EFFECT OF RESVERATROL ON CHLORPYRIFOS-INDUCED COGNITIVE IMPAIRMENT IN SWISS ALBINO MICE

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DECLARATION

I, Bello Yakubu ADAMU declare that the work in this dissertation entitled **"Effect Of Resveratrol On Chlorpyrifos-induced Cognitive Impairment in Swiss Albino Mice"** has been carried out by me in the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University, Zaria, under the supervision of Dr. Saleh M. I. A. and Dr. Alhassan A. W. 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 any university.

Bello Yakubu ADAMU	Signature	Date

CERTIFICATION

This dissertation entitled **"EFFECT OF RESVERATROL ON CHLORPYRIFOS-INDUCED COGNITIVE IMPAIRMENT IN SWISS ALBINO MICE"** by Bello Yakubu ADAMU meets the regulations governing the award of the degree of Master of Science in Human Physiology at Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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This work is dedicated to My Parents.

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I thank God for giving me the privilege to attain this level of my academic pursuit, I return all the glory to Him.

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ABSTRACT

Chlorpyrifos (CPF) is thought to cause oxidative injury in both humans and animals via the formation of free radicals. Exposure to CPF at doses that did not result in overt clinical symptoms has been reported among pesticide applicators and other farm workers thereby constituting an important source of occupational hazards to these groups of individuals. Chlorpyrifos (CPF) has been associated with cognitive and psychomotor impairments in both humans and animal. The aim of this work was to evaluate the beneficial role of resveratrol on chlorpyrifos-induced cognitive impairment in Swiss albino mice. Swiss albino mice were divided into (6) six groups of five mice each (n=5). Group I served as the control and were administered olive oil (2 ml/kg), group II received carboxymethylcellulose (CMC) 10g/L, group III received resveratrol 30 mg/kg, group IV received chlorpyrifos (CPF) 3 mg/kg, group V received CPF (3 mg/kg) after the oral administration of resveratrol (30 mg/kg) and group VI received Vitamin E (Vit E) 100 mg/kg. All administrations were done by oral gavage for 21 days. Cognitive function was assessed using Y-maze recognition memory test. And oxidative stress was evaluated using oxidative biomarkers techniques. The results obtained showed that resveratrol at dose 30 mg/kg significantly ($p<0.05$) improved cognitive impairment. The results showed that resveratrol at dose 30 mg/kg also significantly ($p<0.05$) improved the activities of superoxide dismutase (SOD) as well as catalase (CAT) when compared with the control. Glutathione (GSH) significantly ($p<0.05$) increased in the treated group when compared with the control and malondialdehyde (MDA) concentration significantly decreased ($p<0.05$) when compared with the control. In conclusion, 30mg/kg resveratrol suppressed memory impairment, decreased malondialdehyde levels, increased catalase activity, superoxide dismutase activity and glutathione levels in our chlorpyrifos-induced cognitive impairment mice model.

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LIST OF ABBREVIATIONS

$\cdot O_2$	=	superoxide radical
·OH	=	hydroxyl radical
ATP	=	adenosine triphosphate
CA1	=	conus aminonus area 1
cAMP	=	cyclic adenosine monophosphate
CAT	=	Catalase
CMC	=	carboxymethylcellulose
CoQ	=	coenzyme Q
CPF	=	Chlorpyrifos
DNA	=	deoxyribonucleic acid
Duox	=	dual oxidases
ER	=	endoplasmic reticulum
ERO-1	=	endoplasmic reticulum oxidoreduction-1
ETC	=	electron transport chain
Fe_{2+}	=	iron (ferrous)
GPx	=	glutathione peroxidase
GSH	=	glutathione
H_2O	=	water

H_2O_2	=	hydrogen peroxide
HRP	=	Horseradish Peroxidase
Keap1	=	Kelch-like ECH-associated protein 1
MAPK	=	mitogen-activated protein kinase
MDA	=	Malondialdehyde
NADPH	=	nicotinamide adenine dinucleotide phosphate
NF-κB	=	Nuclear factor-kappa B
Nox	=	nicotinamide adenine dinucleotide phosphate oxidase
Nrf2	=	NF-E2-related factor 2
O_2	=	oxygen molecule
OH-	=	hydroxyl ion
OP	=	organophosphate
PDI	=	protein disulfide-isomerase
Phox	=	phagocytes oxidase
PRX	=	peroxiredoxin
PTZ	=	pentylenetetrazole
ROS	=	reactive oxygen species
RSV	=	resveratrol
SEM	=	standard error of mean

SOD	=	superoxide dismutase
TBARS	=	thiobarbituric acid reactive substances
UV	=	ultra violet
Vit E	=	vitamin E
XDH	=	xanthine dehydrogenase
XO	=	xanthine oxidase

CHAPTER ONE

1.0 INTRODUCTION

There has been a global increase in pesticides usage due to the compelling need to feed the ever-increasing human and animal populations, and to reduce the incidence of food and vector-borne diseases (Ambali, *et al.,* 2012). These health and economic benefits of pesticide usage are achieved not without simultaneous potential health risks and adverse health outcomes in non-target species, including man (Abdollahi *et al.,* 2004).

The adverse effect of some pesticides has necessitated legislation in most developed countries against potentially damaging ones (Czarniewska *et al.,* 2003**)**. However, in most developing nations, regulatory laws governing pesticide production and utilization are almost completely lacking or where available these laws are poorly implemented thereby having a far-reaching effect on the ecosystem (Otitoju and Onwurah, 2005).

Organophosphate (OP) compounds are one of the most widely used pesticides accounting for about 50% of the global pesticide use (Ambali, *et al.,* 2012). This may be of particular concern given the widespread use of OP pesticide in household, agricultural, and commercial environments worldwide (Terry *et al.,* 2007). Human studies in agricultural communities in developing countries have shown that cumulative exposure to OP is associated with cognitive and psychomotor impairments (Kamel and Hoppin, 2004; Kamel *et al*., 2007).

Chlorpyrifos (CPF) is a chlorinated OP insecticide that has enjoyed widespread use in agricultural and domestic pest control (Steenland *et al*., 2000; Ambali *et al*., 2009). It is of public health importance as CPF residues have been detected in poultry egg, meat, cow milk and milk products (Rawat *et al.*, 2003). CPF is thought to cause oxidative stress in both

humans and animals via the formation of free radicals (Terry *et al.*, 2007). Exposure to CPF at doses that did not result in overt clinical symptoms has been reported among pesticide applicators and other farm workers (Farahat *et al.*, 2010) thereby constituting an important source of occupational hazards to these groups of individuals (Ray and Richards, 2001).

However, the body is endowed with enzymatic and non-enzymatic antioxidant systems to counter the lipid peroxidative changes induced by reactive oxygen and nitrogen species. In condition of enhanced free radicals formation (Ambali *et al*., 2010c, 2012), which can also be referred to oxidative insult or stress by CPF these antioxidant systems could be overwhelmed resulting in tissue damage. The brain, due to its biochemical and physiological properties is especially sensitive to reactive oxygen species (ROS), which disrupt its functions and structure (Drewa *et al*., 1998). This is witness in neurological disorders such as Alzheimer's dementia, affecting learning and memory aspect of brain functions.

Learning is the acquisition of new information by the nervous system, resulting in changes in behavior. While memory is the ability to store, process and recall learnt information (Mangina and Sokolov, 2006).

Cognition is the ability to consciously carry out functions utilizing the human brain and includes: visual perception, selective attention, logical reasoning, construction (sentences for example) calculation, attention information processing), planning, problem-solving, comprehension and memory (Warburton, 1995; Claxton, 2002).

For nearly a century, studies of cognition have shown that certain exogenous compounds can improve different types of memory and cognitive functioning in both normal and abnormal animals. However, the cognition-enhancing effects of other agents are only observed in subjects exhibiting poor baseline performance or experimentally induced deficits, whereas providing no measurable effects in normal animals.

Resveratrol (3, 5, 4'-trihydroxystilbene) is a polyphenol that occurs naturally in foods and drinks made from grapes and peanuts, and also in a number of herbal remedies, both alone and as part of plant extracts. Resveratrol was first isolated from the roots of white hellebore (*Veratrumgrandiflorum O. Loes*) in 1940 (Takaoka, 1940) and later, in 1963, from the roots of *Polygonum cuspidatum*, a plant used in traditional Chinese and Japanese medicine. Initially characterized as a phytoalexin (Nonomura *et al.*, 1963), resveratrol is considered to be one of a group of compounds that are produced in plants during times of environmental stress of pathogenic attack (Dercks, and. Creasy, 1989a) or exposure to ultraviolent light (Fremont, 2000).

In the study of Juan *et al*. (2002) 20 mg/kg resveratrol was administered orally to rats for 28 days and reported no treatment-related effects except mild changes in serum liver enzymes. A single dose of 2000 mg/kg resveratrol did not cause any detectable, toxicologically significant changes in the rats. Other published experiments in rats tend to use dose levels less than 20 mg/kg resveratrol and for durations less than four weeks (Turner *et al.*, 1999).

1.1 STATEMENT OF THE PROBLEM

Enhanced formation of reactive oxygen species (ROS) has been associated with the toxicity of various pesticides including organophosphate pesticides (OP). Organophosphate pesticides are known to cause adverse health effects by overwhelming the inherent body antioxidant system thereby causing a further increase in ROS in the body (Farahat *et al.*, 2010).

Nevertheless, humans have evolved with antioxidant systems to protect against deleterious productions of free radicals. These systems include some antioxidants produced in the body (endogenous) and others obtained from diet (exogenous) (Kangralkar *et al.*, 2010). Endogenous antioxidants systems are of particular importance to the brain (Vijayakumar *et al.*, 2012), it comprises of mainly two categories of systems including; enzymatic e.g.

superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase) and non-enzymatic systems e.g. glutathione (GSH), vitamins A, C and E.

However, our endogenous antioxidant defense systems are incomplete without exogenous originating reducing compounds such as vitamin C, vitamin E, carotenoids and polyphenols, playing an essential role in many antioxidant mechanisms in living organisms. Therefore, there is continuous demand for exogenous antioxidants in order to prevent oxidative stress, a disequilibrium of redox state in favor of oxidation (Jaouad and Torsten, 2010).

Resveratrol has been shown to possess potent free radicals scavenging property in several studies (Takaoka, 1939). It has been shown to have protective effect against oxidative stress induced by potassium bromate in rat kidneys, and lipid peroxidation induced by iron and ethanol. Moreover, Resveratrol has also been shown to possess cardio protective effect, which was attributed to its antioxidant property (Floreani *et al.*, 2003; Ellmark, *et al.*, 2005). Resveratrol is now available commercially in pills used as supplements, especially for its ability to increase life expectancy and mostly used to delay undesirable effects of ageing and reversing the complication of obesity.

1.2 JUSTIFICATION OF STUDY

The use of pesticides in developing nations like Africa has increased dramatically in recent times. This has posed negative health impact in man and other non-target organisms. These adverse effects have as well increased. Their toxic effects can manifest in different ways such as bio magnification, chronic toxicity, acute immune response, allergic reaction and, mutagenic, teratogenic and carcinogenic effects (Czarniewska, *et al*, 2003).

The brain is particularly vulnerable to enhanced ROS generation Cadenas and Davies, 2000). This is because the brain mobilizes 20% of the total body oxygen and limited amount of antioxidants capacity. Thus antioxidants may have putative positive benefit in altering, reversing or forestalling the neuronal/behavioral deficits (Olawale *et al*., 2008).

1.3 GENERAL AIM OF THE STUDY

The aim of this study was to evaluate the effect of resveratrol on chlorpyrifos-induced cognitive deficit in Swiss Albino mice.

1.4 SPECIFIC OBJECTIVES OF THE STUDY

I. To determine the effect of CPF on cognitive function in mice.

II. To determine the effect of resveratrol on CPF-induced cognitive dysfunction in mice.

III. To determine the efeect of resveratrol on CPF-induced elevation of oxidative stress biomarkers: Malondialdehyde (MDA), Catalase (CAT), Superoxide dismutase (SOD) and Glutathione peroxidase (GPx)

1.5 RESEARCH HYPOTHESIS

Resveratrol has no effect on chlorpyrifos-induced cognitive impairment in Swiss Albino mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

Cognition is a comprehensive term used to describe the mental processes associated with functions such as attention, perception, working memory, executive function, spatial ability, language, learning and memory (visual and verbal i.e. pattern recognition and learning), abstract reasoning, problem solving, and psychomotor skills (Benefati, 2007).

2.1 LEARNING

Learning is defined as the process involved in acquiring new information (Okano *et al.*, 2000). The ability to learn is a fundamental characteristic of living things (Baddeley and Warrington, 1970).

There are two principal types of learning. They include;

1. Classic Conditioning (passive) learning and
2. Operant Conditioning (active) learning

2.1.1 Classic conditioning (passive) Learning

During a passive learning the subject usually plays a relatively involuntary or passive role, hence this type of learning is considered to represent a relatively primitive, simple or generalized learning.

2.1.2 Operant Conditioning (active) Learning

This is also termed, “trial and error” learning. During Operant conditioning learning the subject exerts a considerable level of voluntary control so that this type of learning is considered to represent a much “higher” level of learning (Baddeley and Warrington, 1970).

2.2 MEMORY

Memory is a critical aspect of cognition. It is composed of numerous component processes that localize to different anatomical sites (Squire, 1992). It is defined as a behavioral change caused by an experience (Okano *et al.*, 2000). Human memory may be regarded as a system that stores and retrieves information acquired through the senses (visual and auditory). Short-term memory implies a memory that lasts a fraction of a second to several seconds (Baddeley and Warrington, 1970), and long term memory describes the ability to identify a stimulus for days, months, or years after exposure. Functionally, memories are stored in the brain by changing the basic sensitivity of synaptic transmission between neurons due to previous neural activity (Baddeley and Warrington, 1970).

2.2.1 CLASSIFICATION OF MEMORY

Memory is classified into three major types based on durations:

I. Long-term Memory

II. Short-term Memory and

III. Sensory Memory

2.2.2 LONG-TERM MEMORY

Long-term memory is further sub-divided into:

1. Implicit/ Non-declarative memory and
2. Explicit/ Declarative memory

2.2.2.1 IMPLICIT (NON-DECLARATIVE) MEMORY

This refers to information stored mainly to perform various reflexive or perceptual tasks. It is referred to as non-declarative or implicit because its recall is subconscious. When using implicit memory, we act automatically and we are not aware of recalling memory traces. It is a heterogeneous collection of memory functions and types of learned behaviors such as reflexive learning (sensitization and habituation), classical conditioning, fear conditioning, procedural memory (for skills and habits) and priming, which is the recalls of words or objects from a previous unconscious exposure to them (Okano *et al*., 2000).

2.2.2.2 EXPLICIT (DECLARATIVE) MEMORY

It is termed declarative because its recall is by a deliberate and conscious effort. It concerns factual knowledge of persons, things, notions and places. Declarative memory can further be divided into episodic or autobiographic and semantic memory (Benfenati, 2007).

I- Episodic Memory

This refers to the memory for recollection of recent information in episodic manner. It is often assessed by delayed recall tasks, such as the ability to recall items from a word list or narrative details from a paragraph story, tested after some delay of minutes or longer. This type of memory depends majorly on the integrity of the hippocampus and the adjacent areas of the medial temporal lobes (Squire, 2009). It allows us to remember personal events and experience. And being a link between what we are and what we have been, gives us the sense of individuality.

II- Semantic Memory

Semantic memory is a sort of public memory for facts and notions be they general or autobiographical. Often over time, autobiographical memory shades into semantic memory so that the experience of an event is remembered as the simple occurrence of such event (Squire, 2009).

While explicit memory fades relatively rapidly in absence of recall and refreshing, implicit memory may last throughout lifetime even in the absence of further practice (Benfenati, 2007).

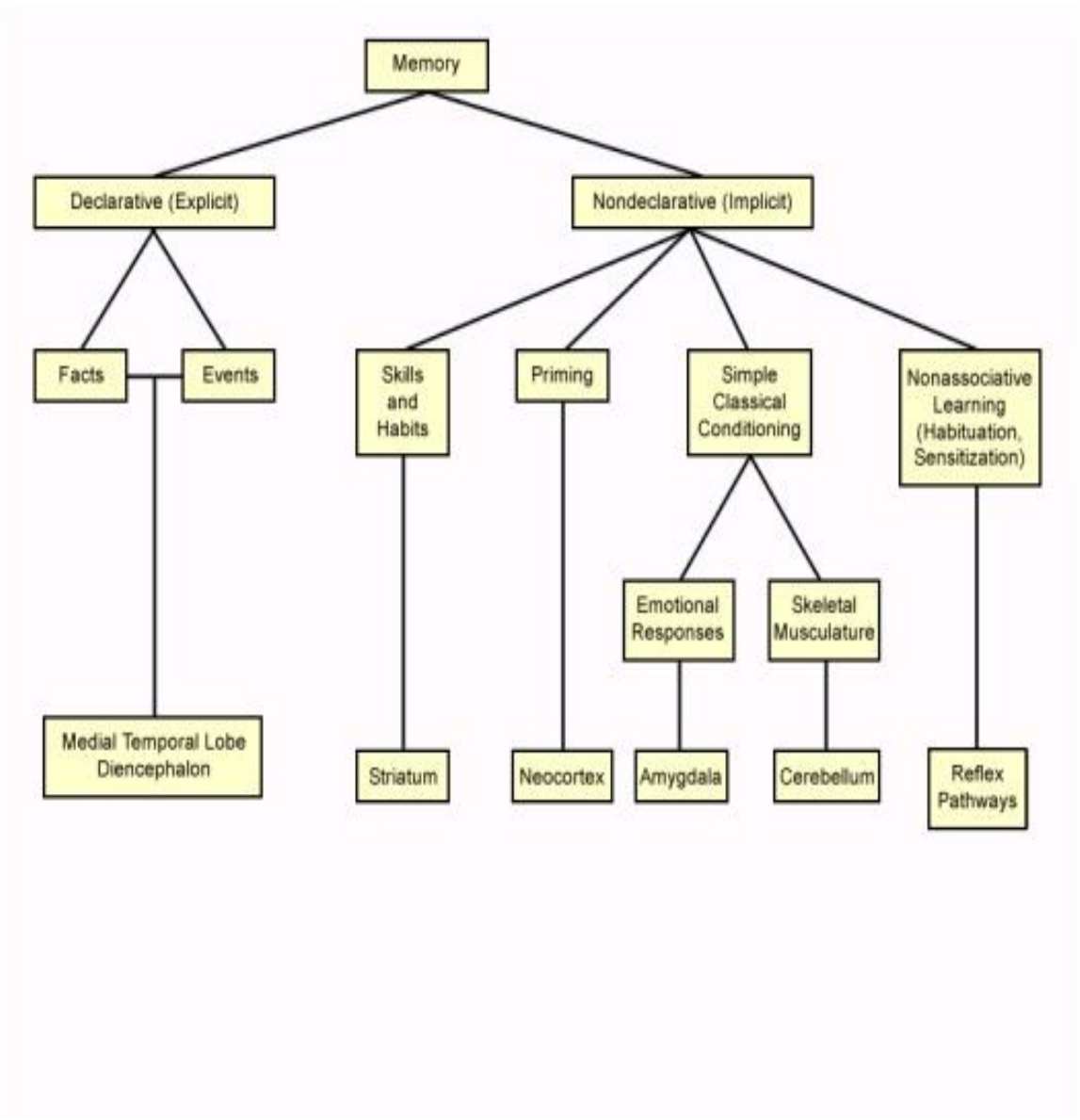

Fig 2.1; The Two Major Divisions of Memory and the Brain Areas Involved in Memory Formation (Benfenati, 2007).

2.2.3 SHORT-TERM MEMORY

Short-term Memory, which includes memories that last for seconds to minutes after which they are converted into long-term memories during which processing in the hippocampus and elsewhere lays down long-term changes in the synaptic strength.

2.2.3.1 Working Memory

Working memory is a form of short-term memory that keeps information available, usually for a very short period of time, while the individual plans actions based on it. It refers to the ability to "hold in mind" and flexibly manipulate information over a short period of time to make a response. It refers to the cognitive demands involved with the temporary storage and manipulating of information. Working memory in humans can be assessed using tests such as digit span backward, Letter-Number Sequencing, serial addition, n-back tests and Brown-Peterson distraction tests (Squire, 2009).

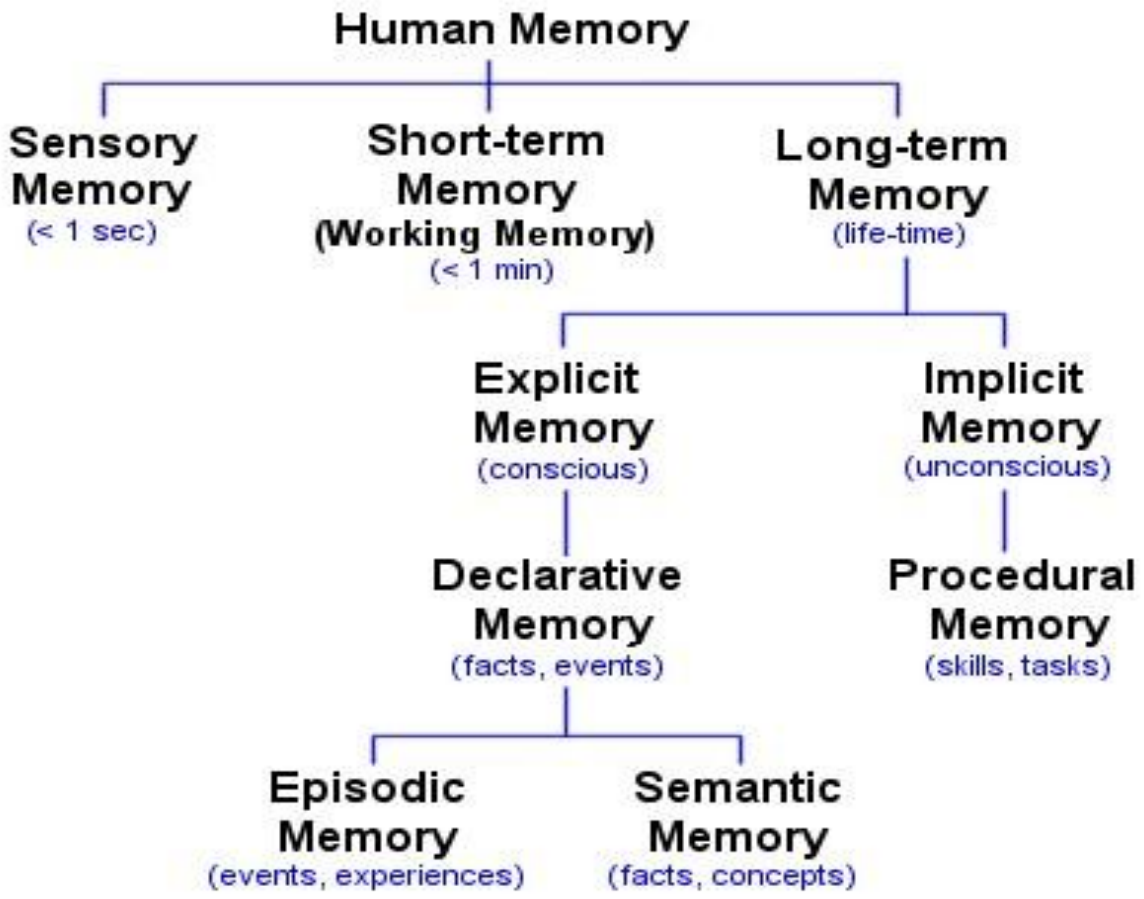

Fig 2.2: Classifications of Memory (Squire, 2009)

2.3 SEX DIFFERENTIATION IN COGNITIVE FUNCTIONS

Although there are no qualitative differences in cognitive skills between the sexes, the quantitative differences have been consistently found. Whereas women tend to excel in tasks of verbal skills and memory, on perpetual skills and accuracy, and on fine motor skills, men tend to excel test of visual memory and on mathematical and spatial ability (Halpern, 1992).

These differences in cognitive function are thought to occur as a result of the exposure of the fetal brain to differential levels of the sex hormones during prenatal life. These so-called "organizational effects" of sex hormones are thought to permanently alter the structure and/or function of specific brain areas during fetal life, perhaps by directing the development of certain neural pathways. Postpubertally, circulating levels of a given sex hormones serve to amplify the neural "hard-wiring" laid down prenatally under its influence, usually referred to as the activational effect of that hormone. Therefore, this psychoendocrine theory proposes that, during prenatal life, the presence of significant quantities of a sex hormones organizes neural substrates for a certain behavior or function that becomes manifest after puberty under the influence of high circulating levels of that same hormone (Sherwin, 2003).

2.4 OXIDATIVE STRESS (EXCESSIVE ACCUMULATION OF ROS)

In a healthy condition, the production of ROS is balanced by various antioxidant systems (Ghandi, 2012; Dasuri *et al.*, 2013). Oxidative stress is induced by an imbalanced redox states, involving either excessive generation of reactive oxygen species (ROS) or dysfunction of the antioxidant system (Ray *et al.*, 2001; Geon *et al.*, 2015). Oxidative stress may be related to cell membrane damage from lipid peroxidation, changes in protein structure and function due to protein oxidation, and structural damage to DNA (Ghandi, 2012).

As the brain is one of the most metabolically active organs in the body, it is vulnerable to oxidative stress particularly because of the following reasons. First, the brain has a high oxygen demand (Geon *et al*., 2015), which constitutes 20% of the body oxygen consumption. Second, the redox-active metals such as iron or copper exist abundantly in the brain and they are actively involved to catalyze ROS formation. Third, the high levels of polyunsaturated fatty acids are found in the brain cell membranes and react as substrates for lipid peroxidation. Fourth, there are relatively low levels of GSH in the brain, which plays a role of endogenous antioxidant in the elimination of ROS (Geon *et al*., 2015).

Many polyphenolic compounds from fruits and vegetables are known for their antioxidant properties, and thus have been considered as therapeutic agents, for example resveratrol [Wassmann *et al.*, 2004].

2.4.1 FREE RADICALS AND REACTIVE OXYGEN SPECIES (ROS)

2.4.2 REACTIVE OXYGEN SPECIES

Oxygen is susceptible to radical formation due to two unpaired electrons present in the outer electron shell (Held, 2012). Reactive oxygen species (ROS) are defined as a group of reactive molecules derived from oxygen (Bolisetty and Jaimes, 2013), which are generally short-lived and highly reactive because of their unpaired valence electrons (Patten *et al.*, 2010). ROS include, but are not limited to free radicals (superoxide, O_2), hydroxyl radical (OH^-), or non-radicals (hydrogen peroxide, H_2O_2) (Gandhi and Abramov, 2012; Bolisetty and Jaimes, 2013; Halliwell, 2006). The consecutive reduction of oxygen through adding electrons cause the formation of a variety of ROS, which include superoxide (O_2^-), hydroxyl radical (OH^-), hydroxyl ion (OH^-) and hydrogen peroxide (H_2O_2). O_2^- is suggested to play a gateway role in ROS production. O_2- may be transformed into the more stable form of H_2O_2 by superoxide dismutase (SOD). It may also be protonated to form HO_2^-. H_2O_2 may have potential to

generate highly reactive hydroxyl radicals ·OH while it can further be divided into H_2O and O_2 by catalase, glutathione peroxidase, and other peroxidases (Song and Zou, 2015). $^{-}$OH is known to be one of the most reactive ROS that are mainly responsible for the cytotoxic effects of ROS (Bolisetty and Jaimes, 2013). ·OH can be generated from H_2O_2 and O_2^{-} and is catalyzed by iron ions through the Fenton reaction that refers to Fe_2-mediated decomposition of H_2O_2 (Song and Zou, 2015).

2.4.3 GENERATION OF ROS

The superoxide (O_2^{-}) is generated from O_2 as a by-product of respiratory chain complex in the mitochondria or by NADPH oxidase. By superoxide dismutase (SOD), the superoxide (O_2-) can be transformed into hydrogen peroxide (H_2O_2).

2.4.3.1 GENERATION OF ROS IN BRAIN

Cellular ROS are usually generated by both exogenous and endogenous sources (Song and Zou, 2015; Mani, 2015). Exogenous sources of ROS generation include ultra violet (UV) ionizing radiation, drugs whose mechanism of action is mediated via ROS production, environmental toxins and chemicals that produce ROS as a by-product of their metabolism (Mani, 2015). Endogenous production of ROS is mediated by mitochondrial and non-mitochondrial ROS-generating enzymes including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox), xanthine oxidase (XO), cytochrome P450 from endoplasmic reticulum (ER), and flavin oxidases from peroxisomes. The major sources of ROS production are the mitochondrial respiratory chain and Nox systems (Song and Zou, 2015; Mani, 2015).

2.4.3.2 MITOCHONDRIAL ROS PRODUCTION

The mitochondrion is the primary source of ROS production in the majority of cells. Under normal physiological condition, up to 2% of the total cellular mitochondrial O_2 consumption may be related to the generation of ROS including O_2^- (Davies, 2000; Orrenius, 2007; Widlansky and Gutterman, 2011). Multiple ways of mitochondrial ROS productions have been proposed which are mainly modulated by the mitochondrial respiratory chain complexes (Cadenas and Davies, 2000; Mancuso *et al.,* 2006; Widlansky and Gutterman, 2011). The mitochondrial electron transport chain (ETC) consists of five multi-subunit complexes including NADH-coenzyme Q (CoQ) reductase (NADH dehydrogenase, Complex I), succinate dehydrogenase (Complex II), coenzyme Q-cytochrome c reductase (Complex III), cytochrome C oxidase (Complex IV), and ATP synthase (Complex V) (Song and Zou, 2012; Mani, 2015).

Complex I is responsible for ROS production of O_2^- (Bolisetty and Jaimes, 2013; Quinlan, 2013) and facilitates electron transfers from NADH to CoQ. During this step, protons are also translocated from the matrix to the intermembrane space (Bolisetty and Jaimes, 2013). Complex II is involved in the reduction of CoQ and is known to be involved in producing low levels of O_2^- (Mclennan and Degli Esposti, 2000; Yankovskaya, 2003). Complex III, on the other hand, is involved in the generation of O_2^- in the intermembrane space. The generation of O_2- is especially enhanced when the electron transfer is reduced with the increased membrane potential (Bolisetty and Jaimes, 2013).

Interestingly, the capacity of these enzymes to produce ROS may vary among the organs or during disease conditions (Turrens, 2003). For instance, Complex I appears to contribute to the production of most of $O2^-$ in the brain, while Complex III is considered as the primary source of O_2^- in the heart and lung (Turrens, 2003). In addition, within mitochondria, ETC

Complex I and III are regarded as the main producers of O_2^- (Song and Zou, 2015). ROS productions from Complex I is approximately one-half of those from complex III in healthy state (Song and Zou, 2015), while Complex I exerts the primary role in ROS productions under pathological conditions ranging from accelerated aging to neurodegenerative diseases (Zorov *et al.,* 2014).

i. NADPH OXIDASES (NOx)

Nox, a transmembrane enzyme complex, is known to be another important endogenous source of O_2^- production as the result of catalyzing the electron transfer from NADPH to oxygen (Babior, 2004; Infanger, 2006). Nox is found highly in phagocytes (neutrophils, eosinophils, monocytes and macrophages, called as Phox or NOx) as well as in the endothelium of cardiovascular tissue (Babior, 2004; Lambeth, 2004). Until now, seven Nox isoforms have been identified in mammalian cells including Nox1 to Nox5 and dual oxidases (Duox1 and Duox2) (Lambeth, 2004). Each Nox isoform has unique cellular localization, regulation, and function (Song and Zou, 2015). For instance, Nox4 and Nox2 are abundant, whereas Nox1 is less in endothelial cells (Görlach *et al.*, 2000; Bengtsson *et al.*, 2003). In contrast, Nox1 and Nox4 are the more highly expressed isoforms in vascular smooth muscle cell than Nox2 (Wingler *et al.*, 2001). Nox2, which is mainly expressed in phagocytes and produces large amounts of ROS, can help kill the foreign organisms as a part of the immune defense system (Babior, 2004; Patten, 2010). On the other hand, Nox produces relatively less ROS at a slow and sustained rate in cardiovascular tissue and exerts a role as intracellular signaling molecules (Patten, 2010). Previous studies have reported that Nox4 is one of the most common isoforms in vascular structures (Van Buul *et al*., 2005; Clempus, 2007; Song and Zou, 2012). In addition, contrary to Nox1 and Nox2, Nox4 is fundamentally active in the cardiovascular systems (Ellmark, 2005; Bedard and Krause, 2007) and the primary source of H_2O_2 production rather than O_2^- production (Dikalov *et al.*, 2008 Gordillo *et al.*, 2010).

ii. XANTHINE OXIDASE (XO)

Xanthine oxidase (XO) and xanthine dehydrogenase (XDH) are inter-convertible forms of xanthine oxidoreductase (Abramov *et al.*, 2007). XO is responsible for the catabolism of purines by converting hypoxanthine to xanthine and xanthine to uric acid (Dasuri, 2013). XO donates electrons to oxygen and subsequently generate O_2^- and H_2O_2 within the cell (Harrison, 2002). In normal conditions, the enzyme is present as a form of XDH (Gandhi and Abramov, 2012). The involvement of XO in ROS-mediated diseases has been proposed with the increased level of intracellular calcium when energy status of the cell decreases and transmembrane gradients are disrupted in such a case of ischemic injury. Increased intracellular calcium may lead to the irreversible conversion of XDH into XO that catalyzes the oxidation of hypoxanthine to xanthine (Harrison, 2004; Gandhi and Abramov, 2012). Particularly during reperfusion, oxygen is reduced to its radical forms, H_2O_2 and O_2^-, in the presence of XO (Harrison, 2002; Harrison, 2004; Gandhi and Abramov, 2012). Increased ROS levels may contribute to further tissue damage.

2.4.3.3 ROS PRODUCTION IN THE ENDOPLASMIC RETICULUM

The endoplasmic reticulum (ER) is the membrane-based intracellular organelle that is primarily related to protein folding and lipid biosynthesis (Mani, 2015; Bhandary, 2012). ER may generate ROS by following two mechanisms (Bhandary, 2012). First, under normal conditions, proper formations of disulfide bonds and protein folding take place in the ER for the stability and maturation of the membrane, which is driven by endoplasmic reticulum oxidoreduction-1 (ERO-1) and protein disulfide-isomerase (PDI) (Bhandary, 2012). As electrons are transferred from protein thiol to oxygen by ERO-1 and PDI, ROS can be produced as a byproduct. Second, ROS is also produced by protein misfolding particularly in cases of glutathione (GSH) depletion (Bhandary, 2012). Oxidized thiols are repaired to

interact with ERO-1 and PDI by glutathione (GSH). These steps would initiate repetitive cycles of breakage and formation of disulfide bond within ER lumen, which more ROS is generating as a byproduct (Bhandary, 2012; Higa and Chevet, 2012). Therefore, proteins which have multiple disulfide bonds may be more vulnerable to producing ROS (Bhandry, 2012). Since GSH is used to reduce incorrectly formed disulfide bonds particularly under oxidizing environment, the level of GSH is further decreased and more ROS can be generated in this situation (Gordillo, 2010). Furthermore, because the process involved in oxidative protein folding in the ER may be highly energy dependent, it should be noted that depletion of adenosine triphosphate (ATP) caused by protein misfolding may elicit oxidative phosphorylation in mitochondria and consequently produce more ROS (Malhotra and Kaufman, 2007; Bhandry, 2012).

2.4.3.4 ROS PRODUCTION IN PEROXISOMES

Peroxisomes are present in most eukaryotic cells and participate in multiple metabolic pathways including fatty acid oxidation, phospholipid biosynthesis, amino acid catabolism, and oxidative part of the pentose phosphate pathway (Schrader and Fahimi, 2006; Mani, 2015). Under normal physiologic conditions, oxygen consumption in the peroxisomes may lead to the production of H_2O_2, but not O_2^-. Peroxisomes is one of the organelles that can generate the majority of H_2O_2 using a number of different oxidases such as acyl-CoA oxidases, D-aspartate oxidase, and urate oxidase, which makes hydrogen to be transferred to O_2 from their respective substrates (Mani, 2015). In addition, peroxisomes also contain catalases that can decompose H_2O_2 and help maintain the balance between the production and removal of ROS (Schrader and Fahimi, 2006; Valko, 2007). When peroxisomes are damaged and the amount of catalases decreases, increased H_2O_2 release into the cytosol can contribute to oxidative stress in the cell (Valko, 2007).

2.5 ANTIOXIDANT PATHWAY

Cellular ROS levels may be reduced through the defense mechanisms of antioxidant enzymes and small-molecule antioxidants. O_2^- radical can be inactivated by SOD to produce H_2O_2. Then H_2O_2 may further be removed by the action of glutathione peroxidases, catalase, and peroxiredoxins (Ghandi, 2012).

2.5.1 SUPEROXIDE DISMUTASE (SOD)

SOD plays a vital role in catalyzing the breakdown of highly reactive O_2^- to less reactive H_2O_2 and oxygen (Dasuri *et al.*, 2013). Cytosolic copper/zinc-SOD (SOD1), mitochondrial manganese SOD (SOD2), and extracellular SOD (SOD3) are three distinct isoforms of SOD that have been identified. SOD1 and SOD2 are mainly involved in the elimination of O_2^- in the cytosol and mitochondria, respectively (Dasuri *et al.*, 2013).

2.5.2 GLUTATHIONE PEROXIDASES (GPX)

Glutathione peroxidase GPX contains a family of multiple isoenzymes which catalyze the reduction of H_2O_2 and lipid peroxides utilizing GSH as an electron donor (Ghandi, 2012; Dasuri *et al*., 2013). GPX is located in both cytosol and mitochondria. In mammals, there are five different isoforms of selenium-dependent glutathione peroxidases (GPX1-4 and 6) and three non-selenium congeners (GPX 5, 7 and 8) that have cysteine instead of selenocysteine. Antioxidant function of GPXs depends on each isoform and location in the cells; GPX1 exists universally in the cytosol and mitochondria, GPX2 does in the epithelium of intestine, and GPX3 does in the plasma. It is noteworthy that GPX1 has been regarded as one of the major antioxidant enzymes in the brain, which is expressed predominantly in microglia but not in neurons. Studies have suggested that up regulation of GPX1 could be one of the protective responses against neuronal injury.

2.5.3 CATALASE

Catalase is responsible for the conversion of H_2O_2 to water and oxygen using either iron or manganese as a cofactor (Ghandi, 2012; Dasuri *et al.*, 2013). Catalase is located in peroxisomes and also found in the cytoplasm and mitochondria. The role of catalase is minor at low levels of H_2O_2, but becomes increasingly important at higher levels of H_2O_2 (Ghandi, 2012).

2.5.4 PEROXIREDOXINS

Peroxiredoxins (PRX) are thiol-specific peroxidases that catalyze the reduction of H_2O_2 as well as other organic hydroperoxides and peroxynitrite (Wood, 2003; Dasuri, 2013). Among the six PRX isoforms, PRX1, 2, and 4 are present in the cytoplasm as well as in the nuclei. In addition, PRX1 is also expressed in the mitochondria and peroxisomes, while PRX4 is found in the lysosomes [Espinosa-Diez, 2015]. PRX3 is exclusively localized in the mitochondria (Espinosa-Diez, 2015), whereas PRX5 is found in the mitochondria, cytoplasm, nuclei, and peroxisomes. All PRX utilize a conserved active-site cysteine residue in order to directly reduce peroxide. Since PRX are abundant in eukaryotic cells, constitute approximately more than 1% of cellular proteins, and show high reactivity, PRX are responsible for the reduction of up to 90% of mitochondrial H_2O_2 and almost 100% of cytoplasmic H_2O_2 (Winterbourn, 2008; Hall *et al.*, 2011).

2.5.5 GLUTATHIONE (GSH)

Glutathione is a tripeptide thiol synthesized by glutamic acid, cysteine and glycine in the pathway of γ-glutamyl cycle (Zhang *et al.*, 2013), and is present in either a reduced (GSH) or oxidized (GSSG) form. Glutathione acts as a strong reducing agent as well as serving as an electron donor for glutathione peroxidase. Glutathione is known to have an important role in

maintaining the intracellular redox state and protecting cells against damage caused by oxidative stress (Zhang *et al.*, 2013).

In the brain, in vivo GSH is produced by the consecutive actions of two enzymes; γ dipeptide of γ-glutamylcysteine is formed by -glutamylcysteine synthetase, using glutamate and cysteine as substrates. And this dipeptide is further combined with glycine by the catalyzing action of glutathionine synthetase to synthesize GSH.

GSH is involved in the following two types of reactions; Firstly, GSH, in its reduced form, is known to non-enzymatically react with ROS such as O_2^- and $\cdot OH^-$ for the removal of ROS. Secondly, GSH is the electron donor for the reduction of peroxides in the GPX reaction. Reaction with ROS firstly oxidizes GSH, which generates glutathione disulfide, the final product of GPX reactions. GSH can be regenerated from glutathione disulfide by the reaction with glutathione reductase that transfers electrons from NADPH to glutathione disulfide (Ghandi, 2012).

Several studies have reported that GSH is involved in inhibiting apoptotic cell death (Abramov, *et al*., 2007) and DNA damage in cells following oxidative stress (Song and Zou, 2015).

2.5.6 VITAMIN E

Vitamin E refers to a group of compounds that include both tocopherols and tocotrienols (Brigelius-Flohé, 1999). Of the many different forms of vitamin E, γ-tocopherol is the most common in the North American diet. The most biologically active form of vitamin E, is the second-most common form of vitamin E in the diet. α-Tocopherol is a form of vitamin E that is preferentially absorbed and accumulated in humans (Rigotti, 2007). Many agencies have set a tolerable upper intake levels (UL) of vitamin E at 1,000 mg. This variant can be found

most abundantly in wheat germ oil, sunflower, and safflower oils. As a fat-soluble antioxidant, it stops the production of reactive oxygen species formed when fat undergoes oxidation. (Herrera E., et al., 2001). Vitamin E is a lipid-soluble antioxidant that can attenuate the effects of peroxide and protect against lipid peroxidation in cell membranes (Ghandi, 2012; Dasuri *et al.*, 2013).

Lipid-soluble vitamin E is concentrated in the hydrophobic interior site of cell membrane and is the principal defense against oxidant-induced membrane injury. Vitamin E donates electron to peroxyl radical, which is produced during lipid peroxidation. Alpha-Tocopherol is the most active form of vitamin E and the major membrane-bound antioxidant in cell. Vitamin E triggers apoptosis of cancer cells and inhibits free radical formations (Masella *et al*., 2005).

Vitamin E has many biological functions although most human supplementation studies about vitamin E have used only alpha-tocopherol as it can affect levels of other forms of vitamin E. The antioxidant function being the most important and/or best known and, that it may not have a significant role in antioxidant metabolism. It has also been suggested that the most important function of vitamin E is in cell signaling.

- Antioxidant function; vitamin E acts as a peroxyl radical scavenger, preventing the propagation of free radicals in tissues, by reacting with them to form a tocopheryl radical which will then be oxidized by a hydrogen donor (such as Vitamin C) and thus return to its reduced state. As it is fat-soluble, it is incorporated into cell membranes, which protects them from oxidative damage (Maheswari *et al.*, 2015).
- Enzymatic activity regulator; for instance, protein kinase C (PKC), which plays a role in smooth muscle growth, can be inhibited by α-tocopherol. α-Tocopherol has a stimulatory effect on the dephosphorylation enzyme, protein phosphatase 2A, which

in turn, cleaves phosphate groups from PKC leading to its deactivation, bringing the smooth muscle growth to a halt (Schneider, 2005).

- It also has an effect on gene expression. Macrophages rich in cholesterol are found in the atherogenetic tissue. Scavenger receptor CD36 is a class B scavenger receptor found to be up-regulated by oxidized low density lipoprotein (LDL) and binds it (Devaraj *et al.,* 2001). Treatment with alpha tocopherol was found to down regulate the expression of the CD36 scavenger receptor gene and the scavenger receptor class A (SR-A) (Devaraj *et al.,* 2001) and modulates expression of the connective tissue growth factor (CTGF). CTGF gene, when expressed, is responsible for the repair of wounds and regeneration of the extracellular tissue that is lost or damaged during atherosclerosis (Villacorta *et al.,* 2003).
- Vitamin E also plays a role in neurological functions, and inhibition of platelet aggregation. Its supplementation in cancer patients showed that it has an important neuroprotective effect as it has the ability to protect neuronal tissue in several neurodegenerative disorders including Alzheimer's disease.
- Vitamin E also protects lipids and prevents the oxidation and deterioration of polyunsaturated fatty acids (PUFAs) that may result to an increased fluidity and viscosity of the lipid membrane bilayer structure (Whitney and Rolfes, 2011).

2.5.7 VITAMIN C

Vitamin C or L-ascorbic acid, or simply ascorbate (the anion of ascorbic acid), is an essential nutrient for humans and certain other animal species. Vitamin C refers to a number of vitamers that have vitamin C activity in animals, including ascorbic acid and its salts, and some oxidized forms of the molecule like dehydroascorbic acid. Ascorbate and ascorbic acid are both naturally present in the body when either of these is introduced into cells, since the

forms interconvert according to PH. Vitamin C has a daily requirement of 90mg to 2000mg. **(**USDA Nutrient Data Laboratory, 2014**)**

Vitamin C is a water-soluble antioxidant, which is involved in the removal of free radicals by electron transfer and also acts as a cofactor for antioxidant enzymes (Song and Zou, 2015; Dasuri *et al.*, 2013). Vitamin C (Ascorbic Acid) Water-soluble vitamin C (ascorbic acid) provides intracellular and extracellular aqueous-phase antioxidant capacity primarily by scavenging oxygen free radicals. It converts vitamin E free radicals back to vitamin E. Its plasma levels have been shown to decrease with age. Vitamin C or L-ascorbic acid, or simply and ascorbic acid are both naturally present in the body when either of these is introduced into cells, since the forms interconvert according to (Higdon, 2006).

2.6 PHYSIOLOGICAL FUNCTIONS OF ROS

Low to moderate levels of ROS are critical in cellular signaling and pro-survival pathways (Patten *et al*., 2010; Ghandi, 2012; Mani, 2015). For instance, Nox-derived ROS play a role in cellular signaling related to the cardiovascular systems (Babior, 2004) and those in phagocytes (Nox2-derived) are involved in defense mechanisms of the immune system against foreign organisms (Babior, 2004). Furthermore, the increased level of Nox-derived ROS activates important survival pathways, such as mitogen-activated protein kinase (MAPK) pathways (Patten *et al.*, 2010). The MAPK, the serine/threonine-specific protein kinases, represents the major redox-regulated signaling molecules in the cardiovascular systems (Manea, 2010). It also modulates various cellular activities including gene expression, mitosis, proliferation, migration, cell survival, and apoptosis (Groeger *et al*., 2009; Patten *et al*., 2010).

ROS can also activate transcription factors that regulate cellular responses to ROS (Patten *et al*., 2010). Increased ROS may therefore promote antioxidant defense processes. An example

is NF-E2-related factor 2 (Nrf2), which is one of major redox-sensitive transcription factors. It is activated by ROS and modulates the expression of several antioxidant enzymes including SOD, PRX, GPX, and heme oxygenases. A suppressor protein, Kelch-like ECH-associated protein 1 (Keap1), which is anchored in the cytoplasm, prevents the translocation of Nrf2 to the nucleus and keeps Nrf2 inactive under normal conditions (Hybertson *et al.*, 2011). Increased ROS production disrupts binding between Keap1 and Nrf2, allowing transcription by activation of Nrf2 (Patten *et al*., 2010). Nuclear factor-kappa B (NF-κB) would be another pro-survival transcription factor that may be activated by ROS. NF-κB is normally present in the cytoplasm as an inactive state by the action of a NF-κB inhibitor. Moderate levels of ROS may induce the phosphorylation and degradation of a NF-κB inhibitor and result in activation of NF-κB. The activated NF-κB transcripts anti-apoptotic proteins and inhibits caspase-dependent cell death pathways. In contrast, high levels of ROS may contribute to inactivation of NF-κB by inhibiting its binding to DNA, attenuate pro-survival pathway, and consequently promote apoptosis. In this regard, the role of NF-κB activation in a survival response to apoptosis depends on the amount of ROS formation (Patten *et al*., 2010).

2.7 RESVERATROL

Resveratrol, a polyphenolic compound, was first reported in 1939 by a Japanese researcher, Dr. Michio Takaoka (Takaoka, 1939; Villaflores, *et al.,* 2012). It is synthesized in several plants in response to adverse conditions such as stress, injury, ultraviolet irradiation and fungal infection (Floreani, 2003). Resveratrol has a wide range of biological effects, including anti-oxidative, anti-inflammatory (Orsu *et al*, 2003; Spanier *et al.*, 2009; Wood *et al.*, 2010; Chang *et al*., 2012) and anticarcinogenic properties (Athar *et al*., 2009), and is largely found in grapes and red wine. Also readily available in boiled groundnut.

Resveratrol exhibits comprehensive beneficial health effects, which include cardioprotective (Sebai *et al.*, 2011; Wu and Hsieh, 2011), antiproliferative (Cui *et al.*, 2010) and neuroprotective properties. Resveratrol was suggested to play a role in the prevention of heart disease as it modulated lipoprotein metabolism and inhibited platelet aggregation. Zamora-Ros*et* carried out a large cross-sectional study using high cardiovascular risk individuals in Spain to investigate the association between total urinary resveratrol metabolites as biomarkers of wine and resveratrol consumption and cardiovascular risk factors. The research found that both resveratrol and wine intake were useful to decrease cardiovascular risk factors, through changing the lipid profiles in blood, fasting blood glucose (only resveratrol) and heart rate. Resveratrol has been proposed as a major constituent of the polyphenol fraction to which the health benefits of red wine consumption are attributed (Lu *et al.*, 2008; Simão *et al.*, 2013).

In vivo and *in vitro* studies have also shown that resveratrol exhibited neuroprotective effects in models of many diseases, such as cerebral ischemia, kainic acid-induced excitotoxicity, Huntington's disease, Parkinson's disease, and Alzheimer's disease (Lu *et al.*, 2008; Simão *et al.*, 2013; Wang *et al.* 2014).

In vivo and *in vitro* studies have also shown that resveratrol plays a role in the prevention of inflammation, atherosclerosis and carcinogenesis (Agarwal *et al.*, 2012; Novaes *et al.*, 2012; Back *et al.*, 2012). Matos *et al.*, (2012) found that resveratrol had significant anti-atherogenic and anti-inflammatory effects in an animal model with rabbits fed a hypercholesterolemic diet (1% (w/v) cholesterol). Back *et al.*, (2012) used human A431 SCC cells to study the effect of resveratrol and found that resveratrol could induce premature senescence in human A431 SCC cells, and that resveratrol-induced premature senescence was associated with blockade of autolysosome formation, as assessed by the absence of co-localization of microtubule-associated protein 1 light chain 3, a specific marker of autophagy, and Lamp-2,

markers for autophagosomes and lysosomes, respectively. Resveratrol also down regulates the level of Rictor, a component of the mammalian target of rapamycin complex 2, leading to a decrease in RhoA-GTPase and altered actin cytoskeleton organization. Resveratrol has also estrogenic, vaso-relaxing activity and cardiovascular benefits (Xu and Si, 2012).

Simao *et al*., (2012) found that resveratrol can prevent damage to CA1 neurons against ischemic injury and that this neuroprotective effect of resveratrol may be mediated by the activation of the PI3-K/Akt signaling pathway, which subsequently down-regulates the expression of glycogen synthase kinase-3 beta and cAMP-response element binding protein. Lu *et al.*, (2012) used a rat brain ischemia model, induced by middle cerebral artery occlusion, and found that resveratrol treatment significantly reduced brain infarct volume and decreased the expression of the pro-apoptotic protein Bax and increased the expression of the anti-apoptotic protein Bcl-2. Resveratrol was also found to provide protection against kainic acid-induced excitotoxicity in the cortex and hippocampus of Wistar rats (Gupta *et al*., 2002). In a global cerebral ischemia model induced by four-vessel occlusion for 10 minutes, resveratrol could modulate membrane lipid composition, as well as the ganglioside profile in ischemia/reperfusion injury (Simão *et al.*, 2013). Resveratrol also had a neuroprotective effect following spontaneous seizures (Wu *et al.*, 2009), Huntington's disease (Ho *et al*., 2010), Parkinson's disease (Kumar *et al*., 2007; Lu *et al.*, 2008; Albani *et al.*, 2009; Bournival *et al*., 2009; Zhang *et al*, 2010) and Alzheimer's disease (Marambaud *et al.*, 2005; Kim *et al*, 2007; Mishra *et al.*, 2009) through its anti-oxidative properties.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS AND HOUSING

Thirty (30) adult Swiss albino mice weighing between 25g and 30g were used for this study. They were purchased from the Laboratory Animal House of the Department of Human Physiology, Faculty of Medicine Ahmadu Bello University Zaria, Nigeria. The animals were housed in laboratory cages and maintained under standard laboratory condition with alternating light/dark cycles. They were fed on standard animal feed, while water was provided *ad libitum*. The experiment was conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

3.2 MATERIALS

- Plastic rat cages
- Feeds
- Drinking bottles
- Sterile disposable syringes
- Cannula
- Conical flask
- Methylated spirit
- Cotton wool
- Hand gloves
- Towels
- Observation cages
- Stop watch
- Normal saline

- Chemicals

3.3 CHEMICALS

Commercial grade CPF (25% EC, Termicot®, Sabero Organics, G limited, Lebanon), were prepared by reconstituting in soya oil (Grand Cereals and Oil Mills Ltd., Jos, Nigeria) to make 10% stock solution and Resveratrol of analytical grade (SIGMA Chemical Company Ltd. St. Louis USA), were dissolved in CMC (SIGMA Chemical Company Ltd. St. Louis USA).

3.4 ANIMAL TREATMENT SCHEDULE

The mice were weighed and then assigned at random into 6 groups of 5 mice in each (n=5) group. Administration was done orally for the period of 21 days.

Group I: Olive oil 2 ml/kg

Group II: Carboxymethyl cellulose (CMC) 2 ml/kg

Group III: Chlorpyrifos (CPF) 3 mg/kg b. w. LD_{50} of 85mg/kg b. w. (Ambali, 2009)

Group IV: Resveratrol (RSV) 30 mg/kg b. w.

Group V: Pre-treated with Vitamin E (100 mg/kg) and 3 mg/kg of CPF was administered 30 minutes later.

Group VI: Pre-treated with resveratrol (30 mg/kg) and 3 mg/kg of CPF was administered 30 minutes later.

All administrations were done orally for 21 days.

3.5 COGNITION TESTS

3.5.1 Y-MAZE MODEL (spatial memory test)

The mice's Y-maze is composed of three equally spaced arms (at 120°, arm's length 50 cm, width 10 cm, and wall height 20 cm). The floor of each arm is made of Perspex. Y-maze is a quick and useful initial test for general cognitive function. This test is based on the innate preference of animals to explore an arm that has not been previously explored (Drewa,

1998). Y-maze function is sensitive to damage in areas concerned with learning and memory functions such as the hippocampus, and is also disrupted by drugs that cause memory loss. The Spontaneous alternation version of Y-maze testing was employed for the study.

3.5.1.1 Spontaneous Alternation Version:

In this version each mouse was placed in the Y-maze for 5 min and the number of arms entered as well as the sequence of entries were recorded and a score was calculated to determine alternation rate. An alternation is defined as entry into all three arms consecutively (Hughes, 2004), for instance if the animal makes the following arm entries; A,C,C.A,B,C,A,C,B,A,B,C,A in this example, the animal made 13 arm entries 7 of which are correct alternations. The number of maximum spontaneous alternations is then the total number of arms entered minus two, and the percentage alternation is calculated as ((actual alternations /maximum alternations) x 100). A high alternation rate is indicative of sustained spatial working memory as the animals must remember which arm was entered last to not re-enter it (Hughes, 2004).

Plate I: Y-maze. (Hughes, 2004)

3.5.2 NOVEL OBJECT MEMORY TASK

The Novel object memory task is an open field assessment of the natural tendency of rodents to investigate a novel object instead of a familiar one as well as their innate tendency to re-start exploring when they are presented with a novel environment or familiar object relocation. The choice to explore the novel object as well as the reactivation of exploration after object displacement reflects the use of learning and (recognition) memory processes (Barker *et al.,* 2007).

3.5.2.1 Temporal Order Task:

This task comprised two sample phases and one test trial. In each sample phase, the mice were placed and allowed to explore two copies of an identical object for a total of 5 min. Different objects will be used for sample phases 1 and 2, with a delay between the sample phases of 1 h. The test trial (5 min) will be given 3 h after sample phase 2. During the test trial, a third copy of the objects from sample phase 1 and a third copy of the objects from sample phase 2 was used. The positions of the objects in the test and the objects used in sample phase 1 and sample phase 2 were counterbalanced between the animals. If learning and recognition memory is intact, the rat will spend more time exploring the object from sample 1 (i.e., the object presented less recently) compared with the object from sample 2 (i.e., the "new" object). The recognition ratio were calculated as the difference in time spent by each animal exploring the object from sample phase 1 compared with the object from sample phase 2 divided by the total time spent exploring both objects in the first minute of the test period (Barker *et al.,* 2007; Baxter, 2010; Gaskin, 2010; Antunes and Biala, 2012).

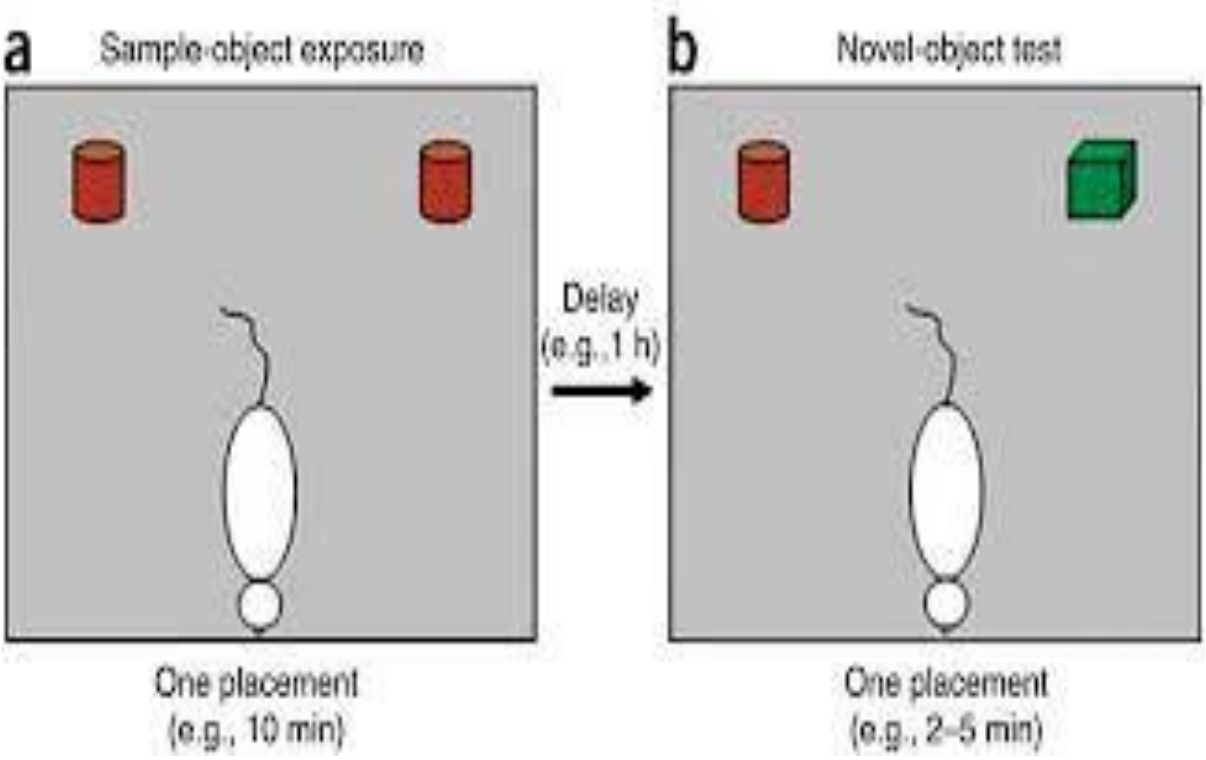

Plate II: The Novel Object Recognition Test (Antunes and Biala, 2012)

3.6 SAMPLE COLLECTION

At the end of the 21 days of administration the animals were sacrificed following chloroform inhalationin a closed chamber. Hippocampal tissues were harvested and homogenized in 7.4 PH phosphate buffer solutions for oxidative stress analysis.

3.7 ANALYSIS OF OXIDATIVE STRESS BIOMARKERS

I. *Assay of catalase (CAT) activity:*

The assay for the catalase activity was carried out according to the principle of colorimetry as described by Beers and Sinha (1952). A monoclonal antibody specific for CAT were pre-coated onto a microplate. Standards and samples (brain homogenates) were pipetted into the wells, gently mixed, covered with a closure plate membrane and were incubated for 30 min. at 37ºC. This allowed any CAT present to be bound by the immobilized antibody. It was afterward removed from the incubator, the wells were washed with a prepared 'wash solution'; the microplate were inverted and thereafter blot-dried by hitting onto an absorbent paper to remove the moisture. An enzyme-linked monoclonal antibody specific for CAT was added to the wells, sealed and, again incubated for 30 min. at 37ºC. Following a wash to remove any unbound antibody-enzyme reagent, substrate solutions (tetra methyl Benzedrine and hydrogen peroxide) were added to the wells and a color was developed in proportion to the amount of CAT bound in the initial step. This was covered and incubated for 15 min. at 37ºC. The colour development was stopped by addition of 'stop solution' and the intensity of the color measured with a microplate reader.

II. *Assay of superoxide dismutase (SOD) activity:*

The assay for the superoxide dismutase activity was carried out according to the principle of colorimetry as described by Martin *et al.*, (1987). This assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for SOD was pre-coated onto a microplate. Standards and samples (brain homogenates) were pipetted

into the wells, gently mixed, covered with a closure plate membrane and incubated for 30 min. at 37ºC. This will allow any SOD present to be bound by the immobilized antibody. It was then afterward removed from the incubator, the wells were washed with a prepared 'wash solution'; the microplate was inverted and thereafter blot-dried by hitting onto an absorbent paper to remove the moisture. An enzyme-linked monoclonal antibody specific for SOD was added to the wells, sealed and, again incubated for 30 min. at 37ºC. It was washed to remove any unbound antibody-enzyme reagent, by adding substrate solutions (tetra methyl Benzedrine and hydrogen peroxide) to the wells and a color was developed in proportion to the amount of SOD bound in the initial step. This was covered and incubated for 15 min. at 37ºC. Color was developed which was stopped by addition of 'stop solution' and the intensity of the color was measured with a microplate reader.

III. ***Assay of glutathione (GSH) activity:***

The assay for the glutathione activity were carried out according to the principle of colorimetry as described by Takebe (2002).This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for GSH was pre-coated onto a microplate. Standards and samples (brain homogenates) were pipetted into the wells, gently mixed, covered with a closure plate membrane and incubated for 30 min. at 37ºC. This will allow any GSH present to be bound by the immobilized antibody. After which it was removed from the incubator, and the wells were washed with a prepared 'wash solution'; the microplate were inverted and thereafter blot-dried by hitting onto an absorbent paper to remove the moisture. An enzyme-linked monoclonal antibody specific for GPx was added to the wells, sealed and, again incubated for 30 mins at 37ºC after which it was washed and blot-dried to remove any unbound antibody-enzyme reagent. Substrate solutions (tetra methyl Benzedrine and hydrogen peroxide) were added to the wells and a color was developed in proportion to the amount of GSH bound in the initial step. This was

covered and incubated for 15 minutes at 37ºC. The color development was stopped by addition of 'stop solution' and the intensity of the color measured with a microplate reader.

IV. *Estimation of lipid peroxidation (malondialdehyde):*

Lipid peroxidation was estimated spectrophotometrically as thiobarbituric acid reactive substances (TBARS). A principal component of TBARS is malondialdehyde (MDA), a product of lipid peroxidation.The assay for malondialdehyde was carried out according to the principle of colorimetry as described by Janero (1990).The assay employed the competitive inhibition enzyme immunoassay technique. A monoclonal antibody specific for MDA was pre-coated onto a microplate. A competitive inhibition reaction was launched between biotin labelled MDA and unlabelled MDA/samples (brain homogenate) with the pre-coated antibody specific for MDA. It was subjected to incubation, after which the unbound conjugate was washed off. Next, avidin was conjugated toHorseradish Peroxidase (HRP) and added to each microplate well and incubated. The amount of bound HRP conjugate was taken to be inversely proportional to the concentration of MDA in the sample. Substrate solution was added which caused development of color. The intensity of color that was developed was measured using a microplate reader and the reading was taken to be inversely proportional to the concentration of MDA in the sample.

3.8 STATISTICAL ANALYSIS

Data were expressed as mean ± standard error of mean (SEM). The values recorded in learning and memory tests were analyzed using the Kruskal-Wallis. The MDA, GPx, CAT and SOD were analyzed using one-way analysis of variance followed by Turkey’s post hoc test using SPSS version 22. Values with ($P < 0.05$) were considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1: Effect of Treatments on Memory of Swiss Albino Mice

Novel Object Recognition Test. Figure 4.1 showed the results obtained in the novel object recognition test, the preference scores (%). A significant decrease ($p<0.05$) in the percentage preference score was observed in the CPF treated group when compared to the control. There was significant improvement in the percentage preference score of the pre-treated (RSV + CPF) group when compared to the CPF group. Significant decrease was also observed in the CMC group when compared to the pre-treated group (RSV + CPF). The Vit E group showed improvement in the preference score when compared to the CPF group, though not statistically significant. But the pre-treated (RSV + CPF) group showed higher improvement in the preference score when compared to the Vit E group.

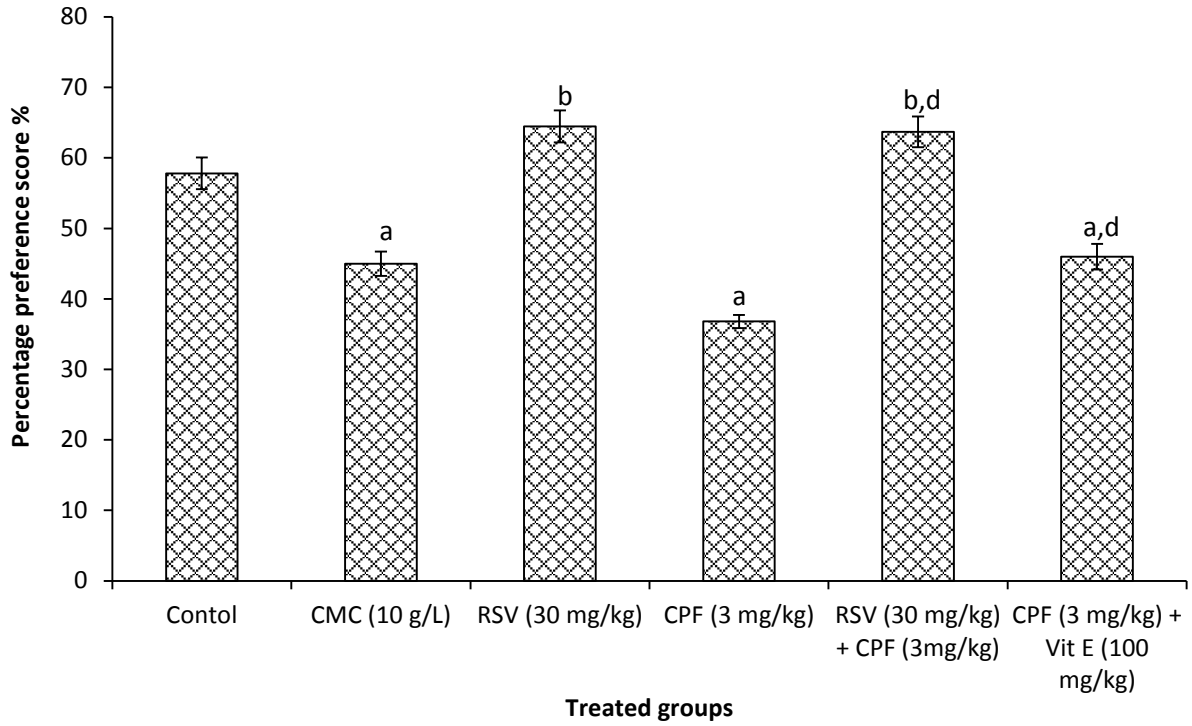

Figure 4.1: Preference scores (%) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days. Superscripts a, b and d indicate statistical significant difference ($P< 0.05$) when compared to Control, CMC and CPF respectively. Result presented as mean ± SEM; n= 5.

Key:

Control= normal saline

CMC= carboxymethylcellulose

RSV= resveratrol

CPF= chlorpyrifos

Vit E= Vitamin E

Figure 4.2: *Y Maze Test.* Figure 4.2 showed the results obtained in the Y-maze test, the mean alternation scores (%). The CPF treated group showed a significantly higher alternation score when compared to the control. The pre-treated (RSV + CPF) group showed a significantly lower alternation score when compared to the CPF treated group. A lower alternation score was observed in the Vit E treated group when compared to the CPF treated group, though not statistically significant. The Vit E treated group also showed a higher alternation score when compared to the RSV pre-treated group.

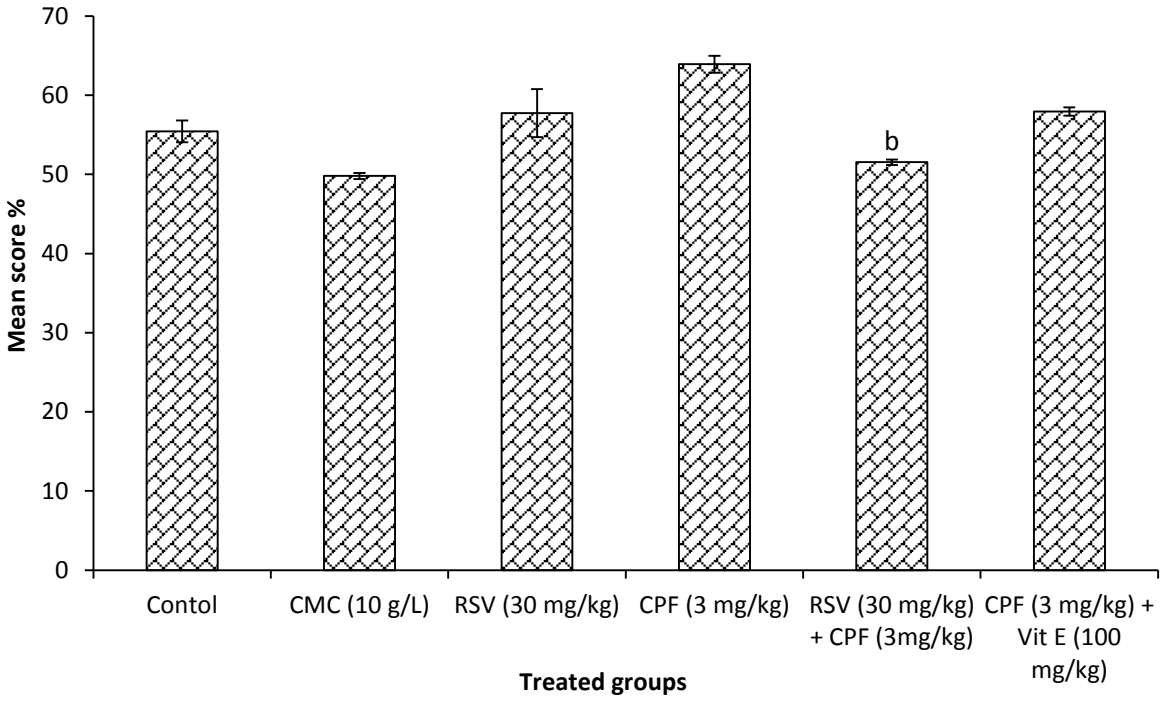

Figure 4.2: Mean scores (%) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days., Superscript b indicates statistical significant difference ($P< 0.05$) when compared to CMC. Result presented as mean ± SEM; n= 5.

Key:

Control (normal saline

CMC= carboxymethylcellulose

RSV=resveratrol

CPF= chlorpyrifos

Vit E= Vitamin E

Figure 4.3: Effect of resveratrol on catalase (CAT) activity in the hippocampus of mice.

Figure 4.3 showed the results obtained in the hippocampal catalase activity. There was no statistical difference in catalase concentration (U/mg) in the control group when compared to the CPF treated group. A significantly increased catalase concentration was observed in the RSV pre-treated group when compared to the control. However, the Vit E treated group showed a significant decrease in catalase concentration when compared to both the control and the CPF treated group. The RSV pre-treated group showed a higher catalase concentration when compared to the Vit E treated group, though not statistically significant.

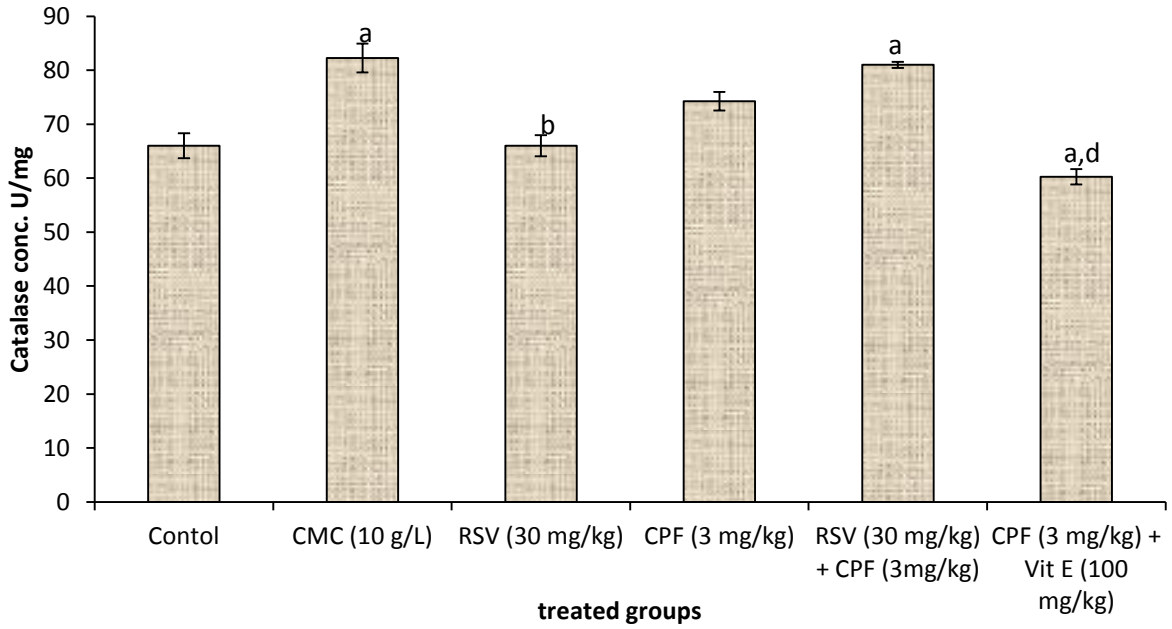

Figure 4.3: Hippocampal catalase concentration (U/mg) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days. Superscripts a, b and d indicate statistical significant difference ($P< 0.05$) when compared to Control, CMC and CPF respectively. Result presented as mean ± SEM; n= 5.

Key:
Control= normal saline
CMC= carboxymethylcellulose
RSV=resveratrol
CPF= chlorpyrifos
Vit E= Vitamin E

Figure 4.4: Effect of resveratrol on superoxide dismutase (SOD) activity in the hippocampus of mice

Figure 4.4 showed the results obtained in the hippocampal superoxide dismutase activity. A significant decrease in the superoxide dismutase concentration (U/mg) was observed in the CPF treated group when compared to the control. Significant decrease in concentration was observed in the RSV pre-treated group when compared to the control. Also, significant decrease concentration of Superoxide dismutase was observed in the Vit E group when compared to the control. The RSV pre-treated group showed significant increase in Superoxide dismutase concentration when compared to the CPF treated group. The Vit E treated group also showed significant increase in superoxide dismutase activity when compared to the CPF treated group. But between the RSV pre-treated and the Vit E treated groups, a similar concentration of superoxide dismutase were observed.

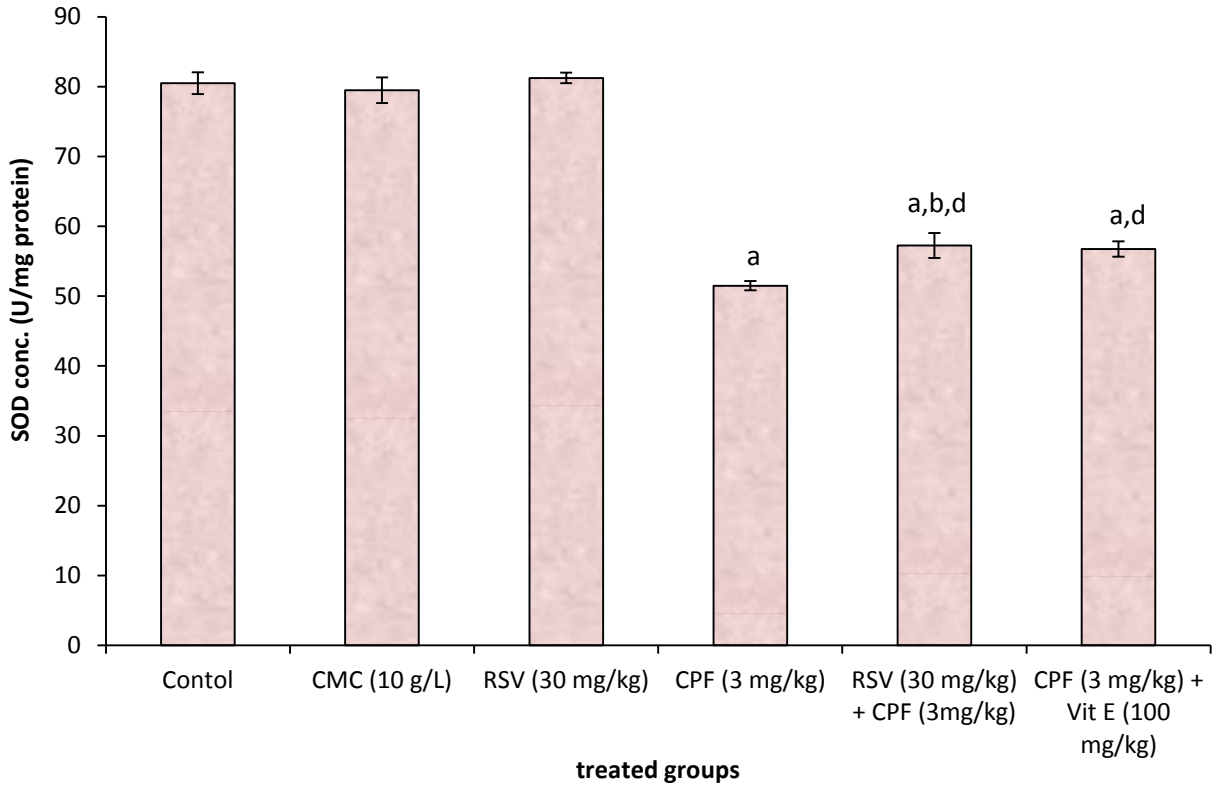

Figure 4.4: Hippocampal superoxide dismutase concentration (U/mg) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days. Superscripts a, b and d indicate statistical significant difference ($P < 0.05$) when compared to Control, CMC and CPF respectively. Result presented as mean ± SEM; n= 5.

Key:

Control= normal saline

CMC= carboxymethylcellulose

RSV=resveratrol

CPF= chlorpyrifos

Vit E= Vitamin E

Figure 4.5: Effect of resveratrol on glutathione peroxidase (GPx) activity in the hippocampus of mice

Figure 4.5 showed the results obtained in the hippocampal glutathione peroxidase activity. A significant decrease in glutathione concentration was observed in the RSV group when compared to the control. A decrease in glutathione concentration was observed in the CPF treated group when compared to the control though, not statistically significant. A significant decrease in glutathione concentration was observed in the RSV pre-treated group when compared to both the control and the CPF treated group, which is similar to that obtained in the RSV group. There was a decreased glutathione concentration in the Vit E group, though not statistically significant when compared to the control. There was an increase in glutathione concentration in the Vit E group when compared to the RSV pre-treated group, though also not statistically significant.

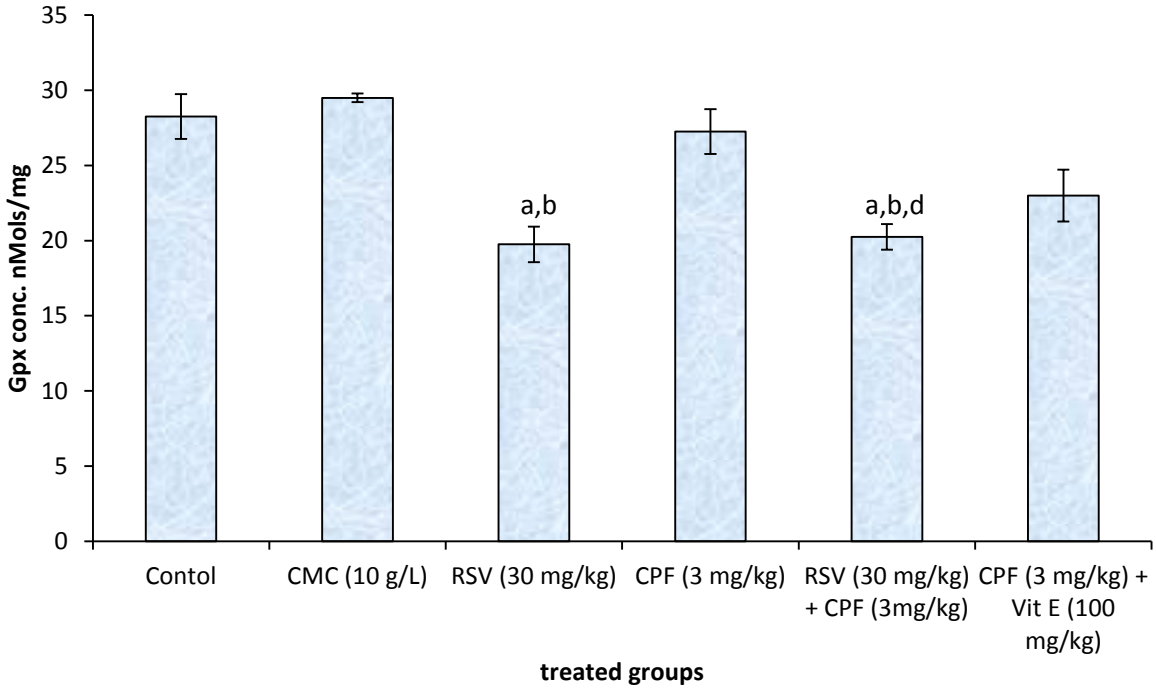

Figure 4.5: Hippocampal glutathione peroxidase concentration (nMols/mg) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days. Superscripts a, b and d indicate statistical significant difference ($P< 0.05$) when compared to Control, CMC and CPF respectively. Result presented as mean ± SEM; n= 5.

Key:

Control= normal saline

CMC= carboxymethylcellulose

RSV=resveratrol

CPF= chlorpyrifos

Vit E= Vitamin E

Figure 4.6: Effect of resveratrol on malondialdehyde (MDA) activity in the hippocampus of mice

Figure 4.6 showed the results obtained in the hippocampal malondialdehyde activity. A significant increase in malondialdehyde level in the CPF treated group when compared to the control. There was also a significant increase in malondialdehyde level in the RSV pre-treated group when compared to the control. A significant decrease was observed in malondialdehyde level in the RSV pre-treated group when compared to the CPF treated group. A significant decrease was also observed in the malondialdehyde level of the Vit E group when compared to the CPF treated group. There was a decrease level of malondialdehyde level in the Vit E group when compared to the RSV pre-treated group, though not statistically significant.

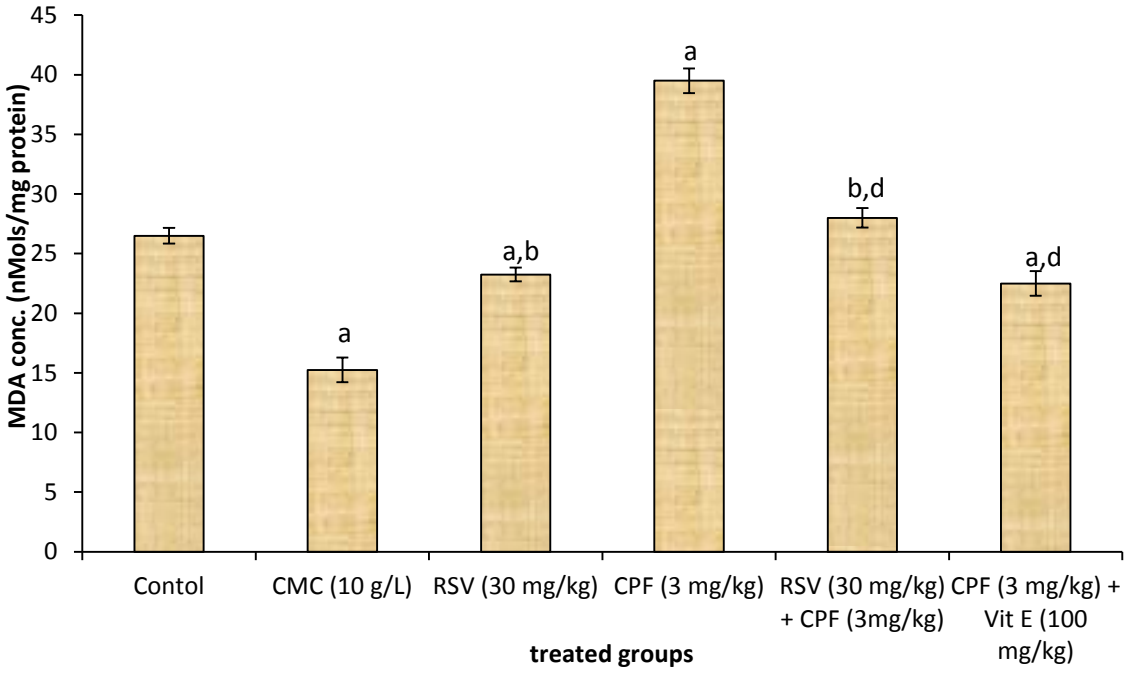

Figure 4.6: Hippocampal malondialdehyde concentration (nMols/mg) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days. Superscripts a, b and d indicate statistical significant difference ($P< 0.05$) when compared to Control, CMC and CPF respectively. Result presented as mean ± SEM; n= 5.

Key:

Control= normal saline

CMC= carboxymethylcellulose

RSV=resveratrol

CPF= chlorpyrifos

Vit E= Vitamin E

CHAPETR FIVE

5.0 DISCUSSION

This study was designed to evaluate the effect of resveratrol on chlorpyrifos-indued cognitive impairment in Swiss albino mice. The Y-maze and the Novel Object Recognition Test (Temporal order task) paradigms were employed to assess the cognitive functions of the mice.

Temporal order task is a method of assessing the recognition memory in rodents. This task comprised two training phases and one test trial. In each training phase, the subjects are allowed to explore two copies of an identical object. Different objects are used for training phases 1 (A1 and A2) and 2 (B1 and B2), with a delay between the training phases of 1 h. The test trial is given 3 h after training phase 2. During the test trial, an objects from training phase 1 (A1) and an objects from training phase 2 (B1) are used. The positions of the objects in the test and the objects used in training phase 1 and 2 are counterbalanced between the animals. If temporal order memory is intact, the subjects will spend more time exploring the object from training 1 (i.e., the object presented less recently) compared with the object from training 2 (i.e., the "new" object). The following parameters are analyzed: the time spent exploring each objects A1 and A2 in the training phase, the time spent exploring each objects B and A2 (object recognition) or objects A1n (A1in its new location) and A2 (object location) in the test phase. The data are expressed as the percentage (%) of time that the animals explore identical objects (tA2/[tA1 + tA2] x 100) during training and the % of time that the animals explore the novel object (tB/[tB + tA2] x 100) in the retention test (Novel Object Exploration -% Time) and total exploration time. The time percentage used for the novel object exploration is considered as an index of memory retention (Carlini, 2011).

The results of the Novel Object Recogntion test showed significant increase in the preference scores in the group that received resveratrol prior to chlorpyrifos (CPF) administration demonstrating improvement in the recognition memory when compared to the CPF treated group. This shows that administration of resveratrol improved recognition memory.

The Y-maze test is based on the innate preference of animals to explore an arm that has not been previously explored (Drewa, 1998). An alternation is defined as entry into all three arms consecutively (Hughes, 2004), for instance if the animal makes the following arm entries; A,C,C.A,B,C,A,C,B,A,B,C,A in this example, the animal made 13 arm entries 7 of which are correct alternations. The number of maximum spontaneous alternations is then the total number of arms entered minus two, and the percentage alternation is calculated as ((actual alternations /maximum alternations) x 100). A high alternation rate is indicative of sustained spatial working memory as the animals must remember which arm was entered last to not re-enter it (Hughes, 2004). Y-maze function is sensitive to damage in areas concerned with learning and memory functions such as the hippocampus.

The results of Y-maze percentage alternation scores demonstrated a rather decrease in the spartial working memory of the RSV pre-treated mice when compared to the CPF treated mice suggesting that resveratrol does not improve spatial working memory.

Lipid peroxidation is often assayed by measuring thiobarbituric acid reactive substances. The end products of lipid peroxidation, such as malondialdehyde assessment, have been widely used to indicate oxidative stress in many studies. Greilberger *et al.*, (2008) used malondialdehyde, carbonyl proteins and albumin-disulphide as oxidative stress parameters to test whether oxidative stress had a primary role in cognitive impairment and Alzheimer's disease. MDA is in many instances the most abundant aldehyde arising from lipid peroxidation (Verma and Srivastava, 2003) and hence is used as an index of oxidative

damage in tissues. The increased MDA concentration indicates an ongoing oxidative damage to the brain tissues. The consequence of CPF-induced increased lipoperoxidation in the tissues is biological dysfunctions as the brain is easily susceptible due to its inherent factors (Eren *et al.*, 2007) as a result of interference with their cellular integrities and alterations in their cytostrutural organizations. In contrast, malondialdehyde levels were decreased significantly after treatment with resveratrol in the hippocampus, suggesting that resveratrol could protect mice from chlorpyrifos-induced cognitive impairment through regulating malondialdehyde levels and inhibiting excessive lipid peroxidation.

The potential toxicity of free radicals is counteracted by a number of cytoprotective enzymes and antioxidants that limit the damage. This protective mechanism functions cooperatively in the form of a cascade, which includes various antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase.

The significant decrease in the hippocampal SOD activities in the CPF group has been reported previously in other tissues (Gultekin *et al.*, 2001; Tuzmen *et al.*, 2008). This indicates that OP either caused the reduction in the enzyme's synthesis or elevated its degradation or even caused its inactivation. Enzymatic antioxidant defence mechanisms are employed by the living organisms to alleviate the cellular assault resulting from interactions between cellular constituents and the ROS (Irshad and Chaudhuri, 2002). SOD is involved in the disputation of O_2 to H_2O_2 and molecular oxygen (Yu, 1994). The low hippocampal SOD activities may be related to the chronicity of the CPF exposure. The persistent increase in lipoperoxidation as exemplified by increased MDA concentration in the CPF group may have eventually resulted in a deficit SOD activities resulting from increased used and subsequent metabolic degradation. Furthermore, CPF-induced oxidative stress has been shown to lead to a shift in the expression of antioxidant genes (Slotkin *et al.*, 2007; Slotkin and Seidler, 2007;

2009). The decrease in the hippocampal SOD activities may also be related to the direct effect of CPF (Oncu *et al*., 2002). After treatment with resveratrol, superoxide dismutase activity was increased when compared with the CPF treated group, indicating that resveratrol protected against persistent increase in lipoperoxidation resulting from enhanced ROS formation thereby preserving the antioxidant defence system.

The significant decrease in the hippocampal CAT activities in the CPF group agreed with the result obtained by previous workers (Gultekin *et al.,* 2001; Altuntas *et al.*, 2002). CAT is involved in a variety of biochemical functions, but more primarily in the break-down of high levels of H_2O_2 (Betteridge, 2000; Abuja and Albertini, 2001) into H_2O and O_2, thereby aiding in the removal of the oxidant (Schneider and De Oliveira, 2004). The decrease in the CAT activity in the CPF group may be linked to a decrease in SOD activity, which reduces the rate of conversion of c to H_2O_2, the substrate for CAT. The lack of substrate for CAT activity eventually results in its reduced activity. Accumulation of O_2^-, through SOD activity inhibition has been shown to inhibit CAT activity (Kono and Fridovich, 1982), since O2•- converts feroxy state of CAT to ferryl state, which is an inactive form of the CAT (Freeman and Crapo, 1982).

GSH, a tripeptide synthesized from glutamate, cysteine, and glycine. It exerts protective function of cell survival against oxidative stress (Ghandi, 2012; Dasuri, 2013).This role suggests that chlorpyrifos-induced oxidative stress injury occurs by reducing glutathione levels, and that resveratrol could protect cognitive impairment in mice by increasing glutathione levels.

Supplementation with resveratrol and vitamin E have however been shown to reduced the level of hippocampal lipoperoxidation and improved SOD and CAT activities. This may be due to the antioxidant properties of resveratrol and vitamin E. Vitamin E, which is a chain

breaking lipophilic antioxidant, performs its free radical scavenging role within the cellular membrane. The lipophilic character of α-tocopherol enables it to locate itself in the interior of cell membrane lipid bilayer. There, it transfers a hydrogen atom with a single electron to a free radical, thus removing the radical before it can interact with the cell membrane (Krishnamoorthy et al., 2007; Ambali, *et al.*, 2009). The implication of this reduced lipoperoxidative changes in the brain tissues is that the antioxidant may mitigate the cognitive impairement challenges associated with CPF exposure. This is thought to be one way via which resvertrol performs its antioxidant activity. The radical scavenging ability of resveratrol may have preserved the antioxidant enzymes, therefore improving their activities.

The present study has shown that chronic CPF exposure causes oxidative changes in the hippocampal tissue. Therefore, we speculate that some of the cognitive deficits that have been previously associated with chronic CPF exposure may be partly due to this oxidative damage. And that supplementation with resveratrol has equally been shown in the present study to mitigate the oxidative damage to the hippocamal tissues and therefore consequently reduced the adverse cognitive dysfunctions outcome associated with CPF exposure.

CHAPTER SIX

6.0 CONCLUSION

In conclusion, 30mg/kg resveratrol suppressed memory impairment, decreased malondialdehyde levels, increased catalase activity, superoxide dismutase activity and glutathione levels in our chlorpyrifos-induced cognitive impairment mice model. The results confirmed the neuroprotective effects of resveratrol on CPF-induced cognitive impairment, and provided novel insights into the neuroprotective effects of resveratrol and its possible therapeutic role in cognitive impairment.

6.1 RECOMMENDATIONS

1. Further studies should be conducted to ascertain the definitive roles play by oxidative injury in causing cognitive dysfunction among CPF users.

2. Awareness campaign should be conducted regularly in the agricultural communities on the dangers and implications of organophosphate pesticides and the use of protective clothing (e.g. face mask, gloves, etc) should be encouraged among both farm and domestic users of chlorpyrifos and other organophosphate pesticides.

3. Further studies should be conducted to ascertain the benefits or otherwise of the long term intake of resveratrol and to check if its beneficial effects are dose-dependent or not.

4. Further studies should be conducted on the effects of long-term intake of resveratrol on other organ-systems of the body to ensure detrimental side effects are avoided.

6.2 CONTRIBUTIONS OF THE STUDY TO KNOWLEDGE

1. Ingestion of low dose of CPF over time causes cognitive dysfunction by inducing oxidative injury and lipid peroxidation in the brain cells.

2. Antioxidants supplementation might provide emerging targets and therapeutic strategies in the treatment of cognitive dysfunction.

REFERENCES

Abdollahi, M., Ranjbar, A., Shadnia, S., Nikfar, S. and Rezaie, A. (2004). Pesticides and oxidative stress: a review. *Medical Science Monitor,* 10 (6).

Abramov, A. Y., Scorziello A., and Duchen M. R. (2007). Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. *Journal of Neuroscience,* 27:1129–1138.

Abuja, P. M. and Albertini, R. (2001). Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins. *Clinical Chim. Acta.*, 306: 1-17.

Agarwal, B., Campen, M. J., and Channell, M. M., (2012). Resveratrol for primary prevention of atherosclerosis: Clinical trial evidence for improved gene expression in vascular endothelium. *International Journal of Cardiology,* pii:S0167.

Albani D, Polito L, and Batelli S. (2009). The SIRT1 activator resveratrol protects SK-N-BE cells from oxidative stress and against toxicity caused by alpha-synuclein or amyloid-beta (1-42) peptide. *Journal of Neurochemistry,* 110 (5):1445–1456.

Altuntas, I., Delibas, N. and Sutcu, R. (2002). The effects of organophosphate insecticide methidathion on lipid peroxidation and anti-oxidant enzymes in rat erythrocytes: Role of vitamins E and C. *Human and Experimental Toxicology*, 21: 681-685.

Ambali, S. F., Abbas, S. O., Shittu, M., Dzenda, T., Kawu, M. U., Salami, S. O. and Ayo, J. O. (2009). Effects of gestational exposure to chlorpyrifos on implantation and neonatal mice. *Journal of Cell Animal Biology,* 3(4): 050-057.

Ambali, S. F., Idris, S. B., Onukak, C., Shittu, M. and Ayo, J. O. (2010c). Ameliorative effects of vitamin C on short-term sensorimotor and cognitive changes induced by

acute chlorpyrifos exposure in Wistar rats. *Toxicology and Industrial Health*, 26(9): 547-558.

Ambali, S. F., Makinde, A. O., Shittu, M. and Adeniyi, S. A. (2012). Alleviating effect of *Phyllanthus niruri* on sensorimotor and cognitive changes induced by subacute chlorpyrifos exposure in Wistar rats. *African Journal of Medicine and Molecular Sciences,* 2(3): 50-58.

Antunes, M. Biala, G. (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive Process*, 13:93–110.

Athar M., Back, J. H. and Kopelovich L. (2009). Multiple molecular targets of resveratrol: Anti-carcinogenic mechanisms. *Arch Biochemistry and Biophysics,* 486(2):95–102.

Babior, B. M. (2004) NADPH oxidase. *Current Opinion Immuno*logy,16:42–47.

Back, J. H., Zhu, Y. and Calabro, A. (2012). Resveratrol-mediated downregulation of Rictor attenuates autophagic process and suppresses UV-induced skin carcinogenesis.*Photochemistry and Photobiology*, 88(5):1165–1172.

Baddeley, A. D. and Warrington, E.K. (1970). Amnesia and the distinction between long-term and short-term memory. *Journal of Verbal Learning and Verbal Behavior,* 9: 176-189.

Baddeley, A. Logie, R., Bressi, S., Della-Salla, S. and Spinler, H. (1986). Dementia and Working Memory. *Journal of experimental Psychology, A*. 38: 603-618.

Barker G. R. J., Bird F., Alexander V. and Warburton E. C. (2007) Recognition memory for objects, place, and temporal order: a disconnection analysis of the role of the medial prefrontal cortex and perirhinal cortex. *Journal of Neuroscience*, 27: 2948–2957.

Baxter M. G. (2010) ‘‘I’ve seen it all before’’: explaining age-related impairments in object recognition. *Behavioral Neuroscience,* 124:706–709

Bedard, K. and Krause, K. H. (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *PhysiologyReview*, 87:245–313.

Benefati, F. (2007). Synaptic plasticity and the neurobiology of learning and memory. *ACTA Biomedical*, 78 (1): 58-66.

Bengtsson, S. H., Gulluyan, L. M., Dusting, G. J. and Drummond, G. R. (2003). Novel isoforms of NADPH oxidase in vascular physiology and pathophysiology. *Clinical and Experimental Pharmacologyand Physiol*ogy, 30:849–854.

Betteridge, D. J. (2000). What is oxidative stress? *Metabolism*, 49: 3-8.

Bhandary, B., Marahatta, A., Kim, H. R. and Chae, H. J. (2012). Involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. *International Journal of Molecular Science,* 14:434–456.

Bolisetty, S. and Jaimes, E. A. (2013). Mitochondria and reactive oxygen species: physiology and pathophysiology. *International Journal of Molecular Science*, 14:6306–6344.

Bournival, J., Quessy, P. and Martinoli, M. G. (2009). Protective effects of resveratrol and quercetin against MPP^{+}-induced oxidative stress act by modulating markers of apoptotic death in dopaminergic neurons. *Cell and Molecular Neurobiology*, 29(8):1169–1180.

Brigelius-Flohé, R. and Traber, M.G. (1999). Vitamin E: function and metabolism". *FASEB Journal*, 13 (10): 1145–55.

Cadenas, E. and Davies, K. J. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free Radical Biology and Medicine,* 29:222–230.

Carlini, V. P. (2011). The Object Recognition Task: A New Proposal for the Memory Performance Study. *InTech*, 978-953-307-222-7

Chang, C. C., Chang, C. Y. and Huang, J. P. (2012). Effect of resveratrol on oxidative and inflammatory stress in liver and spleen of streptozotocin-induced type 1 diabetic rats.*China Journal of Physiology*, 55(3):192–201.

Clempus, R. E., Sorescu, D., Dikalova, A. E., Pounkova, L., Jo, P., Sorescu, G. P., Schmidt, H. H., Lassègue, B. and Griendling, K. K. (2007). Nox4 is required for maintenance of the differentiated vascular smooth muscle cell phenotype. *Arteriosclerosis, Thrombosis and Vascular Biol*ogy, 27:42–48.

Cui, J., Sun, R. and Yu, Y. (2010). Antiproliferative effect of resveratrol in pancreatic cancer cells. *Phytotherapy Research*, 24(11):1637–1644.

Czarniewska, E., Kasprzyk, A. and Ziemnicki, K. (2003). Effect of paraquat and metoxychlor on antioxidant enzymes in frog Rana esculenta L. liver. *Biology Letter,* 2:125-133.

Dasuri, K., Zhang, L. and Keller, J. N. (2013). Oxidative stress, neurodegeneration, and the balance of protein degradation and protein synthesis. *Free Radical Biology and Medicine,* 62:170–185.

Dercks, W., and Creasy, L. L. (1989a). The significance of stilbene phytoalexins in the Plasmopara viticola-grapevine interaction.*Physiologyand Molecular Plant Pathology*, 34 189–202.

Devaraj, S., Hugou, I. and Jialal, I. (2001). Tocopherol decreases CD36 expression in human monocyte-derived macrophages. *Journal of Lipid Research.* 42 (4): 521–527.

Dikalov, S. I., Dikalova, A. E., Bikineyeva, A. T., Schmidt, H. H., Harrison, D. G. and Griendling, K. K. (2008).Distinct roles of Nox1 and Nox4 in basal and angiotensin II-stimulated superoxide and hydrogen peroxide production. *Free Radical Biology and Medicine,* 45:1340–1351.

Drewa, G., Jak.bczyk, M. and Araszkiewicz, A. (1998). Role of free radicals in schizophrenia. *Medical Science Monitor,* 4(6): 1111-1115.

Ecobichon, D.J. (1996): Toxic effects of pesticides In: Casarette and Doull's toxicity. *The Basic Science of Poison*, 26.

Ellmark, S. H., Dusting, G. J., Fui, M. N., Guzzo-Pernell, N. and Drummond, G. R. (2005). The contribution of Nox4 to NADPH oxidase activity in mouse vascular smooth muscle. *Cardiovascular Research,* 65:495–504.

Eren, I., Naziroglu, M., Demirdas¸ A. Celik, O. and Uguz, A.C. (2007). Venlafaxine modulates -induced oxidative stress in brain and medulla of rat. *Neurochemistry Research*, 32: 497-505.

Farahat, F. M., Fenske, R. A., Olson, J. R., Garvin, K., Bonner, M. R., Rohlman, D. S., Farahat, T. M., Lein, P. J. and Anger, W. K. (2010). Chlorpyrifos exposures in Egyptian cotton field workers. *Neurotoxicology,* 31: 297-304.

Floreani, M., Napoli, E. and Quintieri, L. (2003). Oral administration of trans-resveratrol to guinea pigs increases cardiac DT-diaphorase and catalase activities, and protects isolated atria from menadione toxicity. *Life Science,* 72(24):2741–2750.

Freeman, B. A. and Crapo J. D. (1982). Biology of disease: free radicals and tissue injury. *Laboratory Investment*, 47: 412-426.

Fremont, L. (2000). Biological effect of resveratrol. *Life Science*, 66(8): 663-673.

Gaskin, S., Tardif, M., Cole, E., Piterkin, P., Kayello, L. and Mumby, D. G. (2010). Object familiarization and novel-object preference in rats. *Behavioral Process*, 83:61–71.

Gandhi, S. and Abramov, A. Y. (2012). Mechanism of oxidative stress in neurodegeneration. *Oxidative Medicine and Cell Longevity*, 42(80): 10-35..

Geon, H. K., Jieun, E. K. Sandy, J. R. and Sujung, Y. (2015). The Role of Oxidative Stress in Neurodegenerative Diseases.*Experimental Neurobiology*, 24(4): 325–340.

Gordillo, G., Fang, H., Park, H. and Roy, S. (2010). Nox-4-dependent nuclear H2O2 drives DNA oxidation resulting in 8-OHdG as urinary biomarker and hemangioendothelioma formation. *Antioxidants in Redox Signaling*, 12:933–943.

Görlach, A., Brandes, R. P., Nguyen, K., Amidi, M., Dehghani, F. and Busse, R. (2000).A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circle Research*, 87:26–32.

Greilberger J., Koidl C., and Greilberger M., (2008). Malondialdehyde, carbonyl proteins and albumin-disulphide as useful oxidative markers in mild cognitive impairment and Alzheimer's disease. *Free Radicals Research*, 42 (7):633–638.

Gultekin, F., Delibas, N., Yasar, S. and Kilinc, I. (2001). In vivo changes in antioxidant systems and protective role of melatonin and a combination of vitamin C and vitamin

E on oxidative damage in erythrocytes induced by chlorpyrifos-ethyl in rats. *Archive of Toxicology,* 75: 88-96.

Gupta, Y. K., Briyal, S. and Chaudhary, G. (2002). Protective effect of trans-resveratrol against kainic acid-induced seizures and oxidative stress in rats. *Pharmacology and Biochemistry Behavior,* 71(1-2):245–249.

Halliwell, B. (2006). Oxidative stress and neurodegeneration: where are we now? *Journal of Neurochemistry,* 97:1634–1658.

Harrison, R. (2002). Structure and function of xanthine oxidoreductase: where are we now? *Free Radical Biology and Medicine,* 33:774–797.

Harrison, R. (2004). Physiological roles of xanthine oxidoreductase. *Drug Metabolism Review,* 36:363–375.

Held, P. (2012). An introduction to reactive oxygen species: measurement of ROS in cells.*BioTek Instruments, Incorporation: Winooski, VT*.

Herrera, E. And Barbas, C. (2001). Vitamin E action, metabolism and perspectives. *Journal of Physiology and Biochemistry,* 57 (2): 43–56.

Higa, A. and Chevet, E. (2012). Redox signaling loops in the unfolded protein response. *Cell Signaling*, 24:1548–1555.

Higdon, J. (2006). Oxidative stress and cancer: have we moved forward? Role of Oxidative Stress in Parkinson's Disease. *Cytotechnology,* 9: 234-243.

Ho, D. J., Calingasan, N. Y., Wille, E. (2010). Resveratrol protects against peripheral deficits in a mouse model of Huntington's disease. *Experimental Neurology,* 225(1):74–84.

Hughes, R. N. (2004). The value of spontaneous alternation behavior (SAB) as a test of retention in pharmacological investigations of memory. *Neuroscience and Behavioral Reviews*, 28: 497–505.

Infanger, D. W., Sharma, R. V. and Davisson, R. L. (2006). NADPH oxidases of the brain: distribution, regulation, and function. *Antioxidant in Redox Signaling*, 8:1583–1596.

Irshad, M. and Chaudhuri, P. S. (2002). Oxidantantioxidant system: Role and significance in human body. *Indian Journal of Experimental Biology,* 40: 1233-1239.

Jaouad, B. and Torsten, B. (2010). Oxidative Medicine and Cellular Longevity. *Landes Bioscience*, 3:4, 228-237.

Juan, M. E., Vinardell, M. P., and Planas, J. M. (2002). The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful. *Journal of Nutrition*, 132, 257–260.

Kamel, F. and Hoppin, J. A. (2004). Association of pesticide exposure with neurologic dysfunction and disease. *Environmental Health Perspective,* 112(9): 950-958.

Kamel, F., Engel, L. S., Gladen, B. C., Hoppin, J. A., Alavanja, M. C. R. and Sandler, S. P. (2007). Neurologic symptoms in licensed pesticide applicators in the agricultural health study. *Human and Experimental Toxicology,* 26: 243-250.

Kangralkar, V.A., Patil, S. D. and Bandivadekar, R.M. (2010). Oxidative stress and diabetes: *International Journal of Pharmaceutical Applications*, 1(1): 38-45.

Kim, D., Nguyen, M. D. and Dobbin, M. M. (2007). SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. *EMBO Journal,* 26(13):3169–3179.

Kono, Y. and I. Fridovich (1982). Superoxide radical inhibits catalase. *Journal of Biology and Chemistry*, 257: 5751-5754.

Krishnamoorthy, G., P. Venkataraman, Arunkumar A., Vignesh R.C. and Aruldhas M.M. (2007). Ameliorative effect of vitamins (α-tocopherol and ascorbic acid) on PCB (Aroclor 1254) induced oxidative stress in rat epididymal sperm. *Reproductive Toxicology*, 23: 239-245.

Kumar, A., Naidu, P. S. and Seghal, N. (2007). Neuroprotective effects of resveratrol against intracerebroventricular colchicine-induced cognitive impairment and oxidative stress in rats. *Pharmacology*, 79(1):17–26.

Lambeth, J. D. (2004). NOX enzymes and the biology of reactive oxygen. *National Review on Immunology*, 4:181–189.

Lu, K. T., Ko, M. C. and Chen, B. Y. (2008). Neuroprotective effects of resveratrol on MPTP-induced neuron loss mediated by free radical scavenging. *Journal of Agricultural Food Chemistry,* 56(16):6910–6913.

Maheswari, E., Saraswathy, R.G. and Santhranii, T. (2015). Influence of vitamin E on hepatotoxicity and oxidative stress. *International Journal of Research in Pharmacy and Biosciences, 2 (3)*: 30-38.

Malhotra, J. D. and Kaufman, R. J. (2007). Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxidants in Redox Signaling*, 9:2277–2294.

Mancuso, M., Coppede, F., Migliore, L., Siciliano, G. and Murri, L. (2006). Mitochondrial dysfunction, oxidative stress and neurodegeneration. *Journal of Alzheimer's disease*, 10:59–73.

Mangina, C. A. and Sokolov, E. N. (2006). Neuronal plasticity in memory and learning abilities: theoretical revision and selective review. *International Journal of Psychophysiology,* 60: 203-214.

Mani, S. (2015). Production of reactive oxygen species and its implication in human diseases. Free radicals in human health and disease. *New Delhi: Springer*; 3–15.

Marambaud, P., Zhao, H. and Davies, P. (2005). Resveratrol promotes clearance of Alzheimer's disease amyloid-beta peptides. *Journal of Biology and Chemistry*, 280(45):37377–37382.

Masella, R., Di-Benedetto, R., Vari, R., Filesi, C. and Giovannini, C. (2005). Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *Journal of Nutrition and Biochemistry*, 16:577–586.

Matos, R. S., Baroncini, L. A. and Précoma, L. B. (2012). Resveratrol causes antiatherogenic effects in an animal model of atherosclerosis. *Arq Bras Cardiology*, 98(2):136–142.

McLennan, H. R., and Degli-Esposti M. (2000). The contribution of mitochondrial respiratory complexes to the production of reactive oxygen species. *Journal of Bioenergy and Biomembrane,* 32:153–162.

Mishra, R., Sellin, D. and Radovan, D. (2009). Inhibiting islet amyloid polypeptide fibril formation by the red wine compound resveratrol. *Chembiochem*. 10(3):445–449.

Nonomura, S., Kanagawa, H. and Makimoto, L. (1963). A. Chemical constituents of polygonaceous plants. I. Studies on the components of Ko-jo-kon (*Polygonumcuspidatum* Sieb. et Zucc.). *Yakugaku Zasshi*, 83: 988–990.

Novaes, R. D., Peluzio, M. C. and Maldonado, I. R. (2012). Resveratrol causes antiatherogenic effects in an animal model of atherosclerosis. *Arq Bras Cardiology*, 98(6):571–572.

Okano, H. Hirano, T. and Balaban, E. (2000).Learning and memory. *The National Academy of Science*, 97 (23): 12403-12404.

Olawale, O., Ikechuckwu, N. E. O., Grace, T. O. and Chidiebere, E. U. (2008). Oxidative stress and superoxide dismutase activity in brain of rats fed with diet containing permethrin. *Nigerian Society for Experimental Biology*, 20(2): 93-98.

Oncu, M., Gultekin F., Karaoz E., Altuntas I. and Delibas N. (2002). Nephrotoxicity in rats induced by chlorpyrifos-ethyl and ameliorating effects of antioxidant system. *Human and Experimental Toxicology*, 21: 223-230.

Orrenius S. (2007).Reactive oxygen species in mitochondria-mediated cell death. *Drug Metabolism Review,* 39:443–455.

Orsu, P., Murthy, B. V. and Akula, A. (2003) Cerebroprotective potential of resveratrol through anti-oxidant and anti-inflammatory mechanisms in rats. *Journal of Neural Transmission*……

Otitoju, O. and Onwurah, I. N. E. (2005). Superoxide dismutase (SOD) activity and serum calcium levels in rats exposed to a locally produced insecticide "Rambo insect powder. *Journal of Animal Research International*, 2:261-266.

Patten, D. A., Germain, M., Kelly, M. A. and Slack, R. S. (2010).Reactive oxygen species: stuck in the middle of neurodegeneration. *Journal of Alzheimer's disease*, 20(2):S357–S367.

Quinlan, C. L., Perevoshchikova, I. V., Hey-Mogensen, M., Orr, A. L. and Brand, M. D. (2013).Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox Biology,* 1:304–312.

Rawat, D. S., Singh, S. P., Sharma, L. D., Ahamad A. H. and Mehta, G. (2003). Residue analysis of some pesticides in poultry egg and mean samples in Garhwal region of Uttaranchal. *22nd Annual Conference of Society for Toxicology*, 23-24.

Ray, D. E., and Richards, P. G. (2001). The potential for toxic effects of chronic, low-dose exposure to organophosphates. *Toxicology Letter,* 120: 343–351.

Rigotti, A. (2007). Absorption, transport, and tissue delivery of vitamin E. *Molecular Aspects of Medicine,* 28(5–6): 423–36.

Ryan, J., Stanezky, F. Z., Dennerstein, L., Mark, W. J., Clark, M. S., Szoeke, C., Kldea, D. and Henderson, V. W. (2012). Hormone levels and cognitive function in postmenopausal midlife women. *Neurobiology of Aging*, 33: 617e11-617e22.

Schneider, C. (2005). Chemistry and biology of vitamin E. *Molecular Nutrition and Food Research.* 49 (1): 7–30.

Schneider, C. D. and De Oliveira A. R. (2004). Oxygen free radicals and exercise: Mechanisms of synthesis and adaptation to the physical training. *Review de Brasília Medical Esporte*, 10: 314-318.

Schrader, M. and Fahimi, H. D. (2006). Peroxisomes and oxidative stress. *Biochimica Biophysica Acta*. 1763:1755–1766.

Sebai, H., Sani, M. and Aouani, E. (2011). Cardioprotective effect of resveratrol on lipopolysaccharide-induced oxidative stress in rat. *Drug Chemistry and Toxicology*, 34(2):146–150.

Simão, F., Matté, A. and Pagnussat, A. S. (2012). Resveratrol prevents CA1 neurons against ischemic injury by parallel modulation of both GSK-3β and CREB through PI3-K/Akt pathways. *European Journal of Neuroscience*, 36(7):2899–2905.

Slotkin, T. A. and Seidler, F. J. (2007). Comparative developmental neuro-toxicity of organophosphates in vivo: Transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. *Brain Research*, 72: 232-274.

Slotkin, T. A. and Seidler, F. J. (2009). Oxidative and excitatory mechanisms of developmental neurotoxicity: Transcriptional profiles for chlorpyrifos, diazinon, dieldrin and divalent nickel in PC12 Cells. *Environmental Health Perspective*, 117: 587-596.

Slotkin, T. A., MacKillop, E. A., Ryde, I. T. and Seidler, F. J. (2007). Ameliorating the developmental neurotoxicity of chlorpyrifos: A mechanisms-based approach in PC12 cells. *Environmental Health Perspective*, 115: 1306-1313.

Song, P. and Zou, M. H. (2012). Regulation of NAD(P)H oxidases by AMPK in cardiovascular systems. *Free Radicals Biology in Medicine*. 52:1607–1619.

Song, P. and Zou, M. H. (2015). Roles of reactive oxygen species in physiology and pathology. Atherosclerosis: risks, mechanisms, and therapies. *Hoboken, NJ*: *John Wiley & Sons Incorporation*, 379–392.

Spanier, G., Xu, H. and Xia, N. (2009). Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1) and NADPH oxidase subunit (Nox4). *Journal of Physiology and Pharmacol*ogy, 60(4):111–116.

Squire, L. R. (1992). Memory and the Hippocampus: A synthesis from findings with rats, monkeys and humans. *Psychology Reviews*, 99: 195-231.

Squire, L. R. (2009). Memory and Brain systems. *Journal of Neuroscience*, 29: 12711-12716.

Steenland, K., Dick, R. B., Howell, R. J., Chrislip, D. W., Hines, C. J., Reid, T. M., Lehman, E., Laber, P., Krieg, E. F., and Knott, C. (2000). Neurologic function among termiticide applicators exposed to chlorpyrifos. *Environmental Health Perspective*, 108 (4): 293-300.

Takaoka, M. (1939).Resveratrol, a new phenolic compound from Veratrum grandiflorum.*Nippon Kagaku Kaishi,* 60:1090–1100.

Takaoka, M. J. (1940). Of the phenolic substances of white hellebore (*Veratrum grandiflorum* Loes. fil.). *Journal of Faculty Science, Hokkaido Imperial University*, 3: 1–16.

Terry Jr., A. V., Gearhart, D. A., Beck Jr., W. D., Truan, J. N., Middlemore, M., Williamson, L. N., Bartlett, M. G., Prendergast, M. A., Sickles, D. W. and Buccafusco, J. J. (2007). Chronic intermittent exposure to chlorpyrifos in rats: Protracted effects on axonal transport, neurotrophin receptors, holinergic markers, and information processing. *Journal of Pharmacology Experiments and Therapeutics*, 322: 1117-1128.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *Journal of Physiology*, 552:335–344.

Tuzmen, N., Candan, N. Kaya E. and Demiryas, N. (2008). Biochemical effects of chlorpyrifos and deltamethrin on altered antioxidative defense mechanisms and lipid peroxidation in rat liver. *Celullar and Biochemical Functions*, 26: 119-124.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*, 39:44–84.

Van-Buul, J. D., Fernandez-Borja, M., Anthony, E. C. and Hordijk, P. L. (2005). Expression and localization of NOX2 and NOX4 in primary human endothelial cells. *Antioxidants in Redox Signaling*, 7:308–317.

Verma, R. S. and Srivastava, N. (2003). Effects of chlorpyrifos on thiobarbituric acid reactive substances, scavenging enzymes and glutathione in rats tissues. *Indian Journal of Biochemistry and Biophysics*, 40: 423428.

Vijayakumar, T.M., Sirisha, G.B.N., Farzana-Begam, M.D. and Dhanaraju, M.D. (2012). Mechanism Linking Cognitive Impairment and Diabetes mellitus. *European Journal of Applied Sciences,* 4 (1): 01-05.

Villacorta, L., Graca-Souza, A.V., Ricciarelli, R., Zingg, J.M. and Azzi, A. (2003). α-Tocopherol induces expression of connective tissue growth factor and antagonizes tumor necrosis factor-α-mediated downregulation in human smooth muscle cells. *Circulatory Research.* 92 (1): 104–110.

Villaflores, O. B., Chen, Y. J. and Chen, C. P. (2012). Curcuminoids and resveratrol as anti-Alzheimer agents. *Taiwan Journal of Obstetrics and Gynecology,* 51(4):515–525.

Wang J, Zhang HY, Tang XC (2009). Cholinergic deficiency involved in vascular dementia: possible mechanism and strategy of treatment. *Acta Pharmacology Sin,* 30(7):879–888.

Warburton, D. M. (1995). Effect of caffeine on cognition and mood without caffeine abstinence. *Psychopharmacology,* 119, 66-70.

Wassmann, S., Wassmann, K. and Nickenig, G. (2004). Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. *Hypertension*, 44(4):381–386.

Whitney, E. and Rolfes, S.R. (2011). *Understanding Nutrition,* 12th edition. California: Wadsworth, Engaged Learning.

Widlansky, M. E. and Gutterman, D. D. (2011). Regulation of endothelial function by mitochondrial reactive oxygen species. *Antioxidants in Redox Signaling,* 15:1517–1530.

Wingler, K., Wünsch, S., Kreutz, R., Rothermund, L., Paul, M. and Schmidt, H. H. (2001). Up regulation of the vascular NAD(P) H-oxidase isoforms Nox1 and Nox4 by the renin-angiotensin system in vitro and in vivo. *Free Radical Biology in Medicine,* 31:1456–1464.

Wood, L. G., Wark, P. A. and Garg, M. L. (2010). Antioxidant and anti-inflammatory effects of resveratrol in airway disease. *Antioxidants in Redox Signaling*, 13(10):1535–1548.

Wu, J. M. and Hsieh, T. C. (2011). Resveratrol: a cardioprotective substance. *Annual New York Academy of Science,* 1215:16–21.

Wu, Z., Xu, Q. and Zhang, L. (2009). Protective effect of resveratrol against kainate-induced temporal lobe epilepsy in rats. *Neurochemistry Research,* 34(8):1393–1400.

Xu, Q. and Si, L. Y. (2012). Resveratrol role in cardiovascular and metabolic health and potential mechanisms of action. *Nutrition Research,* 32(9):648–658.

Yankovskaya, V., Horsefield, R., Törnroth, S., Luna-Chavez, C., Miyoshi, H., Léger, C., Byrne, B., Cecchini, G. and Iwata, S. (2003). Architecture of succinate dehydrogenase and reactive oxygen species generation. *Science,* 299:700–704.

Yu, B. P. (1994). Cellular defenses against damage from reactive oxygen species. *Physiology Review,* 74: 139162.

Zhang, F., Shi, J. S. and Zhou, H. (2010). Resveratrol protects dopamine neurons against lipopolysaccharide-induced neurotoxicity through its anti-inflammatory actions. *Molecular Pharmacology,* 78(3):466–477.

Zorov, D. B., Juhaszova, M. and Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiology Review,* 94: 909–950.

APPENDIX

Appendix I: Preference scores (%) and mean score (%) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days.

Treatment Groups	Preference Score (%)	Mean Score (%)
Control (Olive oil 2 ml/kg)	57.8 ± 2.24	55.44 ± 1.38
Carboxymethylcellulose (2 ml/kg)	44.99 ± 1.74^{a}	49.8 ± 0.39
Resveratrol (30 mg/kg)	64.47 ± 2.27^{b}	57.73 ± 3.05
Chlorpyrifos (3 mg/kg)	36.8 ± 0.93^{a}	63.91 ± 1.08^{a}
Resveratrol (30 mg/kg) + Chlorpyrifos (3 mg/kg)	$63.69 \pm 2.17^{b,c}$	51.53 ± 0.33^{b}
Vitamin E (100 mg/kg) + Chlorpyrifos (3 mg/kg)	$45.97 \pm 1.82^{a,c}$	57.94 ± 0.54

Appendix I: Hippocampal malondialdehyde concentration (nMols/mg) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days

Treatment Groups	MDA (nMols/mg)
Control (Olive oil 2 ml/kg)	26.50 ± 0.65
Carboxymethylcellulose (2 ml/kg)	15.25 ± 1.03[a]
Resveratrol (30 mg/kg)	23.25 ± 0.58[a,b]
Chlorpyrifos (3 mg/kg)	39.50 ± 1.04[a]
Resveratrol (30 mg/kg) + Chlorpyrifos (3 mg/kg)	28.00 ± 0.82[a,b]
Vitamin E (100 mg/kg) + Chlorpyrifos (3 mg/kg)	22.50 ± 1.04[a,d]

Appendix III: Hippocampal catalase (CAT) concentration (U/mg), superoxide dismutase (SOD) concentration (U/mg) and glutathione peroxidase concentration (nMols/mg) in Swiss albino mice treated with resveratrol, chlorpyrifos and Vitamin E for twenty one (21) days

Treatment Groups	CAT (U/mg)	SOD (U/mg)	GPx (nMols/mg)
Control (Olive oil 2 ml/kg)	66.00 ± 2.31	80.50 ± 1.56	28.25 ± 1.49
Carboxymethylcellulose (2 ml/kg)	82.25 ± 2.66[a]	79.50 ± 1.85	29.50 ± 0.29
Resveratrol (30 mg/kg)	66.00 ± 1.96[b]	81.25 ± 0.75	19.75 ± 1.18[a,b]
Chlorpyrifos (3 mg/kg)	74.25 ± 74.25	51.50 ± 0.65[a]	27.25 ± 1.49
Resveratrol (30 mg/kg) + Chlorpyrifos (3 mg/kg)	81.00 ± 0.58[a]	57.25 ± 1.8[a,b,d]	20.25 ± 0.85[a,b,c]
Vitamin E (100 mg/kg) + Chlorpyrifos (3 mg/kg)	60.25 ± 1.44[a,d]	56.75 ± 1.1[a,d]	23.00 ± 1.73