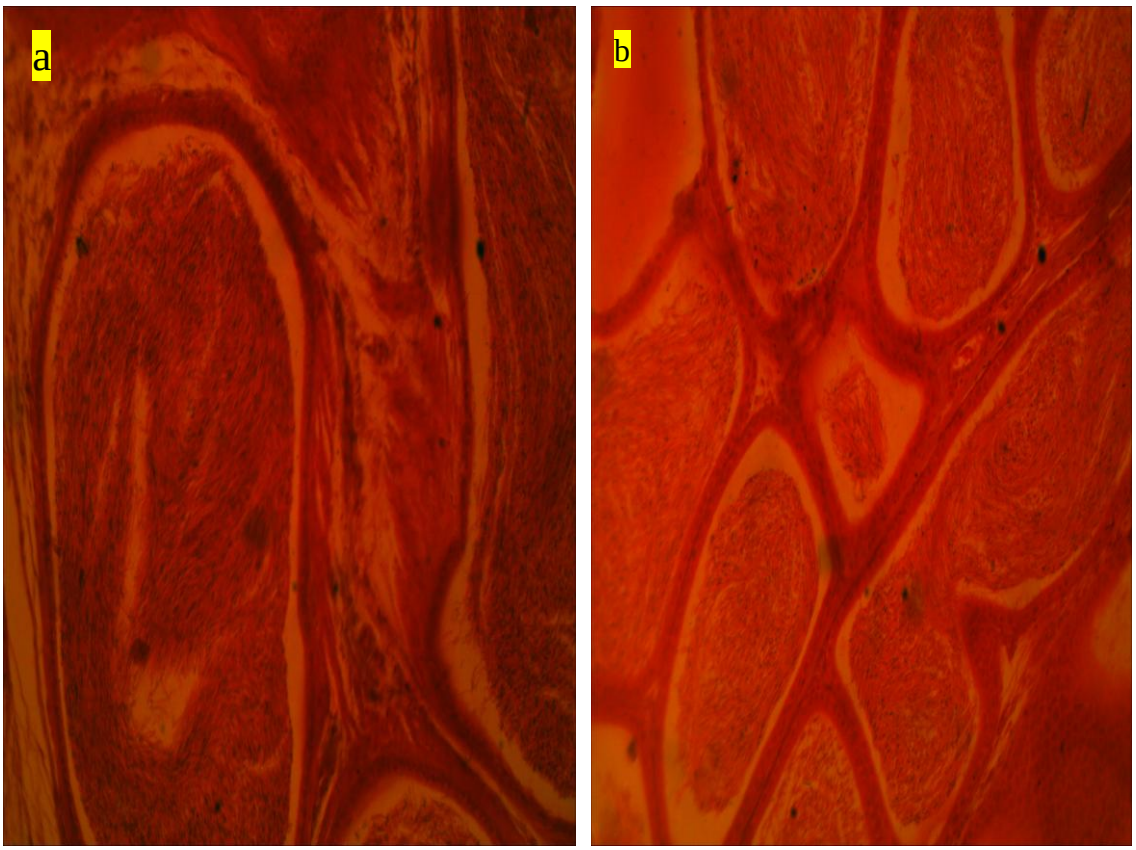


Plate VIa and b: Photomicrograph of the epididymis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and soya oil (b) showing apparently normal cytoarchitecture of the epididymis. Haematoxylin and Eosin × 400.

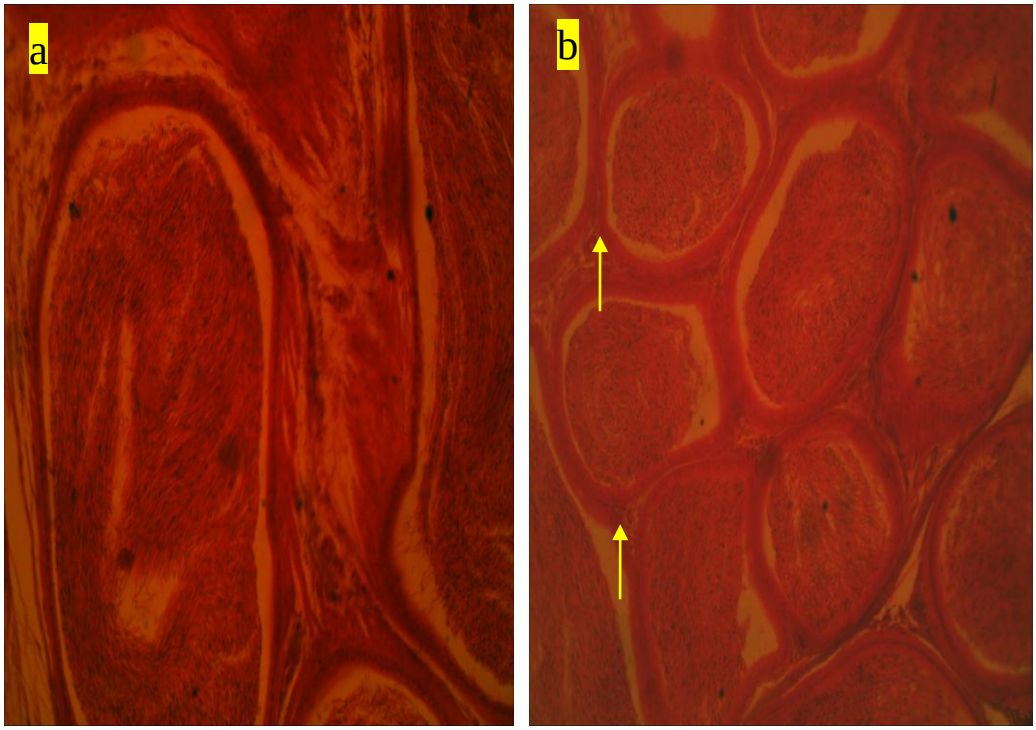


Plate VIIa and b: Photomicrograph of the epididymis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and melatonin (b) showing apparently normal cytoarchitecture of the epididymis (with apparently normal Leydig cells [yellow single arrows]). Haematoxylin and Eosin × 400.

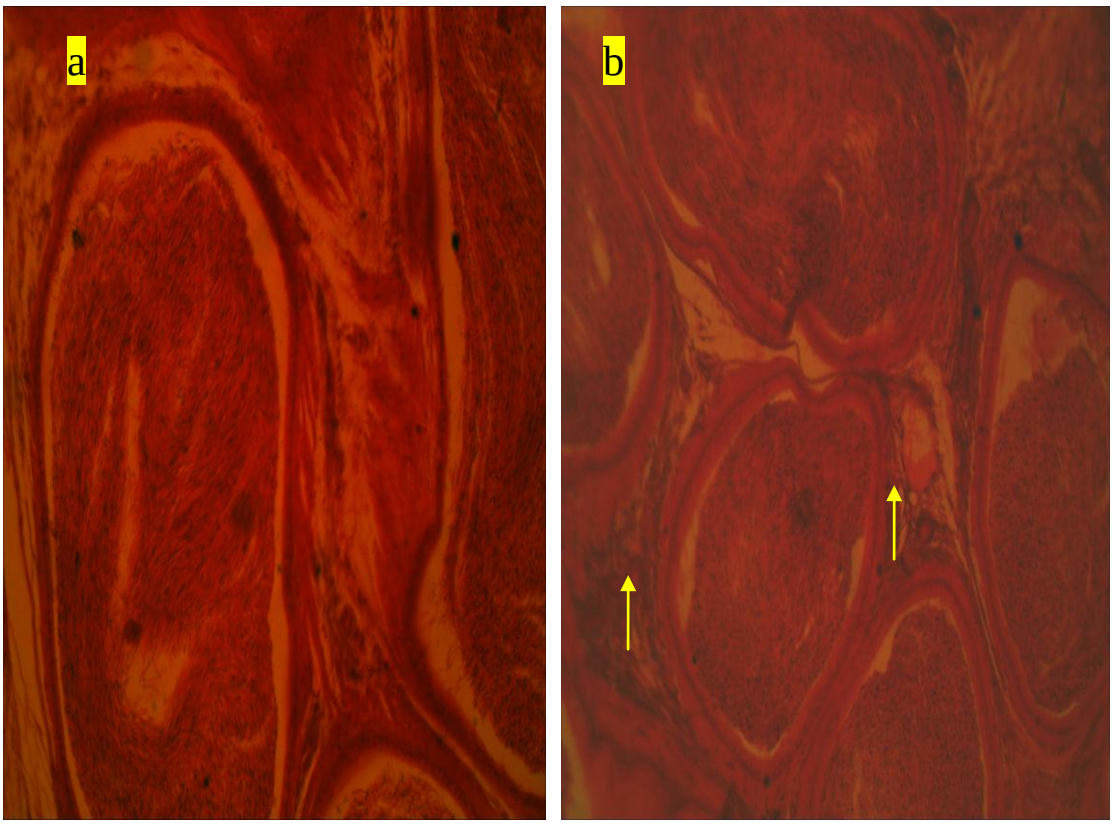


Plate VIIIa and b: Photomicrograph of the epididymis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and chlorpyrifos and cypermethrin (b) showing apparently normal cytoarchitecture of the epididymis but with degenerative changes in the interstitial (Leydig) cells [yellow single arrows]. Haematoxylin and Eosin × 400.

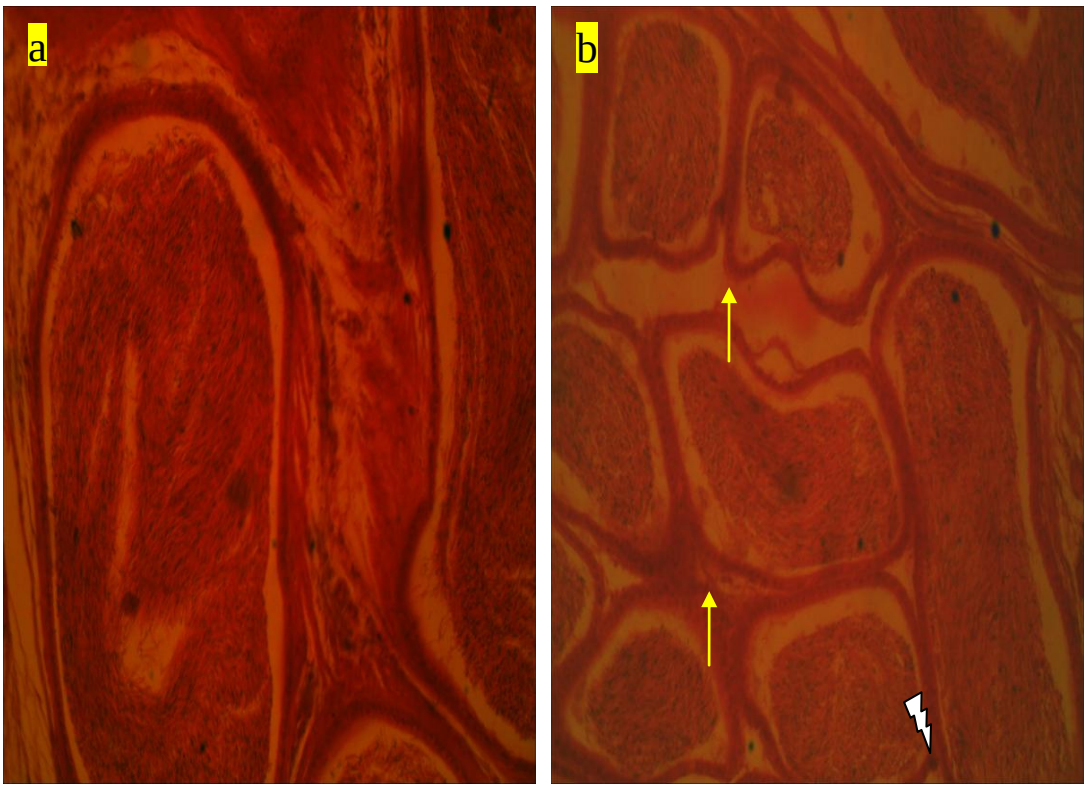


Plate IXa and b: Photomicrograph of the epididymis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and melatonin pretreatment followed by chlorpyrifos and cypermethrin (b) showing apparently normal cytoarchitecture of the epididymis; apparently normal cytoarchitecture of the epididymis with slight congestion (yellow single arrows) and apparently normal Leydig cells (lightening bolt). Haematoxylin and Eosin × 400.

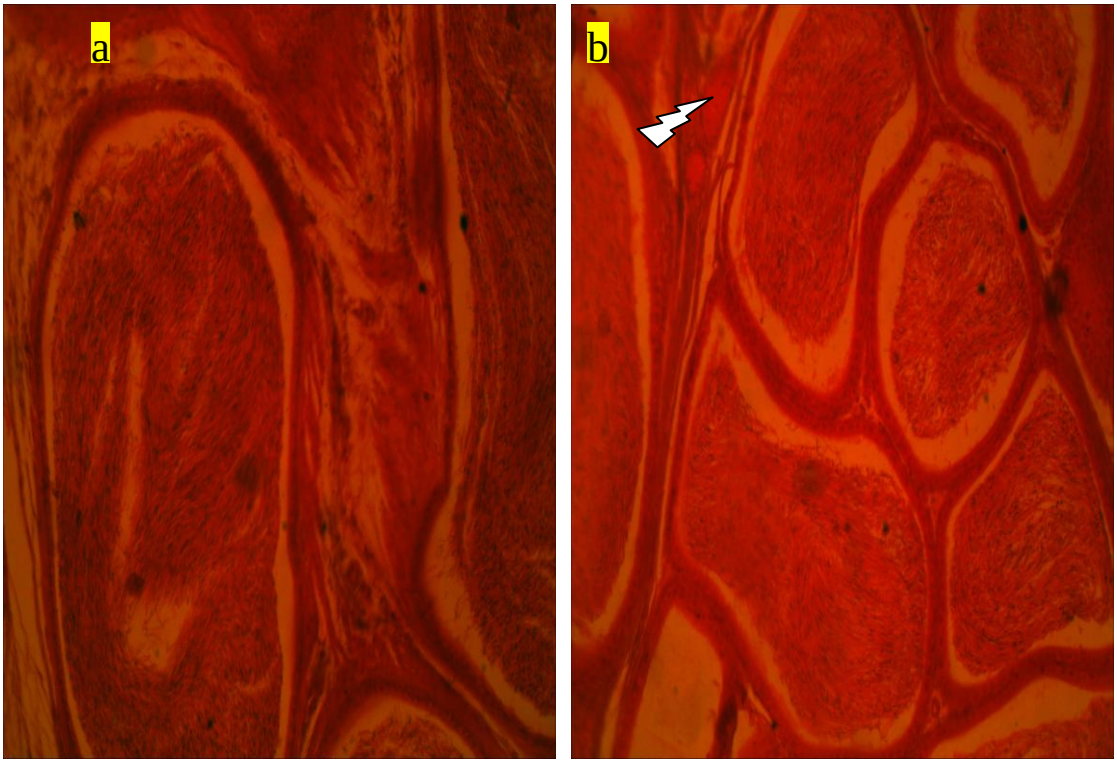


Plate Xa and b: Photomicrograph of the epididymis of F1 male Wistar rat exposed *in utero* and via lactation to distilled water (a) and chlorpyrifos and cypermethrin followed by melatonin showing apparently normal cytoarchitecture of the epididymis; apparently normal cytoarchitecture of the epididymis, but degenerated Leydig cell (lightening bolt). Haematoxylin and Eosin × 400.

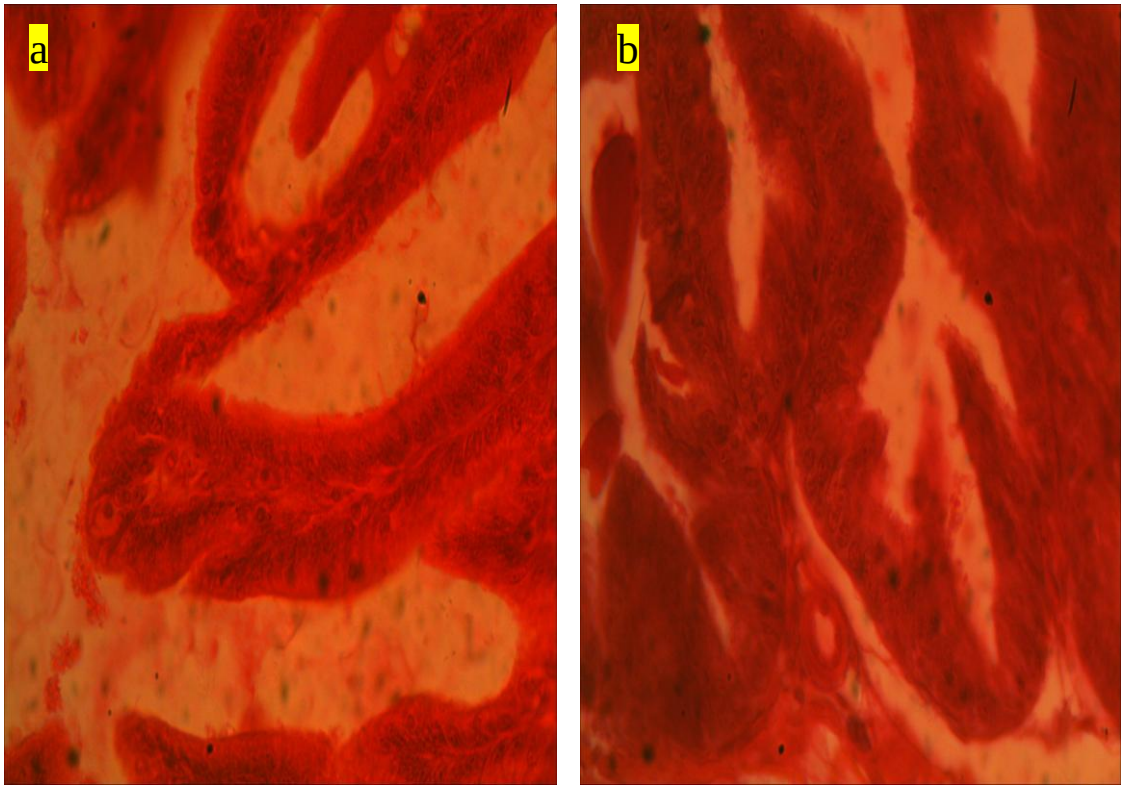


Plate XIa and b: Photomicrograph of the seminal vessicles of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and soya oil only (b) showing apparently normal cytoarchitecture of the seminal vesicle. Haematoxylin and Eosin × 400.

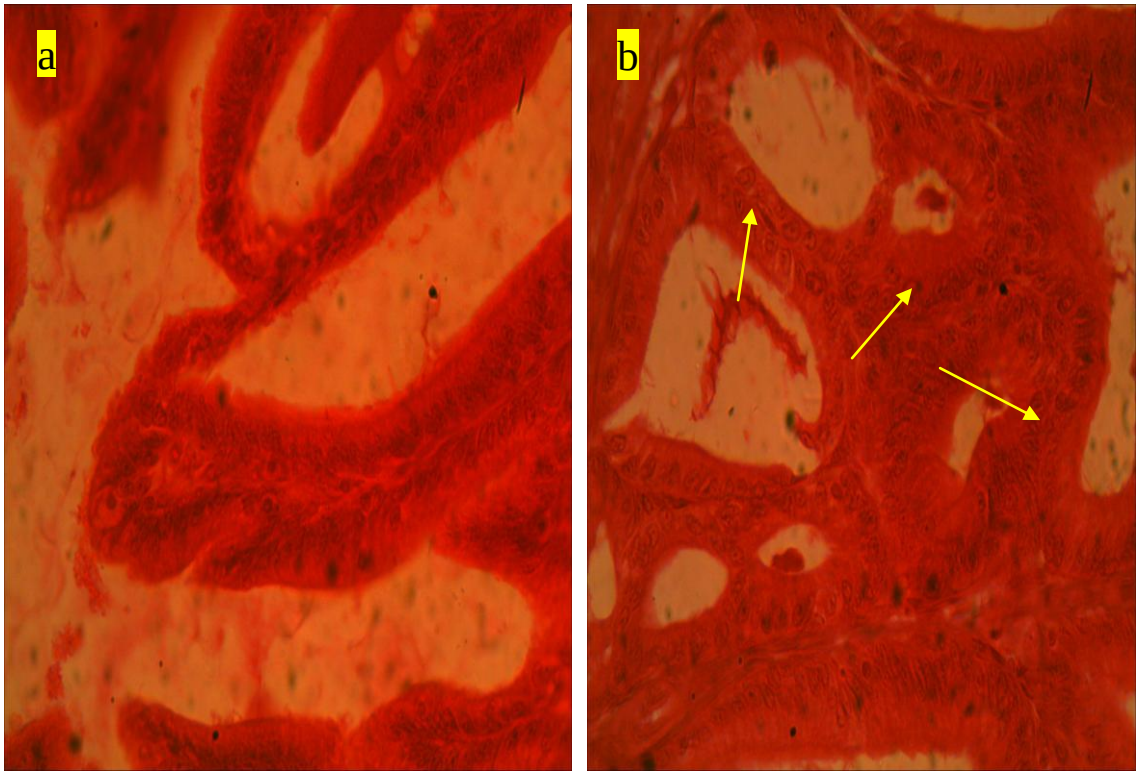


Plate XIIa and b: Photomicrograph of the seminal vessicles of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and melatonin only (b) showing apparently normal cytoarchitecture of the seminal vesicle with healthier epithelial cell linings (yellow single arrows) . Haematoxylin and Eosin × 400.

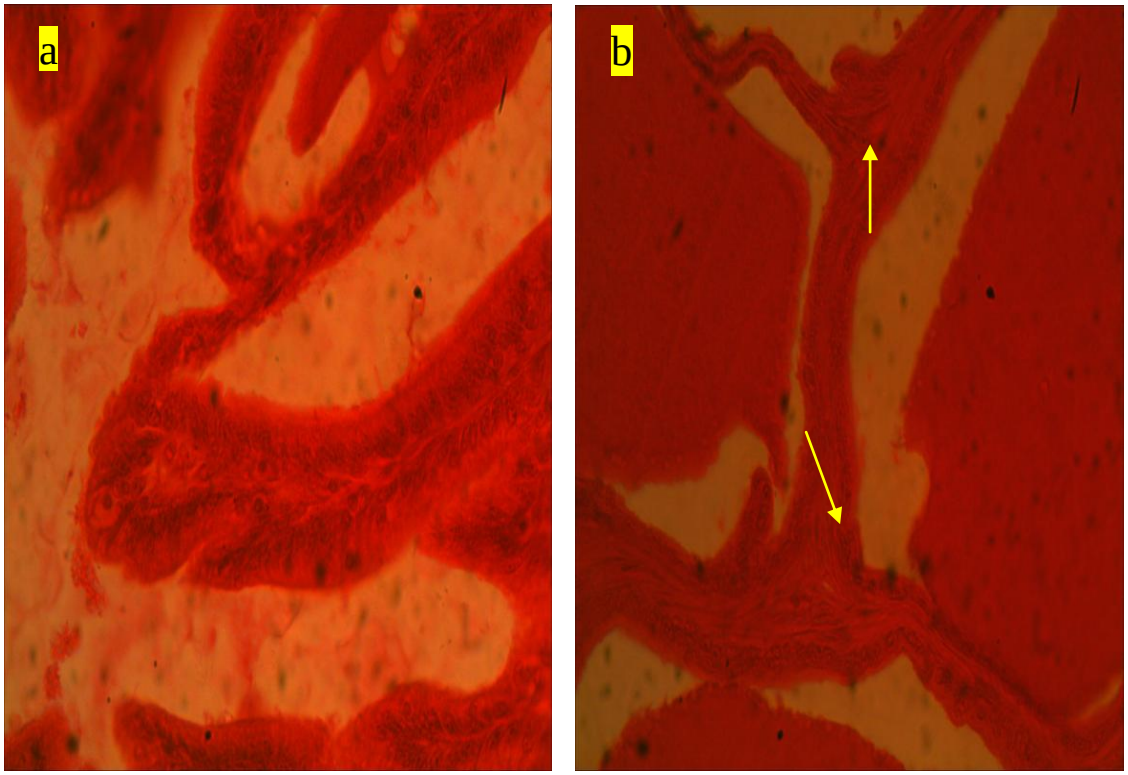


Plate XIIIa and b: Photomicrograph of the seminal vessicles of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and exposure to chlorpyrifos and cypermethrin (b) showing apparently normal cytoarchitecture of the seminal vesicle with some degenerative changes in the epithelial cells (yellow single arrows), respectively. Haematoxylin and Eosin × 400.

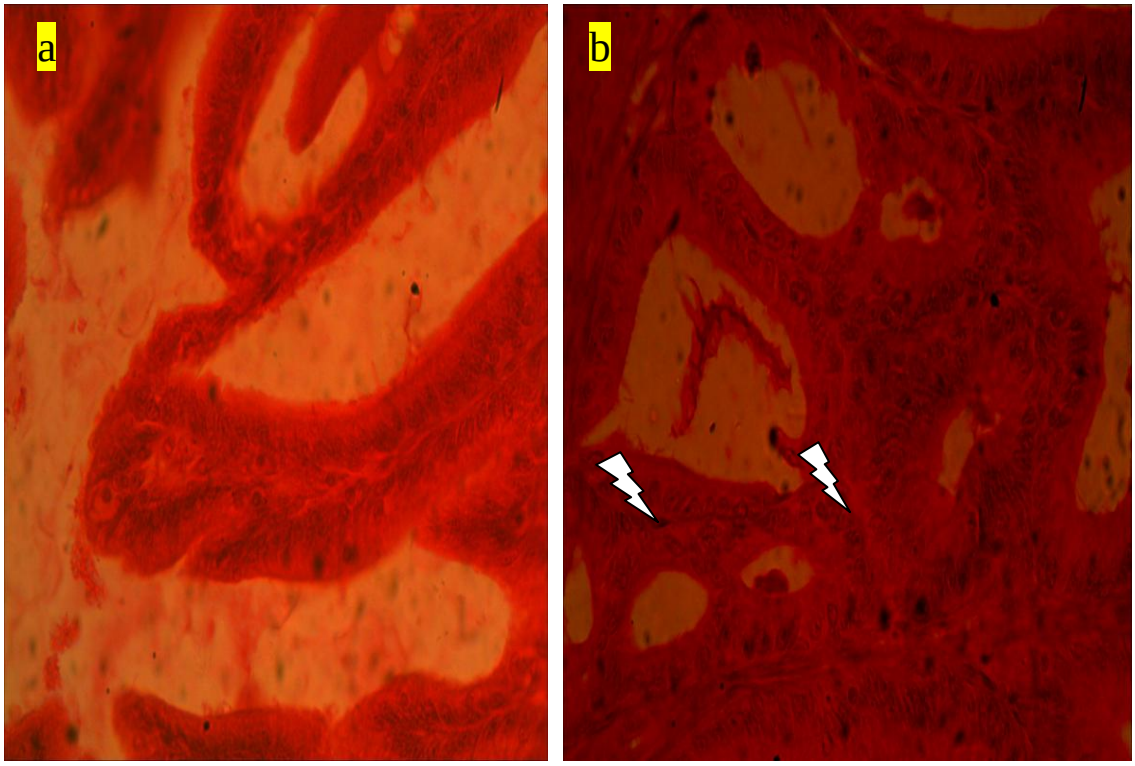


Plate XIVa and b: Photomicrograph of the seminal vessicles of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and melatonin pretreatment before administering mixture of chlorpyrifos and cypermethrin (b) showing apparently normal cytoarchitecture of the seminal vesicle with normal epithelial cell lining [lightening bolt]). Haematoxylin and Eosin × 400.

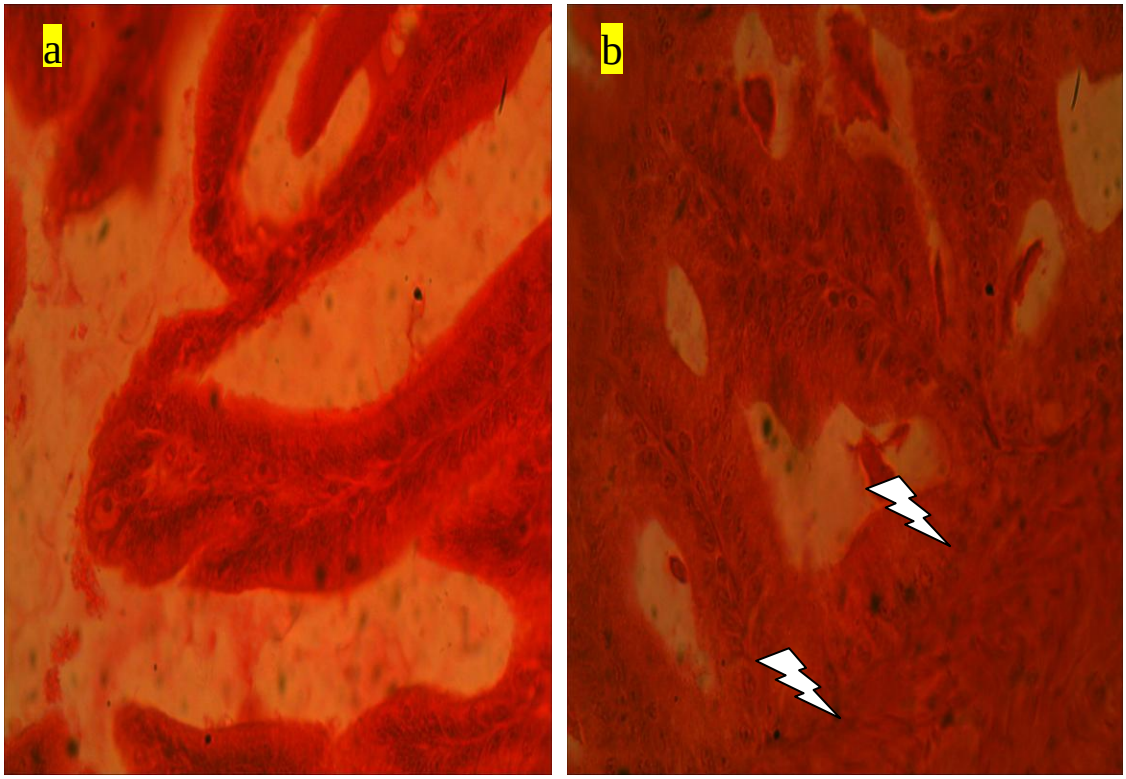


Plate XVa and b: Photomicrograph of the seminal vessicles of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and mixture of chlorpyrifos and cypermethrin followed by melatonin (b) showing apparently normal cytoarchitecture of the seminal vesicle but with some level of epithelial cellular degeneration (lightening bolt). Haematoxylin and Eosin × 400.

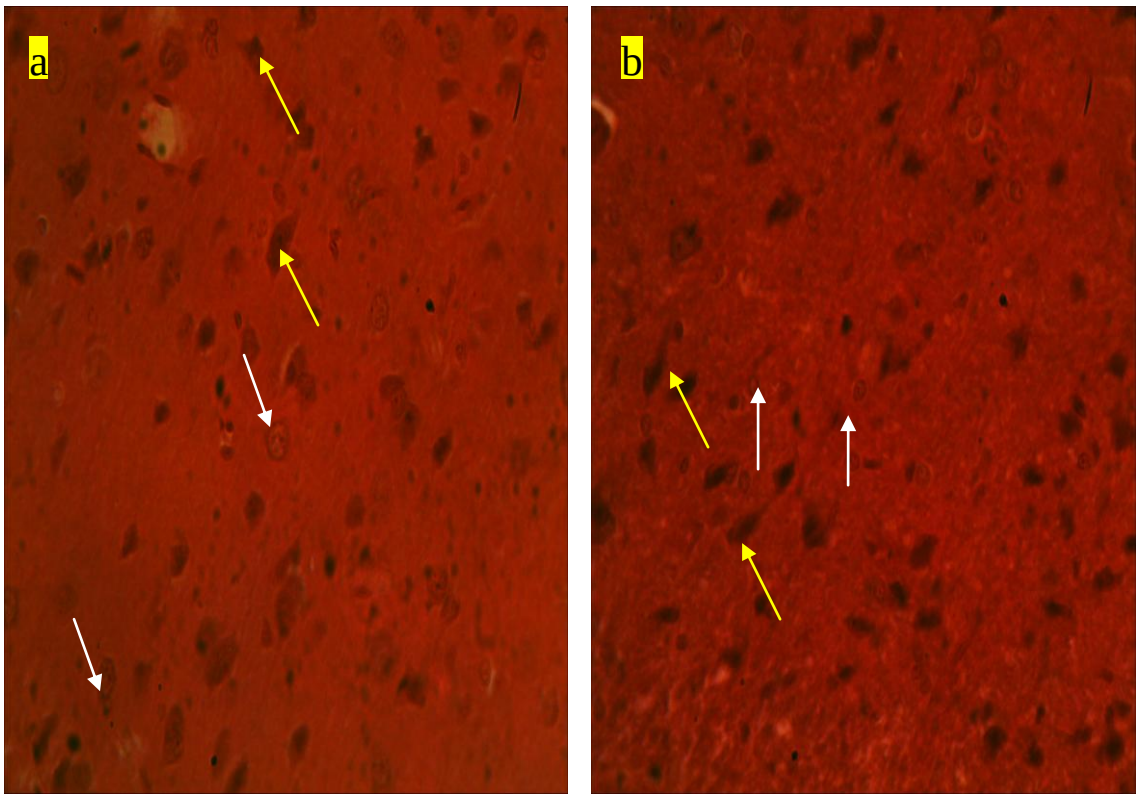


Plate XVIa and b: Photomicrograph of the brain of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and soya oil only (b) showing apparently normal neurones (yellow single arrows) and glial cells (white single arrows). Haematoxylin and Eosin × 400.

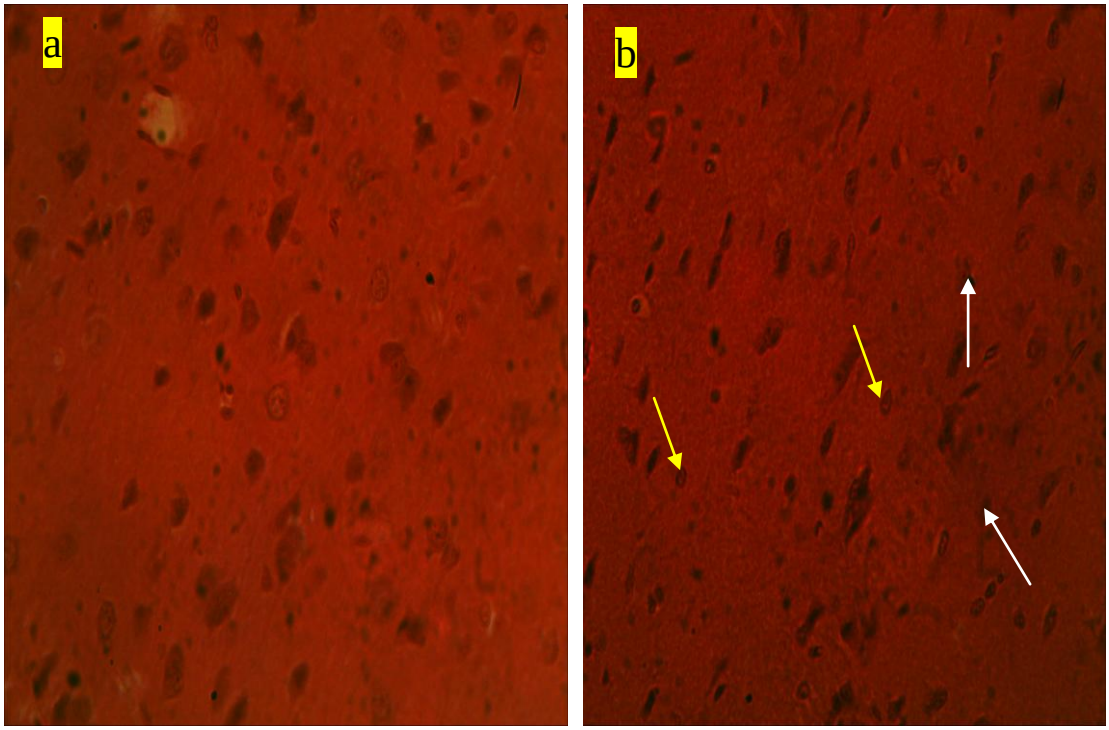


Plate XVIIa and b: Photomicrograph of the brain of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and melatonin only showing apparently normal neurones (white single arrows) and glial cells (yellow single arrows). Haematoxylin and Eosin × 400.

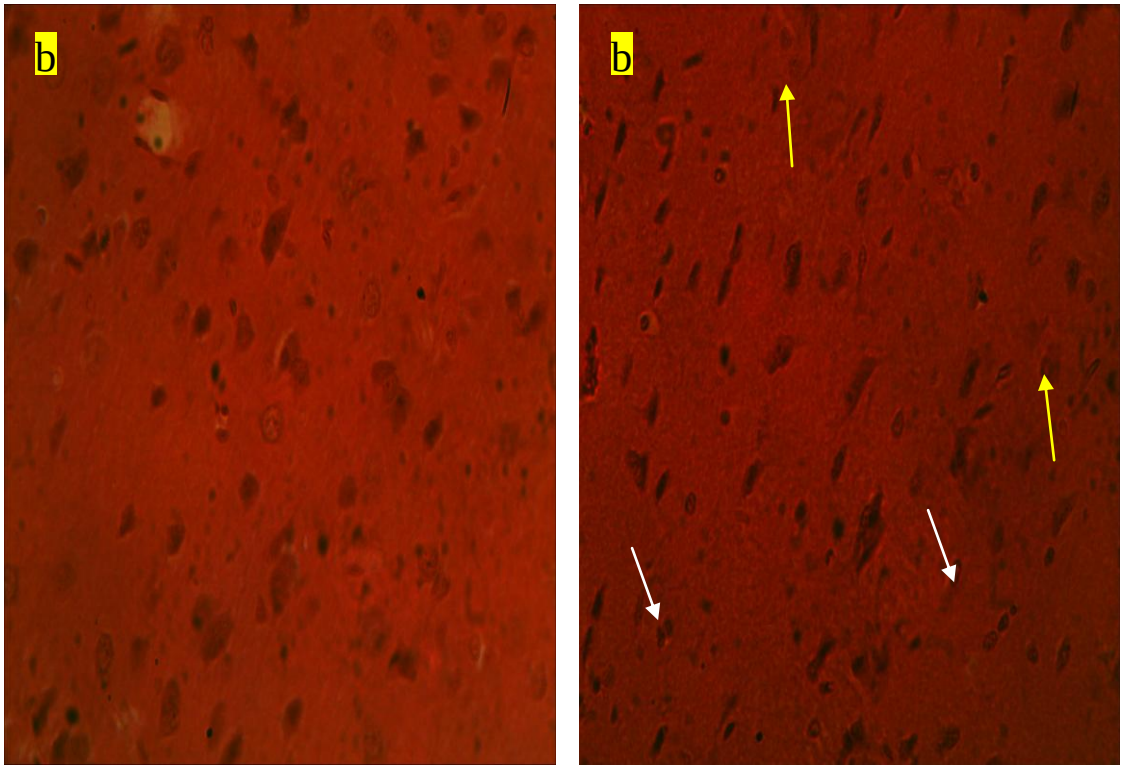


Plate XVIIIa and b: Photomicrograph of the brain of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and chlorpyrifos and cypermethrin only (b) showing apparently normal neurones (a) and glial cells (b); degenerated neurones (white single arrows) and glial cells (yellow single arrows), respectively. Haematoxylin and Eosin × 400.

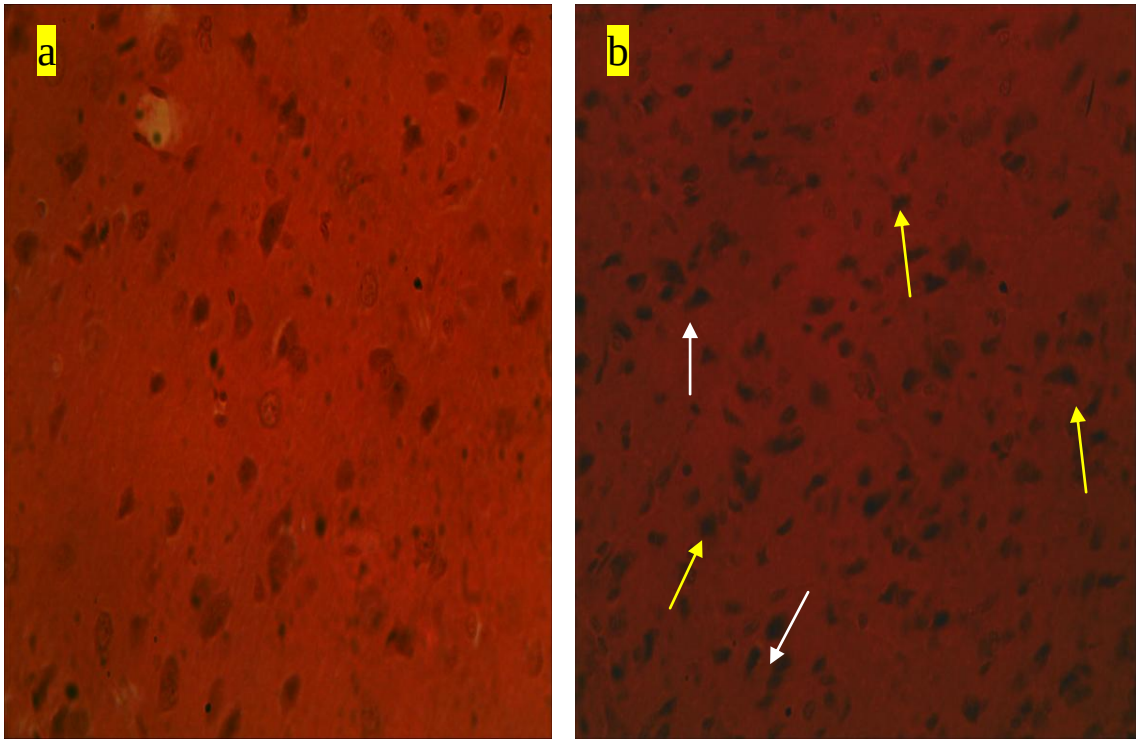


Plate XIXa and b: Photomicrograph of the brain of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and melatonin pretreatment followed by chlorpyrifos+cypermethrin (b) showing apparently normal neurones (white single arrows) and glial cells (yellow single arrows). Haematoxylin and Eosin × 400.

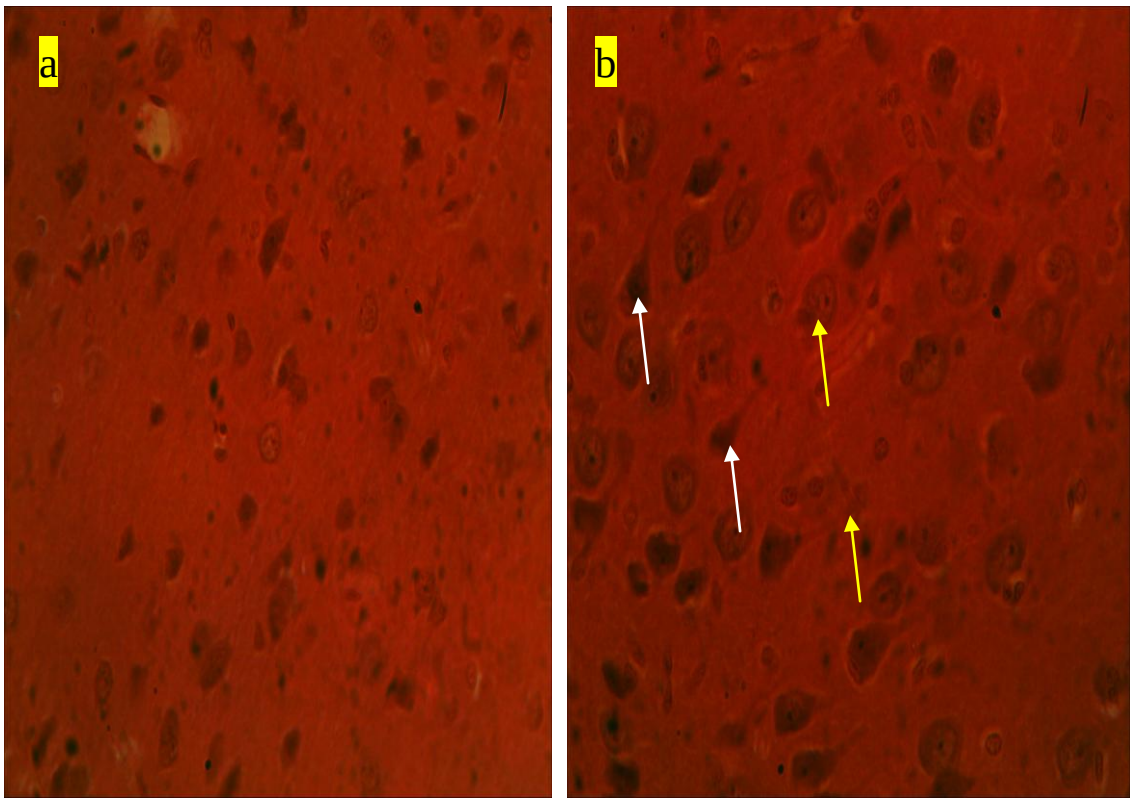


Plate XXa and b: Photomicrograph of the brain of F1 male Wistar rat exposed *in utero* and via lactation to distilled water only (a) and chlorpyrifos+cypermethrin followed by melatonin (b) showing apparently normal neurones (while single arrows) and glial cells (yellow single arrows). Haematoxylin and Eosin × 400.

CHAPTER FIVE

DISCUSSION

The median lethal dose (LD_{50}) for oral toxicity of 95 mg/kg obtained for CPF in the present study agreed with the previous study of 82-350 mg/kg in rats (Drevenkar *et al*., 1993; Ambali, 2009; Savithri *et al*., 2010; Shalaby *et al*., 2013), thus indicating that CPF is a moderately toxic agent (LD_{50} between 50-500 mg/kg) (WHO, 2009). Clinical signs observed include, back-arching, huddling, spastic limb movement, ataxia, convulsion, tachypnoea, paresis, hind limb and quarters paralysis and death. Signs observed in the CPF exposed female rats result from inhibition of AChE causing elevated levels of acetylcholine, a neurotransmitter in synapses of the central and peripheral nervous system, resulting in signs of cholinergic crisis (Shelton *et al*., 2012; Heyer and Meredith, 2017).

The median lethal dose (LD_{50}) of 375 mg/kg obtained for CYP in the present study agreed with the previous study of 82-439 mg/kg (Sheets, 2000; Singh and Saxena, 2014). This confirms the classification of the insecticide as class II moderately hazardous pesticide by the WHO (2009).

The LD_{50} values vary according to the species of animal, sex of experimental animal, age of the animal, amount of food, social environment, routes used, type of agents administered, health status of the animal, physical environment (for example, temperature, relative humidity), and the method used (mathematical and logarithmic) for the LD_{50} determination (Deora *et al*., 2010). The LD_{50} for both chemicals suggest that they are moderately toxic to rats, a non-target organism.

Toxicity signs by dams exposed to CC were muscle tremor, diarrhoea, muscular weakness, huddling, reduced feed intake, convulsion, bloody nasal discharges, salivation, ocular discharges, foetal resorption, cannibalism, dystocia, agalactia and death. This indicates the combined effects of cholinergism and CNS intoxication. In addition, the pups of dams exposed to the combined pesticides showed weakness, bloody nasal discharges, slow reflexes, increased mortality in the CC group relative to the other groups.

The CPF is an OP that is known to cause inhibition of AChE, a β-esterase whose physiological role is that of hydrolysing acetylcholine, a major neurotransmitter in the peripheral nervous system, that is autonomic and somatic nervous systems; and the central nervous system (Costa, 2006). Exposure to CPF during the perinatal period is known to evoke deficits in neuritic outgrowth, specifically including the target of cholinergic projections (Abdelmalek *et al*., 2016).

Cypermethrin is a type-2 moderately toxic lipohilic pyrethroid insecticide that forms long term associations with integral membranous proteins (Michelangeli *et al*., 1990), particularly the ATP-dependent ion channels (Prashanth and David, 2010). Thus, it prolongs the opening of sodium channels in the central nervous system, while simultaneously altering the function of K^+ channels, leading to hypo-polarization and hyper-excitation of the neurones (Smith and Soderlund, 2001; Gowland *et al*., 2002) and causing loss of coordination, muscular tremor, and convulsions (Desi *et al*., 1986). In addition to prolonging sodium channel opening and altering K^+ channels, CYP modulates chloride and voltage-gated calcium channels, alters the activity of glutamate and acetylcholine receptors and adenosine triphosphatases and induces DNA damage

and oxidative stress in the neuronal cells (Singh *et al*., 2012a). Cypermethrin also modulates the level of neurotransmitters, including GABA and dopamine (Singh *et al*., 2012b). In addition, both pesticides have been reported to cross the placenta, apparently due to their lipophilic nature, thereby serving as a source of *in-utero* exposure and interfere with the developing embryo and the foetus (Magnarelli and Guinazu, 2012; Singh *et al*., 2012b; Dewailly *et al*., 2014). The dysgalactia seen in the dam may be due to influence of pesticide on milk let-down, which is directly linked to their interference on hormone regulation. Signs shown by the pups indicate that the combination of CPF and CYP are not selectively toxic to the dam but also developing foetuses following oral exposure. The present study showed that CPF at 1.9 mg/kg and CYP at 7.5 mg/kg induce maternal toxicity with foetotoxicity.

Cannibalism was recorded in rats exposed to combination of the pesticides. It has been reported that maintenance of pregnancy, mammogenesis, lactogenesis, nest building, maternal care and nurturing the neonates is under the influence of the hormone; progesterone (Voci and Carlson, 1973), prolactin (Bole-Feysot *et al.* 1998; Miller *et al.,* 2004) and oxytocin. Thus, behavioural alterations in nest building and nurturing the young ones with considerably high frequency of neonatal cannibalization were considered as outcomes of decreased maternal care that involves alterations in neuro-hypothalamic, pituitary and ovarian hormonal interplay. The incidence of foetal resorption was recorded in the group exposed to CC only. This finding may be attributed to modification of the uterine lining and/or transplacental transfer of the pesticides, with subsequent inhibition of the AChE, and increased opening of the gated channels to influx of Na ions by CPF and CYP (Akhtar *et al*., 2006; Morgan and El-Aty, 2008). Foetal brain AChE has also been reported to have exceptional recovery

capacity when faced with challenge from pesticide exposure and, thus, serve as a form of initial protection (Abdelmalek *et al*.,2016). The amount of pesticide exposure received by the developing foetuses *in-utero* and as neonates via lactation may overwhelm this protective mechanism, thereby resulting in toxic signs recorded in the pups. However, in dams that were pre-treated and post-reated with MEL, the resulting pups showed mild or no apparent signs of toxicity. Melatonin has been reported to reduce the adverse effects, resulting from increased scavenging of ROS generated and increasing the activity and expression of the main body antioxidant enzymes (Tomas-Zapico and Coto-Montes, 2007).

Developmental exposure to neurotoxic chemicals presents significant health concerns because of the vulnerability of the developing central nervous system (CNS) and the immature brain barrier (Lee and Freeman, 2014). Changes in litter size are an important indicator of both developmental (genotoxic, foetotoxic and embryotoxic) and reproductive failure or success. In the current study, oral treatment with CPF and CYP showed a significant decrease in the mean value of litter size in CC group, relative to the other groups. This result is similar to the findings of previous study conducted on CPF (Ambali *et al*. 2009; Shalaby *et al*., 2013) and CYP (Assayed *et al*., 2010b) in rats, which demonstrated decrease litter size. Decrease in the litter size has been documented as a foetotoxic signs of pesticide exposure and attributed to several factors, including the ability of CPF and CYP to cross the placenta barrier and serve as an *in utero* source of exposure (Magnarelli and Guinazu, 2012; Singh *et al*., 2012b; Dewailly *et al*., 2014). These apparently result from the transfer of molecules between the maternal and foetal circulation across membrane of the placenta. Moreover, the placenta interferes with chemical delivery to the foetus, by expressing active membrane transporters and

xenobiotic metabolizing enzymes (Dawson *et al*., 2017). The regulation of these enzymes and transporters and the effects of genetic polymorphisms on their functions may have important implications in foetal and placental exposure to xenobiotics and their potential toxicities (Prouillac and Lecoeur, 2010). Studies have shown that CPF promotes pre-implantation (Ambali *et al*., 2009) and post-implantation (Ambali *et al.*, 2010e) losses, which impact negatively on the litter size. Both CPF (Shittu *et al*., 2014) and CYP (Elbetieha *et al*., 2001; Umosen and Chidiebere, 2014) have been shown to alter sex hormones as a result of oxidative damage to the pituitary gland, thereby altering the release of FSH and LH.

In female animals, FSH and LH play important role in the synthesis of oestrogen and progesterone, respectively. Progesterone and oestrogen coordinate the differentiation of uterus to support implantation and embryo development (Raju *et al*., 2013; Mehl *et al*., 2017). Therefore, pesticides-induced hormonal alterations may interfere with implantation and blastocysts survival, which manifested in lower litter size in the CC group. Furthermore, the ability of OP compounds to cause damage to the Fallopian tube via induction of oxidative stress (Guney *et al*., 2007) may have resulted in creation of unfavourable environment for implantation of the blastocysts, resulting in lower litter size. This is because the Fallopian tube enhances reproductive successes (Talbot and Riveles, 2005) by exclusively conveying gametes in opposite directions and providing a suitable environment for pre-implantation development and transportation of embryo into the uterus for implantation (Guney *et al*., 2007). Exposure of pregnant dams to CPF causes cytogenetic damage to the blastocyst characterised by increased frequency of micronucleus and decrease in embryo cell number (Tian and Yamauchi, 2003). Increased apoptotic damage to embryo observed with exposure to certain pesticides,

including CPF during the pre-implantation and post-implantation period (Rezvanfar *et al*., 2016) may have contributed to the decreased litter size in the pesticide-treated group.

Recent studies indicate that the toxic effects of OPs, including CPF may be associated with the enhanced production of ROS and induction of intracellular oxidative stress, thereby disrupting normal cellular development and differentiation (Altuntas *et al*., 2003, 2004; Gokalp *et al*., 2004), an ongoing process *in utero* and at *post partum*. It has also been reported that CPF generates oxidative stress in various organs in the body through heightened levels of ROS, hydrogen peroxide (H_2O_2), nitrate (NO^-_3) and nitrite (NO^-_2) (Mehta *et al*., 2009). The accumulation of ROS in all the regions of the brain and other tissues may perturb the normal physiological function of a developing foetus and, thus, decrease their survival rates (Nita and Grzyboski, 2016).

Cypermethrin (CYP) can also cross the placental barrier, thereby affecting the physiological functions and the viability of developing foetuses (Dewailly *et al*., 2014). It has been shown to cause morphological and visceral abnormalities in the developing foetuses causing increased post-implantation death (Assayed *et al.,* 2010b). it has been postulated postulated that foetuses are very sensitive to chemical toxicant during critical period of their development (Selevan *et al*., 2000). The present study showed that pre- and post-treatment with MEL improved the litter size in rats, exposed to the pesticide mixture. Previous studies have shown that MEL either alone (Gultekin *et al.,* 2006) or in combination with vitamins C and E (Karaoz *et al.*, 2002) mitigates CPF-induced toxicity, apparently related to its antioxidant and anticholinesterase effects (Karthi and Shivakuma, 2015). Melatonin has also been reported to reduce oxidative assault to the

growing foetus by the pesticide by serving as a guard in a wide range of molecules such as lipids, proteins and deoxyribonucleic acid (DNA) from oxidative damage (Reiter *et al*., 2004). Melatonin acts as a direct ROS scavenger (Allegra *et al.*, 2003; Mihandoost *et al*., 2014) and as an indirect antioxidant, by stimulating antioxidant enzymes production (Reiter *et al.*, 2000; Rodriguez *et al.*, 2004) and synthesis of glutathione (Urata *et al.*, 1999). It also has the capacity to supplement the activities of other antioxidants (Gitto *et al.*, 2001). It has been proven to be greatly valuable in lowering molecular damage under conditions of eminent oxidative stress (Blanco *et al*., 2017). The ability of MEL to mitigate oxidative damage may have provided for conducive environment in the maternal environment for implantation and growth, and reduce deleterious alteration in physiological activities in the developing embryo.

The study showed a significant decrease in the livability of pups from dams co-exposed to CPF and CYP. This result showed that a greater number of rats in the CC treated group were born dead. Cypermethrin has been reported to induce decrease in live born foetuses (Assayed *et al*., 2010b; Madu, 2015). This may be due to the cytotoxic effect of the two insecticides. Cypermethrin has been found to induce DNA damage (Hocine *et al*., 2016) and chromosomal aberrations (Huang *et al*., 2016), disturb the activities of sex steroids (Jin *et al*., 2011; Ahmad *et al*., 2012) and cause hormonal disruptions (Molavi *et al*., 2014; Campos and Freire, 2016). Evidences indicate that CYP exposure inflicts oxidative stress, leading to DNA damage and apoptosis (Jin *et al.*, 2011).

The decrease in livability recorded in CC group may have resulted from the cytotoxic effect of CPF, causing genotoxic effects through DNA damage and cell apoptosis (Mehta *et al.*, 2008; Li *et al*., 2015a). Similarly, CYP has been shown to cause DNA

damage in different animal models and humans (Hocine *et al*., 2016; Vardavas *et al*., 2016). Prenatal exposure to pesticides, including CPF has a residual effect on the foetus (Mullins *et al*., 2015; Rauh *et al*., 2015; Evans *et al*., 2016; Reyna *et al*., 2017; Silver *et al*., 2017**);** thus, exerting negative outcome on developing young animals. Decrease in live/dead ratio may have also resulted from deficient PON-1, an enzyme responsible for the detoxification of pesticides in exposed organisms, and not well developed in children and young animals (Mackness and Mackness, 2015; Furlong *et al*., 2016).

The increase in live/dead ratio in groups pre- and post-treated with MEL shows that the agent has the potential to counteract pesticide-induced toxicity by protecting against ROS-induced DNA damage and protein oxidation (Ghayomi *et al*., 2015). Melatonin has also been reported to decrease the level of certain enzymes; like caspases 3 and 9, responsible for the induction of apoptosis (Espino *et al*., 2011a), thereby reducing apoptotic damage and subsequent death in the offspring.

Foetal development is a dynamic process that includes changes in morphology, anatomy, physiology, biochemistry and general growth (Roger and Kevlock, 2001). A change in litter weight is an indicator of foetotoxicty of a test substance (El-Baz *et al*., 2015). These pesticides may reach the foetus during gestation and after birth (post-natal) via breast-feeding. Result from the present study showed a decrease in the dynamics of litter weight of F1 generation of CC group throughout lactation period. This result was in agreement with previous findings following CPF (Zama *et al*. 2007; Morgan and Abd-El-Aty, 2008; Ambali *et al*., 2009) and CYP (Madu, 2015) exposure. However, Wang *et al*. (2011a) recorded no change in litter weight in mice exposed to CYP. Farag *et al*. (2007) have also observed that CYP, when given orally to female rats resulted in

significant reduction in body weight gain in dams and pups (Al-Hamdani and Yajurvedi, 2010). The young pups are more susceptible to the effect of pesticides. The higher level of sensitivity of the neonatal animals to pyrethroid toxicity has been attributed to partial ripeness of the enzymes, which catalyse the pyrethroid metabolism in the liver of juveniles (Farag *et al*., 2007).

Gestational exposure to CPF has been shown in previous studies to decrease litter size (Ambali *et al*., 2009). Perera *et al*. (2003) found an association between CPF exposure and low-birth weight among African-American population, while Whyatt *et al*. (2004) reported an association between umbilical cord plasma CPF levels and foetal birth weight that decreases among minority women living in New York City during pregnancy. Maternal pesticide exposure can result to a fall in placental efficiency, disproportionate intrauterine growth retardation and a reduction in birth weight. CPF interferes in the critical cellular processes such as cell division (mediated through inhibition of DNA replication), differentiation, gene expression and regulation (Qiao *et al.,* 2001). Therefore, the relative decrease in the pattern of weight changes in the groups co-exposed to the two pesticides may be partly due to cholinergic and oxidative stress, engendered by the pesticides.

Groups pre-treated and post-treated with MEL showed a significant increase in body weight from PND 0 to PND 21. The placenta has been shown to express melatonin receptors and melatonin protects against oxidative damage induced in rat placenta. Maternal treatment with MEL in the present study may have improved placental efficiency and, restored birth weight, probably due to an increase in the expression of placental Mn-SOD and catalase by virtue of the up-regulation of the placental

antioxidant enzymes (Richter *et al*., 2009). In addition, MEL improves blood supply to the developing foetus through improvement in uterine blood perfusion, hence it improves nutrient supply to the growing foetus (Lemley *et al*., 2012). Melatonin may enhance DNA repair capacity by affecting several key genes, involved in DNA-damage responsive pathways (Liu *et al*., 2013). Studies have also shown that MEL may protect DNA from free radical sources (Mishra *et al*., 2015).

Crown-rump length (CRL) comparison is an important parameter that indicate intrauterine growth retardation in response to the foetal exposure to a noxious environmental chemical substance (like insecticides) (Raees *et al*., 2010). The present study showed a decrease in the mean CRL of F1 generation rats in group co-exposed the two pesticides, indicating decreased foetal growth rate. Previous work showed that gestational exposure to CPF (Ambali *et al.,* 2009*;* Shalaby *et al*., 2013) and CYP (Raees *et al.,* 2010) caused decreased CRL. There has been association between foetal growth retardation among women during pregnancy and CPF residue in the umbilical cord (Whyatt *et al*., 2004). These findings indicated embyrotoxicity and foetotoxicity of CPF and CYP. The retarded growth of newborns, whose dams were exposed to OP compounds has been attributed to an alteration of the activity of milk lipase enzyme, with a diminished secretory function in the mammary gland, leading to interference with the nursing of the offsprings (O'Sullivan *et al*., 2015).

In addition, the ability of CPF to cross the placenta barrier owing to its lipophilic nature may have caused direct cytoxicity to the developing foetus thereby impairing its growth and well-being. Furthermore, optimal intrauterine environment is vital for optimal growth and development of the foetus. Chlorpyrifos and CYP, through their ability to

induce oxidative stress and some other forms of stress, may have created unfavourable environment for optimal foetal growth, observed in the present study as reduction in CRL. The lower foetal growth in the CC group may also be related to endocrine-disrupting property of the two insecticides, since decreased anogenital distance (AGD) was recorded in the group. The anti-androgenic effect may have led to oestrogen supervening, as oestrogenisation inhibits pituitary growth hormones (Mnif *et al*., 2011), thereby impairing foetal growth.

The present study, however, showed that pre-treatment and post-treatment with MEL caused significant increase in CRL, relative to the group co-exposed to the insecticides, indicating improvement in the foetal growth and decreased foetotoxicity. The improved foetal growth engendered by MEL may be due to its antioxidant property. Melatonin, a hormone secreted from the pineal gland, has been extensively studied for its antioxidant properties (Perez-Gonzalez *et al*., 2017; Soleimani *et al*., 2017; Mironczuk-Chodakowska *et al*., 2018). It has the ability to up-regulate and down-regulate the activity of various antioxidant enzymes, and it also enhances the actions of other antioxidants such as ascorbate and tocopherol (Hardeland and Pandi-Perumal, 2005). Melatonin, through its antioxidant and cholinesterase restoration properties, may have provided better intrauterine environment, necessary for foetal growth, in addition to reducing cytotoxicity evoked by the insecticides.

Anogenital distance is a sexually-dimorphic measure of genital development and a marker of endocrine disruption in animals and humans, and has been used to gauge reproductive toxicities (Eisenberg *et al*., 2011; Liu *et al*., 2014). The AGD is dependent on prenatal exposure to androgens, which stimulate the growth of the perineum

(Clemens *et al*., 1978; Zehr *et al*., 2001; Bowman *et al*., 2003; Ostby and Gray 2004). As a rule of thumb, the AGD measure in male rats is about two-fold greater than the distance in female rats, and the same applies for humans (Clemens *et al*. 1978, Ostby and Gray 2004, Salazar-Martinez *et al*. 2004). Therefore, AGD is longer in males than in females. When AGD is longer in females, the purported mechanism is by inducing "labioscrotal fusion"; in normal males fusion begins caudally and proceeds ventrally, presumably androgens in females act the same way (Callegari *et al*., 1987). Measurement of AGD in males have been shown to correlate with changes in semen parameters (Eisenberg *et al*. 2011; Mendiola *et al*. 2011).

Although not significant, AGD in the F1 generation of rats exposed to CC in the present study was relatively lower than any of the other groups, including groups treated with MEL. This result indicates that the two pesticides have some degree of anti-androgenic effect, which agrees with findings of previous work in CPF (Ambali *et al*., 2009) and other pesticides (Swan *et al*., 2005) that reported a low AGD. Newborn male rodents have no scrotum, and the external genitalia are undeveloped; only a genital tubercle is apparent for both sexes. The distance from the anus to the insertion of this tubercle, the AGD, is androgen-dependent and about twice as long in males as in females (Swan *et al*., 2005). Anti-androgens are chemicals that interfere with the normal function of androgens and other male hormones. Exposure to such chemicals, particularly during periods of vulnerability prenatally, can result in feminisation of males (Tamura *et al.*, 2001).

In rodents, perineal growth is dihydrotestosterone-dependent (Bowman *et al*., 2003), and males have a greater AGD than the females (Marty *et al*., 2003). Although the

mechanisms of anti-androgenic effect of CPF have not been elucidated, fenitrothion, an OP insecticide, has been shown to bind avidly to the androgen receptor in cell culture, and blocks androgen receptor activity (Tamura *et al*., 2001). Studies have shown that CYP exerts anti-androgen effects in androgen receptor reporter gene assays (Sun *et al*., 2007; Pan *et al*., 2013) and can induce oestrogen receptor (ER) transactivity. The developing foetus is extremely sensitive to the hormonal environment in the uterus, and natural differences in hormone levels surrounding rat or mouse foetuses of only 10 – 12 minutes has been shown to influence the timing of sexual development and the behaviour of the animal in adult life (Tyler *et al.*, 1998). In adult men, shorter anogenital distance predicted poorer semen quality and hypogonadal testosterone levels, while studies have shown that prostate cancer patients had shorter anogenital distances than healthy adult men (Mendiola *et al.*, 2011; Castano-Vinyals *et al*., 2012; Eisenberg *et al*., 2012a). Anogenital distance in humans has been also suggested as a novel marker of adult testicular function (Eisenberg *et al*., 2012a, 2012b, 2012c).

In addition, in a work conducted by Taxvig *et al*. (2013), mixtures of CYP and other pesticides were found to have no effect on AGD. Leydig cells are the only cells that produce testosterone in the testes. They originate from the differentiation of SF-1-positive progenitor cells and begin to produce testosterone around embryonic day 16, which occurs under the influence of testosterone, causing male urethra to begin to develop an increase AGD similar to hypospadias (Li *et al*., 2015b). Although recent findings show that the normal growth of the male urethra does not depend upon testosterone throughout the entire embryonic development period, a programming window exists during which testosterone is critical. Blocking testosterone activity during the developmentally critical window will cause hypospadias and shortened

AGD. However, during other stages, inhibiting testosterone has less influence on urethral development and AGD (Welsh *et al*., 2008). In rats, the critical testosterone reliant stage is generally between embryonic day 16 and 18, therefore it is suggested that exposure to CC delayed testicular maturation, which causes impairment of testosterone secretion during the critical development of urethral development. This findings may have been partly responsible for the apparent decrease in the AGD in the offspring of the rats, exposed to the insecticide mixture.

The apparent restoration of the insecticide-induced deficit in AGD of F1 male rats in group pre- and post-treated groups shows that MEL was able to control the endocrine disruption. This may be partly related to the antioxidant effect of MEL. The ability of MEL to protect the Leydig cells of the developing fetus from insecticide-induced oxidative damage, thereby retaining its capacity to produce testosterone during the critical stage, enhances urethral development and, hence, normal AGD development.

The increase in time of ear opening is an evidence of delay in time of unfolding of the ear. This indicates effects on physical characteristics of somatic development in the neonate (Farkas *et al*., 2009). The result agrees with the result of previous work, which recorded a slight delay in the time of ear unfolding in F1 generation rats gestationally exposed to CPF (Maurissen *et al*., 2000) and CYP (Husain *et al*., 1992; Singh *et al*., 2012b). However, contrary to the observation in the present study, Hoberman (1998) recorded no significant change in the time of pinna opening in the F1 generation, although the period of gestational exposure in his study was two weeks. The delay in the opening of the very important landmark may be attributed to both the maternal and foetal toxicity, engendered by the pesticide. The ear is the vertebrate sense organ of

hearing and balance. Neural crest cells (NCC), notable for varied contributions to other parts of the head and body, contribute multiple tissues to the ear. In higher vertebrates, the ear is composed of three distinct compartments, the inner, middle, and outer ear, each requiring developmental contribution from NCC (Sandell, 2014). Stimuli from the skeletal muscles play a significant role in the formation of the external ear, postulated in the present study to also be responsible for the delay in the time of opening of the ear. Melatonin was able to regularise pinna opening, possibly by reducing both the maternal and foetal toxicity, engendered by the pesticides possibly by its antioxidant properties.

The significant delay in time of eye opening in F1 generation of dams in the CC group indicates that the insecticide combination impaired this important developmental landmark. Husain *et al.* (1992) and Farag *et al*. (2007) demonstrated the ability of CYP to cause delay of eye opening in rats. The delay in eye opening may be partly due to retarded synaptogenesis of primary visual cortex (VI) (Gandhi *et al*., 2005) apparently due to oxidative stress provoked by the insecticide mixture. Oxidative stress plays an important role in synaptogenesis through the activation of mitogen protein activated kinase (MAPK) signalling pathways (Milton and Sweeney, 2012). Such stress-activated pathways include extracellular signal-regulated kinase (ERK), p38 mitogen activated kinase (p38MAPK), and c-Jun-N terminal kinase (JNK) signalling cascades, which are all well-known potent regulators of synaptic function. These signalling pathways are activated and tightly regulated by the action of oxidative stress, particularly JNK, opening the potential for cross-talk between synaptic responses and the oxidative state of the neurone (Milton and Sweeney, 2012). The JNK has many phosphorylation targets and one that is critically important, both in synapse development and the stress response, is AP-1. The AP-1 is a transcription factor composed of heterodimers of the

leucine zipper proteins Fos and Jun. At a cellular level, AP-1 acts downstream of JNK in synapse development to positively regulate both synapse size and strength (Sanyal *et al*., 2002). The ERK is also activated in response to oxidative stress, while also known to regulate synapse development through AP-1 (Milton and Sweeney, 2012). Delay in eye opening as experienced in the CC group has negative consequence on cortical maturation (Gandhi *et al*., 2005). This is because around the time the eyes open, the primary visual cortex (V1) is undergoing considerable synaptogenesis. Reflecting this new synapse growth, the input layer of V1 shows a rapid rise in the frequency of spontaneous excitatory synaptic events (Desai *et al*., 2002). Just as ocular dominance plasticity turns on the cAMP-responsive element (CRE) transcription factor - a molecule that coordinates activity-dependent synaptic rearrangement - in the cortex (Pham *et al*., 1999), so does eye opening (Cancedda *et al*., 2003).

Pretreatment with melatonin was able to reduce the time of eye opening, apparently due to the mitigation of oxidative stress provoked by the insecticide mixture, which allows the normal process of synaptogenesis of the primary visual cortex (VI) to proceed normally. Melatonin may have reduced the activation of MAPK signaling pathways as antioxidants have been shown to reduce the activation of p38 MAPK (Gaitanaki *et al.*, 2006). The implication of the reduction in the time of eye opening in the melatonin-pretreated group is early maturation of the cortex.

The increase in the time of testicular descent in the CC group in the present study indicates changes in physical parameter of sexual maturation (Farkas *et al*., 2009). This shows that CC delays the time of sexual maturation in neonatal rats. The delay in testicular descent observed in the present study might indicate pesticide infraction on

hormone homeostasis, since some pesticides can adversely affect reproduction and development (Sharma *et al*., 2014a). Such effects have been postulated to involve multiple pathways within the reproductive tract and hypothalamic–pituitary–gonadal axis, consequently promoting endocrine-disrupting effects (Gore *et al*., 2015). These findings indicates that pesticides can induce perinatal and postnatal endocrine disruption after gestational and lactational exposure.

In most mammals, the testes must descend from the abdomen to an extracorporeal position to provide a lower ambient temperature for normal spermatogenesis. Sertoli cells produce anti-Mullerian hormone (AMH) during a critical short window in early development to cause regression of Mullerian structures. Leydig cells produce testosterone in large concentrations to act in a paracrine manner to stabilise the Wolffian ducts and systemically to masculinise the external genitalia. Later, high intratesticular concentrations are required for spermatogenesis. All these androgen effects are mediated by the ligand binding to the intracellular nuclear androgen receptor (AR) and activating androgen-responsive genes (Gobinet *et al*., 2002). The Leydig cell also produces insulin-like 3 (INSL3) that is at least partly controlled by human chorionic gonadotrophin (hCG) and luteinising hormone (LH) (Foresta *et al*., 2008). This peptide, by binding to its receptor leucine-rich repeat-containing G protein coupled receptor 8 (LGR8), is also a factor in the control of testicular descent (Hughes and Acerini, 2008). The time of gestation when the testes have completed their descent into the scrotum varies considerably amongst different mammals (Amann and Veeramachaneni, 2007). The human testes descend generally at birth, in common with the rabbit, pig and horse. By contrast, cattle and deer have testes fully descended early in gestation, whereas this is considerably delayed after birth in the dog. Irrespective of the timing, all models

share in common a two-phase process of transabdominal and inguinoscrotal descent, which is controlled by different mechanisms (Hughes and Acerini, 2008). Descent of the testis from an intra-abdominal site in foetal life to an extracorporeal location after birth is a mandatory developmental process to ensure that the mature testes promote normal spermatogenesis. The two phases of transabdominal and inguinoscrotal descent occur approximately during the first and last thirds of gestation respectively. Key anatomical events to release the testis from its urogenital ridge location and to guide the free gonad into the scrotum are the degeneration of the cranio-suspensory ligament and a thickening of the gubernaculum. Androgens play a role in both these processes, particularly with respect to enabling the testis to traverse the inguinal canal in the final phase of descent. Experiments in animals suggest that androgens mediate this effect via the release of calcitonin gene-related peptide by the genitofemoral nerve. The transabdominal phase of descent is under the control of insulin like 3 (INSL3), a product of the Leydig cells. Definitive evidence of its role in rodent testis descent is illustrated by the phenotype of bilateral cryptorchidism in Insl3K/K null mice. Circulating levels of INSL3 are higher in males at puberty, are undetectable in females and are lower in males with undescended testes (Hughes and Acerini, 2008). The delayed testicular descent recorded in the CC group may have been partly due to disruption in androgen synthesis, since its optimal production is essential to avert cryptorchidism. Most importantly, the normal development of the male reproductive system depends on the crucial role of androgen within an early foetal time window, called the masculinisation programming window (Welsh *et al.,* 2008), which influences the reproductive capacity throughout life. Thus, any chemical that disrupts the synthesis or action of androgens, can have deleterious consequences for the developing male genital tract and appear to be risk factors for testicular dysgenesis syndrome (TDS) (Bay

et al., 2006). Therefore, the abnormality in semen parameters recorded in the F1 generation of dams co-exposed to CPF and CYP may have had its genesis from the delayed testicular descent. The ability of both pesticides to induce oxidative damage to the Leydig cells may have also impaired its ability to produce INSL3, further compromising the testicular descent.

The improvement in the time of testicular descent in the FI generation of the group pretreated with melatonin may be due to its antioxidant effect, which protects the organs such as the pituitary gland and the Leydig cells, responsible for the production of androgens and INSL3 from insecticides-provoked oxidative damage. Previous study has shown the ability of melatonin to mitigate CPF-evoked disruption of the function of the pituitary-gonadal axis (Umosen and Chidiebere, 2014). Furthermore, MEL has been reported to influence sexual maturation and reproductive functions via activation of its receptors and binding sites in the hypothalamic-pituitary-gonadal (HPG) axis in both male and female (Shi *et al*., 2013; Jin and Yang, 2014).

The present study shows delay in the surface righting reflex as shown by an increase in the reflex righting time in F1 generation male Wistar rats, exposed to CC compared to other groups. This finding agrees with result of previous studies that CPF (Chanda and Pope, 1996; Dam *et al*., 2000; Carr *et al*., 2001; Ambali and Ayo, 2012) and CYP (Hussain *et al*., 1992; Farag *et al*., 2007; Nair *et al*., 2011) increase surface righting reflex in animal model. The righting reflex requires the integrity of muscular and motor function for the adequate acquisition of symmetrical coordination between the left and right sides of the body (Dierssen *et al.*, 2002). Righting reflex appears soon after birth in

rodents, and its correct performance aparently depends on the integration between vestibular system and the motor system (Fox, 1965; Altman and Sudarshan, 1975). The delay in surface reflex righting time observed in the CC group reflected impairment of integration between the vestibular system and motor system, partly due to the pesticide-induced increased ROS generation, leading to oxidative damage to the neuromuscular system. The delay in righting reflex in the CC group may be due to interference with ACh metabolism arising from AChE inhibition by CPF and CYP insecticide (Ambali and Ayo, 2012; Abreu-Villaca and Levin, 2017). Similarly, lipoperoxidative damage to the muscle may have contributed to the sensorimotor deficits in the CPF group. Therefore, melatonin, a potent antioxidant ameliorated the deficit in the righting reflex caused by the pesticide mixture by its cholinesterase restoration effects. Based on its antioxidant effect, melatonin may reduce the pesticide-evoked impairment of integration between the vestibular system and motor system, thereby restoring the righting reflex.

The decrease in the ability to walk in a 1-minute observatory period in the Fi generation of dam in the CC group between PND 8 to PND 15, compared to other groups signifies progressively impaired neuromuscular coordination and increased sensory motor inhibition. The depressed locomotion may be associated with inhibition of AChE and subsequent accumulation of ACh at neuromuscular junctions and increased neuronal degeneration from increased ROS production by the two insecticides. It may also be associated with changes in the neurochemical capacity of the juvenile rats. In the CNS, ACh plays an important role in the development of the pathways necessary for normal function, including cognitive function and working memory (WM), indicating the ability to maintain and process physically absent information for a short period of time. This vital cognitive function has been related to cholinergic neuromodulation and, in

independent work, to theta (4-8Hz) and alpha (9-14Hz) band oscillations (Winters and Bussey, 2005; Hasselmo, 2006; Benjamin, 2016; Eckart *et al*., 2016). Since the pathways are still developing in juvenile animals, any compound that alters the signals for development may disrupt the establishment of appropriate and functional pathways, necessary for normal physiology and behaviour. Thus, it is possible that exposure of juveniles to OP insecticides like CPF and CYP *in-utero*, which would elevate the levels of acetylcholine during CNS development could result in developmental aberrations of neural pathways, responsible for cognition and overall motor activity levels (Carr *et al*., 2001; Winter and Bussey, 2005). It has been shown that subtoxic doses of CPF are capable of affecting brain development by inhibiting mitosis, eliciting apoptosis, and altering neuronal activity and reactivity (Dam *et al*., 2000). Recent studies in rats have shown that CPF, in doses below the threshold for symptoms of systemic toxicity, nevertheless can compromise the basic cellular processes of brain development, which is even more pronounced in developing and juvenile rats (Mansour and Mossa, 2010). Pesticides induce changes, including inhibition of DNA synthesis, impaired acquisition of new cells, reductions in macromolecules mediating cell differentiation, slowed axonogenesis, altered functioning of neurotrophic signalling cascades, interference with gene transcription and aberrant development of synaptic activity attributed to CPF exposure (Dai *et al*., 2015). Neuronal dynamics of novelty processing are temporally adaptive and flexible with processing enhanced by more of dopaminergic but less cholinergic neurotransmission. This onset of novelty signals is within the medial temporal lobe (MTL). Cholinergic stimulation, on the other hand, has a causal effect in underlying neural substrates from medial temporal to prefrontal brain regions. This incriminates dopamine in regulating the processing speed of novelty-sensitive MTL neurones. Thus the influence of MTL and prefrontal brain regions in novelty processing

is mediated by the balance of dopamine and acetylcholine levels (Eckart and Bunzeck, 2013).

Negative geotaxis refers to an orienting response and movement expressed in opposition to cues of a gravitational vector (Motz and Alberts, 2005). The ability to exhibit negative geotaxis is believed to depend on (a) vestibular or proprioceptive receptor function sufficient to detect a geogravitational stimulus (the incline), (b) central organization, sufficient to process differential inputs that reflect direction of the substrate angle in relation to the gravity vector, and (c) the motor competence to orient and move on the incline (Alberts *et al*., 2004). Negative geotaxis is a response reflecting functioning of the cerebellar and vestibular systems. It is also considered as a diagnostic tool in the determination of the vestibular and/or the proprioceptive function and is useful in the recognition of developmental disturbances, especially coordination (Wiener-Vacher *et al*., 2013)**.** Result of the present study shows a delay in the negative geotaxis in F1 generation of dams, exposed to the insecticide mixture. The result agreed with those obtained from previous studies in mice exposed to CPF (Dam *et al*., 2000; Carr *et al*., 2001) and CYP (Farag *et al*., 2007). The present result showed that the increase in the seconds with which the rats in the CC treated group took to reorient themselves to about 180^{o} reflected possible damage to the motor system of the brain, partly due to oxidative damage by the pesticide mixture. Farag *et al*. (2006) also showed a non-significant alteration in negative geotaxis when permethrin exposed F1 generation mice were compared with those recorded in the controls. The decrease in the number of seconds in group pretreated with melatonin reoriented themselves shows that this agent mitigates insecticide-induced increased negative geotaxis, apparently due to its ability to improve motor coordination in stressed animals (Karadayian *et al*., 2014)

and its antioxidant effect. Melatonin has also been reported to improve neuronal survival, neurogenesis and motor recovery (Kilic *et al*., 2008). It is a potent antioxidant and anti-inflammatory agent. Indeed, increasing body of experimental and clinical evidence has demonstrated its beneficial effects against ROS and RNS status, including that of mitochondrial dysfunction (Acuna-Castroviejo *et al*., 2011).

The aerial righting reflex has traditionally been considered as recovering from an upside-down to a dorso-ventral upward posture during a linearly downwards free-fall. The mechanisms involved in accomplishing such righting depends on the size of the animal and anatomical features, associated with motion of the limbs and body (Jusufi *et al*., 2011). Result from the present study showed an increase in the time it took for the pups of dams exposed to insecticide mixture to reorientate themselves. CPF has been shown to modify spontaneous motor activity in BTBR strain mice, leading to delay motor development in rodents (De Felice *et al*., 2015). Soderlund *et al*. (2002) also showed an alteration in aerial righting reflex in rats dosed with CYP. Decrease in the aerial righting reflex in the combination group may also be adduced to inhibition of AChE, leading to the accumulation of the ACh in the ganglions, hence delay in the motor coordinated transmission of information within the CNS. Aerial righting reflex has been linked with learning and motor coordination (Novier *et al*., 2013)**.** The inability of the pups of dams dosed with insecticide mixture to appropriately orientate themselves mid-air may be due to oxidative damage to some critical portion of the brain responsible for this sensorimotor gating. Pretreatment and post-treatment with MEL were able to restore the deficit in area right reflex response. This result may be due to the ability of melatonin to decrease the pesticide-generated ROS, improve the antioxidant capacity of the body and reactivation of the inhibited AChE.

The present study shows a progressive deficit in the mean angle of slip in the inclined plane performance as an indication of impaired neuromuscular coordination in the CC group, relative to the other groups. This finding agreed with result obtained in rats exposed to CPF (Ambali *et al*. 2010d, 2011, Ambali and Aliyu, 2012; Khokhar and Tyandale, 2012, 2014) and CYP (Rose and Dewar, 1983). Deficits in the inclined plane in the combination group could be attributed to the combined effects of CPF- and CYP-induced oxidative stress to the brain, causing damage to the cerebral cortex, and subsequent neuronal cell death from delayed apoptosis in growing foetus (Zama *et al*., 2007). Similarly, the decrease AChE activity recorded in the offspring of rats in the CC treated group may play a significant role in the decrease in the incline plane, since changes in ACh metabolism may also alter neuronal activity. Chronic exposure to CPF in pesticide applicators have been reported to cause cognitive deficit and other neurotoxicities (Steenland *et al*., 2000), while acute exposure results in long-lasting neurological effects (Terry *et al*., 2003). The improvement in the inclined plane performance in the group pretreated with melatonin may be due to its ability to mitigate the oxidative damage in the brain regions, and improvement in brain AChE activity, thereby reducing neuronal damage. This finding showed that pretreatment with melatonin mitigates deficit in neuromuscular coordination evoked by CC. Although the post-treatment with melatonin did not significantly mitigate CC-induced deficit in inclined plane performance, it however, did demonstrate some level of improvement, compared to the group exposed to CC only.

The deficit in the efficiency of locomotion in the CC group was shown by the number of missed rungs during the test periods. The deficit in ladder walk score manifested by the significantly lower number of missed rungs observed in F1 generation rats gestationally

and lactationally exposed to CPF indicates that the legs of the rats were frequently being held stationary above the rungs for a relatively longer period. This observation demonstrate difficulty in the ability of the rats exposed to the combination only to move fast through the obstacle; and, hence, a deficit in locomotion activity. The locomotion deficit in the CC increased progressively, reaching its peak at PND 60. The relative improvement in the efficiency of locomotion at PND 90 demonstrated tolerance to the pesticide's mechanism of toxicity. Repeated exposure to OPs often results in down-regulation of muscarinic receptors (Nostrandt *et al*., 1997). This down-regulation could be responsible for the development of "tolerance" (that is, decreasing clinical signs in the presence of substantial AChE inhibition). Specifically, marked down-regulation of brain muscarinic receptors has been demonstrated after a single subcutaneous injection of CPF (Chaudhuri *et al*., 1993).

Studies have shown that decrease in movement, which is one of the extrapyramidal symptoms has been observed in humans exposed to non-specific agricultural pesticides, which increase with the duration of exposure (Ritz and Yu, 2000; Alavanja *et al*., 2013). Thus, the locomotion deficit observed in the present study is part of the sensorimotor deficits, occurring in animals exposed to CC. Furthermore, inhibition of AChE activity by both insecticides may have exacerbated the locomotion deficit through persistent decrease in AChE activity at the cholinergic receptors, which may result in initial stimulation and finally results in paralysis (Ambali and Ayo, 2012). Similarly, Ambali and Ayo (2012) recorded decrease in glycogen reserve in the muscle of rats exposed to CPF, which may have reduced the energy essential in the performance of efficient locomotion. Cypermethrin has also been shown to cause neurodegenerative changes in animal models (Mun *et al*., 2005). Furthermore, the increased lipoperoxidative changes

in the muscle may have altered its membrane integrity; hence ionic conductance, which is pertinent to the conduct of efficient locomotion.

The result demonstrated the ability of the two insecticides to provoke oxidative stress in the brain and that oxidative stress critically contributes to the nigro-striatal dopaminergic neurodegeneration; especially in the basal ganglia, leading to bradykinesia, rigidity and postural instability (Dick, 2007; Nasuti *et al*., 2007). The dopaminergic neurones in the brain are particularly prone to oxidative stress due to the presence of ROS generating enzymes such as tyrosine hydroxylase (TH) and monoamine oxidase (MAO) (Johnson *et al*., 2012; Deng *et al*., 2015). It is well known that dopamine system plays an important role in brain activation and therefore agents that cause dopamine depletion exert adverse effects on locomotion. Studies have highlighted the adverse effects of CYP, leading to the reduced striatal dopamine contents (Tripathi *et al*., 2014) and provoking nigro-striatal dopaminergic neurotoxicity (Mun *et al*., 2005). The neurodegeneration induced by CC in the present study, which contributed to the slowness of movement may have been due to induction of apoptosis via activation of pro-apoptotic enzyme capspase-3, and tumour necrotic factor-alpha (TNF-α), observed following exposure to mixture of CPF and CYP (Raszewski *et al*., 2015). All these factors may have been partly responsible for the slowness of locomotion in the CC group recorded in the present study.

The improvement in the ladder walk in groups pretreated with melatonin may be due to its brain antioxidant and AChE restoration properties. In addition, the ability of melatonin to promote the dopamine system (Esteban *et al*., 2010; Deng *et al.,* 2015) may have partly contributed to the improvement in locomotion efficiency, recorded in

the group pretreated with melatonin. Furthermore, evidence abounds to show that melatonin is neuroprotective (Kaneko *et al*., 2011; Voiculescu *et al*., 2014; Watson *et al*., 2016). The prevention of insecticide-evoked glial cell neurodegeneration by melatonin as demonstrated in the present study may have contributed to improvement of movement in the melatonin-pretreated group. Improved glial cell survival after melatonin treatment have also been reported and these may protect injured neurones, as these cells secrete trophic factors such as glial cell line-derived neurotrophic factor (GDNF) with neuroprotective effects (Wang *et al*., 1997; Shinozuka *et al*., 2013). This may have contributed to the enhanced locomotor activity in the group pretreated with melatonin.

The significant increase in the width of slip off the beam by the rats in the CC group was an indication of reduction in the cumulative beam-walk length, thus indicating impairment of motor coordination. The depreciation in beam-walk length in the CC group increased with increase in PNDs with worst deficit observed at PND 60 than in PND 30 and 90. This finding showed that the gestational and lactational exposure to the mixture of the two pesticides caused progressive impairment of motor coordination. CPF (Ambali and Ayo, 2012), and mixture of CPF and nicotine (Abou-Donia *et al*. 2006) have been shown to impair motor coordination in rat model. Control of balance requires communication and integration across the nervous system. Preventing and recovering from loss of balance requires an integration of visual, vestibular, proprioceptive, and other sensory feed-back mechanisms (Faraldo-Garcia *et al*. 2012; Sozzi *et al*. 2012). Beam-walk performance is an integrated form of behaviour that requires pertinent level of consciousness, memory, sensorimotor and proper cortical functions, mediated by the cortical area (Abou-Donia *et al*. 2001). Cortical area

activates long-latency movements that produce a change in base of support, such as stepping. Specifically, a cerebellar-cortical loop has been implemented in integrating prior experience into postural responses, and a basal ganglia-cortical loop is responsible for incorporating sensory information on the current posture (Jacobs and Horak 2007). There have been some imaging studies that have attempted to provide insight into the cortical mechanisms, involved in whole body postural responses (Jacobs *et al*. 2008; Mochizuki *et al*. 2008, 2009a,b; Slobounov *et al*., 2000, 2005, 2006, 2008, 2009). Therefore, the deficit in beam-walk length in the CC treated group may be ascribed to injury to the cortical area of the brain (Abou-Donia *et al*., 2001), perhaps partly due to oxidative damage to the brain. Increase in brain injury by the combination of the two pesticides as shown by cortical neurodegeneration may also be related to the increase in Na^{+}-K^{+}-$2Cl^{-}$cotransporter-1 (NKCC1) disruption of ion homeostasis and enhanced neuronal apoptosis (Hui *et al*., 2016).

The brain is very susceptible to oxidative stress because it contains large amount of oxygen in a relatively small mass, contains significant quantity of pro-oxidant metal (Fe) and peroxidisable lipids; and has fewer antioxidant molecules than other tissues (Chen *et al*., 2012). The increased lipoperoxidation and deficit in antioxidant enzymes in the brain of rats exposed to CC only in the present study may have assisted in the motor coordination impairment in this group.

Pretreatment with melatonin improved the beam-walk length of the rats significantly by decreasing the width at which they slipped off the beam. This showed that melatonin mitigated motor incoordination, provoked by combination of CPF and CYP. This may be possible by decreasing the lipoperoxidative damage to the cortex and the antioxidant

capacitation of the body, restoring AChE activity in the brain, improving the ionic homeostasis and decreasing apoptotic assault by the combination of the two pesticides. Melatonin has been shown to improve motor coordination in ethanol hang-over mice (Karadayian *et al*., 2014).

In the present study, there was a decline in the excitability scores of F1 male rats from dam dosed with CC, indicating impairment of physical and mental alertness, which reflected poor reflex and neuromuscular coordination. The deficit may be attributed to impairment of AChE activity, which finally resulted in decreased impulse propagation (Brocardo *et al*., 2007). In addition, oxidative damage to the brain and muscle provoked by the pesticides may have been partly responsible for the declined excitability score in the CC group. Reduction in muscle glycogen (Ambali and Ayo, 2012) and, therefore, low energy reserve may have contributed to low excitability score. Furthermore, impairment of axonal transport (Terry *et al*., 2003) leading to lower rate of impulse conduction may have contributed to the deficit excitability score.

Pretreatment with melatonin significantly improved the excitability score. This may be due to its antioxidant and AChE restoration activities. Melatonin and its oxidation products, viz; cyclic 3-hydroxymelatonin, N1-acetyl-N2-formyl-5-methoxykinuramine and N1-acetyl-5-methoxykinuramine possess excellent antioxidant properties (Reina and Martinez, 2018). Melatonin's beneficial neuroprotective properties are mostly attributed to excellent ROS scavenging properties. Besides being a strong antioxidant molecule, melatonin is a pleiotropic regulator molecule, which orchestrates multiple functions through all the three melatonin receptors; that is, MT_1, MT_2, and MT_3. Notably, MT_2 receptor agonistic activity is attributed to neuroprotective, hypnotic and

anxiolytic properties, while MT_1 and MT_2 agonistic activities are associated with the clinical efficacy of agomelatine. The third melatonin receptor has been identified as quinone reductase (QR) 2, an enzyme involved in detoxification (Mahmood *et al*., 2016), which may have aided the pesticides detoxification, thereby assisting in improving excitability scores.

The significant reduction in fore-paw grip time, reflecting deficit in fore-paw motor strength following exposure to CC agreed with the findings of earlier study, which showed impaired fore-limb strength following repeated low-dose CPF administration in rats (Terry *et al*., 2003) and other organophosphates like monocrotophos (Kumar *et al*., 2015b). Decrease in fore-paw grip time was also reported in CYP exposed rats (Wolansky and Harrill, 2007). The result of the present study disagrees with the work of Terry *et al*. (2007), where they reported an increase in the grip time. However, in a study conducted by Rajesh and Pilo, (2009), there was no significant difference in the fore-paw strength, when rats given a single dose of combination of cypermethrin and prefenofos, compared to the control animals. The reduced grip time in CC-exposed rats in the present study indicates impaired motor strength. This can be ascribed to decreased possible low gycogen reserve in the muscle of the animals in this group (Ambali and Ayo, 2012), perturbing the energy output required in the performance of this task. This may also be attributed to increased lipoperoxidative damage to the muscle from combined effect of the agents. Similarly, lipoperoxidation has been shown to affect membrane-bound enzymes, including ATPases causing cellular dysfunction due to alteration of cationic transport across the membranes, disturbance in uptake as well as release of certain neurotransmitters (Mehta *et al*., 2005). In addition, the interference with muscle ACh metabolism resulting from AChE inhibition, which both CPF and

CYP affect, may have altered neuronal action potential generation, causing faulty transmission and, hence, reduced motor strength. CPF-induced neurotoxicity may be mediated through ROS formation, reduced antioxidant defence mechanism, and inhibition of AChE activity. The CYP-induced AChE inhibitory activity may be by interacting with the anionic substrate-binding site leading to repetitive nerve impulse generation, which can induce oxidative stress, from excessive ROS generation on CYP exposure (Sharma *et al*., 2014b). The reduced motor strength may be related to homeostatic imbalance due to decreased Ca^{2+}, Mg^{2+}-ATPase activity. The inhibition of Ca^{2+}, Mg^{2+}-ATPase activity by CPF may be due to permeation of sarcoplasmic reticulum, rather than direct enzyme inhibition (Nozdrenko
et al., 2016). This may ultimately cause increased muscle fatigue as a result of imbalanced ionic concentration.

Pretreatment with melatonin was shown in this study to increase motor strength by increasing the fore-paw grip time. This result may be partly due to antioxidant and neuroprotective properties of melatonin. In contrast to many other antioxidants, melatonin crosses all morpho-physiological barriers, that is, the blood-brain barrier and the placenta, and is distributed throughout all the body cells (Rodrıguez *et al*., 2004). Upon oxidation, melatonin converts to a number of antioxidant compounds such as cyclic 3-hydroxymelatonin, N_1-acetyl-N_2-formyl-5-methoxykynuramine and N_1-acetyl-5-meth-oxykynuramine. Therefore, melatonin is considered to be a broad spectrum antioxidant that is more powerful than glutathione in neutralizing ROS, and that it can protect cell membranes from oxidative damage more effectively than other antioxidants (Lu *et al*., 2010).

In the present study, rats from dam co-administered CC had the lowest mobility time compared to the control and the pre- and post-treated groups. This result agreed with those of other workers using CPF (Aldridge *et al*., 2005a) and other OPs like diazinon (Roegge *et al*., 2008). The decrease in mobility time recorded in the CC group indicated an emotional perturbations, which could have been driven by either AChE inhibition or by other non-cholinergic mechanisms, examples include oxidative stress, cannabinoid and dopamine metabolism. The FST has been demonstrated as a parameter indicative of depressive-like behaviour (Lima *et al*., 2013). Chlorpyrifos has been reported to cause increase in despair behaviour in animal models (Chen *et al*., 2011, 2014), with numerous studies showing the potential neurotoxic effect of either acute or chronic exposure to CPF on both humans and animals, especially on developing individuals (Chen *et al*., 2011). The association between emotional disorders and CPF exposure in humans is a major environmental health issue, especially in children, a subpopulation that is highly susceptible to environmental hazards (Chen *et al*., 2014).

Epidemiological studies have demonstrated that pesticide applicators with a history of CPF contact are susceptible to depression (Beseler *et al*., 2006). This may be attributed to the effect of the pesticide through its non-cholinergic mechanism. The effects of CPF are not only confined to cholinergic system, but are involved in a wide variety of neurotransmitter systems; especially the serotonin (5-HT) system (Moreno *et al*., 2008), which leads to long-lasting changes in 5-HT-related emotional behaviours (Chen *et al*., 2011). Serotonin is a critical neurotransmitter in the regulation of anxiety and depression, and OP exposure also increases behaviours associated with anxiety and depression (Aldridge *et al*., 2005a; Furlong *et al*., 2014). Chlorpyrifos has been shown to affect the serotonergic system not only during the critical period of

neurodevelopment (Raines *et al*., 2001; Aldridge *et al*. 2003), but also during later periods of neurotransmission-related refinement of serotonergic circuits (Vester and Caudle, 2016). Apart from subserving important trophic functions that control the differentiation and architectural organization of the developing brain (Rice and Barone, 2000; Tau and Peterson *et al*., 2010), the targeting of 5HT may affect the programming of 5HT function, leading to appetitive and affective disorders, and consequent increases in the incidence of obesity and depression (Aldridge *et al*. 2003; Martin-Gronert *et al*., 2016). Deficiencies of the 5-HT system are frequently associated with emotional disorders, such as depression, anxiety, post-traumatic stress disorder (Toth, 2007). Animal studies have also shown that exposure to CPF produces permanent alterations in serotonergic system (Slotkin and Seidler, 2005); with altered expression of presynaptic 5HT transporter, postsynaptic 5HT receptors (5HT1a, 5HT2), and down-stream 5HT signal transduction in various brain regions, including the fore-brain and brain-stem (Raines *et al*., 2001; Aldridge *et al*., 2003, 2004), and changes in 5-HT-related behaviours (Aldridge *et al*., 2005a). Therefore, the high immobility time in the F1 generation of CC group reflecting depression may have been partly due to alterations in the serotonergic system, provoked by the pesticide.

Furthermore, apart from its role in spatial memory, the dentate gyrus in the hippocampus has been shown to play a significant role in depression **(**Surget *et al*., 2011**).** It is possible that CC-induced oxidative damage to the dentate gyrus in the hippocampus may have partly provoked the depression. Dichlorvos, an OP insecticide has been shown to cause reduced cellularity of the dentate gyrus, with neuronal degeneration and pyknosis of the dentate granule cell (DGC) nuclei (Owoeye *et al*., 2014). In addition, there is possibility that the pesticide mixture caused hyper-

phosphorylation of glycogen synthase kinase -3β (GSK-3β), an enzyme involved in various psychiatric diseases including mood disorders and neurodegenerative diseases (Grimes and Jope, 2001). Phosphorylated-GSK-3 β proteins have been found in post-mortem brain tissues from depressed subjects (Karege *et al*., 2012) and, therefore, may have been partly involved in the depression recorded in the rats gestationally- and lactationally-exposed to CPF and CYP.

Pretreatment with melatonin has been shown in this study to increase mobility time due to its antioxidant and neuroprotective potentials. Recent studies suggest that melatonin exerts a neuroprotective role, and accelerates the early process of neuronal differentiation (Mediavilla *et al*., 2010; Son *et al*., 2014). This may be due to its high lipid solubility, which allows permeation into the interior of the cell (Asghari *et al*., 2017). Melatonin has been reported to decrease generation of ROS in systemic circulation as well as enhancing the acetylcholine function through possible modulation of AChE restoration (Markus *et al*., 2010; Onaolapo *et al*., 2017).

Learning, which is the process by which cellular and molecular mechanisms in the brain convert new information into a form that can be permanently stored as a long-term memory, has been shown to be impaired in the F1 generation males, gestationally and lactationally exposed to CPF and CYP, as shown by the significant increase in the number of foot-shocks in the treated group compared to the others. Similarly, the reduction in the time spent on the platform by the F1 generation gestationally and lactationally exposed to CPF and CYP implies adversity on short-term memory.

At low doses, OPs have several suspected mechanisms of action, including disruption of nuclear transcription factors (Dam *et al*., 2003), interference with neural cell development and neurotransmitter systems (Aldridge *et al*., 2005b), and altered synaptic formation (Qiao *et al*., 2003). Prenatal CPF exposure was shown experimentally to inhibit AChE, to down-regulate muscarinic receptors, inhibit the adenylate cyclase signalling cascade, decrease brain DNA and RNA synthesis, and suppress neurite outgrowth (Rauh *et al*., 2006). Such developmental disruptions have been associated with later functional impairments in learning, short-term working memory, and long-term reference memory (Levin *et al*. 2001). Chlorpyrifos exposure during the perinatal period evokes deficits in neuritic outgrowth, specifically including the targeting of cholinergic projections (Qiao *et al*. 2002, 2003; Howard *et al*. 2005). Dam *et al*. (1999) found a short-fall in choline acetyltransferase (ChAT), which returns choline to the neurone once ACh is broken down by AChE (Qiao *et al*., 2002). Other cholinergic system proteins, such as the vesicular acetylcholine transporter (vAchT), which transports Ach into synaptic vesicles for release; and muscarinic receptors, which interact with acetylcholine once it is released, are affected as well (Richardson and Chambers, 2005).

The initial deficits in the development of cholinergic projections lead to the subsequent emergence of abnormalities of cholinergic innervation. Chlorpyrifos exposure in mice *in utero* is associated with a decrease in activity of the presynaptic ChAT, but this association occurs neonatally, disappears during weaning, and then reappears in adolescence and adulthood (Qiao *et al*., 2003). Given that neurodevelopment proceeds all the way through adolescence and young adulthood, it is likely that cholinergic changes affect neurotransmission across more than one developmental window (Vester

and Caudle, 2016). Indeed, substantial deficits in cholinergic synaptic activity and related-behavioural anomalies in adolescence and adulthood have been observed following neonatal CPF exposure (Slotkin, 1999, 2004). Further studies of postnatal OP exposure also indicate that cholinergic system is affected in ways analogous to prenatal OP exposure; there are reductions in muscarinic and nicotinic receptors that normally interact with acetylcholine, as well as reductions in transporters ChAT and vAchT (Zhang *et al*., 2002; Guo-Ross *et al*., 2007). Acetylcholine is a well-known regulator of cognitive functions such as learning and memory (Mahmoudvand *et al*., 2016) and, in fact, malfunction of the cholinergic system in some mental disorders causes memory impairment (Blokland, 1996; Shinoe *et al*., 2005). Interference with ACh function contributes directly to the emergence of cognitive impairment, resulting from early life exposure to OPs (Levin *et al.*, 2001). Given the diversity of possible mechanisms and target tissues, the developing brain could be vulnerable to OP pesticide exposure from early embryonic life into childhood (Dam *et al*., 1999, 2003; Slotkin, 1999; Qiao *et al*., 2002). Slotkin (2004) stated that the period of vulnerability to CPF extends through the period of synaptic modeling, and this continues well into childhood and even adolescence. In a human study, Rauh *et al*. (2012) found associations between prenatal CPF exposure, brain structure, and neurocognitive alterations at 5.9–11.2 years of age, which suggest that the neurotoxic effects of CPF are long-term, at least extending into the early school years.

The ability of CPF to disrupt glial function may cause developmental changes in the brain, which can be manifested as motor, cognitive or behavioural dysfunctions, since glial cells (specifically astrocytes and oligodendrocytes) are essential to neuronal differentiation, myelination, impulse propagation and homeostatic maintenance

(Saulsbury *et al*., 2009). Chlorpyrifos has been shown to also alter cellular function, viability and gene expression in both astrocytes and oligodendrocytes (Mense *et al*., 2006; Slotkin and Seidler, 2007b). Several studies suggest that exposure to CPF is associated with reductions in the levels of myelin associated glycoprotein (MAG) mRNA (Betancourt *et al*., 2006), inhibition of DNA synthesis in undifferentiated oligodendrocytes (Garcia *et al*., 2001) and astrocytes (Parran *et al*., 2005**)** as well as with alterations in the expression of glial fibrillary acidic protein (GFAP) (Garcia *et al*., 2002) and myelin basic protein (Garcia *et al*., 2003). Chlorpyrifos is also toxic to oligodendrocyte progenitors via the induction of oxidative stress (Saulsbury *et al*., 2009).

Foetuses and babies are thought to be highly susceptible to OP exposure, due to the ready transmission of OPs through the placenta, and the immaturities of metabolic pathways required to process and excrete these compounds (Furlong *et al*., 2014).

Alterations in many of the ubiquitous synaptic proteins have also been attributed to cognitive deficit in prenatal or neonatal CPF exposure. CAMKII expression (Lee *et al*., 2015a), a Ca^{2+}-activated kinase that is highly expressed in glutamatergic neurons (Lisman *et al*., 2002; Giese and Mizuno, 2013) and a key player in the regulation of synaptogenesis, neuronal plasticity, behaviour and formation of memory, has been shown to decrease in the hippocampus of neonates exposed to CPF (Lee *et al*., 2015b). Furthermore, synaptophysin- a CAMKII substrate that regulates the efficiency of synaptic vesicle recycling, which is critical for regulating the efficacy of learning and memory (Gordon and Cousin, 2014), has been shown to decrease in the frontal cortex of

the neonatal mice exposed to CPF (Lee *et al*., 2015a), and may have been partly involved in cognitive deficit in the F1 generation from dams in the CC group.

In the same vein, neonatal exposure to CYP has been shown to impair learning and memory (Lee *et al*., 2015b). CYP has been reported to also have an inhibitory effect on AChE through binding to the anionic site (Marigoudar *et al*., 2009) and alters acetylcholinergic receptors (Singh *et al*., 2012a), thereby altering cholinergic projections, with similar outcome to CPF on cognitive processes. Furthermore, repetitive nerve impulses from AChE inhibition may induce oxidative stress, hence there is possibility of excessive ROS generation on CYP exposure (Sharma *et al*., 2014a). Early-life exposure to CYP induces neuronal cell dysfunctions (Ray and Fry, 2006) that affects synaptic activity and evokes neurodegeneration (Singh *et al*., 2012a). Early childhood exposure to pyrethroid insecticide engenders neurocognitive deficit (Viel *et al*., 2015). CYP has been shown to block heparin-binding epidermal growth factor (HB-EGF)-epidermal growth factor receptor (EGFR) signalling (Maurya *et al*., 2012). HB-EGF functions as an endogenous neuroprotective agent (Opanashuk *et al*., 1999) that regulates memory and cognitive performance (Oyagi *et al*., 2011; Oyagi and Hara, 2012). Therefore, the blockade of HB-EGF may have partly contributed to the impaired learning and memory in the pups, gestationally and lactationally exposed to CYP and CPF.

In addition, exposure to pyrethroids has also been found to influence the neurotransmission processes by altering the levels of neurotransmitters and binding of neurotransmitter receptors (Rao and Jagannatha, 1995; Singh *et al*., 2015a). Indeed, prenatal exposure to low doses of cypermethrin has been shown to induce distinct

alterations in the expression of xenobiotic-metabolising cytochrome P_{450s}, and the marker enzymes involved in the synthesis of specific neurotransmitters in brain regions of offsprings, which persist up to adulthood and produce alterations in the cognitive functions in the exposed offs-prings (Singh *et al*., 2016). Alternatively, accumulation of even low levels of pyrethroids and their metabolites in the brain regions may be sufficient to induce the persistent alterations in the expression of cytochrome P_{450s} and rate-limiting enzymes, involved in the synthesis of neurotransmitters. These alterations may account for the persistence in the alterations in cognitive functions such as learning and memory in the prenatally exposed offs-prings during postnatal development. It has also been shown that brain cytochrome P_{450s} play a crucial role in the cognitive impairments, associated with early dysfunction of the hippocampus (Stapleton *et al*., 1995). Furthermore, cypermethrin exposure in the neonatal period led to alterations in GluR1 in the hippocampus and tau in the frontal cortex (Lee *et al*., 2015b). GluR, an ionotropic receptor belonging to the subgroup of a-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors that helps to facilitate fast excitatory neurotransmissions in the synapses throughout the CNS, has been shown to be necessary for hippocampal synaptic plasticity, as well as for synaptic plasticity in the amygdala (Humeau *et al*., 2007), thereby participating in behavioural modification and learning through AMPA receptor trafficking (Kessels and Malinow, 2009; Henley and Wilkinson, 2013). Tau on the other hand is a microtubule-associated protein and is foremost known for its involvement in neurodegenerative disorders, primarily involved in the assembly and stabilisation of microtubule (Weingarten *et al*., 1975) and to facilitate axonal transport (Vila-Ortiz *et al*. 2001; Dixit *et al*., 2008), but believed to be involved during development in cellular signalling, protein scaffolding and neuronal migration (Morris *et al*. 2011; Sapir *et al*. 2012). Therefore, its alterations contribute to

neuropathogenesis, behavioral and synaptic aberrations. during development or at a later phase of life (Wang and Liu, 2008; Morris *et al*. 2011; Van der Jeugd *et al*. 2011; Kimura *et al*. 2014). It is plausible that the alterations in these two proteins by CYP may have contributed to cognitive and some other neurobehavioural aberrations, recorded in rats gestationally and lactationally exposed to CPF and CYP.

The developing brain is particularly vulnerable to ROS and RNS–mediated damage because of its high concentrations of unsaturated fatty acids, high rate of oxygen consumption, low concentrations of antioxidants, high content of metals, catalysing ROS formation, and large proportion of sensitive immature cells (Ikonomidou and Kaindl, 2011).

Learning and memory are complex functions, involving many areas of CNS including the limbic system. The hippocampus exists in the limbic system and the hippocampal formation has an important role in storing the newly obtained information. Behavioural studies in rodents have identified hippocampus-dependent learning and memory as a target for the neurotoxic effects of repeated subclinical CPF exposure (Terry *et al*., 2003). Chronic CPF administration has been shown to cause reduction in synaptic transmission in the hippocampus, likely due to a corresponding decrease in CA1 pyramidal neuronal synaptic spine density (Speed *et al*., 2012). This may not be entirely unrelated to oxidative damage to the hippocampus by CPF. Induction of oxidative damage to the brain, especially to the hippocampus (the section of the brain responsible for spatial learning and episodic memory) may have partly contributed to cognitive deficit in the pups, gestationally and lactationally exposed to CYP and CPF.

The dentate gyrus of the hippocampal formation, and neuronal stem cells located at the inner border of the granule cell layer (the subgranular zone) continue to produce new granule cells throughout adult life. The dentate gyrus is one of the few regions of the adult brain where neurogenesis takes place. Neurogenesis is thought to play a role in the formation of new memories (Lazarov and Hollands, 2016; Apple *et al*., 2017). Roy *et al*. (2004) observed that neonatal CPF exposure produces subtle morphological changes in the hippocampus that are revealed by quantitative assessment at the cellular and architectural levels. The changes are characterised by neuronal and glial cell loss, preferential targeting of glia, perikaryal enlargement suggestive of cell damage, and thinning of pyramidal cell layers (thereby interfering with axonogenesis and synaptogenesis), many of which intensify in the period after the termination of CPF exposure. The hippocampal damage can be attributed to outright neural cell damage through oxidative stress (Salyha, 2013), interference with transcription factor expression and function (Crumpton *et al*., 2000; Garcia *et al*., 2001; Schuh *et al*., 2002; Dam *et al*., 2003) and promotion of apoptotic signals (Roy *et al*., 1998; Greenlee *et al*., 2004).

In the developing nervous system, CPF has been shown to impair axon growth *in vitro* (Howard *et al*., 2005; Yang *et al*., 2008), while also stimulating dendritic growth (Howard *et al*., 2005) through non-enzymatic mechanisms. The simultaneous effects on neurones and glia contribute to the net outcome of neonatal CPF exposure since glia ordinarily protect neurones by releasing trophic factors, scavenging ROS and RNS, providing nutrients for repair, and guiding axonal outgrowth (Aschner *et al*., 1999; Aschner, 2000). CPF gliotoxicity can, thus, enhance the neuronal damage. Furthermore, CPF has been shown to phosphorylate Ca^{2+}/cAMP response element binding protein (CREB) (Schuh *et al*., 2002) that is critical to several forms of use-dependent synaptic

plasticity and transcription dependent forms of memory (Schuh *et al*., 2002), and also plays a major role in cell survival and differentiation during brain development (Bender *et al.,* 2001). The CPF has been shown to cause impairments in short-term memory and cognition in adults (Ambali *et al*., 2010d; Ambali and Aliyu, 2012; Ambali and Ayo, 2012), and in mature rodents (Canadas *et al*., 2005). Idris *et al*. (2014) also showed that mixture of CPF and CYP in adult rats impaired cognition. Apart from provoking oxidative damage to the hippocampus, pyrethroids such as CYP accumulate majorly in the organ (Anadon *et al*., 1996), with consequent engendering of direct cytotoxicity. Furthermore, evidence has shown the involvement of hippocampal growth hormone (GH) (Sun *et al*., 2005; Laron, 2017) in synaptic plasticity (Zearfoss *et al*., 2008; Vander Weele *et al*., 2013), crucial for cognitive processes. Exogenous growth hormone (GH) also facilitates hippocampal synaptic transmission (Mahmoud and Grover, 2006; Molina *et al*., 2012), which plays an important role in learning acquisition and memory. Growth hormone insulin growth factor-1 (GH-IGF-I) axis may play an important role in CNS functions, including those associated with neuronal growth, development, and protection (Laron, 2017), while also playing a role in influencing aspects of mood and cognition (van Dam and Aleman, 2004). It is, therefore, possible that oxidative and direct cellular damage to the hippocampus, evoked by gestational and lactational exposure to CPF and CYP may have caused deleterious consequences on its expression of hippocampal GH, with consequent adversity on cognition. The GH is important to the development of the CNS, especially in promoting the maturation of the brain, myelin formation, glial cell differentiation, and cognitive function (Zhang *et al*., 2014b). Some studies have shown that the mechanism underlying the effect of GH treatment on cognitive function is associated with a neuroprotective effect, inhibition of the inflammatory response (Dhou *et al.,* 2009; Zhang *et al*., 2014b), and promotion of

angiogenesis (Ying *et al*., 2004) or promotion of cell growth via IGF-1 (Christophidis *et al*., 2009) and relevant neuroprotection (Isgaard *et al*., 2007).

Cognitive impairment recorded in the pups exposed to CPF and CYP *in utero* and during lactation may have been due to impairment of thyroid function as shown by decrease in concentration of T_3 and T_4. In general, thyroid hormones accelerate the myelinating process, influence cell migration and regulate cell differentiation and maturation of specific neuronal populations. In addition, thyroid hormones control the expression of several genes, including neuromodulin/GAP-43, neurogranin/RC3 and CAMKII, which regulate different forms of synaptic plasticity and memory formation (Rivas and Naranjo, 2007). Studies have shown that behavioural and cognitive deficit in the postnatal period, elicited in developmental hypothyroidism is partly due to perturbations in hippocampal synaptic transmission and plasticity in area CA1 and dentate gyrus (Gilbert, 2004; Rivas and Naranjo, 2007). Radial glia maturation in the foetal rat brain is delayed in the hippocampus of hypothyroid rats (Martinez-Galan *et al*., 1997), which affect not only the migration of the neurones, but may also impair neurogenesis.

Furthermore, pesticides may cause neurotoxicity due to their ability to cross the BBB and exert CNS toxicity (Koger *et al*., 2005; Grandjean and Landrigan, 2006, 2014). Since the developing BBB is structurally and functionally immature, toxicant may easily penetrate and impair the barrier, making it more vulnerable to CNS toxicity (Sanders *et al*., 2009). Pyrethroids have been reported to interfere with the normal phosphatidylcholine liposomal membrane structure (Xie *et al*., 2010). CYP is reported to cross the blood brain barrier and cell membrane without any specific or active carrier

transport (Mun *et al*., 2005), hence it could damage the developing nervous system in young rodents in exposure. Similarly, these pesticides may also have effect on the membrane structure of BBB thus may lead to disruption of normal membrane associated functions, resulting in disordered packing of membrane components. Altered membrane properties may facilitate the uptake of toxicant and the unrelated compounds, as well as it may also affect the transport of essential nutrients (Gupta *et al*., 2000). It has been shown that exposure to quinalphos, an OP insecticide and CYP during early postnatal period causes significant impairment in the development and maturation of the BBB that may have adverse consequences on the normal brain functioning with long-term neurotoxic effects. Further to this is the fact that enzymes involved in metabolizing pesticides have been shown to have differential activity and expression levels throughout development, and this may also predispose the developing nervous system to elevated concentrations of toxic chemicals (Vester and Caudle, 2016). For example, the paraoxonase 1 (PON1) enzyme metabolises OP compounds and plasma PON1 levels differ throughout development (Cole *et al*., 2003). Thus, there is both age-related and genetic vulnerability to OP pesticides associated with PON1 expression, which contribute to the evidence suggesting there is neurodevelopmental susceptibility in OP exposures (Vester and Caudle, 2016). Apart from this, there is lower maternal PON1 enzyme activity levels expressed during pregnancy which might potentiate the association between OP exposure during gestation and offspring cognitive development (Eskenazi *et al*., 2014).

Melatonin either used as pre-exposure or post-exposure therapy has been shown in the present study to mitigate cognitive impairment in rats exposed *in utero* and via lactation to mixture of CPF and CYP. This may be partly due to its antioxidant and AChE

restorative activities. Correlation does exist between memory impairment and oxidative stress in the brain (Sharma *et al*., 2011). Melatonin is an endogenous neurohormone, which is appreciated for its powerful ability to scavenge ROS and prevent tissue damage (Campolo *et al*., 2013). It has been shown in the present study to mitigate brain oxidative stress in rats gestationally and lactationally exposed to CPF and CYP. Melatonin exerts its protective effects against neuronal degeneration by decreasing ROS and lipid peroxides formation (De lima *et al*., 2005; Alonso-Alconada, 2013). The antioxidant effect of melatonin has been attributed to its ability to reduce the mRNA expression of various inflammatory cytokines, involved in oxidative and nitrosative stress (Reiter *et al*., 2000), in addition to the up-regulation of antioxidant enzymes expression (Hashimoto *et al*., 2012). High levels of oxidative stress can lead to pro-inflammatory effects such as induced expression of tumor necrosis factor alpha (TNF-α) (Brown *et al*., 2004) and interleukin 1 beta (IL-1β) (Shankar *et al*., 2012). The neuroprotective effects of melatonin on cognitive function were mediated by effectively reducing the hippocampal oxidative stress and successfully enhancing the signalling molecules, participating in bio-energetic activity (New *et al*., 2003; Zhang *et al*., 2013). It has been shown that melatonin effectively increase hippocampal Ca^{2+} expression, enhance Ca^{2+}-mediated molecular element activation, decrease hippocampal oxidative stress, and improve neuronal bio-energetics (Huang *et al*., 2015), modulates calcium signalling (Naziroglu, 2015) and takes part in neuronal plasticity in the hippocampus (El-Sherif *et al*., 2003). Melatonin markedly attenuates formaldehyde-induced hippocampal neuronal death, restored brain melatonin levels, and reversed memory decline (Mei *et al*., 2016). The ability of melatonin to protect the hippocampus from CPF and CYP-induced oxidative damage may have contributed in preserving the integrity of its neurones thereby enhancing hippocampal formation, long- term

potentiation and synaptic plasticity, which are crucial to cognitive processes. The antioxidant effect is further complemented by its ability to readily cross the BBB (Menendez-Pelaez *et al*.,1993), coupled with its higher antioxidant potency than the powerful natural antioxidants, vitamin E and glutathione (Reiter *et al*., 2000). The restoration of brain AChE activity by MEL indicating maintenance of ACh level crucial to regulation of cognitive functions may have partly contributed to improved cognition.

Furthermore, MEL has been shown in the present study to mitigate pesticide-induced hypothyroidism, thereby assisting in the maintenance of hippocampal synaptic transmission and plasticity that is crucial to cognitive functioning. Studies have shown that melatonin mitigates cognitive impairment, evoked in adult rats by phosphamidon (Sharma *et al*., 2011) and mixture of CPF and CYP (Idris *et al*., 2014). Therefore, the ability of MEL to mitigate cognitive impairment, provoked in rats gestationally and lactationally exposed to CPF and CYP, may have been partly due to its antioxidant and cholinesterase reactivating properties, enhancement of hippocampal neuronal transmission and synaptic plasticity and attenuation of hypothyroidism.

The present study showed that breeding of untreated female with F1 male from *in utero* and lactational exposure to CPF and CYP caused shortening of the gestational length, hence pre-term delivery. In USA, pre-term birth is an enormous public health problem that affects over 12% of live births and costing the country over $26 billion in the United States (Murray *et al*. 2010). The shortening of the gestational length is, apparently, due to male factor since that is the sex that was exposed to the insecticide combination. This decrease in gestational length may be attributed to the fertilisation of female ova by defective sperm cells, apparently due to sperm gene defects evoked by

the insecticide combination. The ability of the insecticides to alter the composition of enzymes in the spermatozoa due to oxidative injury might have contributed to defective sperm, culminating in shortening of the gestation period. It is also possible that the insecticides, especially the more lipid soluble CPF may have tied down to the semen (De Coster and van Larebeke, 2012), thereby contaminating the ovum during fertilisation, hence the conceptus. This is especially plausible since CPF is known to induce resetting of neuroendocrine control of pregnancy and parturition, hence, decreasing the gestational period (Ahmad *et al*., 2010).

In addition, contamination of the conceptus by the pesticides may have trigger pre-natal stress, which is known to reduce gestation length (Paris *et al*., 2011). Furthermore, the presence of the CPF and CYP in the conceptus may have polluted the uterine environment resulting in depression of AChE activity and the resulting acetylcholine stimulates uterine contraction, triggering preterm delivery (Eskenazi *et al*., 2004). Findings from the present study showed increase in sperm morphological defects, defective sperm cells may not be able to adequately fertilize an ovum, and when it did, maintaining the pregnancy through its course may prove difficult.

Pretreatment with melatonin have been shown by this study to normalise the gestation length, hence prevented pre-term delivery. This may be linked to the ability of melatonin to protect spermatozoa from genetic damage due to its antioxidant effect. Melatonin has been reported to be a broad-scale epigenetic modulator of gene expression (Hardeland, 2014). Similarly, the apparent protection of the spermatozoa by melatonin from pesticide-engendered sperm defect may have partly been responsible for

the restoration of the gestation length. Melatonin may also have a modulatory effect on the AChE activities, which mediate transmission of stimuli for the initiation of labour.

One of the mechanisms by which a toxic agent can alter the male reproductive function is by altering DNA or its associated proteins in the testes (Wyrobek and Bruce, 1975). Although the mating index of the F1 male offspring in the CC group was not significantly different from those in the other groups, the marginal decrease recorded in this parameter indicated that the insecticide mixture may have some antiandrogenic effect. Pesticides, including CPF (Shittu *et al*., 2014) and CYP (Umosen and Chidiebere, 2014), are known to alter sex hormone concentration via oxidative injury to both the pituitary gland; which produces FSH and LH and regulate the number of Leydig cells and their secretion of testosterone (Ramaswamy and Weinbauer, 2014). Similarly, pesticide-evoked oxidative damage to the testes may have additionally impaired testosterone secretion. At the molecular level, the androgenic activity of testosterone is through the androgen receptor (AR) regulation of gene expression. Testosterone stimulation causes the release of AR from heat shock proteins in the cytoplasm after which the liganded AR translocates to the nucleus and binds to specific DNA sequences called androgen response elements (AREs). The binding of the steroid to its receptor produces conformational changes that result in the formation of a "transformed" or activated receptor that has high affinity for specific DNA-binding sites (Tsai and O'Malley, 1994). When in the proper context, the AR bound to DNA recruits co-activators or corepressors of gene expression (Bagchi *et al*., 1992). In addition, the reduced mating index may be partly attributed to the decrease male response to female pheromone when exposed to insecticides (Delpuech *et al*., 1998) due to effect of the insecticides on nervous transmissions. Pheromone perception involves the excitation of

specific receptors that elicit nerve impulses, interpreted by the CNS hence, any insecticide that perturbs the transmission of nerve impulse may interfere with the perception of pheromones. This could also be attributed to decrease in concentration of hormones such as FSH and testosterone concentration found in the study.

Male infertility is commonly due to deficiencies in the semen and semen quality (Cooper *et al*., 2010; Kumar and Singh, 2015). Decrease in mating index in group exposed to CPF and CYP may also be ascribed to increased production of gonadotropin-inhibitory hormone (GnIH), apparently due to stress (Ubuka *et al*., 2013) evoked by neonates gestationally exposed to the insecticidal mixture. This eventually inhibits hypothalamic-pituitary-gonadal axis (HPG axis) by activating the GnIH system, which ultimately reduces reproductive performance. GnIH neurones regulate not only gonadotropin synthesis and release in the pituitary, but also regulate various hormones in the brain, such as $GnRH_1$, $GnRH_2$, dopamine and kisspeptin neurones. GnIH and G-protein-coupled receptor 147 (GPR147) are also expressed in gonads and they may regulate steroidogenesis and germ cell maturation in an autocrine/paracrine manner (Ubuka *et al*., 2016). Apart from decreasing gonadotropin synthesis and release, GnIH inhibits gonadal development and maintenance (Tsutsui *et al*., 2010). The overall effects are impaired sex hormone synthesis, reduced libido and poor spermatozoa action.

Pretreatment with melatonin has been shown in this study to mitigate the reduced mating index, caused by pesticide mixture. The ROS scavenging and the antioxidant properties of melatonin may have been partly responsible for this effect. Melatonin by protecting the HPG axis from oxidative damage was able to restore testosterone synthesis impaired by insecticide mixture, thereby improving mating index. Melatonin

may protect by virtue of its modulatory effect on agents capable of promoting the fragmentation of sperm DNA (Sarabia *et al*., 2009). The well-demonstrated antioxidant properties of melatonin make it an efficient molecule for the protection of cell macromolecules from oxidative damage. Melatonin is notable for its role in preserving the quality of sperm chromatin, through possible protection of the germinal epithelium of the testes. Studies have shown that melatonin has the potential to counteract CPF-induced toxicity in humans by protecting against ROS-induced DNA damage and protein oxidation (Ghayomi *et al*., 2015).

Melatonin also decreases the level of certain enzymes like caspases 3 and 9, which lead to apoptosis (Espino *et al*., 2011b). It stimulates several antioxidant enzymes such as GPx, SOD, and CAT and promotes gene expression. It also mediates GSH synthesis through stimulating g-glutamylcysteine synthetase (Urata *et al*., 1999). This endogenous agent scavenges ROS and improves activities of antioxidant enzymes, thereby playing a preventive role in phosphine-associated toxicity arising from oxidative stress. Melatonin neutralises the inhibition of respiratory chain complexes and promotes the production of ATP. It also prevents apoptosis by directly inhibiting 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) opening (Reiter *et al*., 2008).

Sex ratio is defined as ratio of number of males to females (Jurewicz *et al*., 2016). World-wide, human sex ratio at birth is fairly constant with male excess (51.4%) (James, 2004). Therefore, in early human development, the sex ratio is female biased. Study conducted by Orzack *et al*. (2015) showed that during pregnancy, there is an increase in the loss of more of male in the first few weeks of pregnancy, primarily due to a higher rate of abnormalities in male embryos. This is followed by an increased loss

of female foetuses later in early pregnancy with an increased mortality of male foetuses from mid-gestation onward (Orzack *et al*., 2015). However, the present study revealed a decreased sex ratio in the CC group, which was indicative of the presence of more females than males. Huang and Li (2014) reported a decrease in male to female ratio following maternal exposure of rats to CYP. Garry *et al*. (2003) have also reported a decrease in sex ratio of children born to pesticide applicators. Studies have shown a decrease in pups sex-ratio (M/F), respectively in spouses of banana workers, exposed to 1, 2-Dibromopropane, and in spouses of pesticides manufacturers and pesticides sprayers (Whorton *et al*., 1979; Ryan *et al*., 2002; Figa-Talamanca *et al*., 2003). These studies concurred with the result of the present study that sex ratio alteration is female-biased. Although study by Venerosi *et al*. (2015) showed a non-significant alteration in sex ratio following CPF exposure in mice, the fact that a mixture of CPF and CYP altered the sex ratio inferred that animals exposed to insecticide combination may be more vulnerable to sex ratio alteration.

In recent years, a trend toward a declining proportion of male births has been noted in several, but not all, industrialized countries (Parazzini *et al*., 1998; Astolfi and Zonta, 1999). The aetiology of alterations in the sex ratio remains uncertain. Many factors have been postulated to affect sex ratio (James, 1987). The sex ratio has been reported to decrease slightly with increasing parental age and increasing rank number of the child (Garfinkel and Selvin, 1976), parental hormone levels (James, 2001), changes in coital rates (Dickinson and Parker, 1996), the length of inter-pregnancy intervals (James, 1996), changes in maternal nutrition (Rosenfeld *et al*., 2003) or multiparity (Juntunen *et al*., 1997). However, the theory that suggests that widespread environmental endocrine disrupting chemicals affecting the male reproductive system has gained prominence

(Parazzini *et al*., 1998; Martuzzi *et al*., 2001). Decrease in the sex ratio in the CC group may also be attributable to effect of both pesticides on gene responsible for determination of sex or gender differentiation during embryogenic development. Mammalian offspring sex ratios can be biased via prenatal and postnatal mechanisms, including sperm selection, sex-specific embryo loss, and differential postnatal investment in males and females (Beery and Zucker, 2012). Several studies on the association between endocrine-disrupting chemicals and the sex secondary ratio have demonstrated possible roles of paternal rather than maternal exposure to select chemicals in offspring sex determination (Terrell *et al*., 2011).

The increased number of female births in the CC group may also be associated with high number of defect in the spermatoazoan in the group. In a recent study, Bae *et al*. (2016) reported that sperm defect is associated with excess number of female births. Given that the Y chromosome is the sex‑determining chromosome in men, a study by Eisenberg *et al.* (2011) in an infertile cohort of 185 men undergoing a semen fluorescence *in situ* hybridisation (FISH) test from 2003 to 2010 in the United States showed that poor semen quality, which was reflected by semen volume, sperm concentration, and total motile sperm count, significantly decreased the odds of having a Y chromosome‑bearing sperm. Bae *et al*. (2016) postulated that sperm motility or morphology may serve as a possible predictor of infant sex, in accordance with an existing theory, focusing on the difference in sperm swimming velocity or size between Y chromosome‑bearing sperm and X chromosome‑bearing sperm as a potential influence of human sex selection (Jongbloet, 2004). Specifically, the "over‑ripeness ovopathy" concept hypothesises that Y chromosome‑bearing sperm are more suitable for navigating non-optimally liquefied cervical mucus accompanied by non-optimally

matured oocytes than are X chromosome-bearing sperm, as Y chromosome-bearing sperm are smaller than X chromosome-bearing sperm in terms of the length, perimeter, and area of sperm head, and the length of sperm neck and tail (Cui, 1997). As a consequence, the preferential fertilization of non-optimally matured oocytes by Y chromosome-bearing sperm may contribute to disproportional loss of male embryos and foetuses, resulting in alterations in the secondary sex ratio. Therefore, intrinsic differences in sperm motility, viability, and fertility potential between Y- and X-chromosome-bearing sperm have been suggested to alter the secondary sex ratio (SSR) on the paternal side (Shettles, 1961; Alminana *et al*., 2014).

In another study (Garry *et al*., 2003), a shift in sex ratio of children born to pesticide applicator families was noted among applicators who applied fungicides in addition to herbicides and insecticides. Reproductive hormone analyses conducted in conjunction with this epidemiological study showed that more female children were born to applicators with lower testosterone levels. Therefore, the higher number of female birth recorded from untreated dam mated by F1 male rats in the CC group in the present study may also be partly due the lower testosterone concentration in the F1 male rats.

Pre-treatment with MEL has been found in this study to normalise the sex ratio altered by the insecticide mixture, when compared to that of the insecticide mixture-treated group. The increase in the sex ratio may be partly due to antioxidant and radical scavenging nature of melatonin that protect the integrity of the spermatozoa and protect it from damage by the insecticide mixture. This is manifested by the lower number of sperm defect recorded in the MCC group, which may have increased the propensity of having Y-chromosome bearing sperm, leading to increase in the number of male births.

Growing body of evidence indicates that melatonin has important effects in the reproduction of some non-seasonal breeding animals. In males, melatonin has effect on the hypothalamus–pituitary axis by inducing an increase in GnRH pulsatile secretion (Lincoln and Clarke, 1997), promotes low prolactin levels and increases in LH, FSH, and testosterone secretion (Webster *et al*., 1991; Rosa *et al*., 2000), and regulates testicular maturation. These imply that melatonin has important effects on the regulation of testicular development and male reproduction (Li and Zhou, 2015).

Semen quality is conditioned by the presence of endogenous MEL, which directly and indirectly acts on some of the most important elements that influence sperm quality parameters, specifically sperm motility, and reducing or delaying apoptosis (Casao *et al.,* 2010). These effects may also be true for exogenous melatonin administration as shown in the MCC group in the present study. It is also documented that melatonin receptors MT_1 and MT_2 are expressed in spermatozoa (Gonzalez-Arto *et al*., 2016), with modulation of this hormone on sperm capacitation mainly via the MT_2 receptor (Casao, Luna *et al.*, 2012). The strengthening of sperm capacitation by melatonin may have also contributed to increasing the sex ratio in the MCC group. Furthermore, the increase in testosterone concentration in the F1 male rats may have played a role in the increased sex ratio recorded in MCC group.

The present study shows a decrease in viability index in group exposed to insecticide mixture. This agreed with the results obtained from previous studies in pups following *in utero* CPF exposure (Farag *et al*., 2003; Morgan and Abd El-Aty, 2008; Shalaby *et al*., 2013). In humans, the aetiology of many developmental disorders is poorly understood; consequently, it is thought that infections, maternal or paternal health and

genetic factors, drugs, over the counter medications, abused substances, environmental or occupational exposures are responsible (Mattison, 2010).

In the present study, the reduction in pups' viability is related to both the direct effect of the pesticides mixture and indirectly due to oxidative stress. During gestation, the maternal mammal is the sole source of nutrients, electrolytes, and oxygen, controls homeostatic mechanisms, regulates fluids and temperature, and provides means for the elimination of metabolic wastes (DeSesso *et al*., 2009). Environmental insults that produce maternal toxicity (for example, reductions in body weight, clinical symptoms) can lead to induction of an acute-phase response and changes in maternal metabolism that result in foetal or pup toxicity (Mink *et al*., 2012). In the same vein, the toxic effect of CPF on suckling pups could be explained by the greater susceptibility of newborns than adult rats to CPF or its metabolites, secreted in milk during lactation.

According to Mansour and Mossa (2010), the transfer of CPF through the mother's milk to the neonates has resulted in oxidative stress as well as some biochemical and histopathological alterations, which may negatively impact on viability. In the present study, the increased level of pesticide-induced ROS production in the embryo may have damage the macromolecules within its cells, resulting in pathological consequences. This may eventually result in the foetus being born weak and non-viable. The decreased foetal viability index in the CC exposed group may also result from the cytotoxic effect of pesticide mixture.

Pre-treatment by melatonin is shown in this study to restore the pups' viability index, altered by the pesticide mixture, partly due to its antioxidant and ROS scavenging

effect. Antioxidants have been shown to neutralise the ROS produced by the pesticide mixture and prevent damage to DNA, lipids, proteins, and other biomolecules (Trushina and McMurray, 2007), thereby preventing ROS induced foetotoxicity. Melatonin diminishes the destruction of DNA, proteins and lipids that occur as a result of their reactions with ROS and RNS (Chojnacki *et al*., 2011) as it easily crosses the cell membranes and the BBB (Hardeland *et al*., 2009). Melatonin along with its metabolites has been shown to scavenge ROS and RNS (Hardeland, 2005). This cascade reaction makes melatonin a highly effective antioxidant, even at low concentrations, in protecting cells from oxidative stress (Tan *et al*., 2007).

It is now well documented that in humans some 30% of implanted embryos are lost, and around two-thirds of these are lost before the woman knows that she is pregnant (Rothman and Greenland, 1998). Male-mediated spontaneous abortion is a well-established phenomenon in some animal experimental studies (Olshan and Faustman, 1993; Marchetti *et al*., 1999). In the present study, the co-habitation of F1 male rats from dams co-exposed to CPF and CYP with an untreated females resulted in an apparent decrease in gestation or pregnancy index. This shows that many of untreated females mated with the F1 males were not able to carry the pregnancy through to parturition, apparently due to defect in the spermatozoa of the F1 male. There is some evidence of epigenetic effects of paternal exposure that can impact on the pregnancy outcome, but human evidence is still not convincing (Cordier, 2008). It has been shown in a human study that loss through miscarriage, prematurity, or still-birth is frequent in spouses of pesticide applicators (Garry *et al*., 2002). The dominant lethal assay detects genetic defects occurring in the male germ cell that allows fertilisation, but results in early embryonic death in the female (Hjollund *et al*., 2004). Recio *et al.* (2001)

demonstrated that OP insecticides significantly increased sperm chromosome nullisomy, involving the sex chromosomes. This abnormality in sperm chromosome may lead to miscarriage or still-birth in females mated with such a deformed sperm. Although sperm chromosome was not analysed in the present study, the increased morphological abnormalities in the CC group of the F1 male may be related to chromosomal aberration, and, hence, the altered gestation index in the CC group.

Pre-treatment with melatonin has been shown in the present study to mitigate the gestation index altered by prenatal exposure of the F1 male rats. This may be due to mitigation of oxidative damage to the spermatozoa of the F1 male rats by melatonin. Melatonin as an antioxidant protects the macromolecules, including DNA from oxidative damage provoked by the pesticide mixture, thereby mitigating apparent chromosomal alterations that may have contributed to relative increase in gestation index in the MCC group.

In the present study, there was a decrease in fertility index in the CC group of F1 male rats relative to the other groups. Decrease in the fertility index in group exposed to insecticide mixture may be due to testicular damage and sperm abnormalities in relation to concentration, morphology and progressive motility. The sperm abnormality may be due to pesticide-provoked oxidative damage to spermatozoa due to *in utero* and lactational exposure to mixture of CPF and CYP. *In utero* exposure to the pesticide mixture may have resulted in oxidative and cytotoxic damage to the germ cells of the spermatozoa. The germ cells determine the fertility potential of an individual because they form the spermatozoa. Failure of the germ cell to survive during the development may lead to defective or lack of gamete production, and hence can lead to infertility

(Sharma *et al*., 2015). CPF has been shown to cause accumulation of exfoliated germ cells within the tubules in addition to the appearance of cytoplasmic vacuolation (Reham, 2013), while deformed and disordered arrangement of germ cells have been observed after exposure of CYP (Fang *et al*., 2013).

Furthermore, oxidative damage to the testis may have also been partly responsible for the lower fertility index of the F1 male rats. The male testes fulfill two essential functions; spermatogenesis in the seminiferous tubules, producing male gametes, and synthesis and secretion of sexual hormones in the interstitium. These two testicular compartments work in collaboration with HPG axis for normal spermatogenesis to occur (Bretveld *et al*., 2007). Any alteration in the complex regulation of hormone production and spermatogenesis can result in impairment or cessation of spermatogenesis, leading to infertility (Sharma and Goyal, 2014). As a result of oxidative damage to the pituitary gland and testis via *in utero* and lactational exposure to the pesticide mixture, the endocrine regulatory system and spermatogenesis have been adversely altered, resulting in the decreased fertility index.

Sertoli cells that create a favourable environment for germ cell proliferation and maturation may have been oxidatively damaged by the pesticide mixture leading to its functional impairment, which is detrimental to spermatogenesis because these cells are essential for the proliferation and differentiation of all spermatogenic cells. In addition, the decreased testosterone concentration in the F1 male rats in the CC group may have been partly due to impaired function of Leydig cells, which in turn can lead to diminished Sertoli cell function and spermatogenesis (Bretveld *et al*., 2007). What is partly responsible for the increased susceptibility of sperm to oxidative stress is the

abundance of oxidative targets such as polyunsaturated fatty acids in the plasma membrane of spermatozoan, which is required for fusion with the oocyte and fertilisation (Sanocka and Kurpisz, 2004). Furthermore, DNA fragmentation resulting from oxidative stress is a major contributor to poor sperm quality and function (Fernando and Rombauts, 2014). DNA damage to the spermatozoan has been shown to have a negative impact on fertilisation, and blastocyst development (Seli *et al*., 2004).

Pre-treatment with melatonin has been shown in the present study to improve fertility index adversely altered by the pesticide mixture partly due to its antioxidant and free radical scavenging effect. Melatonin can also have an indirect action as antioxidant through activation of endogenous antioxidant enzymes, such as SOD, GPx and catalase (Barlow-Walden *et al*., 1995; Oner-Iyidogan *et al*., 2001; Maharaj *et al*., 2002). Apart from its antioxidant and ROS scavenging effects, melatonin has been shown to directly influence the HPG axis. For instance, melatonin receptors (MT_1 and MT_2) have been identified in hypothalamic neurons, governing the release of pituitary gonadotrophs (Roy *et al*., 2001; Dubocovich and Markowska, 2005) in gonadotropins of the anterior pituitary (Johnston *et al*., 2003; Balik *et al*., 2004) and in both female and male gonads (Woo *et al*., 2001; Frungieri *et al*., 2005), as well as in the reproductive adnexae (Kobayashi *et al*., 2003; Ekmekcioglu, 2006). Although MT_1 and MT_2 receptors have been identified in testicular cells, particularly in Leydig cells (Ng and Lo, 1988; Valenti *et al*., 1997; Izzo *et al*., 2010), it is MT_2 receptor that is responsible for enhancement of sperm capacitation. Melatonin also regulates testicular growth and testosterone production in testicular cells (Olivares *et al*., 1989; Valenti *et al*., 1995; Frungieri *et al*., 2005). It has been shown that the addition of melatonin to seminal samples can improve the overall motility and the percentage of progressively motile spermatozoa (Ortiz *et al*.,

2011; Espino *et al* 2011a). The ability of melatonin to stimulate flagellar motility is important given that this response is a requirement for the successful penetration of the *zona pellucidum* and fertilization of the egg (Reiter *et al*., 2009).

Melatonin is also noted to inhibit apoptosis in spermatozoa, with a reduction in early apoptotic events being demonstrated in human sperm thus prolonging sperm survival (Fernando and Rombauts, 2014). These effects would serve to improve sperm quality, therefore increasing the probability of successful fertilisation. Studies in rats also have shown that melatonin has a positive effect on sperm that have been subjected to oxidative stress, improving sperm number, viability and motility (Kim *et al*., 2013; Liu *et al*., 2013; Nasiraei-Moghadam *et al*., 2014). Similar results have been found in a small human study in which *in-vitro* melatonin-treated samples showed a higher percentage of sperm motility and a lower proportion of non-viable spermatozoa (du Plessis *et al*., 2010). Therefore, the improved fertilisation index in the group pretreated with melatonin in the present study was due to its antioxidant and non-antioxidant effects.

It is estimated that there are about 3.1 to 10.7 million perinatal deaths per annum in developing countries alone (Taha and Gray, 1993; Zupan, 2005; Porpora *et al*., 2016). Several factor including, biological and social have been incriminated in perinatal mortality, but with increasing industrial development and modernisation of agriculture, occupational and environmental reasons have taken a lead in terms of importance. The present study showed a decrease in the live birth index in the group exposed to the pesticide mixture only. This agreed with the results obtained in CPF (Farag *et al*., 2010) and a related type-II pyrethroid (*Lambda cyhalothrin*) poisoning in rats (Ratyanake *et*

al., 2007). The decreased live birth index in the CC group is a demonstration of increased prenatal mortality following intrauterine exposure to the pesticide mixture. Decreased live birth index is a parameter used to measure reproductive competence/deficits, associated with a specific agent. It also indicates the vulnerability of subject to test substance.

In the present study the foetal cytotoxicity and oxidative damage to foetal cells macromolecules, including DNA may have been responsible for the decreased live birth index in the F1 male of the CC group. Apart from inducing damage to reproductive organs, CPF is known to induce neurological and organs' and skeletal malformations and micronucleus formation, persistent developmental disorders and maternal toxicity. The binding of CPF with DNA to produce DNA adducts leads to increasing concern about the genotoxic risk of CPF in humans and animals. CPF may induce cytotoxicity through DNA damage and cell apoptosis (Li *et al*., 2015a). This increased apoptotic effects of CPF and in combination with CYP usually occur as a sequelae to the pesticide-induced DNA fragmentation, consequent upon oxidative damage. Pesticides including CPF have been incriminated to significantly increase the probabilities of chromosomal aberrations and the frequency of micronucleus formation in mice spleen cells, rat bone marrow cells, and Chinese hamster pneumonocytes (Bolognesi, 2003; Tian and Yamauchi, 2003). Exposure to CPF, acutely or chronically, also caused a dose-dependent increase in DNA damage in the liver and brains of rats, and also the production of ROS that caused considerable cytotoxicity in PC12 cells (Mehta *et al*., 2008). A primary risk factor of genotoxic agrochemicals is that they act directly or indirectly on chromosomal DNA, causing chronic genotoxicity such as carcinogenic and reproductive toxicity (Luzhna *et al*., 2013; Ibrahim, 2016). The combined effect of these

functional and morphological abnormalities may have resulted in increasing prenatal death recorded in the CC group.

Pre-treatment with melatonin was able to improve the CC-induced live birth index deficit due to its ROS scavenging and antioxidant effect in the developing foetus. Melatonin through its antioxidant mechanism was able to protect the macromolecules from oxidative damage, thereby reducing the number of fragmented DNA, hence genotoxicity.

Across human populations, sperm counts appear to be declining. In 1940 the average human density of sperm was 113 million per millimeter of semen; in 1990 this figure had dropped to 66 million sperm per millimeter of semen. Researchers also estimate that the volume of semen produced by men has dropped to about 20% during the past 50 years, reducing sperm count per ejaculation even further (Ayotte *et al*., 2001; Mehrpour *et al*., 2014).

Male exposures, in isolation from female or foetal exposures, have been shown in experimental studies to be capable of affecting the entire spectrum of reproductive health end-points (Olshan and Faustman, 1993) through mechanisms involving the sperm (Savitz *et al*., 1997). Observations in several Western countries point toward a decline in sperm concentration and semen quality which may be associated with exposure to environmental endocrine disruptors (Diamanti-Kandarakis *et al*., 2009) including pesticides (Roeleveld and Bretveld, 2008). Sperm count is considered to be one of the important factors that affect fertility (Bebb *et al*., 1996; Kumar and Singh, 2015). The present study showed a lower sperm count in the male rats exposed to CPF

and CYP *in utero* and during lactation. Exposure of adult male rats to CPF (Sai *et al*., 2014; Shittu *et al*., 2014; Umosen and Chidiebere, 2014), CYP (Al-Hamdani and Yajurvedi, 2010; Li *et al*., 2013) and mixture OF CPF and CYP (Ikpeme *et al*., 2016; Alaa-Eldin *et al*., 2017) have been shown to negatively alter sperm count. Suppression of gonadotrophins by CPF might have caused decrease in sperm count in testes (Joshi *et al*., 2007; Shittu *et al*., 2014). Perinatal exposure to CYP has been shown to impair testicular development (Huang and Li, 2014) and may have been partly responsible for the decreased sperm count recorded in the CC group. Suppresion of gonadotrophins, FSH and LH with a subsequently decreased testosterone concentration as recorded in the CC group in the present study have been reported to hinder the production and maturation of spermatozoa (Joshi *et al*., 2007; Sharma *et al*., 2014b).

The suppression of the gonadotrophins (LH and FSH) and testosterone may have been due to oxidative damage to pituitary gland and testes, respectively. Although the testicular tissue of an adult rats contains an elaborate array of antioxidant enzymes and ROS scavengers to ensure that the spermatogenic and steroidogenic functions are not disturbed by chronic exposure of xenobiotics (Sharma *et al*., 2014b), this is not the case in the foetus, where the levels of antioxidant enzymes are very low due to immaturity of the organs producing them. Therefore, in the face of increasing oxidative challenge to the testes provoked during *in-utero* exposure to the pesticide mixture, the testicular tissues including the Leydig and the Sertoli cells may be damaged, while also provoking accelerated death of spermatogenic cells with consequent adversity on spermatogenesis in later life.

Furthermore, pyrethroids including CYP cause male reproductive toxicity through neuroendocrine-mediated phenomenon and a hormone-disrupting property. Yousef *et al*. (2003) partly attributed the low sperm count induced by pyrethroids due to their ability to interact competitively with androgen receptors and sex hormone-binding globuli; and thereby cause disruption of endocrine system by mimicking the effect of female hormone oestrogen leading to low sperm counts. It may also be associated with increased on-going apoptotic cascade in the testes as apoptosis occurs at any time in the ontogeny of the testes, especially during the first wave of spermatogenesis (Hou *et al*., 2016; Xu *et al*., 2016).

Pre-treatment and post-treatment with melatonin have been shown in this study to increase epididymal sperm concentration in F1 generation male rats. This may be due to reduced pesticide-provoked oxidative injury to the seminiferous tubules of the testes due to its antioxidant and ROS scavenging properties. Melatonin also appears to inhibit apoptosis in spermatozoa, with a reduction in early apoptotic events being demonstrated in human sperm, thus prolonging sperm survival (Espino *et al*., 2010a,b; Fernando and Rombauts, 2014). These effects would serve to improve sperm quality, therefore increasing the probability of successful fertilisation. Melatonin, through its neutralisation of ROS and RNS, has been shown in both animal and human studies to improve seminal quality *in vitro*. A study investigating the addition of melatonin to semen extender in cryopreserved seminal samples from Holstein bulls resulted in amelioration of the oxidative effects of the freeze-thaw process (Ashrafi *et al*., 2013). Studies in rats also have shown that melatonin has a positive effect on sperm, subjected to oxidative stress, improving sperm number, viability and motility (Kim *et al*., 2013; Liu *et al*., 2013; Nasiraei-Moghadam *et al*., 2014). Similar results have been found in a

small human study in which *in-vitro* melatonin-treated samples showed a higher percentage of sperm motility and a lower proportion of non-viable spermatozoa (du Plessis *et al*., 2010). Melatonin may also produce an increase in sperm concentration, possibly by diminishing the basal levels of apoptosis of the germinal epithelium via diminished ROS levels (Martin-Hidalgo *et al*., 2011).

Sperm motility is an important functional measurement to predict sperm fertilising capacity. Any negative impact on motility would seriously affect fertilising ability (ElMazoudy *et al*., 2011). In the present study, marked inhibition or decrease sperm motility in F1 male rats of the CC group was recorded. Earlier studies have shown that CPF (Joshi *et al*., 2007; Shittu *et al*., 2014; Umosen and Chidiebere, 2014), CYP (Joshi *et al*., 2011; Sai *et al*., 2014) and CPF+CYP (Ikpeme *et al*., 2016) caused decreased sperm motility. The epididymal spermatozoa are highly dependent on testosterone and epididymal protein for their final maturation and development of progressive motility and fertilising capacity (Vawda and Davies, 1986; Baker *et al*., 2014). Therefore, the lower testosterone concentration in the Fi male rats in the CC group may have been partly responsible for the reduced progressive motility. In addition, pesticide-evoked oxidative injury to the spermatozoan may have contributed to the reduced sperm motility in the CC group since increased levels of ROS have been correlated to decreased motility parameters (that is, total motility, progressive motility and rapid motility), which were concluded to have adverse effects on fertility (Padron *et al*., 1997; Saleh and Agarwal, 2002; Aziz *et al*., 2004; Agarwal *et al*., 2014). Although spermatozoa and seminal plasma contain a battery of ROS scavengers, including enzymes such as SOD (Alvarez *et al*., 1987), catalase (Jeulin *et al*., 1989), the glutathione peroxidase/reductase system (Alvarez and Storey, 1989) and also a variety

of substances with SOD- or catalase-like activities (Zini *et al*., 1993; Vernet *et al*., 2004; O'Flaherty *et al*., 2014), such as α-tocopherol, ascorbic acid, glutathione (Halliwell and Gutteridge, 1989), pyruvate (de Lamirande and Gagnon, 1992b), taurine, hypotaurine and albumin (Alvarez and Storey, 1983), these are overwhelmed in the face of pesticide-provoked increased oxidative injury. The ROS also cause loss of sperm motility due to intracellular ATP depletion and insufficient axonemal protein phosphorylation (de Lamirande and Gagnon, 1992a,b). In addition, oxidative effect by the pesticide mixture may cause decreased mitochondrial activity or corrosion of microtubule structure of spermatozoan may be partly responsible for the decrease motility (Uzunhisarcikli *et al*., 2007). Oxidative phosphorolytic process is required for ATP production, a source of energy for the forward movement of spermatozoa (Bebb *et al*., 1996). Full ATP pool is crucial for normal spermatozoa movement and a slight deprivation of ATP leads to reduction in motility, which may cause infertility. Furthermore, reduction in sperm motility in the F1 male rats of CC group could be due to altered fructose synthesis recorded in the present study.

Pre-treatment with melatonin in the present study has been shown to improve the percentage progressive sperm motility. Ortiz *et al*. (2011) and Espino *et al*. (2011a) have shown that the addition of melatonin to seminal samples improve the overall motility and the percentage of progressively motile spermatozoa. Although ROS are known to damage lipids, they also lead to modifications of proteins and DNA; and, thus, ultimately cause sperm death (Agarwal *et al*., 2014). Melatonin increases sperm motility, viability, and survival rates as well as preserving membrane integrity and lowering lipid peroxidation (Ortiz *et al*., 2011; Martin-Hidalgo *et al*., 2011). Moreover, melatonin effectively reduces chromosomal and mitochondrial DNA damage caused by

oxidative stress, and inhibits cellular apoptosis in cryopreserved spermatozoa (Cruz *et al*., 2014). It significantly limits sperm malformations, teratozoospermia and excessive ROS production in mice. In addition, it reduces the degree of lipid peroxidation and improves the stability of sperm DNA in mice exposed to continuous or intermittent hypoxia (Vargas *et al*., 2011). Therefore, inhibition of oxidative stress to the spermatozoa by melatonin may have preserved the ATP and caused sufficient axonemal phosphorylation, consequent to improved progressive motility. Furthermore, the improvement in testosterone concentration in melatonin-pretreated F1 male rats may have contributed to enhanced progressive motility in the group.

The semen quality is determined by the semen volume, sperm motility and by number of normal sperm. Good quality semen enhances the chances of fertilization, and semen with depleted quality generally fails to cause the fertilization (Owen and Katz, 2005). The present study revealed an increase in non-progressive sperm movement in the group exposed to CC relative to other groups. This may be partly due to altered internal milieu of the seminiferous tubules and defective spermatozoa caused by ROS provoked by the insecticide mixture. It is well recognised that oxidative stress is one of the major causes of sperm DNA damage (Aitken *et al*., 1998; Oger *et al*., 2003; Saleh *et al*., 2003a, b). Increased sperm membrane lipid peroxidation has been shown to impede sperm progress motility and increase percentage total sperm abnormalities as well as cause a dramatic loss in the fertilizing potential of sperm (Adamkovicova *et al*., 2016). A link between impaired sperm motility and oxidative stress also extends to the sperm DNA as a recent study has identified a highly significant correlation between oxidation of sperm DNA and reduced motility (Kao *et al*., 2008; Aydos *et al*., 2015). Decrease in sperm movement may also be ascribed to decreased energy in form of ATP that is available for

the sperm to propel itself for forward movement. Sperm motility may be affected by altered enzymatic activities of oxidative phosphorolytic process. Oxidative phosphorolytic process is required for ATP production, a source of energy for the forward movement of spermatozoa (ElMazoudy *et al*., 2011). It may also be related to morphological damage to the sperm mitochondrial section. This could be due to retention of cytoplasmic droplet in sperms in rat's cauda epididymal spermatozoa after treatment with cytotoxic and xenobiotic agents (Akbarsha *et al*. 2000). It is believed that increase in morphological damage to parts of sperm structure may have a damaging effect on their propelling ability and, hence, capacity to fertilise. The decrease in sperm motility could also be due to the disturbance of tail structural protein components in association with or without ATP synthesis (Contreras and Bustos-Obregon, 1999; Mandani *et al*., 2013).

Pre-treatment with melatonin has been shown in the present study to decrease the non-progressive movement, comparable to those of the controls. Melatonin has been shown to decrease effects associated with increased ROS generation in the testicular tissues and create a good medium for optimum maturity and motility sperm (Sarabia *et al*., 2009; Khaksar *et al*., 2017). Since DNA damage to the spermatozoa has been positively correlated with motility, the ability of melatonin to prevent oxidative damage to the DNA may have partly contributed to decrease in non-progressive motility in the pretreated group.

The present study showed an increased in the number of immotile sperm in group exposed to CC compared to other groups. This agreed with the work of Elmazoudy *et al*. (2011) in rats following CPF exposure. The increased number of immotile sperm

concentration may be due to effects of the insecticide mixture on sperm viability partly due to oxidative stress. Increase in immotile sperm cells could also be ascribed to increased number of morphological changes, especially those associated with tail defects recorded in the CC group in the present study. Increase in immotile number could also be due to increased levels of immature sperm cells along the spermatogenic cycle. OPs have been reported to be spermatotoxic manifested through increased sperm broken head, related to the possible pathology in the caput epididymis (Okamura *et al*., 2009; Taib *et al*., 2014). An association between urinary metabolites of pyrethroid insecticides and decreased semen quality, as well as evidence for a relationship between pyrethroid metabolites and sperm DNA damage have been documented (Meeker *et al*., 2008).

Pre-treatment with melatonin was able to decrease the number of immotile spermatozoan due to ROS scavenging properties. The reduction in the number of immature and morphological abnormalities by melatonin may have led to improvement in the level of motility in the melatonin pretreatment group. Melatonin through its antioxidant properties decreases the pesticide-provoked increased seminal oxidative stress, thereby improving the environment for efficient motility since seminal fluid contain "curing factors" that modified the state of sperms motility (Bradshaw *et al*., 1999; Ortiz *et al*., 2011; Saeed *et al*., 2015). Melatonin acts as a direct ROS scavenger (Korkmaz *et al*., 2009; Li and Zhou, 2015) and as an indirect antioxidant via its stimulatory actions on antioxidative enzymes (Anisimov *et al*., 2006; Hibaoui *et al*., 2009). 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 (Bharti *et al*., 2011). Its hydrophilic and lipophilic nature also aids in its antioxidant actions. Its lipophilic nature enhances the easiness at which it crosses the morphophysiological barriers, such as the BBB, and then effectively enters the cells and subcellular compartments (Erukainure *et al*., 2011; Johns, 2011). It is distributed in all cell compartments, being especially high in the nucleus and mitochondria (Srinivasan *et al*., 2011; Venegas *et al*., 2011).

Sperm viability test is an important factor used for *in vivo* and i*n vitro* male fertilization capacity (Khaleghi *et al*., 2016). Decrease in the spermatozoa number and sperm motility and increase in dead and abnormal sperm rate are indicative of damaged testicular structure in rats (El-Gerbed *et al*., 2015). Alteration in sperm live-dead ratio is reported to be associated with damage to the germ cells lining seminiferous tubules arising from oxidative damage by the pesticide mixture. ROS can damage different parts of the spermatozoa, including plasma membrane, and nuclear and mitochondrial DNA (mtDNA), thereby impairing sperm function (Venkatesh *et al*., 2009). ROS-induced lipoperoxidation of the spermatozoa plasma membrane affects membrane fluidity and mobility, in addition to affecting sperm axoneme (Venkatesh *et al*., 2009). ROS also inhibit mitochondrial function and affect the synthesis of DNA, RNA and proteins (De Lamirande and Ganon, 1992a, b). Moreover ROS can cause various types of gene mutations, including deletion, point mutation or polymorphism of both mitochondrial and nuclear DNA of spermatozoa (Spiropoulos *et al*., 2002; Sharma *et al*., 2004). Genetic alteration of mtDNA may have serious consequences on normal spermatogenesis and fertilisation. Several reports have shown that mitochondrial dysfunction could lead to partial or complete spermatogenesis arrest and be an

important causative factor in male infertility (Frank and Hurst, 1996; Cummings *et al*., 1994; St John *et al*., 1997). ROS, triggered by entry into the intrinsic apoptotic cascade, ultimately result in enhanced oxidative DNA damage (Henkel, 2011). The consequence of this oxidative injury to the spermatozoan and mitochondrial DNA couple with seminal fluid oxidation induced by the pesticide mixture may have resulted in the production of defective spermatozoa, with resultant decrease in live-dead ratio.

Pre-exposure and post-exposure treatment with melatonin has been shown in this study to increase the viability of sperm cells. This may be due to the decreased formation of ROS and promotion of the neutralisation of their effects. Melatonin has been reported to prevent OP-induced damage on the testes and sperm DNA (Sarabia *et al*., 2009). The protective effect of melatonin on the oxidative changes in testicular tissue and sperm characteristics was documented (Umosen *et al*., 2012). The consequence of the neutralisation of oxidative stress by melatonin may have been responsible for the preservation of sperm cell integrity and subsequent improvement in live/dead ratio.

Sperm morphology is a more important index of fertility than cell density and the presence of many abnormal forms indicates impaired fertility (Singh *et al*., 2015a). Semen from infertile patients often contains higher abnormal forms than semen from fertile men (Macleod, 1970). The present study showed an increase in the sperm head defects in groups exposed to CC. This agreed with previous studies on CPF (ElMazoudy *et al*., 2011), CYP (Kumar *et al*., 2004) and combination of CPF + CYP (Ikpeme *et al*., 2016). Increased oxidative stress manifested by increase in testicular MDA and decrease in antioxidant status provoked by *in utero* and lactational exposure to CPF and CYP in the present study may be responsible for the increased in defect in sperm morphology.

Increased ROS level in seminal plasma due to oxidative stress may lead to sperm cell membrane defects which may cause morphological deformity in the sperm cells (Singh *et al*., 2015b). Oxidative stress may also cause damage to the DNA, thereby causing morphological abnormalities. Indeed, abnormal sperm morphology has been reported to be associated with high sperm DNA fragmentation in infertile men (Muratori *et al*., 2000; Huang *et al*., 2005; Nicopoullos *et al*., 2008). The integrity of sperm DNA is central to the transmission of genetic information during reproduction, and chromatin abnormalities or DNA damage can result in paternal fertility problems (Evenson *et al*., 2002; Pina-Guzman *et al*., 2005; Sergerie *et al*., 2005; Boe-Hansen *et al*., 2006).

The OPs are considered as potent phosphorylating agents, which are potentially genotoxic to animal sperm by altering the chromatin structure via binding to protamines and DNA, causing DNA to become more susceptible to induced denaturation *in situ* (Pina-Guzman *et al*., 2005). Furthermore, the lower testosterone concentration in the CC group may have contributed morphological defect, since it has been reported that testosterone plays a key role in the development of male reproductive tissues such as the testes and prostate. Testosterone is needed for the continued production of different generation of germ cells in the seminiferous tubules. Therefore, reduction of testosterone level may lead to the separation of germ cells from the epithelium of the seminiferous tubules (Elbertieha *et al*., 2001), resulting in morphological sperm defect. The result agreed with that of previous studies on OP (Yucra *et al*., 2006) and pyrethroids (Mehrpour *et al*., 2014) in adult rats.

Melatonin in the present study reduced the sperm morphological defect following *in utero* and lactational exposure to CPF and CYP in F1 male rats, apparently associated

with reduction of oxidative injury to the membrane of the spermatogenic cells, and reduced damage to the DNA of the spermatozoa. Similarly, the improvement in the testosterone concentration in the melatonin-treated groups may have contributed to the reduction in morphological defect to the spermatozoa.

The present study demonstrated a decrease in the number of normal sperm cells in group exposed to the pesticide mixture only. The decrease in the number of normal sperm cells could be attributed to oxidative injury to the spermatozoa and the testicular tissue. Oxidative stress which has been reported for both insecticides has contributed to declining semen quality (Koureas *et al*., 2012). ROS may also initiate a chain of reactions that ultimately lead to apoptosis of the spermatozoa (Taib *et al*., 2014). The process of apoptosis may also be accelerated by ROS-induced DNA damage ultimately leading to increased morphological defects. Defective spermatozoa are particularly vulnerable to oxidative stress because they contain a super-abundance of free unsaturated fatty acids that trigger ROS generation by the sperm mitochondria and induce high levels of lipid peroxidation (Aitken *et al.,* 2014). Worse still, the products of lipid peroxidation in the form of small molecular mass electrophilic aldehydes such as 4-hydroxylnonenal or acrolein are also capable of triggering ROS generation by the sperm mitochondria (Aitken *et al*., 2012). The only products of apoptosis that can damage sperm DNA are the ROS generated by the mitochondria (Aitken *et al*, 2014). Furthermore, increased seminal oxidative stress may have been partly responsible for the decreased number of normal sperm cells as this environment is hostile to normal spermatogenesis.

Pre-treatment and post-exposure treatment with melatonin was apparently, able to, normalise the number of normal sperm cells relative to that of the controls. This may be attributed to the antioxidant effect of melatonin. Melatonin may have prevented oxidative damage to the spermatozoa membrane and DNA, thereby preserving its morphology.

The present study showed a decrease in the concentrations of FSH in F1 rats co-exposed to CPF and CYP via the intrauterine and lactation routes. Repeated oral exposure of adult male rats to CPF (Joshi *et al*., 2007; Shittu *et al*., 2014; Umosen and Chidiebere, 2014) and CYP (Joshi *et al*., 2011), and combination of CPF and CYP (Ikpeme *et al*., 2016) have been reported to alter the concentration of FSH in male adult animals . The low FSH concentration in the CC group apparently occurred as a result of hormonal dysfunction in all the stages of hormonal regulation: hormone synthesis, hormone release and storage, hormone transport and clearance, hormone receptor recognition and binding, hormone post-receptor activation, thyroid function and the CNS (Bretveld *et al*., 2006). This reduction suggests that the pesticide mxture may also affect the HPG axis (Sharma *et al*., 2014. This may be due to oxidative injury to the hypothalamus that produce GnRH that stimulates the anterior pituitary to produce FSH. The FSH is intimately involved in the control of the growth and reproductive activities of the gonadal tissues, which synthesise and secrete male and female sex hormones.

The FSH controls spermatogenesis via direct stimulation of Sertoli cells, which are essential for the proliferation and differentiation of all spermatogenic cells and sperm maturation. The decreased FSH concentration in the F1 male rats exposed to pesticide mixture prenatally and during lactation may have been partly responsible for their low sperm count. In addition, the increase in ACh levels brought about by OP-induced

inhibition of AChE activates nicotinic and muscarinic receptors, which may alter GnRH release (Krsmanovic *et al*., 1998) and, therefore, affects FSH levels (Sarkar *et al*., 2000a).

Pre-treatment and post-exposure treatment with melatonin resulted in the apparent normalisation of the FSH, relative to that of the controls. This may be due to the ROS scavenging effect of melatonin, which protect the hypothalamus and the anterior pituitary from pesticide-induced oxidative injuries. This leads to elaboration of, apparently, normal FSH concentration in the melatonin treatment groups. Furthermore, the ability of melatonin to restore AChE activity in the pituitary gland as shown in the present study may have led to reduction in the cholinergic effect, hence normalising the gonadotrophin level.

Luteinizing hormone (LH) is a glycoprotein released from the anterior pituitary; it stimulates testosterone production by Leydig cells of the testes in males (Ramaswamy and Weinbauer, 2014). Hypothalamic control of LH appears to be by a common releasing hormone termed gonadoliberin (GnRH, LHRH), with negative feedback control at the hypothalamic level of testosterone in the male (Garcia *et al*., 1984). The present study showed lower serum concentration of LH in the F1 male rats exposed prenatally and lactationally to mixture of CPF and CYP relative to that of that obtained in the other groups. Previous studies have shown that CPF (Joshi *et al*., 2007; Shittu *et al*., 2014; Umosen and Chidiebere, 2014; Ventura *et al*., 2016), CYP (Elbetieha *et al*., 2001; Joshi *et al*., 2011) and their mixture (Ikpeme *et al*., 2016) caused a decrease in LH concentration in adult male rats. This may be partly due to pesticide-triggered oxidative injury to the hypothalamus and pituitary gland as shown by increased lipoperoxidation

and decreased antioxidant status. This results in reduced GnRH and FSH elaboration from the hypothalamus and pituitary gland, respectively. Increase in oxidative damage to the pituitary gland characterised by increase in the level of brain and pituitary gland MDA concentration has been reported in CPF and CYP exposure in rats (Umosen *et al*., 2012; Shittu *et al*., 2014; Idris *et al*., 2014). In addition, the ability of pesticides to suppress genes, involved in gonadotrophin synthesis, or interfere with steroidogenesis (Huang and Li, 2014) may have played a role in the decreased LH concentration. Indeed, CPF has been reported to cause damage to the GnRH gene expression, hence reduction in LH and FSH (Gore, 2001) due possibly to alterations in GnRH (Gore, 2002). The CPF has been reported to present anti-androgenic activity, altering male reproductive abilities and the expression of some steroidogenic enzymes (Viswanath *et al*., 2010). Similarly, anti-androgenic effect of CYP has been reported (Wu *et al*., 2008; Pan *et al*., 2013). Low levels of LH concentration have been shown to have adverse effects on the Leydig cells, involved in testosterone production in males (Shi *et al*., 2017); and may have been partly responsible for the low testosterone in F1 male rats, exposed to mixture of CPF and CYP prenatally and via lactation.

Pre-treatment and post-exposure treatment with melatonin in the present study was able to improve the LH concentration, relative to that of the rats exposed to the pesticide mixture only. This was due partly to the antioxidant effects of melatonin which protects the hypothalamus and the pituitary gland from oxidative injury (Umosen *et al*., 2012). Furthermore, the ability to preserve macromolecule from oxidative injury, its ROS scavenging effect may have played a role in preserving the integrity of the gene, responsible for GnRH production.

Testosterone, the male hormone produced from the Leydig cells in the testis, is the major driver of male reproductive development and function. The present study revealed an apparent decrease in testosterone concentration in male rats following prenatal and lactational exposure to CPF and CYP. Previous studies have shown that exposure of adult male rats to CPF (Joshi *et al*., 2007; Zidan, 2009; Shittu *et al*., 2014; Umosen and Chidiebere, 2014), CYP (Wang *et al*., 2009a; Joshi *et al*., 2011) and combination of CPF and CYP (Ventura *et al*., 2016; Ikpeme *et al*., 2016) resulted in decrease testosterone concentration.. The low serum testosterone may be linked to pesticide-induced oxidative damage to the hypothalamus and pituitary gland, which results in lower level of synthesis of GnRH and LH, respectively. Receptors for LH are present on Leydig cells (Kaur *et al*., 2015).

The role of LH is to induce the biosynthesis and secretion of testosterone. LH acts on enzyme cholesterol demolase activity which results in testosterone synthesis from cholesterol (Kaur *et al*., 2015). Therefore, the lowered LH concentration following prenatal and lactational exposure to CYP and CPF contributed to the impairment of testosterone biosynthesis and secretion. In addition, oxidative damage to the Leydig cells may impair testosterone production. Furthermore, the increase in ACh as a result of pesticide-induced AChE inhibition prevents the release of GnRH and, consequently, hampers the release of LH and FSH. This eventually results into inhibition of gametogenesis and steroidogenesis (Mitsushima *et al*., 1994; Terasawa and Fernandez, 2001). The consequence of impaired testosterone secretion is impairment of spermatogenesis since it is one of its key regulators. Testosterone after being released from Leydig cells diffuses through the interstitial lymphoid spaces and reaches the seminiferous tubules. It is assumed that high amounts of testosterone are needed to

initiate spermatogenesis (Straube *et al*., 1999). Lower testosterone level may also result in erectile dysfunction, since the hormone functions as activator of enzyme nitric oxide synthase, responsible for production of nitric oxide (Zvara *et al*., 1995), which plays a role in relaxation of the smooth muscle and an important neural messenger that mediates penile erection (Ignarro *et al*., 1990; Holmquist *et al*., 1991; Kim *et al*., 1991).

Pre-treatment with melatonin has been shown in this study to restore the pesticide-induced deficits in testosterone concentration. This may be due to its antioxidant effect, which decrease oxidative injury to the hypothalamus, pituitary gland and the testes. Therefore, the levels of GnRH, LH and testosterone biosynthesised and secreted are not compromised. Although under normal circumstance, melatonin inhibits testosterone secretion via its activity on the HPG axis (Yilmaz *et al*., 2000), it seems that under condition of oxidative stress, the melatonin activity is channeled towards mitigating oxidative challenges and, therefore, having positive effect on stereoidogenesis. Melatonin has been reported to have a stabilising effect on the cellular membranes of tissues. Umosen and Chidiebere (2014) showed that melatonin mitigated testosterone concentration following subacute CPF exposure in adult male rats, which was associated with its antioxidant effect. Melatonin, which is associated with the cellular antioxidant defence (Reiter *et al*., 2004) acts at two levels: firstly, as a direct antioxidant, due to its ability to act as a free-radical scavenger; and secondly as an indirect antioxidant, since it is able to induce the expression and/or the activities of the main antioxidant enzymes such as SOD, catalase and GPx (Reiter *et al*., 2013).

The presesnt study revealed a significant decrease in T_3 and T_4 concentration in F1 generation of dam co-exposed to CC. The T_3 has been reported to be a poor indicator of

subclinical or overt hypothyroidism (Ambali *et al*., 2011). Therefore, the marginal decrease in serum T_3 level observed in the F1 male of dam co-exposed to CC may likely be due to the low T_4 level observed in the group rather than a decrease synthesis. This is because T_4 has to be converted to T_3 for the biological effect of the hormone to be manifested. In addition, the relatively lower T_3 level may also be ascribed to deficiency in the synthesis of 5-deiodionase, an enzyme responsible for the conversion of T_4 to the more metabolically active T_3 (Ambali *et al*., 2011). Decreased serum T_4 and T_3 concentrations have been associated with diminished thyroid function (Kendall-Taylor, 1972). CPF has been reported to cause hypothyroidism (Venerosi *et al*., 2008; Ambali *et al.,* 2011). Hypothyroidism in F1 generation male rats may be due to degeneration and apoptosis of follicular cells (Goldner *et al*., 2010), apparently due to oxidative injury to the thyroid gland.

Oxidative stress characterised by increased MDA concentration and decreased activities of SOD and catalase, was recorded in the thyroid gland of F1 males gestationally and lactationally-exposed to CPF and CYP. This vulnerability of the thyroid gland to oxidative stress is attributed to its high metabolic rate, high level of ROS accumulation, and low level of endogenous antioxidants. The consequence of hypothyroidism in a developing mammal is grievous because thyroid gland plays a pivotal role in mammalian prenatal and postnatal development (De Angelis *et al*., 2009). Animal and human studies have shown that thyroid hormones play a role in the development and function of cardiovascular, nervous, immune, and reproductive systems (Choksi *et al.,* 2003). Thyroid hormones regulate a number of biological processes such as neuronal proliferation, cell migration and differentiation (Bernal, 2005, 2015). Many of the genes involved in the processes of brain maturation have been identified as being

regulated by thyroid hormones, through binding to thyroid hormone receptors (Bernal, 2007). Disruption of the thyroid system and change in TH levels during the foetal and postnatal developmental periods are known to cause irreversible mental retardation and neurological deficits (Howdeshell, 2002). Apart from its effect on the gonadotropins, the damage to the thyroid gland may have been partly responsible for the altered reproductive status, reported in the present study.

The significant increase in the TSH concentration in the F1 male of dams co-exposed with CPF and CYP may be due to the attempt by the body to stimulate the thyroid gland to increase the synthesis and elaboration of T_4 in order to compensate for the apparent deficit in the system. The low T_4 concentration in the F1 generation male of dams, co-exposed to CC may have stimulated the hypothalamic neurons to secrete thyrotrophin releasing hormone (TRH), leading to increased stimulation of TSH synthesis (Nillni, 2010; Beck-Peccoz and Mariotti, 2016). Earlier studies have reported an increase in TSH concentration following CPF exposure in rats (Ambali *et al*., 2011; Shittu *et al*., 2014) and CYP in fish (Bhanu and Deepak, 2015).

The pre-treatment with melatonin has been shown in this study to apparently restore the levels of T_3, T_4 and TSH, partly due to its antioxidant effects. The restoration of thyroid hormone levels may have been partly responsible for the improvement in cognitive and reproductive status of the F1 male rats, exposed *in utero* and via lactation to CPF and CYP in the present study. Although, melatonin has been shown in studies to impair thyroid hormone synthesis (Lewinski and Karbownik, 2002; Mogulkoc and Baltaci, 2003; Baltaci *et al.*, 2004). This may not be unconnected to its redox status as the compound can manifest pro-oxidative effect when administered alone over a relatively

longer period of time to animals that have not been oxidatively challenged. Several studies have suggested a paracrine role for this molecule in the regulation of thyroid activity, documenting that administration, as an antioxidant, in thyroid tissues under conditions of increased oxidative stress, could be helpful to reduce the oxidative processes involved in thyroid diseases (Garcia-Marin *et al*., 2015; D'Angelo *et al*., 2016).

The results of the present study showed an increase in the MDA concentration in the brain, pituitary gland, thyroid gland, testicular, prostate, epididymal and seminal vesicles of male rats, prenatally and lactationally exposed to CPF and CYP. This agreed with the result of previous work on CPF (Shittu *et al*., 2014), CYP (Hussein *et al*., 2013) and CPF and CYP (Wielgomas and Krechniak, 2007b; Idris *et al*., 2014) in biological tissues. This indicates lipoperoxidative changes in each of the tissues as a result of ROS generation following exposure to pesticides. ROS may be produced as the result of the metabolism of OP and pyrethroid by cytochrome P450s (Klimek, 1990; Lukaszewicz-Hussain, 2010), which are monooxygenases that catalyse oxidation by addition of one atom of molecular oxygen into a substrate OP by an electron transport pathway (Jakoby and Ziegler, 1990; White, 1991; Chambers *et al*., 2001). The other means of ROS generation in OP toxicity is high energy consumption coupled with inhibition of oxidative phosphorylation, described by Milatovic *et al*. (2006), and increased release of glucose by induction of glycogenolysis in the liver, and subsequent release of ATP to meet the body's energy requirement (Rahimi and Abdollahi, 2007). High energy consumption leads to decreased ability of cells in maintenance of energy levels. For this reason, the excessive amounts of ROS may be generated in different organs (Milatovic *et al*., 2006) as shown in the male rats prenatally and lactationally

exposed to CPF and CYP. Thus, the reaction of ROS interaction with tissue is lipid peroxidation, which finally results in morphological and functional alterations. MDA is a product of lipid peroxidation, which results from the reaction of ROS with polyunsaturated fatty acid residues in membrane phospholipids, shown to damage proteins and DNA (Siddque *et al*., 2012). Changes in MDA concentrations in many instances have been used as an indicator of lipid peroxidative damage (Shittu *et al*., 2012a; Surajudeen *et al*., 2014).

Pre-treatment with melatonin has been shown in the present study to counteract the effect of lipoperoxidative damage recorded in these organs. Melatonin seems to function *via* a number of means to reduce oxidative stress. It is a direct ROS scavenger (Hardeland *et al*., 1993; Allegra *et al*., 2003), by directly interacting with ROS and attenuating their effects. It also has an indirect antioxidant effect by stimulating activities of antioxidant enzymes (Reiter *et al*., 2000; Rodriquez *et al*., 2004), and synthesis of glutathione (an essential intracellular antioxidant) (Urata *et al*., 1999). It has the ability to augment the activities of other antioxidants (or *vice versa*) (Gitto *et al*., 2001), its protection of antioxidative enzymes from oxidative damage (Mayo *et al*., 2002), and its ability to increase the efficiency of mitochondrial electron transport chain (ETC), thereby lowering electron leakage and reducing ROS generation (Acuňa - Castroviejo *et al*., 2002; Okatani *et al*., 2003).

Melatonin along with its metabolites has been shown to scavenge ROS or RNS (Hardeland *et al*., 2009). This cascade reaction makes melatonin a highly effective antioxidant, even at low concentrations, in protecting cells from oxidative stress (Tan *et al*., 2007). Melatonin diminishes the destruction of DNA, proteins and lipids that occur

as a result of their reactions with ROS and RNS (Chojnacki *et al*., 2011), as it easily crosses the cell membranes and the BBB (Hardeland *et al*, 2009).

The development of oxidative stress conditions in multi-tissues by pesticides has been investigated as a possible mechanism for the toxicity and degradation of different tissues in the body (Oruc and Uner, 2000). The SOD intervenes in the first transformation by dismuting the superoxide free radicals (O_2) into H_2O_2 (Dorval *et al*., 2003; Nair *et al*., 2017). The present study showed a decrease in SOD activities in the brain, pituitary gland, thyroid gland, testicular, and an increase in prostate, epididymal and seminal vesicles in rats, prenatally and lactationally exposed to the pesticide mixture. This agreed with work of Hocine *et al*. (2016); Adedara *et al*. (2017), but disagrees with those of Heikal *et al*. (2013) and Chen *et al*. (2017), when cyromazzine and CPF were exposed to rats with an increase in hepatic SOD activities. Superoxide is the primary ROS produced from a variety of sources; and, therefore, its dismutation by SOD is of primary importance for each cell (Birben *et al*., 2012). SOD is generally thought to act as bulk scavengers of O_2^- as it catalyses its dismutation to H_2O_2, and in conjunction with catalase, it is the first line antioxidant defense in the body (Shittu *et al*., 2012a; Umosen *et al*., 2012; Adedara *et al*., 2017). The decreased SOD activity in the tissues of rats prenatally and lactationally exposed to CPF and CYP group may be due to decreased synthesis, increased degradation as a result of increased oxidative challenge or even its outright inactivation. The increase in SOD activities in the group pretreated with melatonin is an indication of the ability of the antioxidant to protect the SOD from the ravaging effect of pesticide-induced ROS, through boosting of the antioxidant reserves in the tissues. Melatonin has also been shown to influence antioxidant enzyme gene expression (Antolin *et al*., 1996; Mayo *et al*., 2002).

The present study showed a decrease in catalase activities in the brain, pituitary gland, thyroid gland, testicular, prostate, epididymal and seminal vesicles of male rats, prenatally and lactationally exposed to CPF and CYP. Exposure of adult male rats to CPF (Umosen *et al*., 2012; Shittu *et al*., 2012a; Adeadara *et al*., 2017) and perinatal exposure to CYP (Hocine *et al*., 2016) have been shown to decrease catalase activity. Catalase is an ubiquitously expressed antioxidant enzyme, primarily located in the peroxisomes that catalyses the decomposition of hydrogen peroxide into oxygen and water (Halliwell and Gutteridge, 1999). Catalase is known to neutralise and detoxify both intracellular and extracellular H_2O_2 to water and oxygen (Dorval *et al*., 2003). The decline in the catalase activity observed in CC group might be due to the reduced conversion of $O_2^{\cdot -}$ to H_2O_2 by SOD, which then leads to the accumulation of O_2-. This accumulation of $O_2^{\cdot}$- has been shown to inhibit the activity of catalase (Corpas and Barroso, 2017) as $O_2^{\cdot -}$ converts ferroxy state of catalase to ferryl state, which is an inactive form of the enzyme (Freeman and Crapo, 1982). On the other hand, the decrease in catalase activity might be due to accumulation of H_2O_2 in the testes. Accumulated H_2O_2 is known to inhibit catalase activity (Latchoumycandane and Mathur, 2002). The ability of melatonin to improve catalase activity is a demonstration of the ability of this antioxidant molecule to boost the endogenous antioxidant reserve and protect catalase from the ROS, produced by prenatal and lactation exposure to the pesticide mixture.

The present study revealed a decrease in GPx activities in the brain, pituitary gland, thyroid gland, testicular, prostate and increase in epididymal and seminal vesicles of male rats, prenatally and lactationally exposed to CPF and CYP. Previous works in adult male showed that CPF (Shittu *et al*., 2012) and CYP (Hussein *et al*., 2013) caused

reduced GPx activity. GPx is a selenium-containing enzyme that protects the cytosol and plasma membrane from lipid peroxidation by catalysing both the reduction of H_2O_2, and organic hydroperoxides produced at the membrane level to water or corresponding alcohols (Pisoschi and Pop, 2015). CPF and CYP are notorious oxidative stress inducers and, as a hydrophobic compound, they easily penetrate and may accumulate in cell membrane and disturb membrane structure (Hussein *et al*., 2013). Decrease in tissue GPx activity may be due to either the inhibition of GSH synthesis or its increased utilisation for detoxification.

Pre-treatment with melatonin increased GPx activities by virtue of its antioxidant properties. Melatonin exhibits indirect antioxidant role by supporting SOD and GPx activities (Reiter *et al*., 2002; 2005b; Rodriguez *et al*., 2004); possibly via epigenetic mechanisms (Korkmaz and Reiter, 2008), which constitutes a specific behaviour for an antioxidant. It also stimulates glutathione production in cells (Winiarska *et al*., 2006) and γ-glutamylcysteine synthase, thereby increasing glutathione level. It promotes the activity of glutathione reductase, which converts oxidised glutathione (GSSG) back to its reduced form (GSH) (Reiter *et al*., 2002).

The present study revealed a decrease in tissue AChE activity in the brain, pituitary gland, thyroid gland, testes, seminal vesicles and increased AChE activity in the prostate gland. Adult male rats exposed to CPF (Shittu *et al*., 2012; Cardona *et al*., 2013; Surajudeen *et al*., 2014; Mamczarz *et al*., 2016) CYP (Sharma *et al*., 2014) and CPF+CYP (Bonansea *et al*., 2016) have shown reduced AChE activity. AChE is an enzyme that is essential for the normal functioning of the central and peripheral nervous system (Hart, 1993), and is widely distributed in the neural and non‑neural tissues

(Friboulet *et al*., 1990). The acute toxicity of CPF results primarily from the irreversible inhibition of AChE, the enzyme that hydrolyzes the neurotransmitter, ACh, resulting in the classical muscarinic, nicotinic and central cholinergic effects (Mamczarz *et al*., 2016). Cypermethrin is suggested to inhibit AChE activity through some kind of interaction with the enzyme (Sharma *et al*., 2014b). Cypermethrin also exerts a neurotoxic effect, manifested by a decreasing of AChE activity in different tissues with the impairment of neural conductivity in the central and peripheral nervous system. However, oxidative stress induction by CPF and CYP has contributed to the AChE inhibition. A relationship between ROS and AChE inhibition has been demonstrated (Tsakiris *et al*., 2000). Oxidative stress is known to affect the activities of various membrane-bound enzymes, including AChE (Mehta *et al*., 2005) via their direct attack by ROS or peroxidation of the membrane lipids in which they are embedded (Souza *et al*., 2010).

Pre-treatment with melatonin has been shown in this study to, apparently, restore the tissues' AChE activities via its membrane stabilising and antioxidative effects. Melatonin scavenges reactive species include hydroxyl radical (HO^{-}), hydrogen peroxide (H_2O_2), nitric oxide (NO^{-}), and various others (Galano *et al*., 2011). Melatonin reacts with agents to form products that are not recycled back to melatonin, making it a "suicidal antioxidant" (Tan *et al*., 2001). At both physiological and pharmacological concentrations, melatonin attenuates or counteracts oxidative stress, and regulates cellular metabolism (Slominski *et al*., 2005, 2008; Korkmaz *et al*., 2009). Some of these protective effects of melatonin are shared by its metabolite, N-acetyl-N-formyl-5-methoxykynuramine (AFMK) (Tan *et al*., 2007). Melatonin, in addition to being a broad-spectrum antioxidant, can also activate cytoprotective enzymes (Rodriguez *et al*.,

2004). By protecting the surface membrane from oxidative injury, melatonin was able to protect damage to the lipids that anchor the membrane-bound enzymes, including AChE. Therefore, the activity of AChE is kept apparently intact.

Sialic acid acts as a lubricant to facilitate the downward movement of sperm and reduce friction among spermatozoa (Gupta, 2001). The synthesis and secretion of sialic acid is under androgen control. Alteration in sialic acid level in reproductive tissues indicate changes in the level of glycoprotein/ FSH and LH, which are needed for normal functioning of gonads and accessory reproductive organs (Gupta *et al*., 2002). In the present study, sialic acid content of testes significantly decreased in the sperm of rats, prenatally and lactationally exposed to CPF and CYP. Previous work in adult rats has shown a decrease in sialic acid following CPF (Joshi *et al*., 2007) and CYP (Joshi *et al*., 2011). Similarly, reduction in testicular sialic acid content may be due to the absence of spermatozoa or reduced androgen production (Levinsky *et al*. 1983).

Apart from playing a role in sperm motility, sialic acid is involved in fertility as it facilitates the binding of the sperm to the zona pelucida of the oocytes (Velasquez *et al*., 2007). Due to its role in cell adhesion, decrease level of sialic acids in the tissue and plasma has a negative effect on the cell adhesion (Ulucam and Bakar, 2016). Moreover, structural integrity of acrosomal membrane depends on sialic acid and any alteration in its content might lead to structural and functional changes in sperm. This may be one of the reasons for the increased morphological alterations in male rats, prenatally and lactationally exposed to CPF and CYP in this study. A decrease in the testicular sialic acid (TSA) levels may be related to deterioration in its biosynthesis due to tissue damage

Improvement in total sialic acid in the group pretreated with melatonin may be due to its free radical scavenging effect, which may have protected the integrity of the surface membrane that contains sialic acid. In the same vein, the improvement in sperm density in the pretreated group might also be involved in the apparent increase in sialic acid. The apparent normalisation of sialic acid might have contributed to the improved sperm motility recorded in melatonin-pretreated group.

The sialic acid maintains osmotic balance in the cauda region, facilitates sperm transport by its lubricating action, and is involved in the maturation of sperm in the epididymis (Dhanju and Navdeep, 2011; Ghosal *et al*., 2015). In addition, the sialic acid, by virtue of its negative charge, is involved in the binding and transport of positively-charged compounds. Furthermore, it influences the conformation of glycoproteins, which is to a large extent, important for the correct positioning of molecules in cell membranes, the maintenance of the activity of glycoprotein enzymes, and resistance to proteolytic degradation (Gautam and Perera, 1992). The decrease in epididymal sialic acid recorded in the group exposed to CC agreed with previous work (Joshi *et al*., 2003; Desai *et al*., 2016). The secretion of sialic acids by the epididymis is an androgen-dependent process. Therefore, the lower level of epididymal sialic acid in the rats, prenatally and lactationally exposed to CPF and CYP may be partly due to alteration in the level of androgens, synthesised as a result of oxidative injury to the HPG axis. Similarly, oxidative injury to the epididymis may have contributed to the lower sialic acid since the epididymal tissue is responsible for its secretion. Furthermore, the lower epididymal sperm count following prenatal and lactational exposure to CPF and CYP may have contributed to the depleted epididymal sialic acid. The lower level of epididymal sialic acid has an adverse consequence on the morphological and functional integrity of

sperm. An optimal level of sialic acid secretion by the epididymal epithelium appeared to be a pre-requisite for the maintenance of the functional integrity of the sperm (Rajalashkmi, 1985). Since sialic acid is also involved in sperm maturation in the epididymis, the lower epididymal sialic acid may interfere with sperm maturity and, thus, involved in altered sperm morphology in the rats, exposed prenatally and lactationally to the pesticide mixture. The depletion of sialic acid is therefore an important landmark for assessing reproductive deficit in male animals. This suggests the fact that decrease in sialic acid content of the epididymis may also be an indicator of reproductive deficits in the group exposed to CC combination.

Pre-treatment and post-exposure treatment with melatonin as shown in this study was able to normalise the sperm sialic acid concentration relative to that of the controls. This result may be due to the antioxidant properties of melatonin, which protected the epididymal membrane from oxidative injury, and confer stability to the epithelium, so as to secrete appropriate amount of sialic acid for optimal sperm function. The antioxidant protection by melatonin on the HPG axis improved the biosynthesis of androgenic hormone, which is a regulator of sialic acid secretion in the epididymis. Furthermore, the improvement in sperm count by pretreatment with melatonin might have contributed to the relative increase in the sialic acid content of the epididymis.

Fructose is considered a measure of seminal vesicle function, because it is an important source of energy for the sperm (Owen and Katz, 2005). Fructose is the primary secretory product of seminal vesicle that offers nutrients for the semen. It is also important for sperm motility and stability of sperm chromatin (Ghosh *et al*., 2016). Fructose is also involved in protein complexes, particularly in coagulated semen (Montagnon *et al*, 1982). The

present study revealed marginal decrease in the epidydimal fructose concentration in male rats, prenatally and lactationally exposed to CPF and CYP. This finding agreed with that of ElMazoudy *et al*. (2011) in CPF and Ikpeme *et al*. (2016) in CPF+CPY poisoning. The decrease may be attributed to reduced testosterone concentration (ElMazoudy *et al*., 2011), since fructose formation by the accessory glands is dependent on secretion of testosterone by the testes (Desai *et al*., 2016). Therefore, the reduced testosterone concentration in male rats, prenatally and lactationally exposed to CYP and CPF may have been partly responsible for the lower fructose level in the group. Furthermore, since fructose is a seminal vesicle secretion, the reduced fructose content in the exposed male rats indicated that the secretory ability of the seminal vesicle was hampered by the insecticide mixture, apparently due to oxidative injury. The function of fructose is to induce the glycolytic metabolism of spermatozoa; therefore, the depletion of fructose content impairs the glycolytic metabolism of spermatozoa, resulting in abnormal sperm functions, which ultimately leads to complete male sterility (Sarkar *et al*., 2000b). It is well known that the function of seminal vesicles is under androgen control and a direct association exists between serum testosterone, seminal fructose and spermatozoa motility/fertility (Gonzale, 2001).

Pre-treatment and post-exposure treatment with melatonin was able to marginally improve the epididymal fructose concentration, relative to that of the pesticide-exposed group. The improved fructose concentration might have been due to an improved level of testosterone, recorded in these groups as a result of protection by melatonin on pesticide-mediated oxidative injury to the hypothalamo-pituitary-gonadal axis. Similarly, pesticide-mediated oxidative injury to the seminal vesicle might have been

protected by melatonin due to its antioxidant and ROS scavenging, therefore resulting in improved fructose elaboration in the seminal vesicle.

CHAPTER SIX

6. 0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

Alterations of some foetal parameters by decreasing litter size, foetal live-dead ratio, litter weight, CRL and anogenital distance, in addition to prolongation of the time of testicular descent, ear and eye openings in the offspring of dams co-exposed to CPF and CYP were mitigated by pretreatment with melatonin.

Gestational and lactational co-exposure to CPF and CYP caused alteration in sensorimotor and cognitive parameters by increasing the time to walk , impairment of spontaneous movement, motor coordination, neuromuscular coordination, motor strength, sensorimotor reflex, locomotion efficiency, learning and memory. In addition, the co-exposure caused depression in F1 male rats, which were ameliorated by pretreatment with melatonin.

The F1 male rats exposed *in utero* and via lactation to CPF and CYP altered some reproductive parameters by causing reduction in cauda epididymal sperm concentration, active progressive sperm movements, sperm live/dead ratio, concentration of normal sperm cells, testicular total and free sialic acid, and epididymal fructose concentrations; serum FSH, LH and testosterone concentrations. It also increased the morphological abnormalities in the spermatozoa. The adverse effects were all mitigated by pretreatment with melatonin. Furthermore, prenatal and lactational exposure to CPF and CYP decreased T_3 and T_4 concentrations; but increased the TSH level, which were attenuated by pretreatment with melatonin.

Mating a sexually-mature nulliparous female rats with the F1 male rats exposed *in utero* and via lactation to CPF and CYP reduced gestation length, mating index, pregnancy index, male/female ratio, live birth ratio and viability index, which were mitigated in F1 male rats of dams pretreated with melatonin. Alterations of oxidative stress parameters (MDA, SOD, CAT and GPx) in the brain, pituitary gland, thyroid glands, testes, prostate gland, epididymis and seminal vesicles of F1 male rats gestationally and lactationally exposed to CPF and CYP were mitigated by treatment with melatonin. Similarly, alterations of AChE activities in the brain, pituitary gland, thyroid glands, testes and seminal vesicles of F1 male rats gestationally and lactationally exposed to CPF and CYP were restored by pretreatment with melatonin.

Melatonin was also shown to have protected the tissue from cellular injury by preventing the pesticide-evoked degeneration of the neurones, glial cells, spermatocytes, spermatid and Leydig cells, apparently due to its antioxidant properties.

6.2 Conclusions

The present study has shown that:

1. Gestational and lactational co-exposure to CPF+CYP caused developmental toxicity in F1 generation male rats.
2. Gestational and lactational co-exposure to CPF+CYP induced sensorimotor and cognitive changes in F1 generation male rats.
3. Gestational and lactational co-exposure to CPF+CYP induced reproductive toxicity in F1 male rats.

4. Oxidative stress was involved in the mechanism underlying developmental, sensorimotor and reproductive toxicity, associated with gestational and lactational co-exposure to CPF and CYP in F1 male Wistar rats.
5. Pre-exposure and post-exposure treatment with melatonin mitigated the deficits in the developmental, neurobehavioural and reproductive toxicity from gestational and lactational co-administration to CPF and CYP in F1 male Wistar rats.

6.3 Recommendations

Specific Recommendations

1. Pregnant and lactating mothers and dams should be cautioned in any activity that involves the application of pesticides to avoid foetal and neonatal exposure, which can later affect the developmental, sensorimotor, cognitive and reproductive potentials of such offspring.
2. Caution should be exercised in exposing highly prized pregnant and lactating animals to pesticides to avoid cognitive deficits in their offsprings, which is crucial in the performance of their task since most of their activities are related to optimal cognitive function.
3. Where it is inevitable to prevent pregnant or lactating individuals and dams from having contact with pesticides, pre-exposure treatment with melatonin is recommended to mitigate foetal and neonatal toxicity.
4. Further studies using toxicogenomic/toxicoproteomic tools be conducted to shed more light on the molecular mechanism of toxicity associated with co-exposure to CPF and CYP, and ameliorative potentials of melatonin.

General Recommendations

1. Efforts should be geared towards reducing the use of chemical pesticides in homes, gardens and farms by engaging in biological control and integrative farming, respectively.

2. There is the need for Nigeria and other developing countries to have National Pesticide Policy to provide legislative framework for the manufacture, importation, sales and use of pesticides.

3. There should be general enlightenment and awareness of pregnant and nursing mothers and farmers on the danger of pesticide exposure to the developing foetus and new born. Farmers should also be enlightened on the need to abide with the withdrawal period of pesticides as stated by the manufacturers when they are used on stored farm produce.

4. Regulatory agencies of government such as Standard Organisation of Nigeria and National Agency for Food and Drug Administration and Control should be alive to their responsibilities of making sure that pesticides in foods consumed within the country are within the internationally recognized acceptable limit.

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APPENDICES

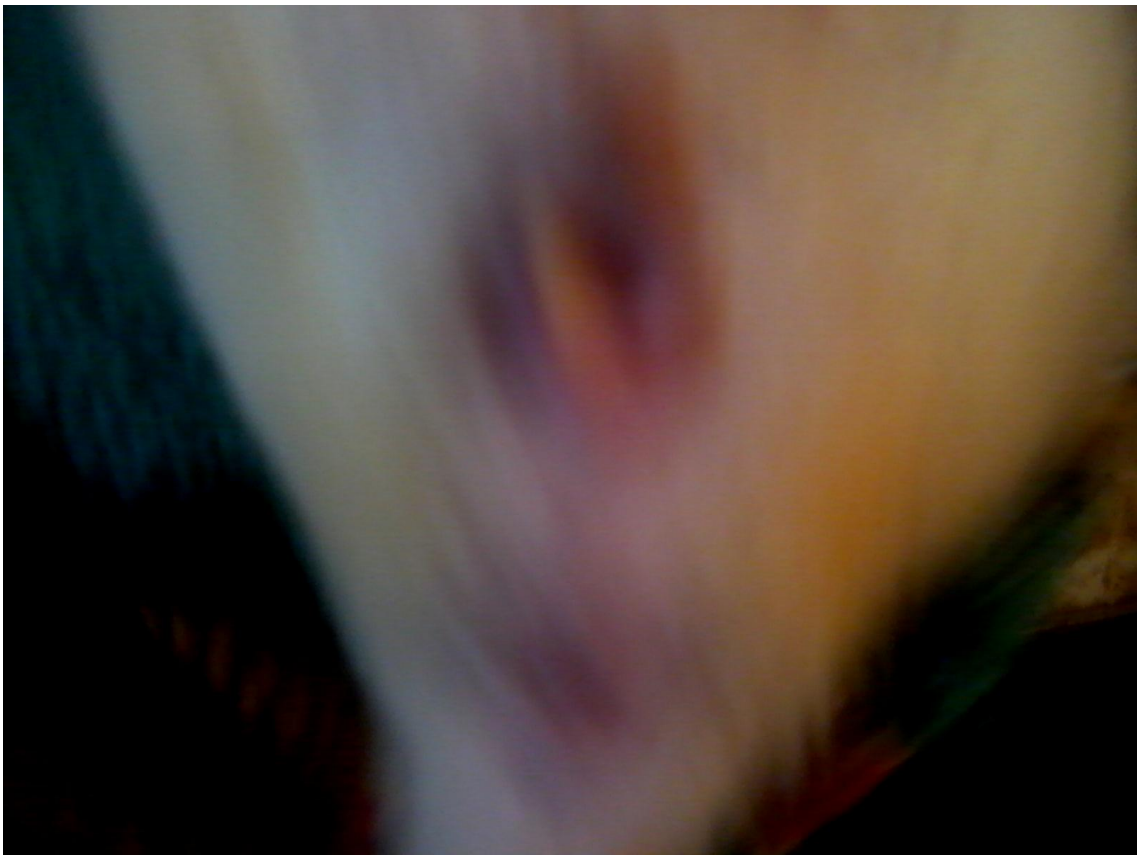
APPENDIX I

Insecticides (Chlorpyrifos and Cypermethrin)

APPENDIX II

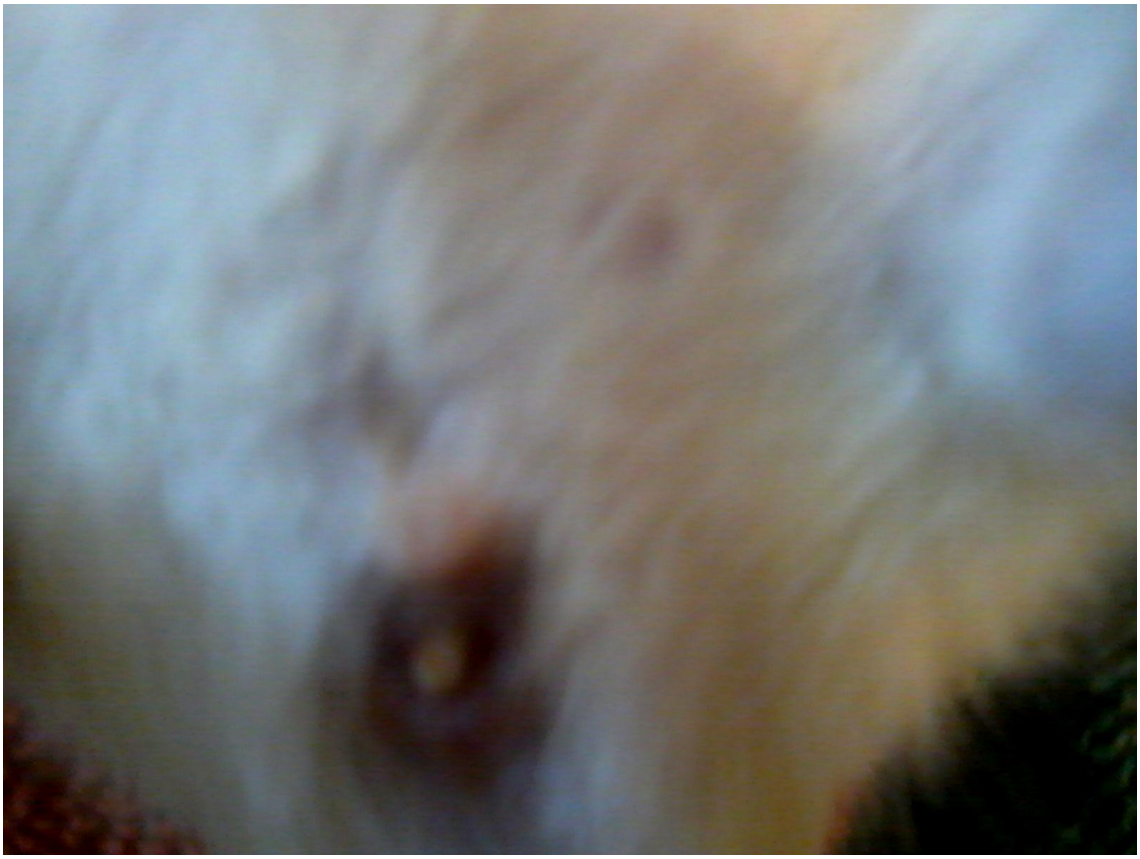
Melatonin – Antioxidant used

APPENDIX III



Vaginal plug for comfirmation of mating

APPENDIX IV

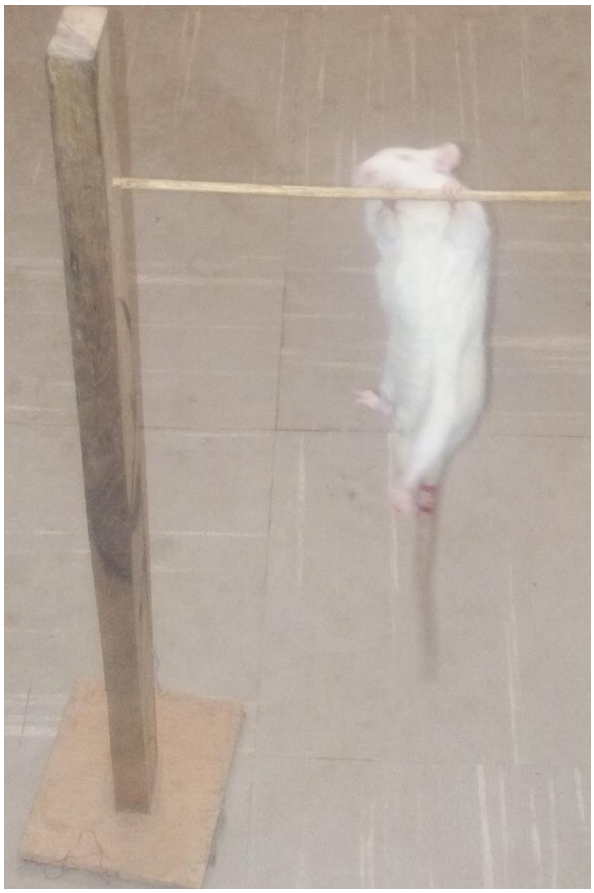


Vaginal plug for confirmation of mating

APPENDIX V

Vernier caliper for measurement of anogenital distance

APPENDIX VI – Forelimb grip test. Was used for evaluating motor strength



APPENDIX VII – Ladder walk. Was used for determinig efficiency of locomotion

APPENDIX VIII – Inclined plane. Was used for determining neuromuscular co-ordination

APPENDIX IX – Beam walk. Was used for determining motor coordination

APPENDIX X – Step down inhibitory apparatus. Was used for determining cognitive ability