PROTECTIVE EFFECT OF VITAMIN E ON ENDOSULFAN-INDUCED OXIDATIVE CHANGES AND ASSOCIATED NEUROBEHAVIOURAL DEFICIT IN MALE WISTAR RATS

BY

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DECLARATION

I declare that the work in this dissertation, entitled 'Protective Effect of Vitamin E on Endosulfan-Induced Oxidative Changes and Associated Neurobehavioural Deficit in Male Wistar Rats' has been carried out by me in the Department of Human Physiology, Ahmadu Bello University, Zaria under the supervision of Professor A. Mohammed and Dr. Y. Tanko. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

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CERTIFICATION

This dissertation entitled "PROTECTIVE EFFECT OF VITAMIN E ON ENDOSULFAN-INDUCED OXIDATIVE CHANGES AND ASSOCIATED NEUROBEHAVIOURAL DEFICIT IN MALE WISTAR RATS" by Hajara Ali UMAR meets the regulations governing the award of the degree of Master of Science in Human physiology of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to my late parents Alhaji Ali Umar Baba and Hajiya Safiya Ali Baba, for their love and support in all my endeavours and to my husband, Alhaji Abdullahi Lawal Saulawa for his love, support and unwavering belief in my abilities.

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ABSTRACT

Endosulfan is an organochlorine pesticide commonly used to control pests in vegetables, cotton, fruits and other agricultural produce. It is a contact and stomach insecticide toxic to mammals, fish and other aquatic organisms. Several reports have shown that it is a highly neurotoxic agent. The aim of this study was to evaluate the role of brain lipoperoxidation and oxidative stress in sensorimotor and cognitive changes, arising from endosulfan exposure in Wistar rats and the ameliorative potentials of vitamin E. Thirty two (32) male Wistar rats were randomly divided into four groups of eight (8) rats each: group I rats received soya oil at 2 ml/kg body weight; group II rats received vitamin E at a dose of 30 mg/kg body weight; group III rats received endosulfan at a dose of 12 mg/kg; and group IV rats received vitamin E (30 mg/kg) followed by endosulfan (12 mg/kg) after 30 mins. The regimens were administered orally by gavage once daily for 28 days. The rats were examined for neurobehavioural parameters: assessing motor endurance and neuromuscular coordination at weeks 0, 2 and 4; and for cognition 48 hrs to the termination of the study. At the end of the study period, the rats were euthanized. Brain homogenate samples were evaluated for malondialdehyde (MDA) concentration and activities of superoxide dismutase (SOD) and catalase (CAT). The results showed significant elevation ($p < 0.05$) in MDA concentration in the endosulfan-treated group compared to the vitamin E pre-treated group; a significant increase in CAT activity in the endosulfan-treated group ($p < 0.05$) compared to the control group, and no significant change in SOD activity in all the groups. Endosulfan-treated rats showed decreased motor endurance and neuromuscular coordination, compared to that of the control rats and rats in other treatment groups; although the difference was not significant ($p > 0.05$). No significant difference ($p > 0.05$) was recorded for the learning acquisition in all treatment groups, although rats in the

endosulfan-treated group showed an impaired ability to learn. However, the ability of the endosulfan-treated rats to retain memory was significantly impaired ($p < 0.05$) when compared to the vitamin E treated rats. The results showed that endosulfan induced oxidative stress, impaired motor skills and short term memory acquisition and retention, which were ameliorated by vitamin E treatment in male Wistar rats.

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ABBREVIATIONS

Ach	Acetylcholine
AchE	Acetylcholinestrase
ADI	Allowed Daily Intake
ANOVA	Analysis of Variance
ATPase	Adenosine triphosphatase
Ca^{2+}	Calcium ions
CAT	Catalase
CDC	Centre for Disease Control
CGC	Cerebellar Granular Cells
Cl^{-}	Chloride ion
CN	Cortical Neurons
CNS	Central Nervous System
CPF	Chlorpyrifos
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
END	Endosulfan
ER	Estrogen Receptors
FAO	Food and Agriculture Organisation
GABA	Gamma-amino-butyric acid
$GABA_A$	Gamma-amino-butyric acid A
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
H_2O	Water

H_2O_2	Hydrogen per oxide
HCH	Hexachlorocyclohexane
HDL	High density lipoprotein
HIV/AIDS	Human immunodeficiency virus/Acquired immunodeficiency syndrome
K^+	Potassium ion
LD_{50}	Mean lethal dose
LDL	Low density lipoprotein
LPO	Lipoperoxidation
MDA	Malondialdehyde
Mg^{2+}	Magnesium ion
Na^+	Sodium ion
O_2	Oxygen
O_2^-	Super oxide ion
OCP	Organochlorine pesticide
OP	Organophosphates
PCBs	Polychlorinated biphenyls
ROS	Reactive Oxygen Species
SH-SY5Y	Human derived cell line (used in in-vitro research)
SOD	Superoxide Dismutase
TBA	Thiobarbituric acid
US EPA	United States Environmental Protection Agency
VLDL	Very low density lipoprotein
W.H.O	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The subtle impact of chemicals in our environment upon animals was first highlighted by Rachel Carson with her work on Dichlorodiphenyltrichloroethane (DDT) in the 1950s and 1960s. More recently, studies have shown clearly the impact of man-made chemicals and many pesticides. These may interfere with or disrupt normal body functions, exacerbating the development of cancers, reducing sperm counts, interfering with normal development, among other health impacts (Colborn *et al.,* 1996). In recent years, there has been a tremendous increase in the use of these chemicals without paying much attention to the adverse effects they may have due to the toxic ingredients (Yekeen and Adeboye, 2013).

Pesticide-related adverse effects in humans, animals and birds remain a serious public health concern (Farag *et al.,* 2014). Pesticides are a group of chemicals made for the purpose of killing or otherwise deterring "pest" species. The word *pesticide* may refer to insecticides, herbicides, fungicides, or other pest control formulations. Pesticides are known to be the most important tool for the production of adequate food supply for an increasing world population and for the control of vector-borne diseases (Mahugija *et al.,* 2017), they are however, inherently toxic and often pose significant toxicological risks to a myriad of non-target organisms (US EPA, 2009; Ondarza *et al.,* 2014). Pesticides applied to crops and livestock can remain on or in our food (Colborn *et al.,* 1996; Mahugija *et al.,* 2017), finally finding their way to the food chain threatening the ecological balance and the biodiversity of nature (Konstantinou *et al.,* 2006). Most of the chemicals which are used as

pesticides are not highly selective, but are generally toxic to non-target species, including man and other animals (Chitra *et al.,* 1999). Their very nature of killing organisms renders them harmful to humans and other living beings (Bamigboye *et al.,* 2017). Among the pesticides that have a detrimental effect on human health, organochlorine pesticides (OCPs) top the list, owing to their high transport potential and molecular properties (Sebastian and Raghavan, 2015).

Organochlorine pesticides like endosulfan, hexachlorocyclohexane (HCH) and dichlorodiphenyltrichloroethane (DDT) are ubiquitous in the environment because they break down very slowly and have the potential to be bioaccumulated in the environment (Pathak *et al.,* 2010; Yekeen and Adeboye, 2013; Huang *et al.,* 2014; Ozdal *et al.,* 2017). These chemicals, still broadly used in many developing countries, are resistant to degradation, have long half-lives and are highly lipid soluble (Pathak *et al.,* 2010; Mehr *et al.,* 2017). The toxicity of these agents varies according to their molecular size, volatility, and effects on the central nervous system (CNS). In general, they cause either CNS depression or stimulation, depending on the agent and dose (Bhalla and Thami, 2004). Exposure to OCPs may also be associated with adverse reproductive outcomes (Siddiqui *et al.,* 2003; Torres *et al.,* 2003), hormonal dysfunction (Liu *et al.,* 2005; Windham *et al.,* 2005), oxidative stress (Banerjee *et al.,* 2001) and immunotoxicity (Koner *et al.,* 1998).

Endosulfan is a persistent broad spectrum contact and stomach organochlorine insecticide and acaricide registered for use on a wide variety of vegetables, fruits, cereal grains, and cotton, as well as ornamental shrubs, trees, vines, and in ornamentals used in commercial agricultural settings (Singh and Singh, 2014; Ozdal *et al.,* 2017). It is a highly toxic

substance, classified as a category II (moderately hazardous) pesticide, by the World Health Organisation (WHO, 2002); and as a category 1b (highly hazardous) pesticide by the United States Environmental Protective Agency – US EPA (PAN-AP, 1996; Mahran, 2013). The intensive use of endosulfan affects public health, beneficial biota and contaminates the ecosystem (Ozdal *et al.,* 2017).

Vitamin E (α-tocopherol) is a natural component of the membrane lipid bilayer (Jalili *et al.*, 2007). Studies carried out with the antioxidant α-tocopherol, have shown that it inhibits free radical formation and may effectively minimize lipid peroxidation in biological systems by scavenging free radicals. It may also be useful in controlling the toxic effects of pesticides and chemicals (Mahran, 2013).

1.2 Statement of Research Problem

The use of pesticides has become an integral part of modern day agricultural practice in food production worldwide (Bamigboye *et al.,* 2017). Children born in agricultural areas where pesticides were applied were found to have lower performance on numerous neurobehavioural assessments, when compared to children not born in an agricultural region (Ribas-Fito *et al.,* 2006). Exposure to OCPs has been linked to decreased psychomotor function and mental activities, including memory, attention, and verbal skills (Jurewicz and Hanke, 2008; Korrick and Sagiv, 2008). There is also some evidence that OCP exposure is associated with the development of autism (Roberts *et al.,* 2007). Endosulfan is known to exert adverse health effects, such as reproductive defects, neurobehavioural disorders, and endocrine and immunological toxicity (Mrema *et al.*, 2013; Sebastian and Raghavan, 2017). Endosulfan has been shown to induce oxidative

stress in human cell cultures (Sohn *et al.*, 2004), and human leukemic cell line (Kannan *et al.,* 2000). Reactive oxygen species (ROS), such as hydrogen peroxide, produce oxidative stress and cause a significant increase in intracellular chloride (hyperpolarization), through GABA-gated chloride channels in brain slices (Sah *et al.*, 2002). The generation of ROS may occur by uncoupling oxidative phosphorylation and reducing $GABA_A$-gated chloride channel function (Kannan *et al.,* 2000). The prolonged persistence of endosulfan in the environment and its bioaccumulation through the food chain remains a global health concern affecting millions of people in both developed and developing countries (Du *et al.*, 2015). The role of brain lipoperoxidation and oxidative stress on impaired sensorimotor and cognitive functions induced by endosulfan exposure and the possible ameliorative potentials of antioxidants are what this study attempted to further explore.

1.3 Justification

Although prohibitions on uses of OCPs have been implemented in developed nations, a number of them are still in use in some African countries (Mansour, 2009). For instance, DDT has been legalised for use by the World Health Organisation (WHO, 2006), and is currently in use in some Eastern and Southern African countries to control mosquitoes, under strict laws of usage (WHO, 2006). Endosulfan is still widely used for agricultural purposes in most of the developing countries because of its effectiveness and low application cost (Ondarza *et al.*, 2014; Sabzar *et al.*, 2015). Therefore, it still remains a serious global environmental concern due to its persistence and slow degradation processes in the environment (Sabzar *et al.*, 2015); and the increasing trade of agricultural products (Sebastian and Reghavan, 2017). Nigeria is a developing nation where there is a high level

of illiteracy and malnutrition, which may exacerbate the effects of pesticide exposure. There is also lack of pesticide awareness, coupled with porous borders through which pesticides are imported into the country, due to ineffective or total absence of laws and regulations (Adegboye, 2009). All of these factors put Nigerians at great risk of exposure to this pesticide and its consequent devastating effects. Therefore, measures aimed at combating the toxicity by this pesticide are pertinent. Studies by Zaidi *et al.* (1985) and Seth *et al.* (1986) showed that endosulfan causes neurotoxicity. Since oxidative stress has been implicated in the molecular mechanizations of endosulfan toxicity, the present study explores the role of vitamin E, a lipid soluble antioxidant vitamin in ameliorating the possible sensorimotor, cognitive and biochemical changes arising from its repeated exposure.

1.4 Aim of the Study

The aim of the study was to evaluate the role of brain lipoperoxidation and oxidative stress in sensorimotor and cognitive changes, arising from endosulfan exposure in male Wistar rats; and the ameliorative potentials of the antioxidant vitamin E.

1.5 Specific Objectives

The specific objectives were as follows:

1. To evaluate the sensorimotor and cognitive changes induced by endosulfan exposure in male Wistar rats.
2. To evaluate oxidative stress (OS) changes due to endosulfan exposure by assessing malondialdehyde (MDA) and enzyme activities of superoxide dismutase (SOD) and catalase (CAT) in male Wistar rats.

3. To assess the ameliorative potentials of vitamin E on endosulfan–induced sensorimotor and cognitive changes; and changes in biomarkers of OS in male Wistar rats.

1.6 Alternate Hypotheses

i. Endosulfan administration may impair sensorimotor and cognitive functions by inducing oxidative stress.

ii. Vitamin E may ameliorate the toxic effects of endosulfan.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Pesticides

Pesticides are unique among toxic substances. Most environmental toxins are an unwanted by-product of another process (for example, outflow from a manufacturing plant or emissions from an automobile engine), but pesticides are chemicals designed to be harmful to a target pest and purposely introduced into the environment to do their job of managing insects, bacteria, weeds, rodents, or other pests (US EPA, 2002). They encompass a large group of molecules that are widely used around the globe whose benefits are many a times overshadowed by harmful effects on non-target organisms including humans (Sebastian and Raghavan, 2015).

A pesticide is any substance or mixture of substances intended for: preventing, destroying, repelling, or mitigating any pest. Though often misunderstood to refer only to insecticides, the term pesticide also applies to herbicides, fungicides, and various other substances used to control pests (US EPA, 2012). Pesticides are occasionally used indiscriminately in large amounts causing environmental pollution; the human population is usually exposed to low doses of these pesticides simultaneously via food and, to some extent, via inhalation and cutaneous contact, and therefore, are a cause of concern (Savary *et al.*, 2014). Residual amounts of organochlorines (OC) and organophosphate (OP) pesticides have been detected in the soil, water bodies, vegetables, grains and other foods products (John *et al.*, 2001; Kalender *et al.*, 2004; Fang *et al.*, 2017).

Pesticides play an important role in modern agriculture by providing dependable, persistent and relatively complete control against harmful pests with less expense and effort. They have, no doubt, increased crop yields and food production during the last century by killing different types of pests, which are known to cause substantial or total crop damage. At the same time, these chemicals are considered as potent pollutants of the environment with undesirable effects on non-target organisms (Rehman, 2005; Kafilzadeh *et al.*, 2015; Carvalho, 2017). But, the current need to increase food production to feed a rapidly growing human population maintains pressure on the intensive use of pesticides (Carvalho, 2017). Among pesticides, the chlorinated hydrocarbon insecticides are generally much more persistent and have long residual properties that have placed them in excessive usage in agriculture and forestry throughout the world (Rehman, 2005). These contaminators of the environment have been well documented worldwide and constitute a major issue that give rise to concerns at local, regional, national and global scales (Lazartigues *et al.*, 2013).

2.1.1 Uses

Pesticides are used to control organisms that are considered to be harmful. For example, they are used to kill mosquitoes that can transmit potentially deadly diseases like West Nile virus, yellow fever, and malaria. They can also kill bees, wasps or ants that can cause allergic reactions. Insecticides can protect animals from illnesses that can be caused by parasites such as fleas (Whitford *et al.*, 2006). Pesticides can prevent sickness in humans that could be caused by moldy food or diseased produce. Herbicides can be used to clear roadside weeds, trees and brush. They can also kill invasive weeds that may cause environmental damage (Helfrich *et al.*, 1996). Uncontrolled pests such as termites and

mould can damage structures such as houses. Pesticides are used in grocery stores and food storage facilities to manage rodents and insects that infest food such as grain. Each use of a pesticide carries some associated risk. Proper pesticide use decreases these associated risks to a level deemed acceptable by pesticide regulatory agencies (Whitford *et al.*, 2006; Bamigboye *et al.*, 2017).

2.1.2 Classification of pesticides

Pesticides are often classified according to the type of pest they control. Another way to think about pesticides is to consider those that are chemical pesticides or are derived from a common source or production method. Other categories include biopesticides, antimicrobials, and pest control devices (US EPA, 2012).

2.1.2.1 Chemical pesticides

The following are examples of chemically-related pesticides:

- **Organochlorine pesticides:** were commonly used in the past, but many have been removed from the market due to their health and environmental effects and their persistence (e.g. DDT and chlordane). These pesticides affect the nervous system mainly by blocking Cl^- channels linked to $GABA_A$ receptors in the brain, resulting in generalized uncontrolled excitation (US EPA, 2012).
- **Organophosphate pesticides:** These pesticides affect the nervous system by disrupting the enzyme that regulates acetylcholine, a neurotransmitter. Most organophosphates are insecticides. They were developed during the early 19th century, but their effects on insects, which are similar to their effects on humans,

were discovered in 1932. Some are very poisonous (they were used in World War II as nerve agents). However, they usually are not persistent in the environment (US EPA, 2012).

- **Carbamate pesticides:** affect the nervous system by disupting an enzyme that regulates acetylcholine, a neurotransmitter. The enzyme effects are usually reversible. There are several subgroups within the carbamates (US EPA, 2012).
- **Pyrethroid pesticides:** were developed as a synthetic version of the naturally occurring pesticide pyrethrin, which is found in chrysanthemums. They have been modified to increase their stability in the environment. Some synthetic pyrethroids are toxic to the nervous system (Soderlund, 2010; US EPA, 2012).

2.1.2.2 Biopesticides

These are certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals (US EPA, 2012). For example, canola oil and baking soda have pesticidal applications and are considered biopesticides (US EPA, 2009). At the end of 2001, there were approximately 195 registered biopesticide active ingredients and 780 products (US EPA, 2012). Biopesticides fall into three major classes:

- **Microbial pesticides:** consist of a microorganism (e.g., a bacterium, fungus, virus or protozoan) as the active ingredient. Microbial pesticides can control many different kinds of pests, although each separate active ingredient is relatively specific for its target pest(s) (Borgio *et al.*, 2011). For example, there are fungi that control certain weeds, and other fungi that kill specific insects (US EPA, 2012).

- Plant-incorporated-protectants (PIPs)**:** are pesticidal substances that plants produce from genetic material that has been added to the plant (US EPA, 2012).
- **Biochemical pesticides:** are naturally occurring substances that control pests by non-toxic mechanisms. Conventional pesticides, by contrast, are generally synthetic materials that directly kill or inactivate the pest. Biochemical pesticides include substances, such as insect sex pheromones that interfere with mating as well as various scented plant extracts that attract insect pests to traps (US EPA, 2012; Pal and Kumar, 2013).

2.2 Organochlorine Pesticides (OCPs)

Chlorinated hydrocarbon (organochlorine) insecticides, solvents, and fumigants are widely used around the world. This class comprises a variety of compounds containing carbon, hydrogen, and chlorine. These compounds can be highly toxic, and the overwhelming majorities have been universally banned because of their unacceptably slow degradation and subsequent bioaccumulation and unexpected toxicities to non-target organisms in the environment (Stockholm Convention, 2009; Lee *et al.*, 2013; Fang *et al.*, 2017). The toxicity of these agents varies according to their molecular size, volatility, and effects on the CNS. In general, they cause either CNS depression or stimulation, depending upon the agent and dose (Bhalla and Thami, 2004).

2.2.1 Classification of OCPs

OCPs can be classified into 5 groups, as follows:

i. Dichlorodiphenyltrichloroethane (DDT) and analogues (e.g. dicofol, methoxychlor);

ii. Hexachlorocyclohexane (i.e. benzene hexachloride) and isomers (e.g. lindane, gamma-hexachlorocyclohexane);

iii. Cyclodienes (e.g. endosulfan, chlordane, heptachlor, aldrin, dieldrin, endrin, isobenzan);

iv. Chlordecone, kelevan, and mirex;

v. Toxaphene (Wong *et al.*, 2012).

2.2.2 Pharmacokinetics of OCPs

Absorption and metabolism

OCPs are well absorbed orally and by inhalation (Reigart and Roberts, 1999; Fang *et al.*, 2017). Transdermal absorption is variable. For example, DDT is poorly absorbed transdermally, whereas cyclodienes have significant transdermal absorption rates (Reigart and Roberts, 1999; CDC, 2005). Cyclodienes have high absorption levels when taken orally as in the case of food contamination with these pesticides (CDC, 1984). Lindane is known to be absorbed after topical application, but oral ingestions are not rare (CDC, 2005). Studies have shown that the young, malnourished, and those with frequent application are at increased risk for toxicity (Pramanik and Hansen, 1979).

OCPs are strongly lipid soluble and sequester in body tissues with high lipid content, such as the brain and liver. Consequently, blood levels tend to be much lower than fatty tissue levels (Reigart and Roberts, 1999). The lipophilic tendency of OCPs accounts for prolonged systemic effects in overdose. The half-life of DDT has been measured in months or years, whereas other organic chlorines are metabolized faster, like lindane, with a half-life of 21 hours (Mortensen, 1986).

2.2.3 Mechanisms of OCP toxicity

Sahoo *et al.* (2000), suggested that the disruption of the organisation of plasma membrane is the primary action of biologically active OCP isomers that may affect cell survivability and increase lipid peroxidation. OCPs have also been known to cause inhibition of acetylcholinesterase activity in the target tissues and effect Na^{+}- K^{+} ATPase, Mg^{2+}ATPase

and Ca^{2+} activities of cells (Ansari *et al.,* 1987; Dikshith *et al.,* 1988; Naqvi and Vaishnavi, 1993; Kalender *et al.,* 2004).

Toxicity in humans is largely due to stimulation of the central nervous system. Cyclodienes (such as endosulfan), hexachlorocyclohexanes (such as lindane), and toxaphene predominately are GABA antagonists and inhibit calcium ion influx, but also may inhibit Ca^{+}- and Mg^{2+}-ATPase; causing calcium ion accumulation at neuronal endplates, thereby causing sustained release of excitatory neurotransmitters (Roberts *et al.,* 2004; Karatas *et al.,* 2006). DDT affects potassium and voltage-dependent sodium channels. These changes can result in agitation, confusion, and seizures. Cardiac effects have been attributed to sensitization of the myocardium to circulating catecholamines (Wong *et al.,* 2012).

Some of the more volatile organochlorines can be inhaled in vapour form or swallowed while in liquid form. Inhalation of toxic vapours or aspiration of liquid after ingestion may lead to atelectasis, bronchospasm, hypoxia, and a chemical pneumonitis. In severe cases, this can lead to acute lung injury (ALI), hemorrhage, and necrosis of lung tissue. In liquid form, they are easily absorbed through the skin and gastrointestinal tract (GIT) (Wong *et al.,* 2012).

2.2.4 Adverse health effects

There is mounting evidence that organochlorine compounds can act as hormones. These compounds, including DDT, PCBs, and endosulfan, may also be part of the cause for the decrease in the quality of semen, increase in testicular and prostate cancer, increase in

defects in male sex organs, and increased incidence of breast cancer which has been observed in the last fifty years (Hileman, 1994).

2.3 Endosulfan

Endosulfan (6, 7, 8, 9, 10, 10–hexachloro–1, 5, 5a, 6, 9, 9a–hexahydro–6, 9–methano–2, 4, 3–benzodioxathiepin–3–oxide), patented in 1956, is an organochlorine (OC) insecticide that belongs to the cyclodiene group. It is one of the most commonly used pesticides to control pests in grains, vegetables, tea, cotton, tobacco and fruits. It is also used as a wood preservative (Chan *et al.,* 2006; Hwang *et al.,* 2015; Patočka *et al.,* 2016). It is a cream to brown chemical which smells like turpentine (Patočka *et al.,* 2016). Endosulfan is a contact and stomach insecticide for food and non–food crops and it is toxic to mammals, fish and other aquatic organisms (Romero-Aguilar *et al.,* 2014). It is a lipophilic insecticide which causes severe health issues due to its environmental stability, toxicity and biological reservation in organisms (Kafilzadeh *et al.,* 2015). The commercial product is a mixture of two stereoisomers α-: 70% and β-: 30%. α-endosulfan is more thermodynamically stable, while β-endosulfan slowly converts irreversibly to the α-form at room temperature (Patočka *et al.,* 2016); and is now being detected as an important cause of pesticide poisoning in many countries (Pathak *et al.,* 2010; Sabzar *et al.,* 2014; Sabzar *et al.,* 2015).

The α-isomer (melting point 108-110°C) is more bioaccumulative and about three times more toxic to insects and mammals than the β-isomer (melting point 208-210°C). The reported mean lethal dose in rats (LD_{50}) after oral exposure is between 76 mg/kg (α-endosulfan) and 340 mg/kg (β-endosulfan) (Ozdal *et al.,* 2017). The endosulfan residues of toxicological concern are α-endosulfan, β-endosulfan and endosulfan sulfate. The sulfate

which is regarded as being equally toxic and of increased persistence in comparison with the parent isomers has been identified as the dominant product of endosulfan degradation in soil (US EPA, 2010; Fang *et al.*, 2016).

2.3.1 Uses of endosulfan

Endosulfan is a pesticide for use against invertebrate animals (Besbelli, 2000). It controls a wide range of sucking and chewing insect pests notably of the orders of Lepidoptera, Coleoptera, Heteroptera, Homoptera, Tysanoptera, Diptera, and some species belonging to the order of Acarina. It is especially used on non-food crops such as cotton and tobacco and on food crops such as vegetables, fruits, corn, cereals, tea, and coffee (Naqvi and Vaishnavi, 1993).

Endosulfan has also been used in wood preservation, home gardening, and tse-tse fly control (Stockholm Convention, 2009). Because of its unique mode of action, it is useful in resistance management; however, because it is non-specific, it can negatively impact populations of beneficial insects (Mossler *et al.*, 2006). It is, however, considered to be moderately toxic to honey bees and less toxic to bees than organophosphate insecticides (US EPA, 2002).

2.3.2 Chemical structures

Endosulfan is a synthetic product with structural name: 6, 7, 8, 9, 10, 10 - hexachloro - 1, 5, 5a, 6, 9, 9a - hexahydro - 6, 9 - methano - 2, 4, 3 -benzodioxathiepine -3 – oxide, molecular formula: $C_9H_6Cl_6O_3S$ and molecular weight: 406.9 (Besbelli, 2000). β-endosulfan is a

symmetrical compound, whereas α-endosulfan is asymmetric and exists as two twist chair forms (Patočka *et al.,* 2016).

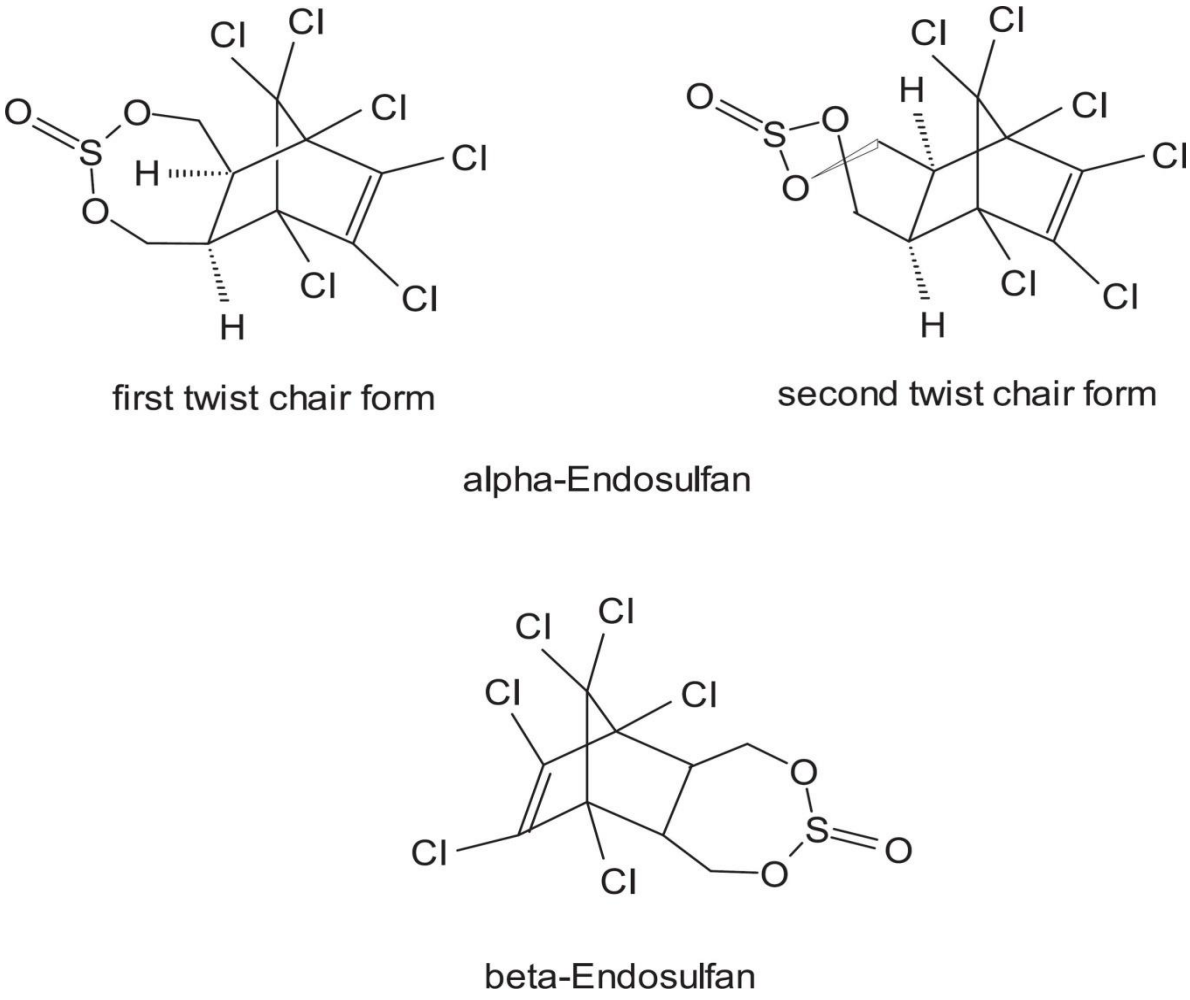


Figure 2.1: Chemical structures of alpha- and beta- endosulfan (Patočka *et al.,* 2016).

2.3.3 Routes of endosulfan exposure

Short term toxicity with endosulfan is high and influenced by the solvents and emulsifiers used to dissolve it. Endosulfan is easily absorbed by the stomach, lungs and through the

skin – all routes of exposure pose a hazard and proper protective clothing is needed to prevent poisoning when handling the pesticide (Amizadeh and Saryazdi, 2011).

2.3.3.1 Dietary exposure

i. Ingesting food that has been sprayed with endosulfan
ii. Drinking water from contaminated ground or surface stores (US EPA, 2002).

The acceptable daily intake (ADI) for endosulfan according to FAO and WHO, (2010) is 0.006 mg/kg body weight.

2.3.3.2 Occupational exposure

Skin exposure or inhalation during pesticide mixing, loading and/or applying a pesticide or re-entering treated sites (US EPA, 2002).

2.3.3.3 Accidental exposure

Skin exposure or inhalation due to proximity to endosulfan use (US EPA, 2002).

2.3.4 Mechanisms of endosulfan action

Endosulfan binds to and blocks the Cl^- channel linked to the gamma (γ)–amino–butyric $acid_A$ ($GABA_A$)-receptor. It does not affect the GABA recognition site and so can be termed a "non-competitive GABA antagonist" (Gant *et al.,* 1987; Ffrench-Constant, 1993). Because $GABA_A$ receptors are the principal inhibitory neuroreceptors in the mammalian brain, the antagonism of GABAergic neurons within the central nervous system (CNS) causes generalized brain stimulation (Gant *et al.,* 1987; ATSDR, 2015). When GABA binds to its receptor ($GABA_A$), the Cl^- ion channels are opened, leading to an influx of Cl^- into

neurons through an electrochemical gradient. The result is hyperpolarization of the cell membrane and inhibition of neuronal firing. Endosulfan prevents Cl^- from entering neurons, thus blocking the effect of GABA binding to its $GABA_A$ receptor, resulting in uncontrolled excitation (Silva and Gammon, 2009).

The Na^+-K^+ ATPase and Ca^{2+}- Mg^{2+} ATPase are also potently inhibited. The latter's inhibition in terminal boutons results in accumulation of intracellular calcium with promotion of Ca^{2+} mediated neurotransmitter release. Subsequent depolarization of adjacent neurons propagates the stimulation throughout the CNS (Amdur *et al.,* 1991).
A study carried out on animals showed a significant reduction in acetylcholinesterase (AChE) activity (Naqvi and Vaishnavi, 1993; Mor and Ozmen, 2010). This suggests that inhibition of AChE may be another mechanism of endosulfan action.

However, there is some evidence that endosulfan-induced oxidative stress, due to decreased activity of antioxidant enzymes and increased lipid peroxidation, are involved in the manifestation of alterations seen in organs and systems following exposure to endosulfan (Omurtag *et al.*, 2008; Pal *et al.,* 2009; Ozdem *et al.,* 2011; Alva *et al.,* 2012; Mahran, 2013; Aly and Khafagy 2014; Wang *et al.,* 2016).

2.3.5 Endosulfan contamination

Once endosulfan is released into the environment, it is highly persistent and mobile:

i. Endosulfan is transported long distances in the atmosphere and has been detected in areas far from use sites, including national parks and the Arctic.

ii. The half-life of combined residues of endosulfan varies from 9 months to 6 years.

iii. The main degradation product, endosulfan sulphate, is equally toxic to the parent compound and even more persistent (USEPA, 2002; US EPA, 2010).

2.3.6 Vulnerable groups

Some populations are particularly sensitive to endosulfan's neurotoxic effects; these include unborn children, infants and the elderly (Gude and Bansal, 2012). Certain medical conditions also make people particularly sensitive to its adverse affects. The Agency for Toxic Substances and Disease Registry (ATSDR) identifies people with liver or kidney disease; pre-existing anaemia or haematological disorders; neurological problems especially seizure disorders; people with HIV/AIDs and people with protein-deficient diets such as the malnourished poor, chronic alcoholics and dieters, as vulnerable groups (ATSDR, 2000; 2015).

2.3.7 Bioaccumulation

Endosulfan stores easily within the fatty tissues of living organisms, and it accumulates in concentration whilst exposure continues – that is, the organism absorbs endosulfan at a greater rate than it can be excreted. Studies have shown that both aquatic and terrestrial species can accumulate concentrations of endosulfan to a significant extent (Naqvi and Vaishnavi, 1993; PAN, 2000), but the susceptibility to bioaccumulation varies greatly between species – for example, oysters and bivalves appear to accumulate very little endosulfan, whilst some fish species accumulate endosulfan much more readily (UNEP, 2008). Terrestrial species show a greater relative potential for accumulation than aquatic

species, and monitoring data has shown that concentrations of endosulfan have increased over time in beluga whale blubber samples from the Canadian Arctic (Stern and Ikonomou, 2003), the tissue of freshwater tetra in Brazil (Jonsson and Toledo, 1993) and even in plants. This characteristic, together with endosulfan's high toxicity, means there is significant potential for damage.

2.3.8 Biodegradation

Although endosulfan is biodegradable in nature, its residues are found in the environment (Weber *et al.,* 2010). Biodegradation of endosulfan in soil and water by photolysis, chemical reactions and biotransformation is governed by a number of physicochemical environmental parameters such as energy source (electron donors), nutrients, pH, temperature, substrate concentration, and the type of microorganisms present (Besbelli, 2000; Hussain *et al.,* 2009; Singh and Singh 2011; Kong *et al.*, 2013).

Biotransformation is mainly due to oxidation (producing endosulfan – sulfate) and hydrolysis (producing endosulfan – diol) (Weber *et al.,* 2010). The diol can be converted to endosulfan ether (Hussain *et al.,* 2007) or endosulfan hydroxyether (Lee *et al.,* 2003) and then endosulfan lactone (Awasthi *et al.,* 2003). Hydrolysis of endosulfan lactone yields endosulfan hydroxycarboxylate (Walse *et al.*, 2003).

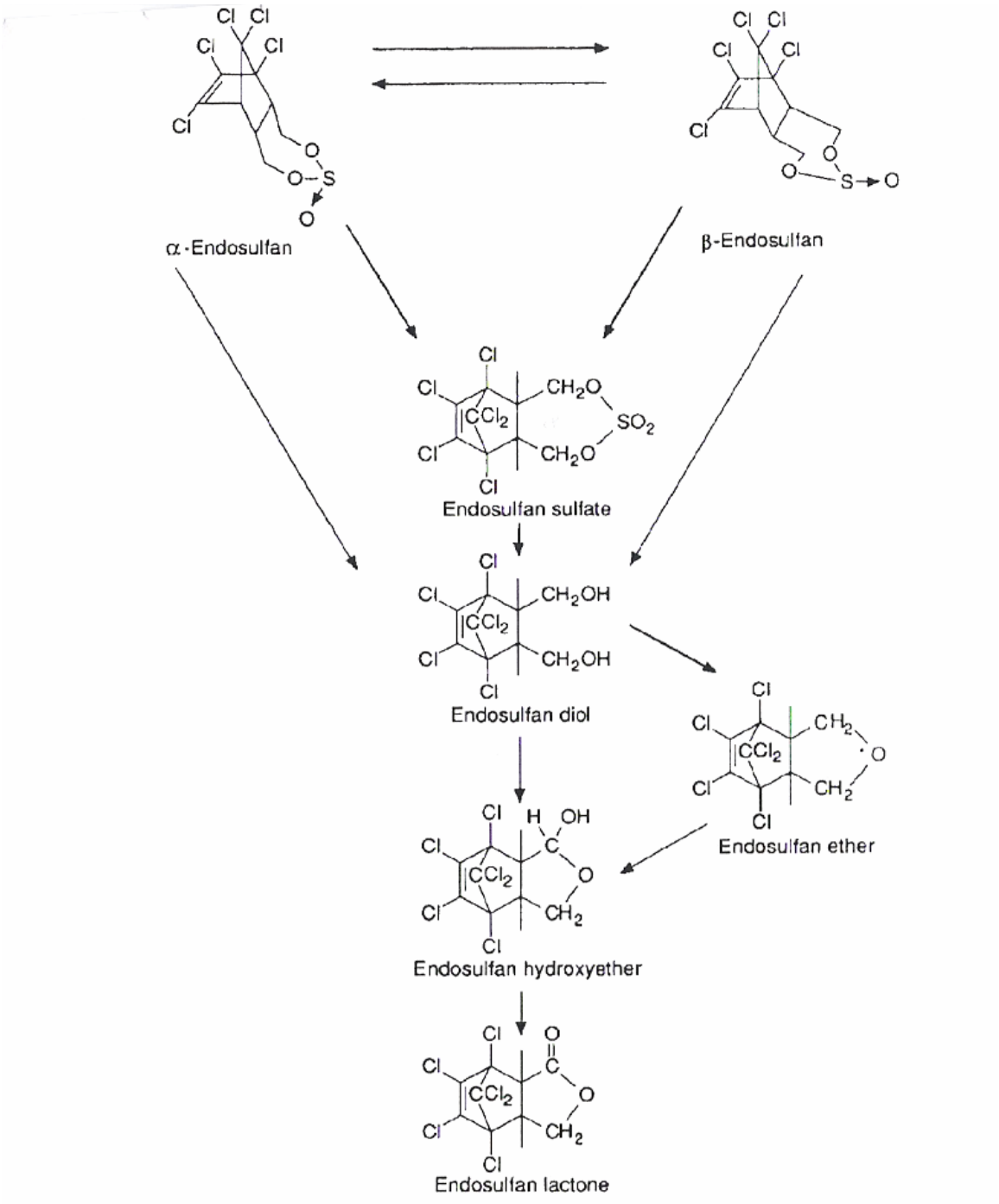


Figure 2.2: Schematic pathway of endosulfan degradation (Shivaramaiah and Kennedy, 2006).

2.3.9 Toxic effects of endosulfan

Toxicity studies of Endosulfan have been conducted in animals. These studies are carried out to identify the target organs of toxicity and possible spectrum of effects. The effects of any chemical substance are determined by the dose, duration and the time of exposure. There is a close similarity between the spectrum of health effects observed in the human population exposed to endosulfan and those described in animal experiments. It has been demonstrated that much lower doses of toxicants may result in adverse health effects manifesting as functional or organic disorders in later life if the exposure takes place during the early developmental phase (Amizadeh and Saryazdi, 2011).

The health and ecological hazards caused by exposure to endosulfan has been a global concern. Endosulfan persists in the environment and bioaccumulates in animals and plants, leading to instances of food contamination and eventually dietary exposure in humans (Briz *et al.,* 2011). Endosulfan's predominant toxicological effect is over-stimulation of the central nervous system, with little or no peripheral component. Endosulfan generally has been shown to have higher acute oral and inhalation toxicity than dermal toxicity (US EPA, 2010). Absorption of endosulfan through the gastrointestinal tract is extremely efficient – around 90% is absorbed. Similarly, absorption through the skin can be high; as much as 50% (GFEA-U, 2007).

2.3.9.1 Acute toxicity

Stimulation of the central nervous system is the main characteristic of endosulfan poisoning. Acute endosulfan poisoning can cause convulsions, psychiatric disturbances, epilepsy, paralysis, brain oedema, impaired memory and death (PAN-Int., 2008).

Endosulfan is highly toxic and can be fatal if inhaled, swallowed or absorbed through the skin. Acute oral toxicity is higher than dermal toxicity (Sang and Petrovic, 1999).

Symptoms of acute exposure include hyperactivity, excitement, dyspnea, apnea, salivation, anemia, loss of consciousness, diarrhea, nausea, vomiting, insomnia, blurred vision, cyanosis, foaming at the mouth, tremor, dry mouth, lack of appetite, irritability, head ache, decreased respiration, loss of memory, haematuria, albuminuria, confusion, dizziness, imbalance and lack of coordination. Blindness has been observed in cows, sheep and pigs which have grazed in fields sprayed with endosulfan (IPCS., 1984; ATSDR, 1995; Sang and Petrovic, 1999; Anonymous, 2000; Patočka *et al.*, 2016).

2.3.9.2 Chronic toxicity

Subacute and chronic toxicity studies of endosulfan in animals suggest that the liver, kidneys, immune system and testes are the main target organs. A number of neurotoxicity studies are available, primarily in the rat. An acute oral neurotoxicity study in rats, showed a greater sensitivity of females compared to males (ATSDR, 2000; 2015). Long-term oral and dermal exposure in rats has been found to result in rapidly progressive glomerulonephritis (a renal disease that affects the small blood vessels in the kidneys) and in male rats, has been observed to cause aneurysms (blood-vessel dilations which if ruptured can even lead to death) (Galatone, 2009). There is some indication that endosulfan can have adverse effects on the immune system at low levels of exposure (ATSDR, 1993; 2015). Long term exposure has been linked to immunosuppression, neurological disorders, congenital birth defects, chromosomal abnormalities, mental retardation and impaired learning and memory (PAN-Int., 2008). Consumption of endosulfan at low doses for a long

duration ultimately led to differential alterations of monoamines (an important class of neurotransmitters) in various regions of the rat central nervous system (Lakshmana and Raju, 1994).

2.3.9.3 Neurotoxicity

Endosulfan has been shown to produce neurotoxicity effects in laboratory animals, which are believed to result from over-stimulation of the central nervous system. Possible mechanisms of neurotoxicity include (a) alteration of neurotransmitter levels in brain areas by affecting synthesis, degradation, and/or rates of release and reuptake, and/or (b) interference with the binding of neurotransmitters to their receptors (POPRC, 2009; ATSDR, 2015). Those exposed over prolonged periods have been found to experience cognitive and emotional deterioration, severe impairment of memory and inability to perform most daily tasks. Repeated exposure to a tolerated dose of endosulfan resulted in a deficit of behavioural responses involving both learning and memory (Lakshmana and Raju, 1994; Paul *et al.*, 1994; Chitra *et al.*, 1999; Sinha *et al.*, 2001).

A serotonergic mechanism appears to be involved significantly in endosulfan-induced neurotoxicity as repeated exposure to endosulfan caused a significant increase in 3H-serotonin binding and brain levels (Agrawal *et al.*, 1983; Zaidi *et al.*, 1985; Seth *et al.*, 1986; Paul *et al.*, 1993; Paul *et al.*, 1994; Cabaleiro *et al.*, 2008). Studies have shown the involvement of serotonergic receptors in endosulfan-induced learning impairment, negligibly in its memory disrupting action (Paul *et al.*, 1994; Chitra *et al.*, 1999; Sinha *et al.*, 2001) and also in the alteration of behaviour (especially foot-shock-induced fighting

behaviour) influenced by neurotransmitters and neuroendocrine pathways (Agrawal *et al.*, 1983; Zaidi *et al.*, 1985; Seth *et al.*, 1986; Paul *et al.*, 1993).

Lakshmana and Raju (1994) suggested that monoaminergic systems in the developing rat brain respond to endosulfan by undergoing something like 'a reorganization'. However, such changes do not ameliorate certain functional losses following the exposure to endosulfan as operant conditioning revealed deficits in acquisition as well as retention of memory.

Exposure has also been linked to conditions such as cerebral palsy, epilepsy and it may increase the risk of Parkinson's disease. Some have also experienced gross impairment of visual-motor coordination (Jia and Misra, 2007). Hyperemia, hemorrhages, degenerations, gross lesions and slight gliosis of the brain and cerebellum were found in rabbits exposed to sublethal dose of endosulfan (Mor and Ozmen, 2010). Behavioral and biochemical studies carried out by Anand *et al.* (1986) on pigeons given a single dose of endosulfan indicated the involvement of a cholinergic (muscarinic) transmitter system in endosulfan-induced neurotoxicity. Investigation by Srikanth *et al*. (1989) showed that endosulfan inhibited the activity of calmodulin-dependent Ca^{2+}-ATPase to an extent in rat brain, suggesting that endosulfan may modulate calmodulin-related events in neurons and result in neurotoxicity.

2.3.9.4 Reproductive and developmental toxicity

There has been a major concern regarding the effect of endosulfan on the reproductive system. It harms the reproductive system by affecting sperm count and testicular weight (Umar *et al.*, 2012), semen quality, spermatogonial cells, sperm morphology and other

defects in male sex hormones (Pandey *et al.*, 1990). Pandey *et al.*, (1990) also confirmed that endosulfan exposure displayed dose-dependent deformities, behavioural abnormalities and mortality. Detailed studies in adult rats exposed to endosulfan for 5 days per week for 10 weeks showed reduced intratesticular spermatid counts, sperm abnormalities, and changes in the marker enzymes of testicular activities, providing further evidence of effects on spermatogenesis (Khan and Sinha, 1996; Du *et al.,* 2015). There is experimental evidence of adverse effects of endosulfan on the male reproductive system, delaying sexual maturity and interfering with the sex-hormone synthesis (Saiyed *et al.*, 2003). It has also been suggested that endosulfan can cause cell death in exposed sertoli-germ cells due to higher oxidative damage with the activation of intrinsic cell death pathway which may affect the production of quality gametes (Rastogi *et al.*, 2014).

Studies have shown that $GABA_A$ receptors are found in rat interstitial testicular cells, most of which are testosterone-producing Leydig cells (Geigerseder *et al*., 2003) and spermatozoa (Hu *et al*., 2002; Li *et al*., 2005). If endosulfan blocks the GABA receptors for chloride channels, many aspects of spermatogenesis would also be blocked, such as regulation of endocrine function in the testis through Leydig cells, maturation and differentiation of germ cells and initiation of cell proliferation and differentiation in the interstitial testicular compartment (Giergerseder *et al.*, 2003). In addition, GABA receptors on sperm play a role in triggering the acrosome reaction whereby the sperm become active (Ritta *et al.*, 1998; Hu and Yan, 2002; He *et al.*, 2003).

A study by Guo *et al.* (2017) found that endosulfan decreased the cell viability, damaged cell membranes; and induced apoptosis, oxidative stress and DNA damage in spermatogenic cells.

They suggested that endosulfan could lead to apoptosis of spermatogenic cells by activating the negative regulation factors of the cell cycle, and also by the death receptor pathway, causing oxidative stress.

Pre- and post-natal exposures to endosulfan have been confirmed by measures of residues in human breast milk, placenta, cord blood, and adipose tissue (Campoy *et al.,* 2001; Cerrillo *et al.,* 2005; Shen *et al.,* 2008; Du *et al.,* 2015). This has been associated with pre-term delivery (Pathak, 2010). A study carried out in 2006 by Damgaard *et al.*, suggested an association between congenital cryptorchidism and persistent endosulfan residue in mother's breast milk due to prenatal exposure.

A relationship has been established between maternal exposure and foetal malformations in the skull, ribs and spine of rats (Singh *et al.,* 2007); and impaired behavior in rat pups (Zaidi *et al.,* 1985). Both acute and chronic studies on various animal species during development suggested that endosulfan prolonged time to metamorphosis, increased incidences of mouth malformations as well as mortality, and reduced body weight (Brunelli *et al.,* 2009), skeletal malformations, as well as **morphological and morphometric malformations (**Brunelli *et al.*, 2009; Brunelli *et al.*, 2010; Mobarak **and** Al-Asmari**,** 2011) . Physical malformations observed in humans include cleft palates, harelips, club feet, limb malformations, eye deformities and extra fingers and toes (Rupa *et al.*, 1991).

Studies have also shown that perinatal exposure to endosulfan can produce neurochemical alterations in brain of the offspring. In mice, maternal exposure to 1 mg/kg/day endosulfan in the diet during gestation and lactation resulted in alterations in the expression of proteins

in the frontal cortex of 12-week-old male offspring (females were not tested) that are critical for the normal functioning of gabaergic, glutamatergic, and dopaminergic pathways (Wilson *et al.,* 2014a). The same exposure protocol also resulted in alteration in dopaminergic pathways in the striatum of 12-week-old offspring. Furthermore, transplacental and/or lactation exposure to endosulfan rendered the offspring more susceptible to the dopaminergic neurotoxin, MPTP, than mice that were exposed to MPTP as adults (Wilson *et al.,* 2014b). Similar exposure of rats to endosulfan also resulted in alterations in the concentration and/or metabolism of dopamine, norepinephrine, and serotonin in the striatum of male and female 60-day-old offspring (Lafuente and Pereiro 2013). Gender differences in response were evident in this study, suggesting that male offspring were more susceptible to early life exposure than females.

2.3.9.5 Cardiotoxicity

Some studies have shown the cardiotoxicity of endosulfan on animals. Jalili *et al.* (2007) in their study, carried out on rats, showed endosulfan to cause severe heart congestion, haemorrhages with interstitial oedema, diapedesis of leukocytes in some places, different degrees of myocardial degeneration, granular myofibrils with pyknotic nuclei and the thickening of arterial walls. They also suggested that endosulfan-induced heart injury may be due to oxidative tissue damage (Jalili *et al.,* 2007). Studies by Kalender *et al.* (2004) also suggested that the cardiotoxicity induced by endosulfan was more pronounced in its effects on myocardial cells.

Wei *et al.* (2017b) in their study provided a new insight for the mechanism of endosulfan-induced cardiotoxicity. They suggested that exposure to endosulfan resulted in injury of

cardiac tissue with impaired mitochondria integrity by inducing DNA damage and activated DNA damage response signaling pathway; also by promoting cell apoptosis through death receptor pathway resulting from oxidative stress.

2.3.9.6 Immunotoxicity

The immune system is adversely affected by endosulfan because exposure decreases the white blood cell count. These cells are vital for functions such as fighting infections, allergies and for tumour suppression (Galatone, 2009). Endosulfan inhibits leucocytes and macrophage migration (this is the inhibition of the natural immune system by disrupting anti-body protection) causing adverse effects on humoral and cell-mediated immune system (Sang and Petrovic, 1999).

Study carried out by Pistl *et al.* (2003) showed that endosulfan suppressed both the metabolic activity of phagocytes and mitogenic activation of lymphocytes; while Lafuente *et al.* (2006) suggested that exposure during pregnancy and lactation in adult rats could modify humoral immunity in offspring adulthood, and that females are more susceptible to endosulfan because their cellular immunity was also altered.

2.3.9.7 Carcinogenicity and genotoxicity

There is considerable evidence that endosulfan is genotoxic. In general, one of the main mechanisms underlying endosulfan toxicity is the induction of several genotoxic insults (Romero-Aguilar *et al.,* 2014). It has been found that exposure to sublethal doses of endosulfan and its metabolites induce DNA damage (strand breaks) and mutation (Bajpayee *et al.,* 2006; 2013). Genotoxicity has been displayed in HepG2 cells (Human

liver carcinoma cells) (Li *et al.*, 2011), hepatocyte-derived transformants (Hashizume *et al.*, 2010) and bone marrow micronuclei (Manjula *et al.,* 2006). Endosulfan has caused mutations in bacterial and yeast cell. It is also known to cause mutations in mammals (Quijano, 2000). It has been suggested that endosulfan is considered to be genotoxic on the basis of evidence of genotoxicity in tests for gene mutation, chromosomal aberration and DNA damage (Silva and Beauvais, 2010; Romero-Aguilar *et al.*, 2014).

A re-analysis data from a 1978 National Cancer Institute, US (NCI) study in Osborne-Mendel rats has revealed that endosulfan induced malignant neoplasms at all sites in male and female rats and endocrine organs in male rats. Both sexes developed lymphosarcomas (a diffused malignant cancer of lymphatic cells of the immune system) and female rats had neoplasms (tumour) of the reproductive system. Endosulfan is also carcinogenic for the liver of female mice (Reuber, 1981). Study by Fransson-Steen *et al*. (1992) in rats, suggested that endosulfan and its two stereoisomers are potential liver tumour promoters as they promoted the development of altered hepatic foci. Endosulfan has also been reported as a tumor promoter, causing a significant increase in hepatocytes and rapidly inhibiting gap junctional intercellular communicationin liver cells (Patočka *et al.,* 2016).

No accurate data related to the carcinogenicity of endosulfan in human is available but from field level reports, endosulfan can be highly suspected for having carcinogenic properties in human beings, especially in cases of chronic exposure. In some reports it is referred to as having possible carcinogenic effects, effects in human immune and reproductive systems (Anon., 2002). Studies have also shown that it induces proliferation of human breast estrogen sensitive MCF7 cells in vitro which may lead to greater breast cancer risk (Soto *et*

al., 1994; Preziosi, 1998). Studies also indicate the contribution of endosulfan in the combined effect of environmental estrogens in inducing breast cancer (Ibarluzea *et al.,* 2004).

2.3.9.8 Endocrine disruption

There are several studies that prove that endosulfan is an endocrine disruptant. The commission of European Union has published a priority list of endocrine disrupting substances, placing endosulfan in Group II substance, i.e. a High Production Volume Chemical with a potential for endocrine disruption (Knauf and Schulze, 1973). It has potential to induce hypo thyroidism (Anon., 2001; Coimbra *et al.,* 2005). It competes for estradiol for binding to estrogen receptors, thereby inhibiting hormonal function. The estrogenic potential of endosulfan increases in the presence of other estrogenic organochlorines (Grumfeld and Bonefeld-Jorgensen, 2004). It potentiates the 17-estradiol induced cell proliferation (Andersen *et al.,* 2002). It has been shown to modify the expression and release of leuitinizing hormone, growth hormone, prolactin and thyroid stimulating hormone (Caride *et al.,* 2010).

Endosulfan has been shown to have hormone disruption activity on diverse animals ranging from newts to zebra fish. There are disturbing reports suggesting endocrine effect of pesticides including endosulfan on humans. Studies have linked endosulfan with abnormal breast development in pre-pubertal females, earlier menarche, thelarche and pubic hair stage; while in males it slows down the timing of reproductive maturation and decreses penile length (Hond and Schoeters, 2006; Roy *et al.,* 2009). Endosulfan has even shown endocrine-disrupting activity in two different neuronal populations, Cerebellar Granular

Cells (CGC) and Cortical Neurons (CN) through their direct interaction with neuronal Estrogen Receptors (ER) (Briz *et al.,* 2011).

2.3.9.9 Teratogenicity

Few studies suggest the teratogenic properties of endosulfan on rats and mice (Anon., 2001). One study by Singh *et al*. (2007) showed that endosulfan induced gross fetal anomalies the most important of which include: internal hydrocephalus, cerebellar hypoplasia, microphthalmia, contracted and notched kidneys, multilobulated liver, dilated renal pelvis, sacral and caudal vertebrae agenesis, rib anomalies and incomplete ossification of skull bones; at a very low dose.

2.3.9.10 Cytotoxicity

A number of epidemiological studies have demonstrated a strong association between the incidence of cytotoxicity and pesticide exposure. Endosulfan has been shown to induce cytotxicity in SH-SY5Y neuronal cells via both apoptotic and necrotic pathways (Jia and Misra, 2007), in the B cells of pancreatic islets as well as exocrine acini (Kalender *et al.*, 2004) and in human red blood cells by membrane damage (Daniel *et al.,* 1986).

Endosulfan has been shown to inhibit endothelial cell viability by increasing the release of lactate dehydrogenase (LDH), arresting cell cycle, inducing apoptosis; damaging microfilaments, microtubules and nuclei; arresting mitosis, and significantly inducing oxidative stress in the cells (Li *et al.*, 2016; Wei *et al.*, 2017a)

2.4 Oxidative Stress

Oxidative stress, is an adverse reaction resulting from the exposure of molecules, cells or tissues to excess levels of free radical oxidants, especially reactive oxygen species (ROS) (Li *et al.,* 2009; Velisek *et al.,* 2012; Lushchak, 2014). The term "oxidative stress" has been coined to represent a shift towards the pro-oxidants in the pro-oxidant/antioxidant balance that can occur as a result of one of three factors: (i) an increase in ROS, (ii) an impairment of antioxidant defence systems; and (iii) an insufficient capacity to repair oxidative damage (Mahran, 2013). Increased oxidative stress at the cellular level can also come about as a consequence of many other factors, including exposure to alcohol, medications, trauma, cold, heat, ultrasound, infections, poor diet, toxins, radiations, or strenuous physical activity (Halliwell *et al.*, 1992; Percival, 1996). Oxidative stress causes peroxidation of lipids and formation of other reactive intermediates that may also result in increased oxidative damage of biomolecules, as well as formation of protein carbonyl adducts (Pathak *et al.*, 2010).

Oxidative damage to DNA, proteins, and other macromolecules has been implicated in the pathogenesis of a wide variety of diseases, most notably heart diseases, cancer, diabetes, Alzheimer's disease, Parkinson's disease, and eye diseases such as cataracts and age-related macular degeneration (NCCAM, 2012). The brain is particularly vulnerable to oxidative damage since (i) it contains a high proportion of polyunsaturated fatty acids, which are highly susceptible to lipid peroxidation, and catecholamines prone to autoxidation; (ii) it has a high metabolic activity that requires large amounts of oxygen (the brain uses approximately 20% of the oxygen consumed by the resting body); (iii) it is relatively deficient in antioxidant systems with a lower activity of glutathione peroxidase (GPx) and catalase (Cat) compared to other organs; and (iv) it contains redox-active metals

(copper and iron) that can promote the production of free radicals (Kedar, 2003; Nunomura *et al.*, 2006).

2.4.1 Free radicals and reactive oxygen species

Free radicals and reactive oxygen species (ROS) are molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals, which characterises free radicals with high reactivity (Halliwell and Gutteridge, 1999). Cells generate small amounts of free radicals or reactive oxygen species (ROS) during their normal metabolism. Although low levels of ROS are essential in many biochemical processes, accumulation of ROS may damage biological macromolecules, that is, lipids, proteins, carbohydrates and DNA (Mates *et al.*, 1999; Schieber and Chandel, 2014).

Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves. Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, causing a chain reaction to occur. And until subsequent free radicals are deactivated, thousands of free radical reactions can occur within seconds of the initial reaction (Percival, 1996; NCCAM, 2012). Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction (Sies *et al.*, 1992; Miranda *et al.*, 2013; Zhai *et al.*, 2013). Overall, free radicals have been implicated in the pathogenesis of at least 50 diseases (Langseth, 1993; Halliwell, 1994).

Reactive oxygen species (ROS) is a term which encompasses all highly reactive, oxygen-containing molecules, including free radicals, which are by-products of aerobic metabolism. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage (Percival, 1996; Schieber and Chandel, 2014). This damage can compromise cell viability or induce cellular responses leading to cell death by necrosis or apoptosis (Valko *et al*., 2007). Exogenous agents (such as photochemical smog, ozone, pesticides, xenobiotics and ionizing radiation) and a variety of endogenous processes (for example, mitochondrial respiration, cytochrome P-450 detoxification reactions, phagocytic oxidative bursts, and peroxisomal leakage) can generate significant amounts of ROS in the human body (Chakravarti and Chakravarti, 2007).

2.4.2 Endosulfan and oxidative stress

Pesticides have been known to alter oxidant/antioxidant status and induce oxidative damage leading to the production of **free radical**s and changes in enzymatic and non-enzymatic antioxidants or the oxygen free-radical scavenging enzyme system (Mossa *et al.,* 2015; Badgujar *et al.,* 2016; Swelam *et al.,* 2017). Endosulfan has been reported to induce lipid peroxidation and oxidative stress (by generation of free radicals) in mammals and other organisms (Aggarwal *et al.,* 2009; Pathak *et al.,* 2010; Ahmed *et al.,* 2011; Shao *et al.,* 2012; Mahran, 2013) and oxidative stress has been shown to have a pivotal role in endosulfan toxicity in multiple organs including the brain, liver, kidney, spleen and testis (Wu *et al.,* 2012; Mahran, 2013; Du *et al.,* 2015). Study by Ballesteros *et al*., 2008

suggested that the brain was the most sensitive organ to oxidative damage. A possible mode of action is that endosulfan can uncouple oxidative phosphorylation (Kannan *et al*, 2000) to create ROS and oxidative stress (Sohn *et al*, 2004) which can produce apoptosis in immune cells (Kannan *et al*, 2000), testicular damage (Rao *et al*, 2005) and neuronal damage (Sah *et al*, 2002). These modes of action studies may explain the results of the in vivo studies demonstrating testicular damage (Chitra *et al*., 1999; Sinha *et al*., 2001), neurobehavioural disruptions (Zaidi *et al*., 1985; Seth *et al*., 1986; Paul *et al*., 1992; 1993; 1994), and immune toxicity (Banerjee and Hussain, 1986; 1987) in rodents. Effects on these organ systems are consistent with endocrine disrupting activity through inhibition of cellular respiration and/or antagonism of $GABA_A$ receptors for chloride channels that leads to alterations in development in the young (Knadle and Chan, 2006; ATSDR, 2015).

A general pathway of toxicity for many environmental pollutants is mediated by the enhancement of intracellular ROS, which modulate the occurrence of cell damage via initiation and propagation of lipoperoxidation (LPO) (Oruc and Usta, 2007). LPO is a complex process in which polyunsaturated fatty acids in the biological membrane systems undergo changes by chain reactions and form lipid hydroperoxides, which decompose double bonds of unsaturated fatty acids and disrupt membrane lipid (Radwan *et al*., 2010). Previous investigations have also reported the induction of LPO by pesticides such as chlorpyrifos (Bebe and Panemangalore, 2003; Ambali *et al.*, 2010; Ambali *et al.*, 2011), methyl parathion (Celik and Suzek, 2008) and tebuconazol (Toni *et al*., 2010).

2.5 Antioxidants

To protect the cells and organ systems of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals (Jacob, 1995).

2.5.1 Types and classification of antioxidants

Antioxidants are generally classified based on their endogenous and exogenous origins. Those of endogenous origin include the Antioxidant enzymes, e.g., catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase, which catalyze free radical quenching reactions (NCCAM, 2012). Those of exogenous origin include: (i) Nutrient-derived or dietary antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids, and other low molecular weight compounds such as glutathione and lipoic acid; (ii) Metal binding proteins, such as ferritin, lactoferrin, albumin, and ceruloplasmin that sequester free iron and copper ions that are capable of catalyzing oxidative reactions; and (iii) Numerous other antioxidant phytonutrients present in a wide variety of plant foods (Percival, 1996; NCCAM, 2012).

2.5.2 Vitamin E

Vitamin E, the principal lipid-soluble antioxidant, was first discovered in 1936 (Chow, 2001). Vitamin E includes eight naturally occurring forms; namely, the alpha, beta, gamma and delta classes of two families of compounds - the tocopherols and the tocotrienols, collectively known as tocols (Shekelle *et al.,* 2003; Rizvi *et al.,* 2014). Specifically, vitamin E is able to extinguish single oxygen species as well as to scavenge peroxyl radicals and

terminate free-radical chain reactions (Giugliano, 2000), and is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation (Mahran, 2013). Apart from antioxidant properties, more recent studies have clearly demonstrated that vitamin E also possesses important non-antioxidant cellular and molecular functions such as regulation of platelet aggregation and protein kinase C activation (García, 2013; Rizvi *et al.,* 2014), regulation of cell survival, enhancement of endothelial function, regulation of inflammatory processes (Vardi *et al.,* 2013), and its deficiency has been related to neurological disorders and female infertility (García, 2013). It has been suggested that all of the other biological functions of vitamin E are actually a result of its antioxidant activity (Vardi *et al.,* 2013).

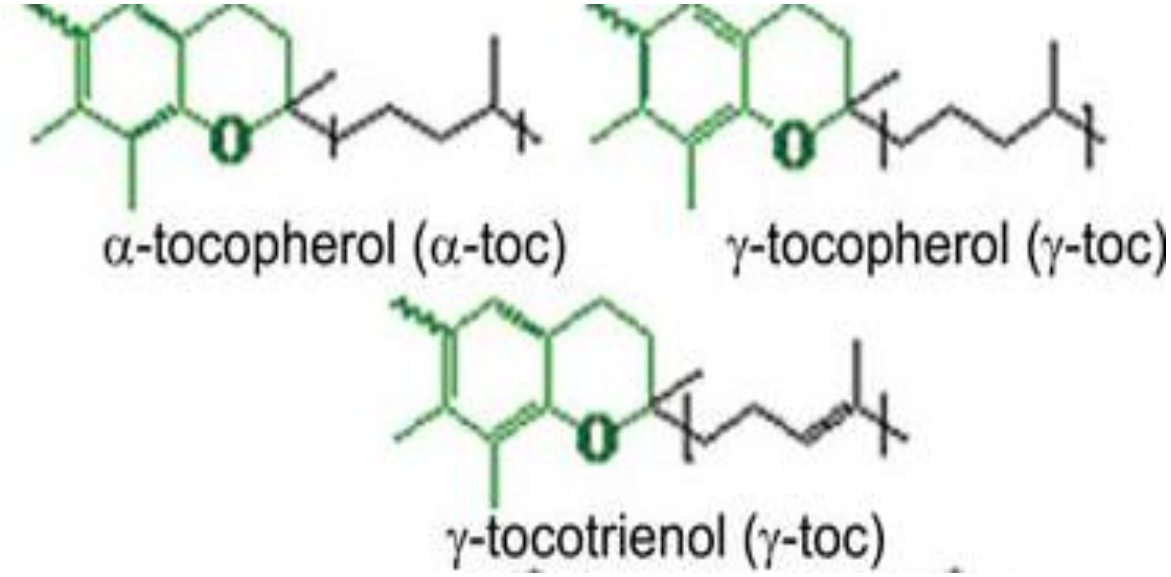


Figure 2.3: Structures of α-tocopherol, γ-tocopherol and γ-tocotrienol. Green indicates the chromanol ring with the free OH^- functional domain (Augustyniak, *et al.,* 2010).

Alpha-tocopherol (α-Tocopherol) has been shown to have the highest biological activity of all the tocopherols and tocotrienols and is the major vitamin E compound found in tissues of humans and animals (Vatassery *et al.,* 1999; Vardi *et al.,* 2013). It acts as an antioxidant either by donating a hydrogen radical to remove the free lipid radical, reacting with it to form non-radical products, or simply trapping the lipid radical (Upston *et al.,* 1999). It is thought to exert its primary protective effects via the protection of low density lipoproteins (LDL) from oxidation (Shekelle *et al.,* 2003). Antioxidant vitamins generally, have been shown to interfere with the oxidation of LDLs. Of these, vitamin E may be the most potent inhibitor of lipid oxidation because it is fat-soluble and constitutes part of the LDL molecule (Pryor, 2000). Vitamin E supplementation has been shown to decrease the oxidation of LDL, measured in prolongation of lag-time before LDL oxidation, often experimentally induced by heavy metals such as copper (Suzukawa *et al.*, 1998). Studies by Subramaniam *et al.* (1998) have shown that vitamin E protected the brain from lipoperoxidation and the consequent oxidative stress. Following its oxidation, vitamin E can be recycled back to its native unoxidized form by various soluble antioxidants such as vitamin C and ubiquinol. This process prevents the accumulation of vitamin E radicals and their subsequent peroxidation of lipids, and is considered by some to be critical for the antioxidant activity of vitamin E (Gey, 1998; Brigelius-Flohé, 2009; Vardi *et al.,* 2013).

2.5.3 Sources of vitamin E

Vitamin E is mainly found in most plant oils like soya oil, sunflower oil, olive oil, corn oil, peanut oil, palm oil, cotton seed oil, safflower oil, almond oil and wheat germ oil; most vegetables like spinach, asparagus, tomatoes, sweet potato, avocado and turnip greens; in meat, fish, poultry and eggs; in some fruits like apples and mangoes; and in some seeds and

nuts like peanuts, almonds, hazelnuts and walnuts; and fortified cereals (COMA 1991; Rizvi *et al.,* 2014).

2.5.4 Pharmacokinetics of vitamin E

α-Tocopherol is a superb chain-breaking antioxidant that appears to be the only major lipid-soluble antioxidant of its kind in mammalian membranes and lipoproteins. It is absorbed rather inefficiently from the gut into the lymphatic system and is transported from the intestine in chylomicrons. During chylomicron catabolism by lipoprotein lipase, vitamin E can be transferred to other lipoproteins and to the tissues. Tocopherol reaches the liver by uptake of chylomicron remnants. It is secreted from the liver into the plasma in very low density lipoprotein (VLDL), the metabolism of which effects transfer to LDL and HDL, as well as to tissues. The liver shows a strong preference for secretion of natural *RRR*-α-tocopherol (Burton and Traber, 1990).

Studies of the biokinetics and bioavailability of vitamin E have shown that vitamin E turnover is slowest in neural and brain tissues. It is therefore interesting to note that vitamin E plays a very important role in maintaining neurological function in man (Sokol, 1989; García, 2013).

2.6

Neurobehavioural Assessment Paradigms

Neurobehavioural science is the study of processes and mechanisms underlying normal and abnormal behaviours (Crawley and Holmes, 2011). Neurobehaviour is the final output of the nervous system at the level of the whole organism. Animal behaviours are used to test hypotheses about anatomical, neurochemical, neurophysiological, and genetic substrates of behaviour. The appropriate animal neurobehaviour paradigms provide the critical functional tools for translational research, moving discoveries at the molecular level toward clinical applications (Crawley and Holmes, 2011). There are several neurobehavioural paradigms used to assess motor and cognitive functions; some of which assess both motor and cognitive functions at the same time.

2.6.1 Paradigms for assessment of motor functions

Assessing locomotor activity is among the most basic exercises in neurobehavioural study. The study for locomotor activity in rodents does not involve the extensive learning and conditioning required by other neurobehavioural tasks (Pierce and Kalivas, 2007). Some of the models used in assessing motor functions include:

i. **The open field activity test** is used to assess spontaneous locomotor activity, including rearing, grooming, stereotypic behavior, ambulatory activity, exploration of a novel environment and habituation (Pierce and Kalivas, 2007).

ii. **The rotorod test** is a test which involves the ability of rats to walk and balance on a rotating rod. This test is widely used to assess balance and coordination or ataxia in animals. Performance is measured by the duration the animal stays on the drum as a function of drum speed. Dunham and Miya (1957) first described the roto-rod to

evaluate strength and coordination in animals (Carter *et al.,* 2001; Jeon *et al.,* 2009).

iii. **The forepaw grip time test** involves having rats hang from a thin rod. This test is used in assessing motor strength and endurance. Performance is measured by the duration the rat hangs-on before releasing its grip (Abou-Donia *et al.*, 2001)

iv. **The inclined plane test** involves placing rats on a rough plane inclined at various angles. This test is used in assessing neuromuscular coordination. Performance is measured by the highest angle the rat is able to remain on the platform for 3 secs without sliding off (Ambali *et al.,* 2010).

v. **The beam-walking test** involves placing rodents on an elevated narrow beam to traverse and reach an enclosed escape platform. This is test is used in assessing motor coordination, gait and balance. Performance is measured by number of footslips and latency to traverse the beam (Carter *et al.,* 2001).

2.6.2 Paradigms for assessment of cognitive functions

Some of the models used for assessing cognitive functions include:

i. **The Morris water maze task** involves the rodent being placed in a large circular pool of opaque coloured water and is required to find an invisible or visible platform that allows it to escape the water using various cues. There are three basic tactics for the rats to escape the maze – (i) a praxic strategy (remembering the movements needed to get to the platform), (ii) a taxic strategy (the rodent uses visual cues to reach their destination), and (iii) a spatial strategy (using distal cues as points of reference to locate themselves). The test is used to assess spatial learning and memory and performance is measured by latency to escape to the

platform. The test can also be used to assess sensorimotor activity (Carter and Shieh, 2015; Hubbard and Chaudry, 2017).

ii. **The step-down inhibitory avoidance learning task** involves the use of footshocks to invoke an unconditioned response in the rats during the learning phase and a conditioned avoidance response 24 hrs later during the memory phase of the test. The number of footshocks received by each rat is used as a measure of learning acquisition and the time spent avoiding the footshocks is used as a measure of memory retention (Zhu *et al.*, 2001).

iii. **The Barnes maze task** involves placing the rodent on a circular table with holes around the circumference, placed in a room with visual cues in the periphery. Most of these holes lead to an open drop to the floor but a single hole leads to dark drop box where the rodent can hide. The rodent is placed in the centre of the table and must find the drop box on its own. The task is used to assess spatial learning and memory. Performance is measured by latency to find the correct hole, the number of incorrect holes explored and the length of the exploratory path (Carter and Shieh, 2015).

iv. **The radial arm maze task** is used to measure spatial learning and memory (working and reference memory) in rats. It involves placing the rat on the apparatus which consists of eight horizontal equidistant arms radiating froma small circular central platform elevated about 70 cm off the floor. At the end of each arm is a cup that may or may not contain a food reward and the rats are trained to recognise that only one of the arms will contain food. Performance is measured by the latency to find the arm leading to food and the number of times the rat traverses an arm it has previously visited (Carter and Shieh, 2015; Brolese *et al.*, 2017).

v. **The beam-walking test** can also be used as a learned avoidance test. A pre-training session is preceded by a negative reinforcement paradigm in which termination of adverse stimuli (noise and light) serves as a reinforcement reward. The time taken to traverse the beam and enter a darkened goal box in order to terminate the loud white noise and bright light is measured to assess memory, motivation, attention, somatomotor and locomotor functions (Jeon *et al.,* 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Animals

Thirty two (32) male Wistar rats weighing an average of 100 ± 25 g were obtained from the animal house of the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria. They were weighed, housed in metal cages, fed on standard feed and allowed access to fresh water *ad libitum.* They were allowed to acclimatize for two weeks prior to commencement of the study.

3.2 Chemicals

Commercial grade endosulfan (35% EC; Endocel®, Excel Crop Care Limited, Mumbai, India) was reconstituted in Soya oil (Grand Cereals Limited, Jos, Plateau State) to a 10% solution prior to use. Vitamin E (Archy's®; Futurebiotics LLC, Hauppauge, New York) was reconstituted in Soya oil to make a 20 mg/ml (Ambali *et al.,* 2011) solution, prior to use.

3.3 Mean Lethal Dose (LD_{50})

The mean oral lethal dose (LD_{50}) for endosulfan in Wistar rats was calculated using the acute toxicity study method described by Lorke (1983). The method uses a total of thirteen (13) Wistar rats and the test is carried out in two phases. In the first phase, the range of doses producing the toxic effects was established. In this phase, nine (9) rats were divided into three (3) groups of three (3) rats each and administered three (3) different doses of endosulfan at 10, 100 and 1,000 mg/kg of body weight, respectively. In the second phase, specific doses were administered, based on the results obtained in the first phase, to

calculate an LD_{50}. Here the proposed doses were given to four (4) groups consisting of one (1) animal each. To estimate the LD_{50}, the geometric mean of the highest dose that recorded no mortality (D_0) and the lowest dose that recorded 100% mortality (D_{100}) was calculated using the formula:

$$\mathbf{LD_{50} = \sqrt{D_0 \times D_{100}}}$$

3.4 Treatment Protocol

The rats were divided at random into four groups of 8 rats each and treated as follows:

- Group 1: Rats were given soya oil at 2 ml/kg body weight (Ambali *et al.,* 2010).
- Group 2: Rats were administered Vitamin E at a dose of 30 mg/kg body weight (Pal *et al.,* 2009).
- Group 3: Rats were administered endosulfan at a dose of 12 mg/kg (10% of the calculated LD_{50} of 120 mg/kg).
- Group 4: Rats were pre-treated vitamin E (30 mg/kg) followed by endosulfan (12 mg/kg) 30 mins later.

The regimens were administered orally by gavage once daily for 28 days. The animals were then observed for clinical signs and possible death throughout the study period. The rats were also examined at various intervals during the study period, for various neurobehavioural parameters such as sensorimotor performance and cognition. The animals were managed in conformity with the Ahmadu Bello University Animal Research Ethics Committee and in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

3.5 Neurobehavioural Assessments

All behavioral parameters were assessed by two trained observers oblivious to the animals' treatment status in order to eliminate bias.

3.5.1 Forepaw grip time

The forepaw grip time as described by Abou-Donia *et al.* (2001) and modified by Ambali *et al.* (2010) was used to evaluate the effect of treatments on motor strength of the rats. This was assessed by having the rats hang from a 5 mm diameter wooden dowel gripped with both forepaws. The time spent by each rat before releasing their grip was recorded in seconds and this was tried twice during each test session. The results of the two trials were averaged for each testing session. This assessment was done at weeks 0, 2 and 4.

3.5.2 The inclined plane

The effect of treatments on neuromuscular coordination was assessed using the performance on inclined plane as described by Ambali *et al.* (2010). The inclined plane performance was evaluated by placing each rat on an apparatus made with an angled rough wooden plank with thick foam pad at its bottom end. The plank was raised to 35° and thereafter, gradually increased stepwise by 5° until the subject could no longer stay and be situated horizontally on the plank for three (3) secs, without sliding down. Angles were measured and marked on the apparatus beforehand and were obtained by propping the plank against a vertical bar with several notches. The test was performed with the head of the rat first facing left and then right. The highest angle at which each rat stayed

horizontally facing each direction was recorded. Two trials were performed for each testing period. This assessment was done at weeks 0, 2 and 4.

3.5.3 Step-down inhibitory avoidance learning task

The effect of treatments on learning and memory was tested using a step-down inhibitory avoidance learning task as described by Zhu *et al.* (2001). The apparatus used was a 40 × 25 × 25 cm acrylic chamber consisting of a floor made of parallel 2-mm-caliber stainless steel bars spaced 1 cm apart. An electric shock was administered through the floor bars. A 2.5 cm high, 8 × 25 cm wooden platform was placed on the left extreme of the chamber. Each rat immediately received a single 80-volt footshock. If the rat does not return to the platform, the footshock was repeated every 5 secs. A rat was considered to have learned the avoidance task if it remained on the platform for more than 2 mins. The number of footshocks was recorded as an index of learning acquisition. To assess memory, the rats were again placed gently on the platform after 24 hrs. The time a rat remained on the platform was recorded as an index of memory retention. Staying on the platform for 2 mins was counted as maximum memory retention (ceiling response). This assessment was done 48 hrs to the termination of the study.

3.6 Biochemical Assays

3.6.1 Assessment of brain lipoperoxidation

The level of thiobarbituric acid reactive substance, malondialdehyde (MDA), as an index of lipid peroxidation was evaluated in brain samples. Brain samples were collected from each animal immediately after decapitation, weighed and then homogenized in a known volume of ice-cold phosphate buffer to obtain a 10% (w/v) homogenate which was then centrifuged at 600 g for 10 min to obtain the supernatant (Freitas *et al.*, 2005). Quantitative measurement of lipid peroxidation of MDA was determined using NWLSS™ MDA assay kit (Northwest Life Sciences Specialities, Product NWK-MDA01, Vancouver WA, Specificity: Malondialdehyde, Sensitivity: 0.08 μM). The principle is based on the reaction of MDA with thiobarbituric acid (TBA); forming an MDA-TBA2 adduct that absorbs strongly at 532 nm (Janero, 1990).

3.6.2 Determination of brain antioxidant enzymes activity

The assessment of superoxide dismutase (SOD) and catalase (CAT) activities was used to determine brain antioxidant enzymes activity. The 10% (w/v) homogenate, as obtained above, was centrifuged at 800 g for 20 min and the supernatant obtained was used to assay SOD and CAT activities (Freitas *et al.*, 2005).

3.6.2.1 Superoxide dismutase activity

Activity of SOD in the rat brain was determined using NWLSS SOD assay kit (Product NWK-SOD02, Specificity: Cu/Zn, Mn and Fe Superoxide Dismutase, Sensitivity: 5 U/mL). The assay kit is based on the principle of superoxide inhibition of autooxidation of hematoxylin as described by Martin *et al*. (1987).

3.6.2.2 Catalase activity

CAT activity was assessed using NWLSS CAT activity assay kit (Product NWK-CAT01, Specificity: Catalase, Sensitivity: 6.0 U Catalase/mL). Catalase enzyme activity was measured based on the principle of catalase consumption of H_2O_2 substrate at 240 nm (Beers and Sizer, 1952).

3.7 Statistical Analysis

Results are expressed as mean ± SEM. Repeated measure ANOVA followed by Tukey's multiple comparison post hoc test was used to analyse the sensorimotor performances, while single factor ANOVA followed by Tukey's test was used to evaluate the cognitive and biochemical parameters. All statistical analysis was evaluated using SPSS version 17.0 software and Microsoft Excel (2007). *P*-values $\leq$ 0.05 were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Mean Lethal Dose (LD_{50})

The LD_{50} was determined using the method of Lorke (1983). In the initial phase, % mortalities were recorded as follows (Table 4.1):

i. 0% (0/3) mortality in the group administered 10 mg/kg;
ii. 66.67% (2/3) mortality in the group administered 100 mg/kg;
iii. 100% (3/3) mortality in the group administered 1000 mg/kg.

In the second phase, endosulfan was administered orally to the four (4) groups at 20 mg/kg, 40 mg/kg, 90 mg/kg and 160 mg/kg; and % mortalities were recorded as follows (Table 4.2):

i. 0% (0/1) mortality in the groups administered 20 mg/kg, 40 mg/kg and 90 mg/kg;
ii. 100% (1/1) mortality in the group administered 160 mg/kg.

The rats in both phases, were monitored for clinical signs of toxicity (which included tremor, restlessness and excessive grooming), over 24 hours. The LD_{50} was calculated to be 120 mg/kg as shown below:

$$\mathbf{LD_{50}} = \sqrt{\mathbf{D_0 \times D_{100}}}$$

$$= \sqrt{\mathbf{90 \times 160}}$$

$$= \sqrt{\mathbf{14400}}$$

$$= \mathbf{120\ mg/kg}$$

Table 4.1: Percentage mortality of Wistar rats following administration of different doses of endosulfan in first phase of acute toxicity studies.

Groups	Doses (mg/kg)	Number of Recorded Deaths	% Mortality
1	10	0/3	0
2	100	2/3	66.67
3	1000	3/3	100

Table 4.2: Percentage mortality of Wistar rats following administration of different doses of endosulfan in second phase of acute toxicity studies.

Groups	Doses (mg/kg)	Number of Recorded Deaths	% Mortality
1	20	0/1	0
2	40	0/1	0
3	90	0/1	0
4	160	1/1	100

4.2 Clinical Signs

Some clinical signs observed in the endosulfan - treated rats (group 3) include tremor, hyperactivity and excessive grooming. Mild tremor was observed in the vitamin E pre-treated + endosulfan rats (group 4) for just few seconds, while there were no apparent clinical signs observed in groups 1 (control) and 2 (vitamin E).

4.3 Neurobehavioural Assessments

4.3.1 Forepaw grip time

The changes in motor endurance as measured by the forepaw grip time test is shown in table 4.3. The dynamics of the test reveal that there was a decrease in grip time in all the groups from weeks 0 to 2. Rats in the endosulfan group showed a further slight decrease in grip time by week 4, while rats in the soya oil group showed an increase in grip time with only a slight increase in the vitamin E and vitamin E + END groups. The mean grip time was lowest in the endosulfan group (20.167 ± 1.9718 secs) compared to the soya oil group (33.042 ± 7.673 secs); although the difference was not statistically significant ($p > 0.05$).

Table 4.3: Effect of treatment with vitamin E on motor endurance in male Wistar rats exposed to endosulfan (n=8/group).

Groups	Treatment	Week 0 (secs)	Week 2 (secs)	Week 4 (secs)
1	**Control (Soya oil) (2 ml/kg)**	39.75 ± 4.313	22.5 ± 4.468	36.875 ± 14.238
2	**Vitamin E (30 mg/kg)**	47.625 ± 4.330	16.375 ± 2.970	17.50 ± 2.659
3	**Endosulfan (12 mg/kg)**	42.75 ± 3.081	9.25 ± 1.485	8.50 ± 1.349
4	**Vitamin E (30mg/kg) + Endosulfan (12 mg/kg)**	46.875 ± 3.372	24.125 ± 5.383	24.875 ± 12.998

4.3.2 Inclined plane

The effect of treatments with endosulfan and vitamin E on neuromuscular coordination in the inclined plane test, showed no significant difference ($p > 0.05$) for the angles at which rats slipped off the plane, between the groups. However, the END group showed a slight decrease in the angle at which rats slipped off the inclined plane $(50.42 \pm 1.742)^{o}$ compared to the vit.E + END group $(54.79 \pm 1.843)^{o}$ (Table 4.4).

Table 4.4: Effect of treatment with vitamin E on neuromuscular coordination in male Wistar rats exposed to endosulfan (n=8/group).

Groups	Treatment	Week 0 (º)	Week 2 (º)	Week 4 (º)
1	**Control (Soya oil) (2 ml/kg)**	59.38 ± 1.815	44.69 ± 1.526	55.94 ± 1.886
2	**Vitamin E (30 mg/kg)**	59.06 ± 1.699	43.44 ± 1.049	56.56 ± 0.937
3	**Endosulfan (12 mg/kg)**	58.75 ± 2.004	43.75 ± 1.056	48.75 ± 2.165
4	**Vitamin E (30 mg/kg) + Endosulfan (12 mg/kg)**	58.75 ± 2.315	47.19 ± 1.451	58.44 ±1.763

4.3.3 Step-down inhibitory avoidance learning task

The effect of treatment with endosulfan and vitamin E on learning acquisition, as assessed by the number of foot shocks received by the rats in each group is shown in table 4.5. The rats in the END group received a slightly higher number of foot shocks (2.25 ± 0.25) compared to the soya oil group (1.63 ± 0.38), the vit. E and vit. E + END groups (1.50 ± 0.19); although the difference was not statistically significant ($p > 0.05$).

The effect of treatment with endosulfan and vitamin E on memory retention as measured by the duration the rats in each group stayed on the platform is also shown in table 4.5. The duration rats in the vit. E group stayed on the platform (63.75 ± 18.43 secs) was significantly ($p < 0.05$) longer than that of rats in the END group (6.63 ± 2.35 secs).

Table 4.5: Effect of treatment with vitamin E on short-term learning acquisition and memory retention in male Wistar rats exposed to endosulfan (n=8/group).

Groups	Treatment	Number of foot shocks/2mins	Duration on platform (secs)
1	**Control (Soya oil) (2 ml/kg)**	1.625 ± 0.375	45.625 ±17.729
2	**Vitamin E (30 mg/kg)**	1.50 ± 0.189	63.75 ± 18.433[a]
3	**Endosulfan (12 mg/kg)**	2.25 ± 0.250	6.625 ± 2.345[b]
4	**Vitamin E (30 mg/kg) + Endosulfan (12 mg/kg)**	1.50 ± 0.189	18.625 ± 14.495

[a,b]: values with different superscript letters are significantly different ($p < 0.05$).

4.4 Biochemical Assays

4.4.1 Assessment of brain lipoperoxidation

The MDA concentration used as an index of brain lipoperoxidation, was significantly higher ($p < 0.05$) in the END group (2.06 ± 0.15 nmol/mg) compared to the vit. E + END group (1.46 ± 0.16 nmol/mg). The soya oil and vit. E groups showed MDA concentrations of (1.62 ± 0.116 nmol/mg) and (1.74 ± 0.081 nmol/mg) respectively, which were lower than but not significantly different ($p > 0.05$) from the concentration seen in the END group (Table 4.6).

Table 4.6: Effect of treatment with vitamin E on brain malondialdehyde concentrations in male Wistar rats exposed to endosulfan (n=5/group).

Groups	Treatment	Malondialdehyde (nmol/mg protein)
1	**Control (Soya oil) (2 ml/kg)**	1.62 ± 0.116
2	**Vitamin E (30 mg/kg)**	1.74 ± 0.081
3	**Endosulfan (12 mg/kg)**	2.06 ± 0.150[a]
4	**Vitamin E (30 mg/kg) + Endosulfan (12 mg/kg)**	1.46 ± 0.157[b]

[a,b]: values with different superscript letters are significantly different ($p < 0.05$).

4.4.2 Determination of antioxidant enzymes activity

4.4.2.1 Superoxide dismutase activity

The effect of treatment with endosulfan and vitamin E on SOD activity showed that the highest value was obtained in the END group (1.88 ± 0.22 IU/L) while, the lowest was obtained in the soya oil group (1.64 ± 0.10 IU/L); although, the difference was not statistically significant ($p > 0.05$) (Table 4.7).

4.4.2.2 Catalase activity

The effect of treatment with endosulfan and vitamin E on CAT activity showed that the antioxidant enzyme activity in the endosulfan-treated group was significantly higher ($p < 0.05$) when compared to that of the soya oil group, with values of 45.60 ± 1.78 (IU/L) and 36.00 ± 2.00 (IU/L) respectively; (Table 4.7).

Table 4.7: Effect of treatment with vitamin E on antioxidant enzymes activity in male Wistar rats exposed to endosulfan (n=5/group).

Groups	Treatment	Superoxide dismutase (IU/L)	Catalase (IU/L)
1	**Control (Soya oil) (2 ml/kg)**	1.64 ± 0.103	36.00 ± 2.00[a]
2	**Vitamin E (30 mg/kg)**	1.68 ± 0.166	38.80 ± 1.463
3	**Endosulfan (12 mg/kg)**	1.88 ± 0.097	45.60 ±1.778[b]
4	**Vitamin E (30 mg/kg) + Endosulfan (12 mg/kg)**	1.66 ± 0.075	40.00 ± 2.345

[a,b]: values with different superscript letters are significantly different ($p < 0.05$).

CHAPTER FIVE

5.0 DISCUSSION

The clinical signs observed in the endosulfan group are consistent with continuous CNS stimulation due to antagonism of GABAergic neuroceptors (Brickely and Mody, 2012). The mild toxic signs observed in the group pretreated with vitamin E showed its ability to attenuate the endosulfan-induced toxic signs which further demonstrated the possible involvement of oxidative stress in the toxic manifestations observed in endosulfan poisoning. This observation is in agreement with earlier findings by Kalender *et al.* (2004) and Saxena *et al.* (2011). The ability of vitamin E to reduce these toxic signs may be due to its capacity to scavenge lipid peroxyl radicals (Saxena *et al.,* 2011; Takhshid *et al.,* 2012).

Neurobehavioural effects of pesticide exposure in humans include psychomotor and cognitive deficits; it also affects vibration sensitivity and balance (Gupta, 2006). Neurobehavioural effects of endosulfan exposure have been documented (Rehman, 2005; Pathak *et al.,* 2010; Lee *et al.,* 2015). Neurotoxicity is the primary effect observed both acutely and chronically in both humans and animals (Amizadeh and Saryazdi, 2011; Raj *et al.,* 2013). Documented human data have shown that CNS hyper-stimulation (with little or no peripheral component) is the predominant toxicological effect of endosulfan mediated by inhibition of calmodulin-dependent Ca^{2+}, Mg-ATPase and antagonism of chloride ion transport in $GABA_A$ receptor/ionophore complex, which releases the synaptic inhibition on neurons (Satar *et al.,* 2009; Gude and Bansal, 2012; Raj *et al.,* 2013). Continued CNS toxicity can be explained by its property of being both a substrate and inhibitor to the cellular efflux transporter p-glycoprotein (p-gp), which allows endosulfan to enter and remain in the CNS (Gude and Bansal, 2012). In the present study, grip time and inclined

plane models were used to assess motor strength and neuromuscular co-ordination respectively. While, the step-down inhibitory avoidance learning task (foot shocks) was used to assess short-term learning and memory.

The ability of pesticides to adversely affect motor strength has been well documented (Terry *et al.,* 2003; Ambali, 2009; Ambali *et al.,* 2010). Exposure to endosulfan has also been associated with reduced motor functions (Castillo *et al.,* 2002; Rehman, 2005). Results of receptor binding and biochemical studies have shown that endosulfan has a potential to alter the activities of central cholinergic, dopaminergic and serotonergic mechanisms (Amizadeh and Saryazdi, 2011). In their studies with chlorpyrifos, Prendergast *et al*. (2007) attributed reduced motor strength to reduction in neuronal viability, probably resulting from impaired microtubule synthesis and/or functions. Their findings were consistent with the results of this study where we recorded decreased forepaw grip strength in the rats administered endosulfan. The slightly improved motor strength in the vitamin E + endosulfan group demonstrated the ameliorative effect of this vitamin partly due to its antioxidant properties. The antioxidant vitamin may have protected the neuron from Endosulfan-induced oxidative damage to the brain and the peripheral nervous tissue.

The angles at which rats in the endosulfan group slipped off the inclined plane were lower than those of the other groups, indicating a deficit in incline plane performance and hence, in neuromuscular coordination. This result agrees with those obtained by Ambali *et al.* (2009, 2010) in rats following CPF exposure but contrary to results obtained by Paul *et al*. (1992), where they observed no effect on coordination in rats exposed to endosulfan. Rats pre-treated with vitamin E showed an improved performance in the inclined plane

compared to the control group, signifying amelioration of the neuromuscular coordination deficits induced by endosulfan exposure. The deficit in neuromuscular coordination observed in endosulfan group may be partly due to induction of oxidative stress, which was ameliorated by vitamin E.

The rats exposed to endosulfan only, received a higher number of foot shocks compared to other groups, though the results obtained showed no significant difference within the groups. This implies a deficit in learning acquisition in this group. The present study also demonstrated that exposure to endosulfan causes short-term impairment in memory retention as indicated by the duration the rats in the endosulfan group remained on the platform, which was significantly lower than for those in the vitamin E group. This is in agreement with findings obtained by Paul *et al*. (1992; 1994) and; Lakshmana and Raju (1994), in their studies with endosulfan; and Ambali *et al*. (2010), in their study with chlorpyrifos, who observed deficits in acquisition and retention of memory in animals after pesticide treatment. The results obtained in the present study may be partly due to induction of oxidative stress in the relevant portions of the brain responsible for learning acquisition and memory retention (such as the cerebral cortex and the hippocampus), as the deficits could possibly be associated with the increased brain lipid peroxidative changes. This was further supported by the slight improvements in both learning acquisition and memory retention in rats pre-treated with vitamin E. Paul *et al*. (1994) in their study suggested that the impairment in memory and learning was due to a motivation deficit rather than to motor impairment; also that endosulfan produced a learning and memory deficit by affecting serotonergic activity.

Several studies have linked cognitive deficits to alteration in ACh metabolism. The central cholinergic system has been linked with synaptic plasticity, learning and memory processes (Hasselmo, 2006; Pereira *et al.,* 2012). Since it has been shown that endosulfan has a potential to alter the activities of the central cholinergic system (Amizadeh and Saryazdi, 2011; Pereira *et al.,* 2012), this may also be a possible mechanism involved in the impairment of cognition seen in this study.

The results obtained for the MDA showed a significant increase in MDA levels in the endosulfan treated rats compared to those pre-treated with vitamin E. The high MDA concentration in the brain samples of rats in the endosulfan group demonstrated the role of lipid peroxidation in the endosulfan-induced neurotoxicity obtained in the present study. The increase in MDA levels can be as a result of impairment in antioxidant enzymes due to ROS formation that attacks the cell membrane, with direct consequences on cell integrity and cell function (Sandrini *et al.,* 2006; Sabzar *et al.,* 2015). The MDA has been recognized as a reactive product of membrane lipid peroxidation that plays a vital role in the pathogenesis of several diseases and inflammatory processes (Zhang *et al.,* 2002, Ambali *et al.,* 2011). While, the main function of vitamin E is as a chain – breaking free radical trapping antioxidant in cell membranes and plasma lipoproteins, which reacts with lipid peroxide radicals formed by peroxidation of polyunsaturated fatty acids before they can establish chain reactions (Murray *et al.,* 2007).

The decreased MDA level in vitamin E pre-treated rats reinforced the fact that the tissue protective effect of this antioxidant vitamin is due to suppression of lipoperoxidative damage. In agreement with these results, a study by Zervos *et al*. (2011) and Dale *et al.*

(2017) showed that vitamin E protected the brain from oxidative stress induced by endosulfan. This is also supported by other studies which showed the protective role of vitamin E (Kalender *et al.,* 2004; Sohn, *et al.,* 2004; Pal *et al.,* 2009; Takhshid *et al.,* 2012) and other antioxidant compounds such as melatonin (Omurtag *et al.,* 2008) and 5-aminosalicic acid (Jaiswal *et al.,* 2005) in endosulfan–induced oxidative stress in other experimental systems. However, a non-antioxidant activity of this vitamin, like its ability to enhance cholinesterase reactivation (Yavuz *et al.,* 2004; Ambali *et al.,* 2010; 2011) may have contributed to the mitigation of endosulfan-induced neurotoxicity observed in the present study.

Antioxidant status results showed an increase in SOD activity in the endosulfan-exposed rats compared to rats in other groups, although there was no significant difference. This is contrary to results obtained by Alva *et al*. (2012) in rats and Mansour *et al.* (2014) in mice, where there was reduction of SOD activity. While, there was a significant increase in CAT activity in the same endosulfan-exposed rats compared to rats in the control group. This is contrary to results obtained by Alva *et al*. (2012) in rats where there was a decrease in CAT activity and in agreement with those obtained by Dale *et al.* (2017) in mice. The increase in antioxidant enzymes could be as a result of compensatory changes in response to an increase in the production of ROS. The antioxidant enzymes play a crucial role in the cellular defence against ROS. SOD is a group of metallo-enzymes that constitute a first line of defense against the natural or chemically induced production of ROS (Bernabucci *et al*., 2002; Deviller *et al.,* 2005; Sabzar *et al.,* 2015). The function of intracellular SOD is to scavenge superoxide (O_2^-) produced by cellular metabolism, and catalyze dismutation of superoxide to oxygen (O_2) and hydrogen peroxide (H_2O_2) (Bernabucci *et al*., 2002; Das,

2011). An increased dismutation of O_2^- by SOD leads to an increase in production of H_2O_2, which is further detoxified by CAT to H_2O and O_2 (Das, 2011; Sabzar *et al.*, 2015). The significant increase in CAT activity, as depicted by the present study, may also be in response to increase in H_2O_2 produced by SOD activity.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, the results obtained from the present study demonstrate that endosulfan exposure induces oxidative stress in male Wistar rat brain. The high level of oxidative stress could have contributed significantly to the endosulfan-induced neurobehavioural deficits, which were attenuated by vitamin E administration. Enzymatic antioxidants also play an important defence mechanism against increased oxidative stress induced by endosulfan exposure.

6.2 Recommendations

Based on the findings of this study, the following recommendations were made:

i. Daily supplementation with vitamin E (or other lipid–soluble antioxidants) by farmers, their families and people living in farm areas should be encouraged.
ii. Further studies should be carried out on the long term effects of endosulfan exposure, long after its use has stopped.
iii. Government should put in place an aggressive awareness campaign on the risks of exposure to pesticides at the grass root levels.

6.3 Contributions to Knowledge

1. Endosulfan impaired short-term memory retention (sec); (6.63 ± 2.35) in the END group compared to (63.75 ± 18.43) in the control ($p < 0.05$).
2. Endosulfan increased brain malondialdehyde concentration (nmol/mg protein); (2.06 ± 0.15) compared to (1.62 ± 0.12) in the control group ($p > 0.05$).
3. Vitamin E protected against endosulfan-induced brain lipid peroxidation (nmol/mg protein); (1.46 ± 0.16) in the vitamin pre-treated group compared to (2.06 ± 0.15) in the endosulfan-treated group ($p < 0.05$).

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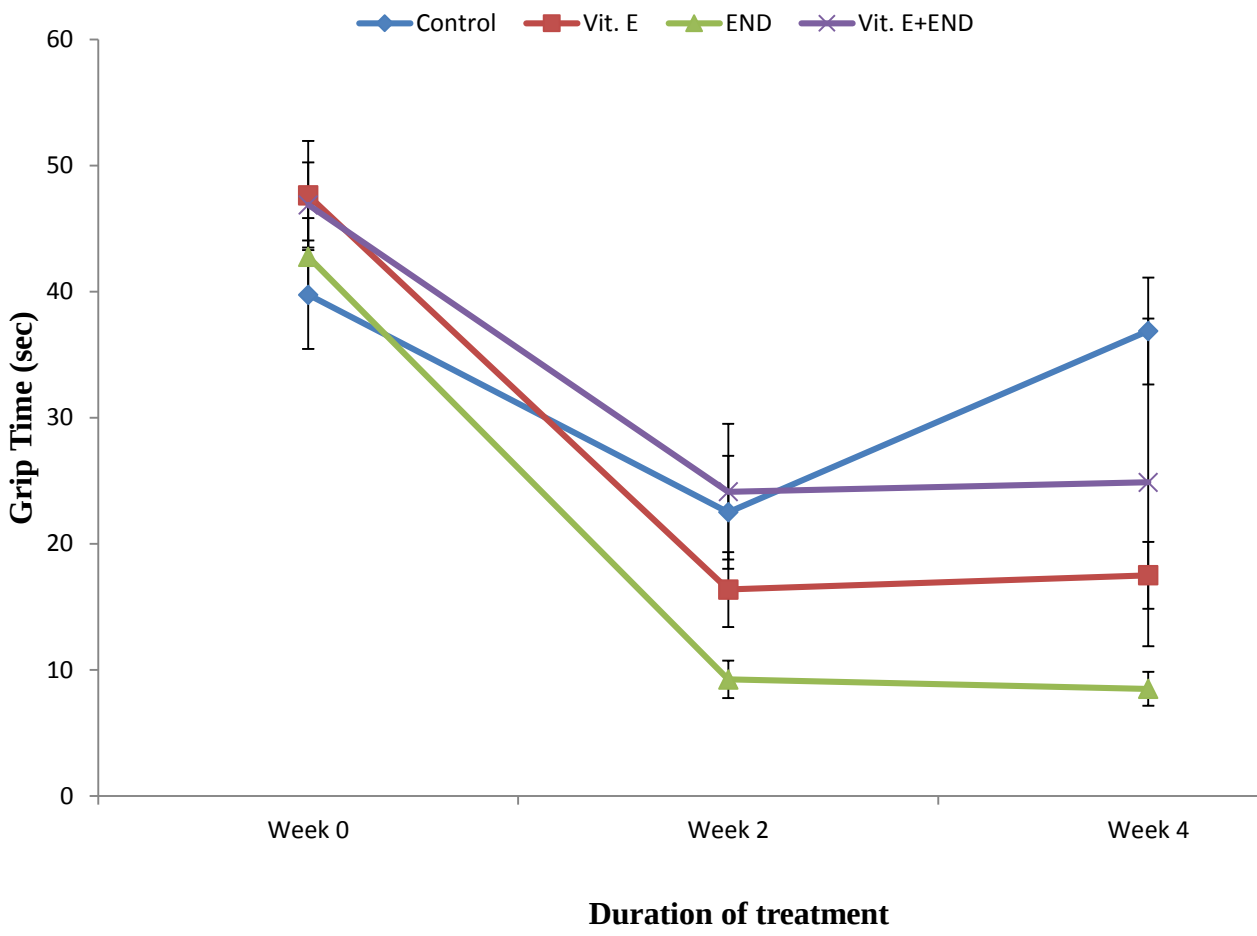
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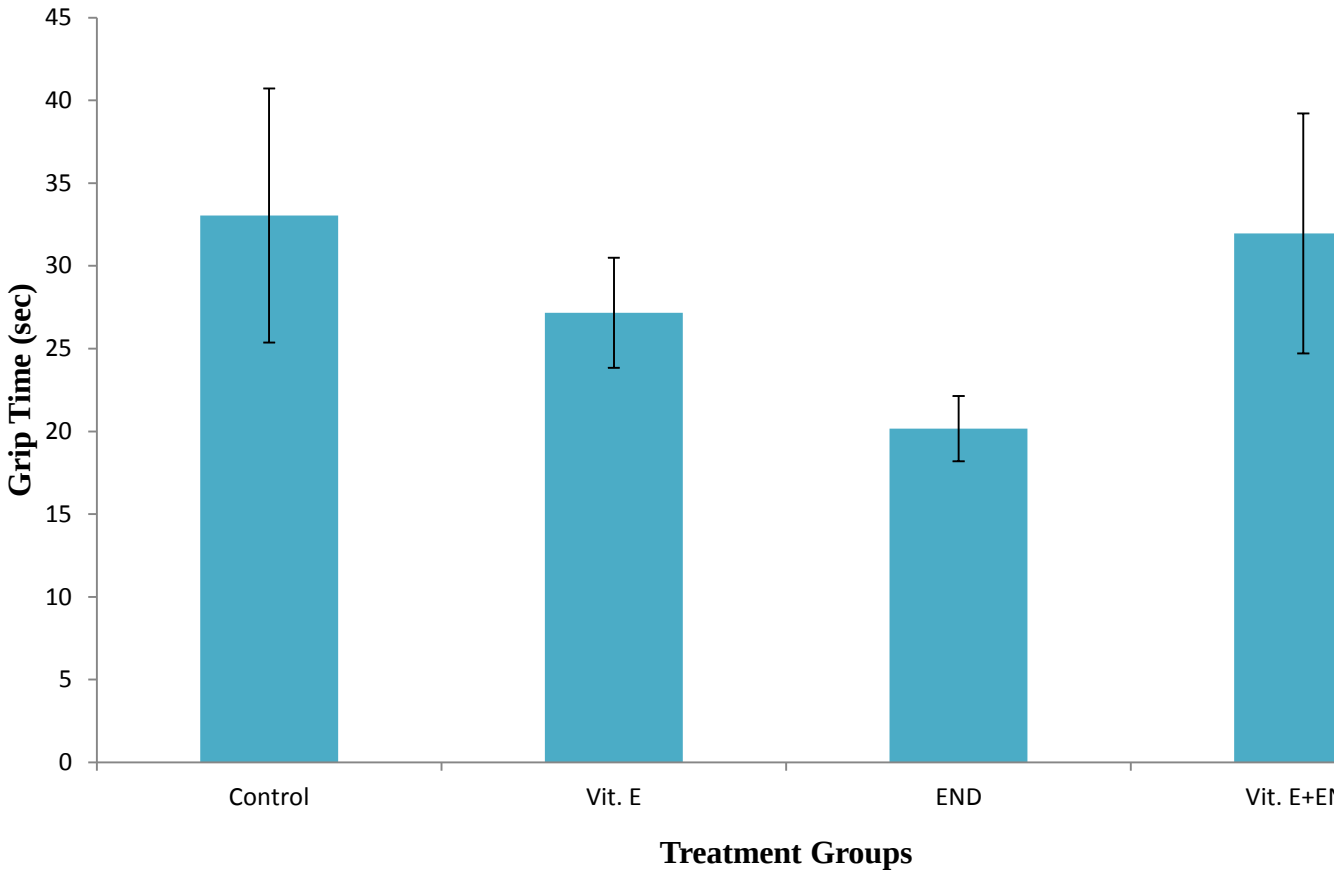
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Appendix 1



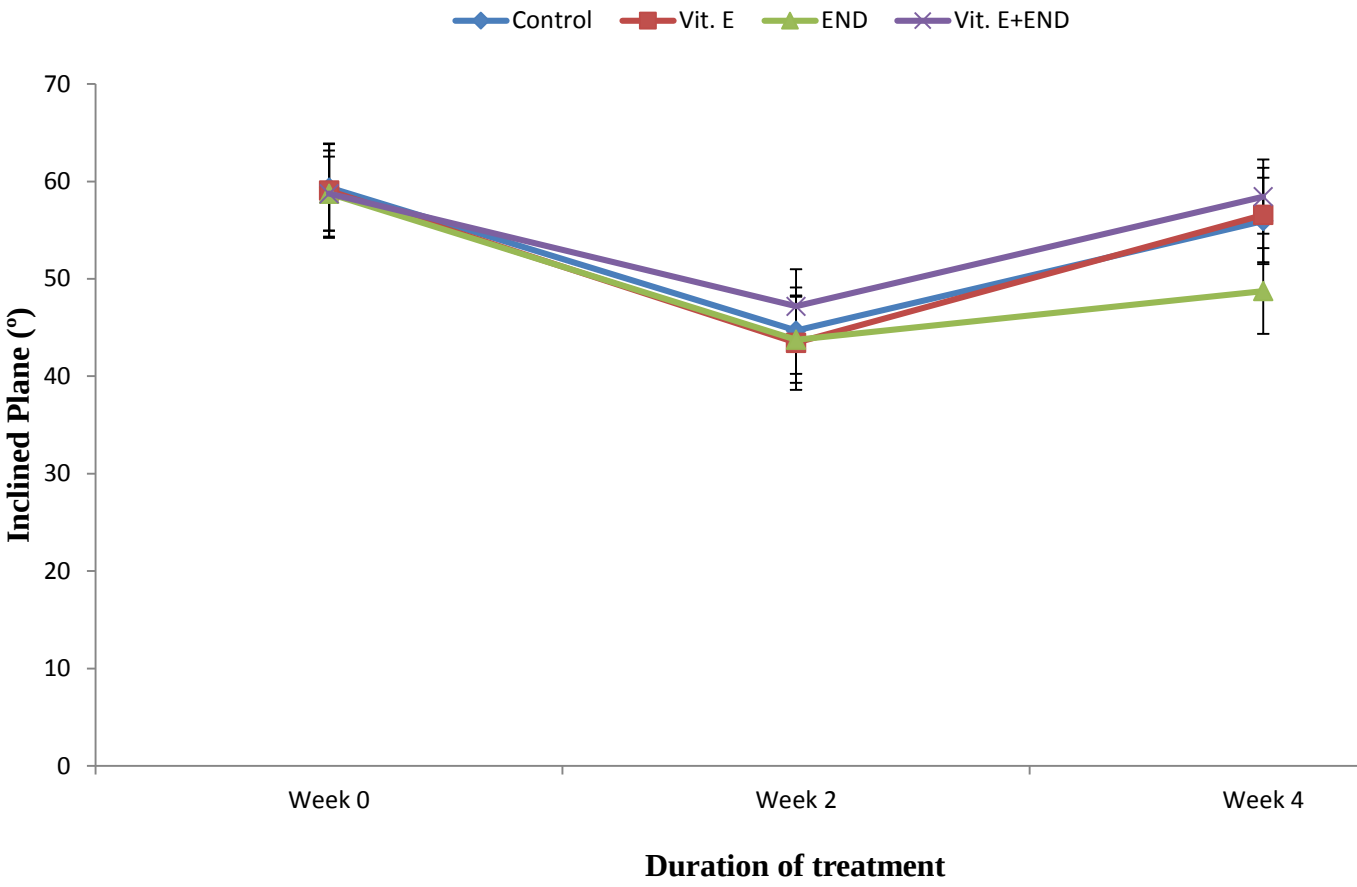
Effect of treatment with vitamin E on the dynamics of motor endurance in male Wistar rats exposed to endosulfan (n=8/group).

Appendix 2



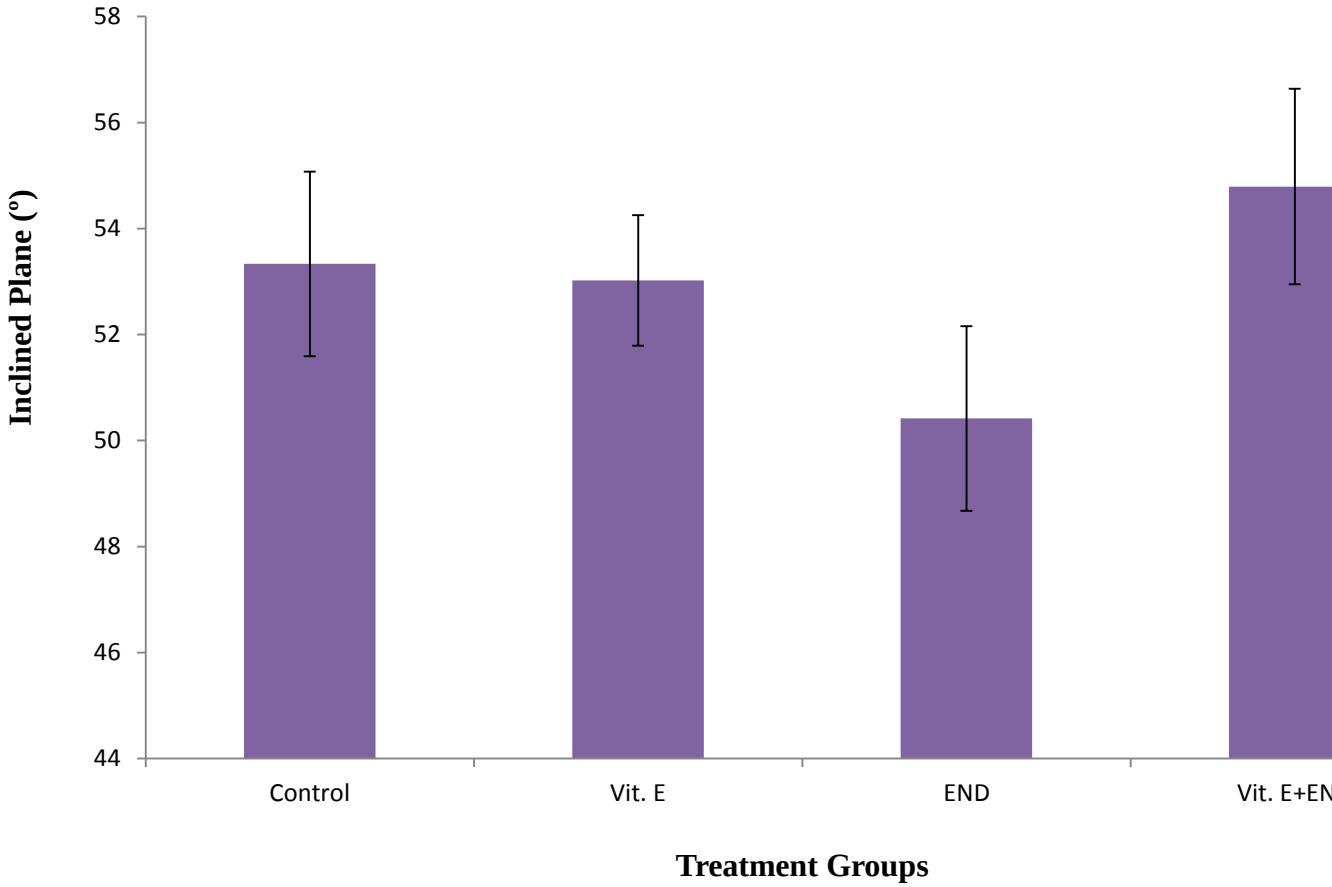
Effect of treatment with vitamin E on motor endurance in male Wistar rats exposed to endosulfan (n=8/group).

Appendix 3



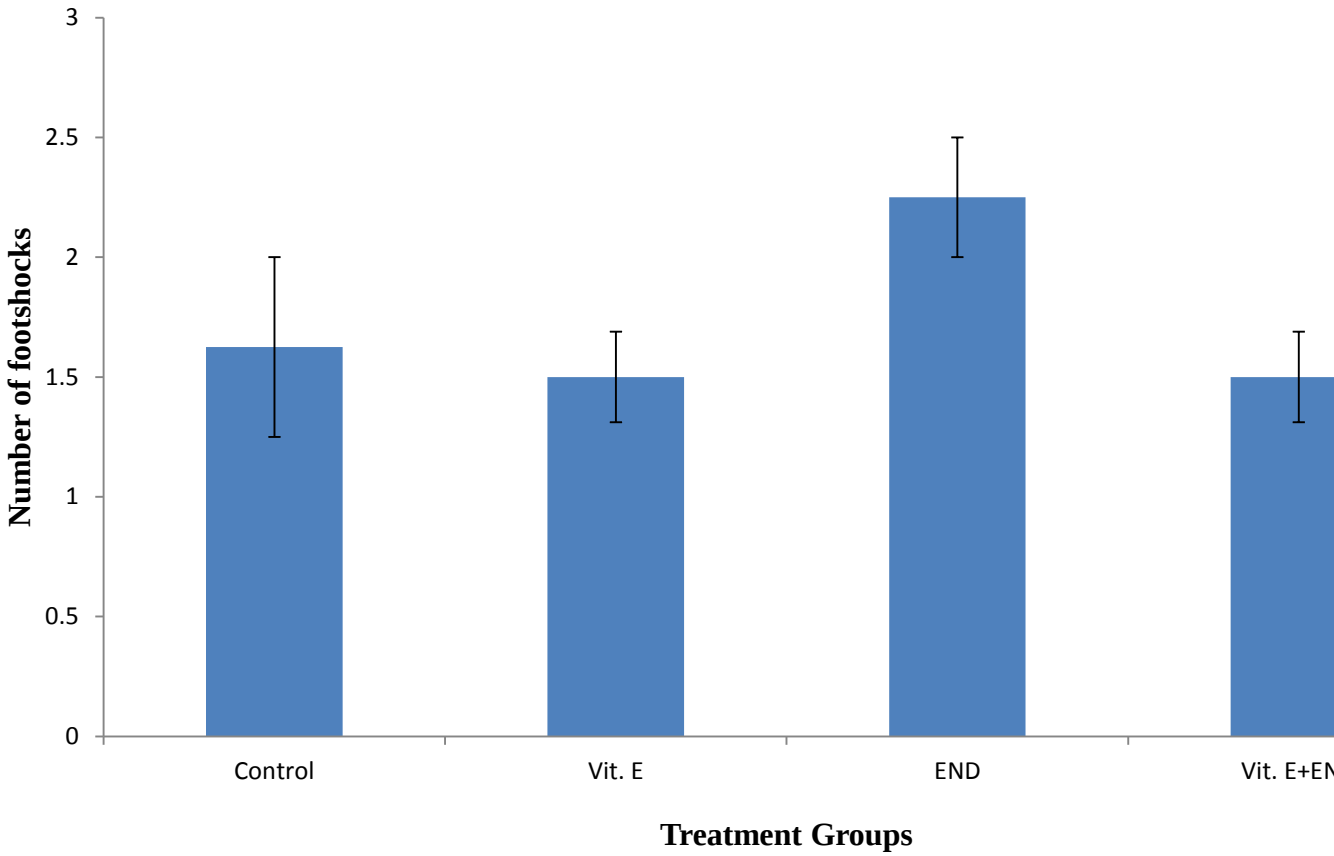
Effect of treatment with vitamin E on the dynamics of neuromuscular coordination in male Wistar rats exposed to endosulfan (n=8/group).

Appendix 4



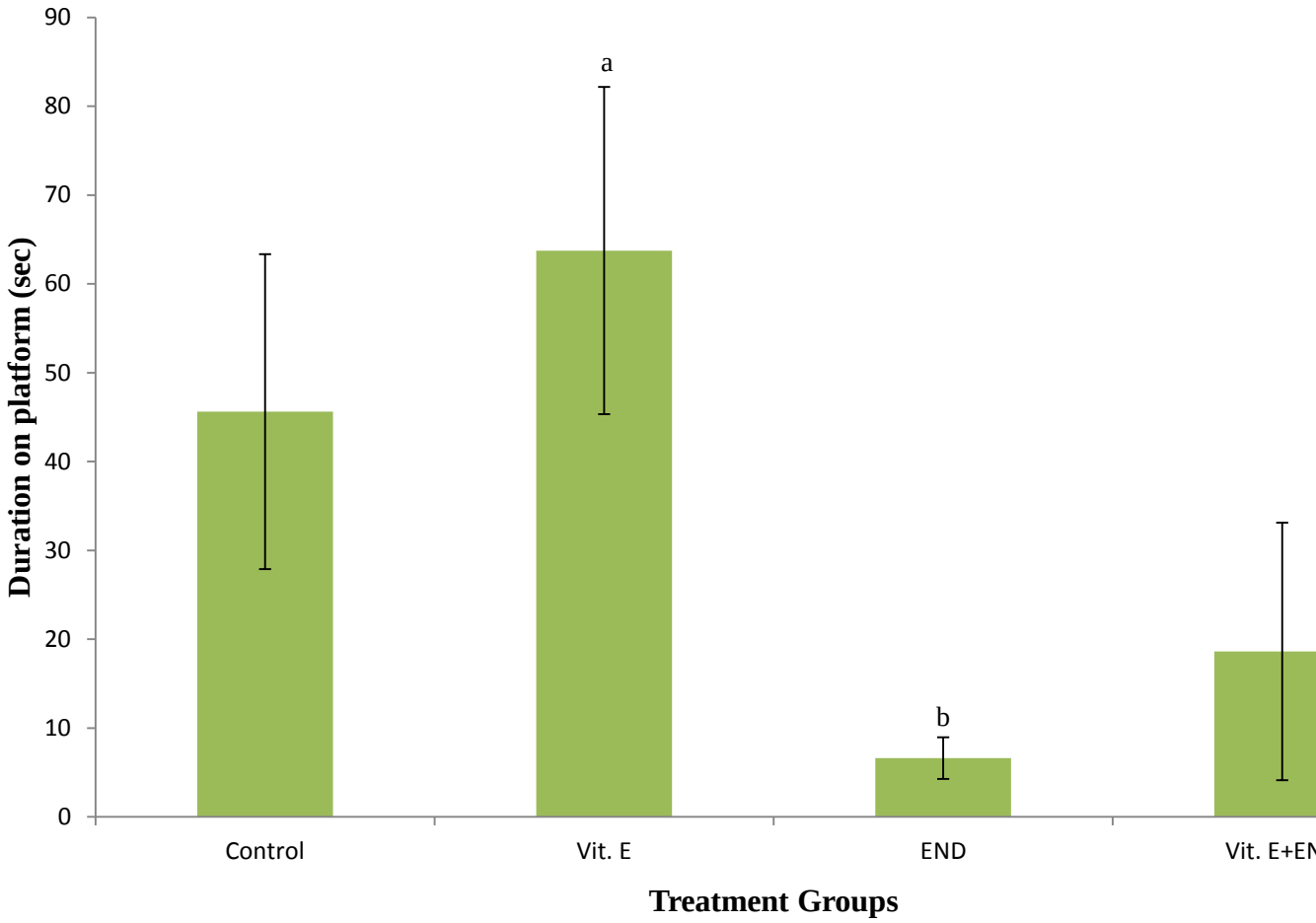
Effect of treatment with vitamin E on neuromuscular coordination in male Wistar rats exposed to endosulfan (n=8/group).

Appendix 5



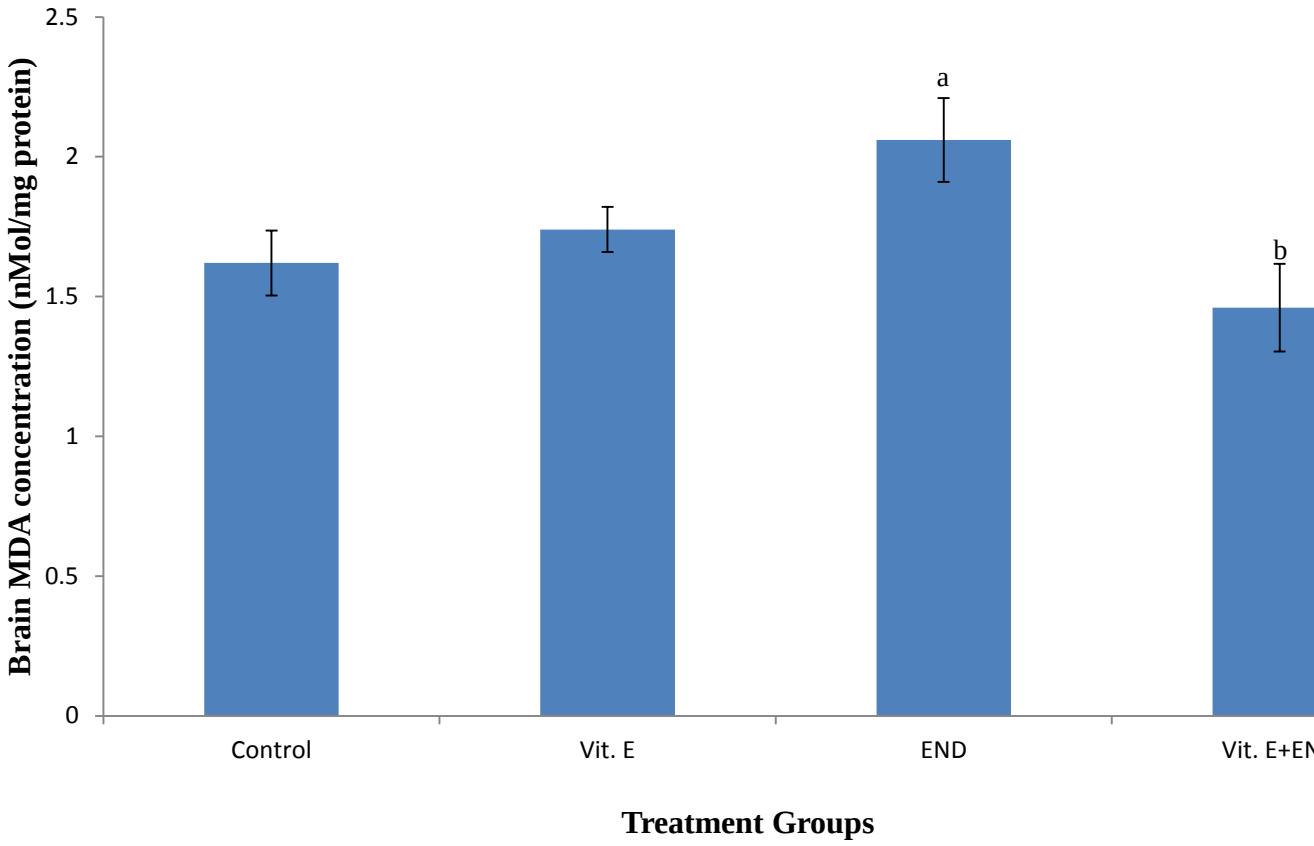
Effect of treatment with vitamin E on short-term learning acquisition in male Wistar rats exposed to endosulfan (n=8/group).

Appendix 6



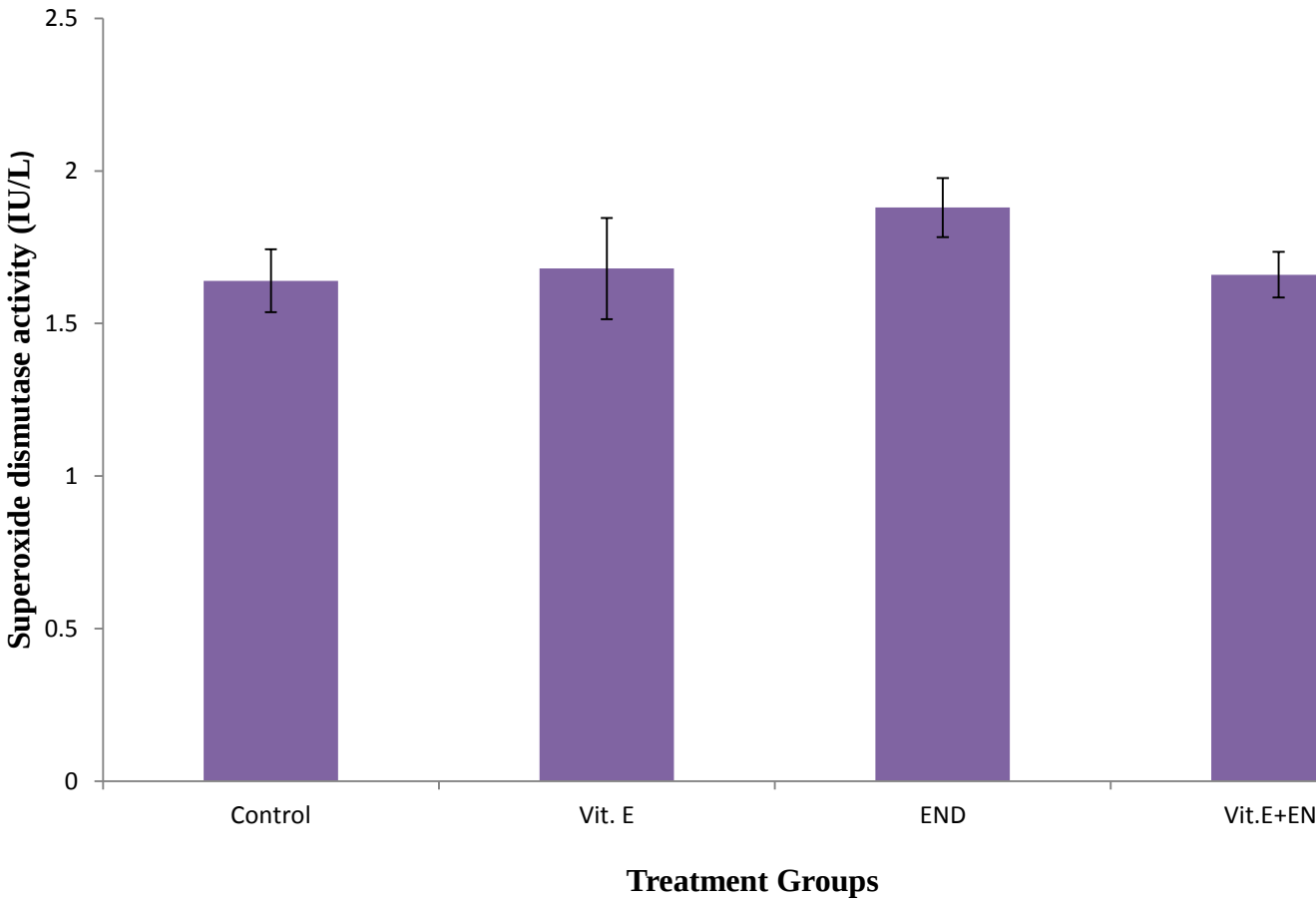
Effect of treatment with vitamin E on memory retention in male Wistar rats exposed to endosulfan (n=8/group).[a,b] : values with different superscript letters are significantly different ($p < 0.05$).

Appendix 7



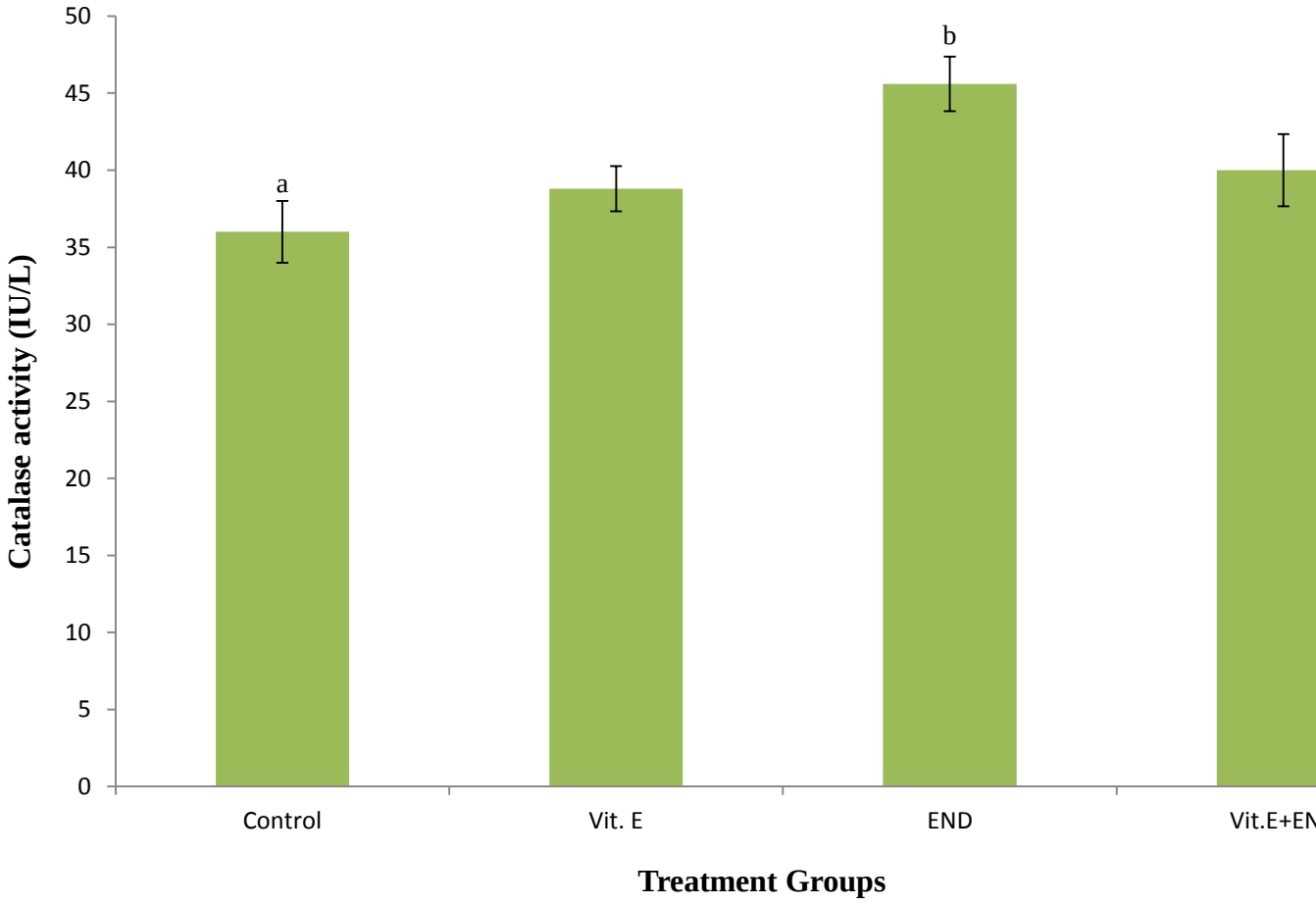
Effect of treatment with vitamin E on brain malondialdehyde concentrations in male Wistar rats exposed to endosulfan (n=5/group). [a,b] : values with different superscript letters are significantly different ($p < 0.05$).

Appendix 8



Effect of treatment with vitamin E on brain superoxide dismutase activity in male Wistar rats exposed to endosulfan (n=5/group).

Appendix 9



Effect of treatment with vitamin E on brain catalase activity in male Wistar rats exposed to endosulfan (n=5/group). [a,b] : values with different superscript letters are significantly different ($p < 0.05$).