EFFECT OF VITAMINS C AND E ON MEMORY IN ADULT MICE

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DECLARATION

I declare that the work in the thesis, entitled: **"**Effect of vitamin C and E on memory in adult mice" was performed by me in the Department of Human Physiology, under the supervision of Dr. Rabiu A. Magaji and Dr. Yusuf Tanko.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any University.

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CERTIFICATION

This Thesis, entitled "**EFFECT OF VITAMIN C AND E ON MEMORY IN ADULT MICE**", by Sheba DARIYA (M.Sc/MED/4651/2010-2011) meets the regulations governing the award of degree of Master of Science of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my mother, Mrs. H.Z.S. Dariya, who believes in me and is a strong backbone in my pursuit of a career in Human Physiology. And in loving memory of my father- Mr. Z.S. Dariya, and sister- Miss Sophie Dariya, your memories are evergreen in my heart.

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ABSTRACT

Vitamins C and E are naturally present in some foods and are available as dietary supplements. While vitamin C is also known as L-ascorbic acid, vitamin E is a generic name for tocopherols and tocotrienols each with α, β, γ and δ subunits. Neurobehavioural models have been used to study behaviour in animals with models specific for each animal species and behaviour. The aim of the study was to investigate the effect of vitamins C and E on memory and serum biochemical changes in adult mice. Twenty male and twenty female mice weighing 16-35 g were divided into 5 groups of eight mice each. The first group served as the control and received distilled water (1 ml/kg); mice in the second group served as the positive control and received soya oil (1 ml/kg), animals in group three received vitamin C (100 mg/kg), group four received vitamin E (100 mg/kg) and the last group received both vitamins C and E (100 mg/kg). The drugs were given once daily orally for a period of 21 days. Learning and memory were assessed using the elevated plus maze (EPM), object recognition and location models for memory (ORT and OLT) at the end of the experimental period. Memory index was calculated. The mice were sacrificed on day 22 and serum estrogen and testosterone levels and catalase, superoxide dismutase and glutathione peroxidase activities were also evaluated. Lipid peroxidation was determined by measuring the malondialdehyde concentration in the brain sample. The relationship between the gonadal hormone levels and the performance of animals in each of the neurobehavioural models for memory was positive. There was no significant difference in the malondialdehyde concentration ($P < 0.05$) of all groups in the males but in females between the control and vitamin C-treated group. The activities of superoxide dismutase showed no significant difference in the males but showed significance ($P < 0.05$) between control and treatment groups in the females, catalase showed no significant difference in the females but showed significant difference ($P < 0.05$) between control and all groups and glutathione peroxidase showed no significant difference between all groups ($P < 0.05$) of both males and females. The memory index for the EPM also showed significant difference ($P < 0.05$) between the vitamin E-treated group and the soya oil and vitamin C-treated groups on the first day and between vitamin E and C-treated group on the second day in the females. In the males the significant difference was observed between control and treatment groups on both days. In the ORT model, discriminatory and recognitive index showed a significant ($P < 0.05$) difference in the vitamin E and E+C treated groups. In the OLT model discriminatory and recognitive indices showed a significant difference ($P < 0.05$) in the vitamin E and vitamins C+E treated groups. In conclusion, administration of vitamin C and E improved memory indices and there was a positive relationship between endogenous gonadal hormones and recognition.

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LIST OF ABBREVIATIONS

ROS	-	Reactive Oxygen Species
MDA	-	Malondialdehyde concentration
CNS	-	Central Nervous System
HRT	-	Hormone Replacement Therapy
EPM	-	Elevated Plus Maze
ORT	-	Object Recognition Test
OLT	-	Object Location Test
SOD	-	Superoxide Dismutase
CAT	-	Catalase
GPx	-	Glutathione Peroxidase
FR	-	Free Radical
RNA	-	Ribonucleic Acid
ROS	-	Reactive Oxygen Species
RNS	-	Reactive Nitrogen Species
DNA	-	Deoxyribonucleic Acid
PUFA	-	Polyunsaturated Fattty acids
CSF	-	Cerebrospinal Fluid
GST	-	Glutathione Sulphur Transferase
AA	-	Ascorbic Acid
USDA	-	United States Department of Agriculture
FDA	-	United States Food and Drug Administration
DV	-	Daily Value
GIT	-	Gastro-intestinal Tract
DHAA	-	Dehydroascorbic Acid
SVCT	-	Sodium-dependent vitamin C Transporter
GLUT	-	Glucose Transporter
ATP	-	Adenosine Triphosphate
IUPAC	-	International Union of Pure and Applied Chemistry
VLDLP	-	Very-Low Density Lipoprotein

HDL	-	High-Density Lipoprotein
USP	-	United States Pharmacopeia
RDA	-	Recommended Daly Allowance
ER	-	Estrogen Receptor
mRN	-	Messenger Ribonucleic Acd
chAT	-	Choline Acetyl Transferase
AD	-	Alzheimer's Disease
MAPK	-	Mitogen Activated Protein Kinase
IA	-	Inhibitory Avoidance
TL	-	Transfer Latency
D_1	-	Difference
DI	-	Discriminatory Index
RI	-	Recognitive Index
MWM	-	Morris Water Maze
Ach	-	Acetylcholine
RAM	-	Radial Arm Maze
WM	-	Working Memory
OVX	-	Ovariectomize
LP	-	Long-day Photoperiod
SP	-	Short-day Photoperiod
DHT	-	Dihydrotestosterone
ANOVA	-	Analysis of Variance
E_2	-	Estradiol

CHAPTER ONE

1.0 INTRODUCTION

Vitamin C, also known as L-ascorbic acid, is a water-soluble vitamin that is naturally present in some foods, added to others, and available as a dietary supplement and is destroyed by heat or reduced by prolonged storage (Weinstein *et al*., 2001). Humans, unlike most animals, are unable to synthesize vitamin C endogenously, so it is an essential dietary component (Naidu, 2003; Li, 2007). Sex steroids are hormones produced mainly by the reproductive glands, either the ovaries or testes, which share a similar basic structure of three hexane rings and a pentane ring (Gasbarri, 2010). The primary role of the gonadal steroid hormones in mammals is to regulate reproduction and related behaviours; however, both androgens and estrogens are also integrally involved in mediating higher brain function and processes, including cognition, neural development and neural plasticity (Dohanich, 2002). The hippocampal system plays an important role in memory function. Neurohormones like androgens and oestrogens are present in the hippocampus and have important roles in learning and memory (Talebi *et al.*, 2010).

Oestrogens act on the central nervous system (CNS) both through genomic mechanisms, modulating synthesis, release and metabolism of neurotransmitters, neuropeptides and neurosteroids, and through non-genomic mechanisms, influencing electrical excitability, synaptic function and morphological features. Therefore, oestrogen's neuroactive effects are multifaceted and encompass a system that ranges from the chemical to the biochemical to the genomic mechanisms, protecting against a wide range of neurotoxic insults (Genazzani *et al*., 2007).

Vitamin C, also known as ascorbic acid AA is required for the biosynthesis of collagen, L-carnitine, and certain neurotransmitters; vitamin C is also involved in protein metabolism (Carr and Frei, 1999). Collagen is an essential component of connective tissue, which plays a vital role in wound healing. Vitamin C is also an important physiological antioxidant (Carr and Frei, 1999) and has been shown to regenerate other antioxidants within the body, including alpha-tocopherol (vitamin E) (Jacob, 2002). In addition to its biosynthetic and antioxidant functions, vitamin C plays an important role in immune function (Jacob, 2002) and improves the absorption of non-haeme iron, the form of iron present in plant-based foods. Insufficient vitamin C intake causes scurvy, which is characterized by fatigue or lassitude, widespread connective tissue weakness, and capillary fragility (Weinstein *et al*., 2001; Wang, 2007).

Vitamin E is a generic name for tocopherols and tocotrienols. It is a family of α, β, γ and δ tocopherols and corresponding tocotrienols. Tocopherol contains saturated phytol side chains and tocotrienol have 3 double bonds in the side chain (Blatt *et al*., 2001; Dietrich *et al*., 2006). The alpha-tocopherol form of vitamin E is an important lipid-soluble antioxidant. In the brain and other tissues, alpha-tocopherol has a key role in preventing oxidant-induced lipid destruction and is, therefore, vital in maintaining the integrity of cell membranes (Blatt *et al*., 2001; Dietrich *et al*., 2006). Accordingly, vitamin E deficiency causes lipid peroxidation in brain tissues. Severe vitamin E deficiency results mainly in neurological symptoms, including impaired balance and coordination (ataxia), injury to the sensory nerves (peripheral neuropathy), muscle weakness (myopathy), and damage to the retina of the eye (pigmented retinopathy) (Traber *et al*., 2006). The

developing nervous system appears to be especially vulnerable to vitamin E deficiency. For instance, children who have severe vitamin E deficiency from birth and are not treated with vitamin E rapidly develop neurological symptoms. In contrast, individuals who develop malabsorption of vitamin E in adulthood may not develop neurological symptoms for ten to 20 years (Drake, 2011).

Gender differences in cognitive function tests have been widely reported: men tend to excel in fields of spatial cognition, whereas women show greater abilities in verbal fluency (Halpern, 1992). Many factors influence the development of these abilities, such as environment, education, cultural background and inherited factors, but sex hormones also play a role in development and maintenance of cognitive functions. Variation and overlapping scores between genders show the limited extent of this impact; to a much higher degree, an individual's cognitive abilities are derived from experience, education and inheritance (Zitzmann, 2001).

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

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

Biological systems have evolved endogenous defence mechanisms to protect against FR-induce cell damage (Singh *et al*., 2004). These are called antioxidants. Antioxidants were originally defined as any substance that when present in low concentration, compared with that of oxidizable substrate, significantly prevent the pro-oxidant-initiated oxidation of that substrate (Halliwell and Gutteridge, 1985; Black, 2004). However, because of the imperfection in the definition in relation to the concentration of antioxidants (Halliwell

and Gutteridge, 2007), Halliwell and Gutteridge (2007) redefined antioxidants as any substance that delays, prevents or removes oxidative damage to a target molecule. Higher organisms have developed a remarkably efficient antioxidants defense system over the course of evolution. The antioxidants neutralize FR by donating an electron to stabilize the FR (Chihuailaf *et al*., 2002), the antioxidant molecules "internalize" the loss of an electron through resonance between carbon bonds. The antioxidant systems can prevent oxidative damage by (Sen. 1995):

(a) Scavenging of reactive oxygen species (ROS) and other reactive derivatives;

(b) Decreasing the conversion of less reactive ROS to more reactive ROS

(c) Facilitating repair of damage caused by ROS; and

(d) Providing a favourable environment for the activity of other antioxidants

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

effectiveness varies with the stage of development and other physiological aspect of the organism (Halliwell and Gutteridge, 1999; Livingstone *et al*., 2001). The antioxidants require micronutrients such as selenium, zinc, iron, copper, and manganese as co-factors for optimum catalytic activity and effective defence mechanisms (Halliwell and Gutteridge, 1992; Halliwell, 2001).

1.1 Statement of Research Problem

The need to preserve cognitive functioning is present at every stage in life, whether at childhood when the focus is cognitive development or at adulthood when the need is protection or alleviation of short-term or long-term cognitive decline. This has led to promotion of the use of prescription drugs, herbal supplements, among others, in order to promote or preserve cognitive performance levels (Schmitt, 2010). Starting from adulthood, there is an age-related decline in the level of gonadal hormones, which has been correlated with impairment in some cognitive tasks (Leonard, 2011). Hence, the need for a substance that can promote cognitive development as well as protect against cognitive decline (Schmitt, 2010). Micronutrient supplementation has been shown to be associated with less cognitive decline (Morris, *et al*., 2002). It is reported that treatment with ascorbic acid significantly improved cognitive function in rats (Shahidi *et al*., 2008) and it ccould enhance learning and memory processes (Cho, *et al.*, 2003). While a lot of work has been done on the effects of antioxidant supplementation in improving cognitive functions in aged rodents and humans (Durga, *et al.*, 2007; Saleem, *et al.*, 2012) there is paucity of information on effects of vitamins C and E supplementation on serum testosterone and estrogen levels as well as on learning and memory in adult mice of both sexes. Thus, the question this study seeks to answer is whether vitamins C and E affect

the level of the testosterone and estrogen which in turn affect learning and memory, and whether endogenous levels of these hormones can be correlated to the optimum cognitive capacity observed in adulthood.

1.2 Justification

The maintenance of brain health underpinning intact cognition is a key factor to maintaining a positive, engaged, and productive lifestyle (Stough *et al.*, 2012). There is an age-related decline in the level of gonadal hormones, which has been correlated with impairment in some cognitive tasks such as learning and memory (Leonard, 2011).

It is reported that treatment with ascorbic acid significantly improved cognitive function in aged rats (Shahidi, *et al.*, 2008), and it can enhance learning and memory processes (Cho, *et al.*, 2003). Vitamin E intake, from foods or supplements, was also shown to be associated with less cognitive decline with age (Morris *et al.*, 2002). The combined effects of multiple antioxidant nutrients might be more influential on cognition than a single antioxidant as reported by Devore *et al.* (2010).

1.3 General Aim

The general aim of this study was to investigate the effect of vitamins C and E on learning and memory in healthy adult mice.

1.3.1 Specific objectives

The specific objectives of this work were as follows:

1. To assess the effect of administration of vitamins C and E on learning and memory using the elevated plus maze for memory (EPM), object recognition test (ORT) and object location test (OLT) in mice.
2. To determine the effect of vitamins C and E on lipid peroxidation; malondialdehyde (MDA), and enzymatic activities; superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in mice.
3. To relate the effect of endogenous testosterone and estrogen with animals performance in the EPM, ORT and OLT models for memory.

1.4 Hypothesis

Ho: Vitamins C and E have no effect on learning and memory in adult mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Oxidative Stress and the Nervous System

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

The sources of ROS in the brain include inefficiency of the electron-carrying components, the mitochondrial, nitric oxide synthase, arachidonic acid metabolism, xanthine oxidase, monoamine oxidase and cytochrome P_{450} enzymes (Schulz *et al*., 2000). Oxidants can be

produced essentially in all cells in the brain. For example the NADH oxidase, the superoxide generating enzymes in the phagocytes is expressed by microglial, astrocytes and the neurones (Tammariello *et al*., 2000). Although superoxide can lead to generation of OH^-through slow process of Fenton and Haber-Weiss reactions, respectively (Beckman, 1991), the toxicity of superoxide can be increased by its interaction with nitric oxide to produce Peroxynitrite ($ONOO^-$) that modifies protein to form nitrotyrosine, essential for the induction of neuronal injury (Brennan *et al*., 2002). The role of Fe^{2+} in the oxidative damage in the brain cannot be over-emphasized as it serves as catalyst in the formation of FR. For Fe^{2+} to perform its catalytic role, it must not be bound to protein. Since, unlike most extracellular fluids, the cerebro-spinal fluid (CSF) has low concentration of ferrous-binding protein, Fe^{2+} released from cells is more readily available to catalyze the formation of OH^-. Fe^{2+} can also donate electron to H_2O_2 (a non-radical, non-polar compound that can easily cross the membrane to from $OH^\bullet$ (Schmidley,1990).

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

Perry *et al*. (2002) has established a connection between antioxidant depletion and brain degeneration. Mutation of a-tocopherol transfer protein gene resulted in retinal degeneration and gait abnormalities in homozygous mice at one year of age, which

correlated with degeneration of the posterior column of the spinal cord (Yokota *et al*., 1998). In addition, mice devoid of SOD develop gait abnormalities at 12 days of age, associated with vacuolization in the brain stem and cortical region (Melov *et al*., 1998). These indicate that the loss of antioxidant gene can lead to neuronal degeneration.

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

2.2 Vitamin C in the Nervous System

Vitamin C accumulates in the central nervous system, with neurons of the brain having especially high levels (Bourr*e,* 2006*)*. Vitamin C is an important antioxidant that is required for the synthesis of the neurotransmitter, norepinephrine (Groff *et al*., 1995). Animal studies have shown that ascorbic acid protects the brain from damage by serving as a metabolic switch modulator in neuronal metabolism between rest and activation periods, helping the neuron to utilize lactate by stimulating the lactate transport instead of glucose (Castro *et al*., 2009; Narayanan *et al*., 2010).

Parle *et al*. (2003) reported that ascorbic acid (60, 120 mg/kg) injected for 3 and 8 consecutive days improved learning and memory of aged mice as indicated by decreased transfer-latency

and increased step-down latency. It also provided protection to the young animals from scopolamine and diazepam-induced impairment of memory. Narayanan *et al*. (2010) also observed that ascorbic acid (100 mg/kg) significantly prevented the neurobehavioural changes induced by Monosodium Glutamate (0.002 mg/kg) with the underlying mechanism of action as a memory enhancer being attributed to its antioxidant properties.

2.3 Vitamin E and Memory

Vitamin E supplementation has been shown to reduce cognitive impairment in rats, probably due to reduced oxidative stress in the cerebral cortex and hippocampus (Fukui, 2002). There is a link between endogenous plasma concentration of vitamin E and cognition status (Alzoubi *et al*., 2012). Vitamin E exerts its effect on memory through its antioxidant function by normalizing reductions in endogenous antoxidant enzymes like glutathione peroxidase and superoxide dismutase which are induced by mental stress, cerebral ischaemia and sleep deprivation among others (Aiguo *et al*., 2010).

The brain contains high levels of unsaturated lipids and is responsible for 20 to 25% of the body's oxygen consumption, conditions that favor the occurrence of oxidative stress (Drewa, 1998; Diniz *et al*., 2009). Quantitatively, vitamin E is the predominant encephalic lipophilic antioxidant, whose main function is to protect brain lipids from oxidative damage (Kontush and Schekatolina, 2004). Thus, vitamin E supplementation improves memory.

2.4 Sex Hormones and Memory

Sex hormones are linked with memory. The primary role of the gonadal steroid hormones in mammals is to regulate reproduction and related behaviours; however, both androgens

and estrogens are also integrally involved in mediating higher brain function and processes, including cognition, neural development and neural plasticity (Leonard and Winsauer, 2011). Cognitive ageing affects episodic memory and executive functions, and these vulnerable domains are postulated to be modulated by endogenous and exogenous estrogen exposures (Henderson and Popat, 2011). There is convincing evidence that steroid sex hormones play an organizational role in brain development, affecting cognition in men and women (Ulubaev *et al*., 2009). Specifically, ovarian steroids estradiol and progesterone have been shown to modify neuronal and synaptic architecture in a variety of brain regions: the hypothalamus, lateral septum, midbrain central gray and hippocampus (Sandstrom and Williams, 2001). The presence of high density of steroid hormone receptors in the structures of temporal lobe, involved in learning and memory, such as the hippocampus, perirhinal cortex, entorhinal cortex and amygdaloid complex, shows that there must be a direct relationship between gonadal hormones and organizational effects of steroid hormones in those structures during development of the nervous system (Guruzu *et al*., 2008). There is a theory that facilitation of learning and memory by the gonadal hormones is through modulation of the structure and function of the hippocampus. Indeed, the gonadal hormones do affect the density and growth of dendritic spines and synapses in the pyramidal cells of the hippocampus, and the growth and modification of these structures appear to be integral in the formation and maintenance of learning and memory processes (Geinisman *et al*., 2001; Kasai *et al*., 2003; Lang *et al*., 2004).

A study conducted by Barrett-Connor *et al*. (1999) supports an association between endogenous sex hormone levels and cognition in older men. Frye and Koonce (2007)

concluded based on the data obtained from their study that cognitive-enhancing effects of steroids may be mediated through ERα and or ERβ. Further confirming the conclusion of Rissman *et al*. (2002) that the cognitive effects of oestrogen are through the activity of ERα and ERβ.

The results of a study conducted on the effect of oestrogen and progesterone on scopolamine-induced impairment of spatial memory indicated that progesterone alone, or in combination with oestrogen, can improve the impairments of working memory and reference memory induced by scopolamine (Tanabe *et al*., 2004). The presence of estrogen receptors in the hypothalamus and frontal cortex has also led to the hypothesis that the hormone could protect against cognitive decline as seen in ageing (Rissman *et al*., 2002; Genazzani, *et al*., 2007). Recent findings suggest that testosterone modulates learning and memory in males through an interaction with the cholinergic system (Leonard, 2011). Results obtained from a study on the associations between serum testosterone fall and cognitive functions in prostate cancer patients suggest selective association between testosterone decline and cognition (Salminen *et al*., 2004).

2.4.1 Neurobiology of oestrogen

The identification and mapping of oestrogen receptors (ER) in the brain led to the discovery that they are concentrated in the hypothalamus, hypophysis, cerebral cortex, mid-brain, and brainstem (Micevych and Mermelstein, 2008). The nuclear ERs are ligand-activated transcription factors, belonging to the steroid hormone receptors and included in the nuclear receptor super family (Osterlund and Hurd, 2001). Two types of ERs are known: ERα and ERβ; which are similar in their structural organization into

domains, but differ in their binding affinities for diverse ligands and selective ER modulators (Gruber *et al*, 2002; Rehman and Masson, 2005). ERα and ERβ are products of different genes and show tissue- and cell-type specific expression (Pettersson and Gustafsson, 2001). Both ERs are widely distributed throughout the body (Rehman and Masson, 2005) and have also been localized in several cerebral areas, such as the cortex, amygdala, hippocampal formation, basal fore-brain, cerebellum, nucleus ceruleus, rafe, and central grey matter, confirming an involvement of oestrogen in controlling cognitive functions in both physiological and pathological conditions (Sherwin, 1997; Sherwin and Henry, 2008). The cerebral distribution of ERα has been well established by steroid autoradiography, immunocytochemistry, and hybridization (Osterlund and Hurd, 2001). Many studies have shown nuclear and extranuclear ERβ immunoreactivity in several brain regions, especially the hippocampus (Mitra *et al*, 2003; Milner *et al*, 2005).

The ERα mRNA expression prevail in the hypothalamus and amygdaloid complex, suggesting that the α-subtype could modulate neuronal populations, involved in autonomic and reproductive neuro-endocrine functions as well as emotional processes. On the contrary, in the thalamus, entorhinal cortex and neocortex, there is a prevalence of ERβ, indicating a putative role for ERβ in cognition, non-emotional memory and motor functions (Osterlund and Hurd, 2001). The co-localization of ERβ mRNA with cell nuclear ERβ immunoreactivity was revealed in the cerebral cortex, paraventricular nuclei and preoptic area of hypothalamus in the rat (Shughrue and Merchenthaler, 2001). Oestrogen effects on the brain include complex cellular mechanisms, ranging from classical nuclear to non-classical membrane-mediated actions. Both forms of cell signaling could be activated separately, even though there is evidence that they are

intertwined at several cellular instances and can influence each other reciprocally, yielding synergic effects (Genazzani *et al.*, 2007). Due to the widespread presence of the ERs in their different forms throughout the brain, oestrogen actions are also widespread and affect many neurotransmitter systems, including the cholinergic, catecholaminergic, serotonergic, and gamma-aminobutyric acid systems (McEwen, 2002). The influence of oestrogen on cerebral structures and functions offer possible explanations for the mechanisms of action by which this steroid hormone could affect cognitive functions in women. For example, it was reported that one of the effects of oestrogen is to enhance the density of dendritic spine on CAl hippocampal neurones within 24–72 h after its acute administration (Woolley *et al.*, 1990). Moreover, oestrogens increase the concentration of choline acetyltransferase critically involved in memory functions and whose levels are markedly decreased in Alzheimer's disease (Gibbs and Aggarwal, 1998). The neuroprotective action of oestrogen could also be exerted through a modulator effect on molecules involved in apoptosis (Pike, 1999).

2.4.2 Oestrogen and cognition

The term cognition indicates the totality of human information processing, including psychomotor skills, pattern recognition, attention, language, learning and memory, problem-solving, abstract reasoning or higher-order intellectual functioning. In female mammals, including rodents and non-human primates, oestrogen effects on non-reproductive behaviours include, anxiety, depressive-like and cognitive behaviours (Spencer *et al.*, 2008; Walf and Frye, 2006) When administered to ovariectomized rats, estradiol decreased anxiety and depressant behaviour in laboratory tests (Walf and Frye, 2006).

2.4.3 Oestrogen in learning and memory

Studies in knock-out mice, using the selective oestrogen receptor modulators, suggest that ERα and ERβ contribute differently to memory mechanisms (Rissman *et al*, 2002; Rhodes and Frye, 2006). Several studies have shown oestrogen regulation of ERα (Hart *et al*., 2001; 2007). There is scientific evidence that rapid improvements in cognition could be mediated by membrane-associated oestrogen receptors, activating mitogen-activated protein kinase signaling pathways in specific neural sites (Bryant *et al*., 2006). For example, oestrogen enhances performance in tasks such as inhibitory avoidance (IA), object recognition and placement within 4 h of treatment; a post-training paradigm evidenced that these effects are due to the facilitatory action of oestrogen, on memory (Rhodes and Frye, 2006; Walf and Frye, 2006; Frye *et al*., 2007; Luine, 2008).

Previous memory studies hypothesized that newly-acquired information is transferred to long-term memory over time, and work done by McGaugh (2000) has shown that consolidation takes place within 1–2 h post-training. In addition, the impairment or improvement of the consolidation process due to drugs or hormones can occur; if they are given within this time, but not later. Oestrogen-related enhancement of consolidation, utilizing post-training paradigms have been shown in some memory tasks, such as Morris water maze (MWM), Inhibitory Avoidance (IA), Object Recognition and Object Placement (Rhodes and Frye, 2006; Walf and Frye, 2006; Frye *et al*., 2007; Luine *et al*., 2008). Administration of the powerful oestrogen agonist, diethylstilbestrol, either immediately before or immediately after the presentation of objects, increased

discrimination between previously viewed and never viewed items in the recognition trial. Therefore, temporal relations between hormonal application and performance enhancements are in agreement with memory improvement (Frye *et al*., 2007).

Oestrogen not only modulates memory formation and maintenance processes in some contexts, but also biases the learning strategy utilized to solve a task. Thus, oestrogen changes what and how information is learned, and therefore not only how much is learned, that is, the strength of the memory (Gasbarri *et al.*, 2010, Pompili *et al.*, 2010). Rats with high estrogen levels out-perform hormone-deprived rats on tasks requiring the configuration and use of extra-maze cues for successful completion. On the contrary, rats with low estrogen levels tend to use response or egocentric strategies on tasks where the use of a directional turn, for example left or right, is required for acquisition (Korol, 2004). Taking into account the actions of oestrogen across a large range of neural systems, its modulation on cognition could be exerted by altering the relative involvement of specific memory systems, acting much like a conductor, orchestrating the dynamics, timing and coordination of multiple cognitive strategies during learning (McGaugh, 2000).

Influences on neurotransmitters, such as acetylcholine (ACh), regulating other processes, like inhibitory tone and excitability, reflect one of the mechanisms by which oestrogen may orchestrate learning and memory. In fact, the ACh system is also activated by oestrogen in cerebral areas that are important for memory, such as the basal fore-brain and its ACh-containing projections to the hippocampal formation and frontal cortex (Gibbs *et al*., 2004; Luine, 2008). Even though gonadal hormones influence cognition, these

hormone-induced changes are not large (Luine, 2008), and they occur especially when function is compromised by ageing or lesions (Gulinello *et al*., 2006; Scharfman *et al*., 2007). However, they do not improve all the different aspects of cognition such as, for example, acquisition during memory processes (Dohanich, 2002; Luine, 2007).

Rodents have been evaluated in different tasks, utilizing several kinds of mazes, and they rely on diverse re-inforcements or contingencies (positive food rewards or aversive electric shocks) for the learning phase. The tasks measure different kinds of memory, such as spatial memory, which requires the establishment of relationships between distant cues in the environment and the reinforcement site (Gasbarri *et al*., 2012). Other tasks use visual memory, based on visual associations. Nonetheless, many studies show positive effects of oestradiol on cognition (Dohanich, 2002). Spatial memory, which is dependent on the hippocampal formation, has been extensively evaluated using the radial arm maze (RAM) and morris water maze (MWM). Studies conducted in ovariectomized subjects show enhancements in performance during the acquisition (Dohanich, 2002; Luine *et al*., 1998); but, after learning how to solve the task (reference memory), estradiol no longer enhances performance (Luine *et al*., 1998; Fader *et al*., 1999) and could even impair spatial memory (Dohanich 2002). Consistent estrogen-related improvements are reported in studies utilizing spatial tasks for the evaluation of working memory (Daniel and Dohanich, 2001; Sandstrom and Williams, 2001, 2004; Scharfman *et al*., 2007; Luine, 2008). Working memory is defined as the ability to retain information in the face of potentially-interfering distraction, in order to guide behaviour and make a response (Baddeley, 1992, 1998).

Results of studies, assessing hormonal effects on learning and memory, have shown evidence on the importance that context and or experience can have on performance, and these considerations may account, at least in part, for some inconsistency in the literature. Therefore, it is hypothesized that stress experienced during task performance may interfere with oestrogen enhancements of some spatial tasks (Frick *et al*., 2004; Engelmann *et al*., 2006). Recognition memory tests, where subjects have to discriminate between familiar and unfamiliar objects or objects in familiar or unfamiliar locations, appear to be quite consistently improved by oestrogen and its agonists in ovariectomized (OVX) rats (Luine, 2008) or mice (Fernandez and Frick, 2004; Li, 2004). In agreement with OVX models, pro-oestrous rats show evidence of better recognition memory compared to rats in a different phase of the oestrous cycle (Frye *et al*., 2007; Walf and Frye, 2006), and mice show better spatial memory in the pro-oestrous phase (Frick *et al*., 2001). However, rats in pro-estrous phase are often impaired during acquisition (Frye, 1995; Warren and Juraska, 1997; Bowman *et al*., 2001). Other researchers did not show modifications over the cycle (Stackman *et al*., 1997). This inconsistency could be explained taking into account that the evaluated reference memory, seems to be insensitive to hormones after acquisition.

2.4.4 Testosterone and cognition

Similar to oestradiol, testosterone also maintains normal dendritic spine synapse density on CA1 pyramidal cells in the hippocampus of male rodents (Leonard and Winsauer, 2011). Photoperiodic changes in testosterone concentrations are correlated with variation in hippocampal spine density as well. In seasonally breeding rodents, testosterone concentrations are elevated during the breeding season and under long-day photoperiod

(LP) conditions, whereas during the non-breeding season and under short-day photoperiod (SP) conditions testosterone concentrations are at their nadir (Pyter *et al*., 2005). Thus, testosterone in males can modulate certain structural components of the hippocampus in a manner similar to oestradiol in females, through both organizational and activational mechanisms. Moreover, it appears that these effects are mediated through testosterone's action at androgen receptors (either directly and/or through conversion to dihydrotestosterone) rather than at oestrogen receptors via aromatization to estradiol (Leonard and Winsauer, 2011). Positive associations have consistently been observed between endogenous testosterone and various tests of visuospatial ability in young men (Hooven *et al*., 2004; Driscoll *et al*., 2005; Thilers *et al*., 2006)

Research has shown that a reduction in testosterone concentration as a result of gonadectomy can disrupt various spatial working memory processes in male rodents, demonstrating that such processes are dependent on the presence of circulating testosterone. These findings parallel the effects of ovariectomy on spatial working memory in female rodents in that the subsequent loss of oestrogens after ovariectomy often negatively affects the performance of tasks that are dependent on spatial working memory (Sandstrom and Williams, 2001). There is difficulty in ascertaining the mechanisms by which testosterone affects learning and memory in that testosterone is readily metabolized by the 5α-reductase enzyme to dihydrotestosterone (DHT), which also binds to androgen receptors (Andriole *et al*., 2004), and is reported to have higher affinity (Wilbert *et al*., 1983) and efficacy at androgen receptors than testosterone (Deslypere *et al*., 1992). Thus, there is the possibility that testosterone could affect

cognitive functioning not only directly but also indirectly via conversion to an active metabolite such as DHT (Leonard and Winsauer, 2011).

Testosterone may also mediate learning and memory through aromatization to estradiol by the aromatase enzyme (Naftolin, 1994). The effect of oestradiol on learning and memory in males has been reported to range from enhancement to impairment of responding on certain tasks. For instance, oestradiol attenuated the acquisition deficits shown after gonadectomy in male rats (Kritzer *et al*., 2007).

2.4.5 Gonadal hormones and the cholinergic system

The neurotransmitter, acetylcholine (ACh), has been implicated in the modulation of various learning and memory processes by a large number of studies (Gold, 2003; Hasselmo, 2006). In general, increased ACh release in areas of the brain such as the hippocampus and prefrontal cortex is associated with enhanced response under various learning and memory tasks (Fadda *et al*., 2000; Hironaka *et al*., 2001; Arnold *et al*., 2002; McIntyre and Pal *et al*., 2002), whereas decreased synaptic levels of ACh are associated with reduced response (Lehmann *et al*., 2002). The effects of testosterone in males may be mediated largely through potentiation of cholinergic neurotransmission in the septo-hippocampal pathway and other related brain areas (Mitsushimet *et al*., 2009, 2010).

2.5 Cognition

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; that is, pattern recognition and

language), abstract reasoning, problem solving and psychomotor skills (Sherwin, 2003; Heury and Min, 2010).

2.6 Memory

Memory is a critical aspect of cognition. It is composed of numerous component processes that localize to different anatomical sites (Squire, 1992). Memory is defined as a behavioural 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 visual memory implies that the visual memory trace decays after a fraction of a second to several seconds (Baddeley and Warrington, 1970), and long-term visual memory describes the ability to identify a visual stimulus for days, months, or years after exposure. The same is true for verbal memory; that is, short-term verbal memory describes the situation in which a verbal stimulus (for example, a word) is held in memory for seconds, whereas long-term verbal memory describes the ability to recognize or remember a verbal stimulus or association over an extended period of time (Sherwin, 2003). Physiologically, memories are stored in the brain by changing the basic sensitivity of synaptic transmission between neurones as a result of previous neural activity (Kandel, 2009).

2.6.1 Classification of memory

Two major types of memory exist, one for skills and one for knowledge.

1. **Implicit/Non-declarative memory**: This refers to information storage to perform various reflexive or perceptual tasks. It is referred to as non-declarative or implicit memory because it is recalled unconsciously. When implicit memory is used, it acts automatically and there is no awareness of recalling memory traces. Implicit

memory is a heterogeneous collection of memory functions and types of learned behaviours such as reflexive learning (sensitization, habituation), classical conditioning, fear conditioning, procedural memory (for skills and habits) and priming (the recall of words or objects from a previous unconscious exposure to them) (Okano *et al.*, 2000; Kandel, 2009).

2. **Explicit/Declarative memory**: This second form of memory is called declarative or explicit memory because it is recalled by a deliberate and conscious effort. It concerns factual knowledge of persons, things, notions and places. Declarative memory can be further classified as episodic or autobiographic memory and semantic memory (Benfenati, 2007).

Episodic memory refers to the ability to recall in a deliberate manner recent episodes or events. 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. Normal episodic memory performance depends particularly on integrity of the hippocampus and adjacent areas of the medial temporal lobes (Squire, 2009). It allows indivituals to remember personal events and experience, and, being a link between what the indivitual's present, history and, gives one the sense of individuality. On the other hand, 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 and rapidly in the absence of recall and refreshing, implicit memory is much more robust and may last for the entire life-span, even in the absence of further practice (Benfenati, 2007).

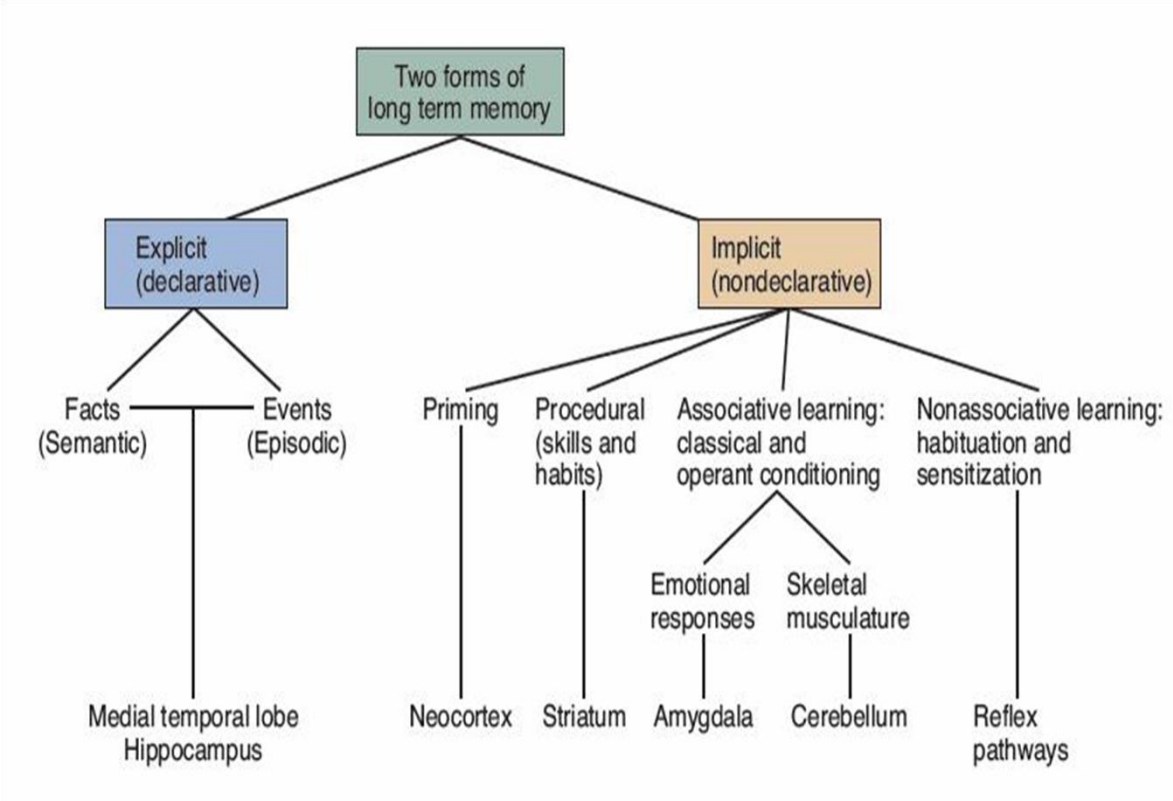


Figure 2.6.1: A schematic diagram showing the two major divisions of memory and the brain areas involved in them. (Benfenati, 2007)

Memory is also classified based on duration as follows:

1. ***Short-term memory***, which includes memories that last for seconds or at most minutes. They may be converted into longer-term memories during which processing in the hippocampus and elsewhere lays down long-term changes in synaptic strength.
2. ***Intermediate long-term memories***, which last for days to weeks, but then fade away.
3. ***Long-term memory***, which, once stored, can be recalled up to years or even a life-time later (Ryan, 2012).

Working memory is a form of short-term memory that keeps information available, usually for very short periods, while the individual plans action based on it (Ryan, 2012). It also 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 with such tests as digit span backward, Letter-Number Sequencing, serial

addition, n-back tests, and Brown-Peterson distraction tests (Baddeley *et al*., 1986; Ryan *et al*., 2012).

2.7 Learning

Learning is defined as the processes involved in acquiring memory (Okano *et al*., 2000). It is the alteration of behaviour or modification of innate responses by experience and training. The ability to learn is a fundamental characteristic of man and most animals (Hojjatallah, 2002). There are two principal types of learning processes as follows:

1. **Classical conditioning (passive) learning**: During passive learning, the subject plays a relatively involuntary or passive role, hence, this type of learning is considered to represent a relatively primitive, simple or generalized learning.
2. **Operant conditioning (active) learning**: During operant conditioning also known as trial-and-error learning, the subject exerts a considerable level of voluntary control so that this type of conditioning is considered to represent learning at a much "higher" level than classical conditioning (Hojjatallah, 2002).

2.8 Sex Differences in Cognitive Functions

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

These sex differences in cognitive functioning are thought to occur as a result of the exposure of the foetal 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 foetal life, perhaps by directing the development of certain neural pathways. Post-pubertally, circulating levels of a given sex hormone 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 psycho-endocrine theory proposes that during prenatal life, the presence of significant quantities of a sex hormone organizes neural substrates for a certain behaviour or function that becomes manifest after puberty under the influence of high circulating levels of that same hormone (Sherwin, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Site of Experiment

The experiment was carried out in the Physiology Research Laboratory of the Department of Human Physiology and the Neurobehaviour Research Laboratory of the Department of Pharmacology, Ahmadu Bello University, Zaria, (11^0 $10^/$ N, 07^0 $38^/$ E) at the elevation of 650 m above the sea level (Zaria) located in the Northern Guinea Savannah zone of Nigeria (Akpa *et al*., 2002), with an annual rainfall of 1107 mm (Abdulwahab *et al*., 2010). The experiment started on the 26th of April 2013 and terminated on the 17th of May 2013.

3.2.1 Experimental animals

Forty (40) eight-week-old albino mice of both sexes, weighing between 16 and 35 g used for this study were obtained from the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria.

3.2.2 Housing and grouping

The animals were kept in the Animal House of the Department of Human Physiology, Ahmadu Bello University, Zaria for two weeks to acclimatize before they were used for the experiment. They were fed on pellets made from grower mash and water *ad libitum.*

The animals were randomly divided into five groups of eight with four males and females each as follows:

Group I: Control - Animals were orally administered with distilled water at 1 ml/kg.

Group II: Positive Control - Animals were orally administered with soya oil per body weight 1 ml/kg.

Group III: Vitamin C - Animals were orally administered 100 mg/kg of vitamin C each (Bashandy, 2006; Narayanan et al., 2010).

Group IV: Vitamin E - Animals were orally administered 100 mg/kg of Vitamin E each (Knight and Roberts, 1985; Bashandy, 2006).

Group V: Vitamins C and E - Animals were orally administered 100 mg/kg each of both vitamins (Bashandy, 2006).

The regimens were given for a duration of three weeks (Narayanan *et al*., 2010

3.3 Drugs and Soya Oil Preparations

3.3.1 Drugs and preparation

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

Vitamin E (100 mg/capsule) with brand name ACAVITAMIN E (Medizen Pharmaceutical Industries for ACA PHARMA-Cairo-Egypt) was obtained from a reputable Pharmacy store in Kaduna, Nigeria. Prior to daily use each capsule was aspirated into a syringe and then reconstituted with soya oil to 100% v/v.

Soya oil (Grand Cereals and oil Mills Limited, Jos, Nigeria) was obtained from a provision store in Samaru market, Zaria, Nigeria.

3.4 Neurobehavioural Assessments

3.4.1 Elevated plus maze for memory (EPM)

The elevated plus maze was used to evaluate long-term spatial memory (Itoh *et al.,* 1990). The elevated plus maze for mice consists of two perpendicular open arms (21.5 x 7.5 cm) and two closed arms (21.5 x 7.5 x 20 cm), which were extended from the central (7.5 x 7.5 cm) platform. The platform and floor was made from wood, and the lateral walls of the closed arms were made of wood painted black. The maze was elevated 38 cm above the floor as illustrated in plate I. On the 1st day (aquisition), each animal was placed at the end of one open arm, facing away from the central platform. The latency of the mouse to move from the open to the enclosed arms within 90 seconds was recorded. Following entry into the arm, the animal was allowed to explore the apparatus for 30 s (Blatt and Takahashi, 1998).

Twenty-four hours later, the second trial (retention test) was performed and recorded for each animal. If the animal failed to enter into the enclosed arms within 90 s, it is gently pushed into the enclosure and a transfer latency of 90 s is then recorded for that animal. After each trial

the maze was cleaned by wiping with a cloth dipped in 70% ethyl alcohol and allowed to dry to remove any olfactory cue (Ninan, 1999).

3.4.2 Object recognition test (ORT)

Object recognition test was performed by the method described by the Ennaceur and Delacour, (1988); De Rosa *et al*. (2005) and Zhao *et al*. (2007). The test apparatus was a transparent, open-field plastic box (60 cm × 50 cm × 40 cm) placed in the laboratory during day light. Discriminated objects (A, B and C) used were identically-sized objects. Objects A and B were transparent cylindrical containers filled with sand to ensure stability, whereas object C was a blue cuboid box of approximately equal size with objects A and B (figure 3.3.2; plate II & III). Each of the three objects was sufficiently heavy to prevent the mice from moving it.



Plate I: The Elevated Plus Maze neurobehavioural model for mice

The ORT consisted of two phases: the sample trial and the test trial. In order to acclimatize the mice, each mouse was first placed into an empty box for 5 minutes. Subsequently, during the sample trial the two identical objects A and B known as familiar objects were placed on opposite sides of the apparatus. To start a 5-minute period of exploration, each animal was placed in the middle of the two objects. After this period, the mice were returned to their home cages for an interval of 24 hours (Ennaceur and Delacour, 1988; Fernandez *et al*., 2005; Wang *et al*., 2009; De Oliveira *et al*., 2010; Antunes and Biala, 2012).

During the test trial, object B was replaced by object C, which is novel to the mice and different from either object A or B. The mouse was then left to explore objects A and C for 5 minutes. The animals were observed and the exploration time, of the sample and test phases for each of the three objects were recorded. Thereafter then difference of time

spent between familiar and novel object was computed as well as the discriminatory and recognitive indices using the following formulae:

I Difference

The difference D_1 is the first measure for habituation. It is the difference in exploration time for novel versus familiar object as shown in the formula above (Ennaceur and Delacour, 1988; Wang *et al*., 2009).

Difference $\mathbf{(D_1) = T_n - T_f}$

Where $\mathbf{T_n}$ = Time (s) spent with new object.

$\mathbf{T_f}$ = Time (s) spent with familiar object

II Discriminatory Index (DI)

This is the second index measured. It quantifies discrimination between the novel object and familiar objects. It was computed as follows:

$$\mathbf{DI = T_n - T_f \div T_n + T_f}$$

T_n and T_f are same as above.

III Recognition Index (RI)

This is the percent of time spent exploring the novel object relative to the total time spent exploring both objects. This is the measure of novel object recognition. It is the main index of retention. It was calculated, thus:

$$\mathbf{RI = T_n \div T_n + T_f}$$

Where T_n and T_f are same as above.

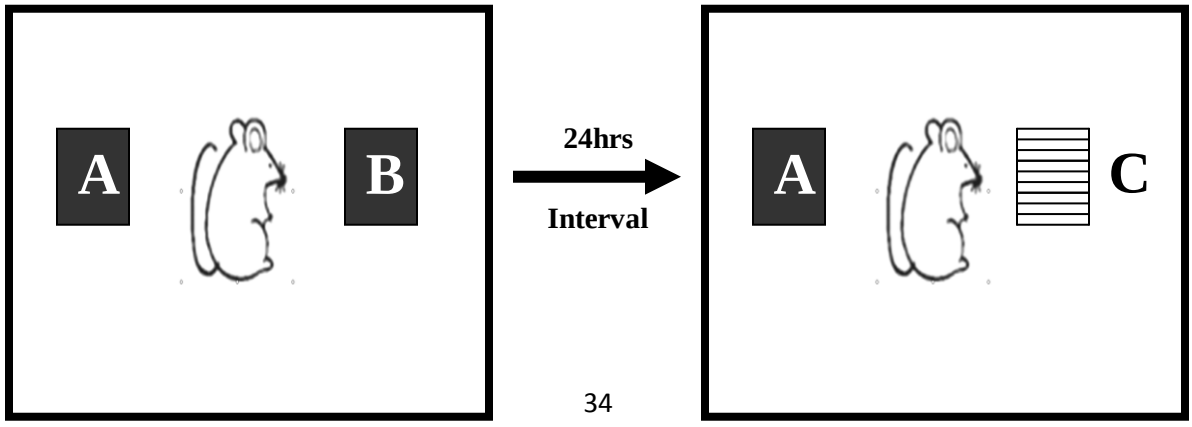
Note - However, that the formulae for calculating indices for OLT are same as that of ORT, but T_n becomes T_R, and T_f becomes T_L; where T_R is the exploration time of object on the right side, and T_L is the exploration time for object on the left (Ennaceur and Delacour, 1988).

The ORT and OLT models for memory are functional on the basis of the innate tendency of rodents to differentially explore novel objects over familiar ones (Arque *et al*., 2008).

3.4.3 Object location test (OLT)

The apparatus used for this test was the same box that was used in the ORT. The discriminated objects, D and E, were two identically sized cylindrical containers. The period of acclimation was performed as in the ORT. In the sample trial, objects D and E were placed in the apparatus as described in the ORT. After 5-min object exploration, the mice were returned to their home cage for a 24-hour interval. Subsequently, in the test trial, object E was moved to a location that was diagonally opposite to object D, and the mouse was left in the box for 5-min exploration (figure 3.4.3; plate IV).

ORT Protocol



Sample Phase Trial (5 mins) **Test Phase Trial (5 mins)**

Figure 3.3.2: Schematic representation for object recognition test (ORT) by mice (adopted from Wang *et al*., 2009)



Plate II: A picture of the object recognition test model used in the present study for the acquisition phase



Plate III: A picture of the model for object recognition by mice test used in the current study for the retention phase

The exploration time of both the sample and test phases were recorded. The criterion used to assess exploration in both the ORT and the OLT was that the nose of the mouse was oriented towards the objects at a distance of less than 2 cm. Periods in which the rat moved around, climbed over or sat on the objects was not recorded. To prevent olfactory cues, the objects and the test box were cleaned with 75% ethanol after each trial. A discrimination index (DI), difference (D_1) and recognitive index (RI) were calculated just as in the ORT (De Rosa *et al*., 2005; Zhao *et al*. 2007).

3.5 Biochemical Tests

3.5.1 Effect of drugs on brain lipid peroxidation

The level of thiobarbituric acid reactive substance, MDA an index of lipid peroxidation, was evaluated in the brain sample using NWLSS^{TM} NWK-MDA01 Malondialdehyde Assay kit (Northwest Life Science Specialties, LLC, Vancouver, WA 98662), as stated by the manufacturer. The principle is based on the method of Janero (1990) which involves spectrophotometric measurement of the colour developed during the reaction of thiobarbituric acid (TBA) and MDA, which forms a TBA-MDA adduct that absorbs strongly at a wavelength of 532 nm. Brain samples were collected from each animal immediately after decapitation at the termination of the experiment on the 17th of May, 2013. They were weighed and then homogenized in a known volume of ice-cold phosphate buffer to obtain a 10% (w/v) homogenate, into which butylated hydroxytoluene (BHT) and EDTA are added to the sample to minimize oxidation of lipids that occurs during the sample processing and the TBA reaction. The sample is then vortexed vigorously (5-count) and incubated for 60 minutes at 60°C and centrifuged at 10,000 x *g* for 2-3 minutes. The supernatant was transferred to a mixture cuvette and the spectra absorbance was recorded from the spectrophotometer at wavelength of 532nm. The result was expressed as μmol/ml.

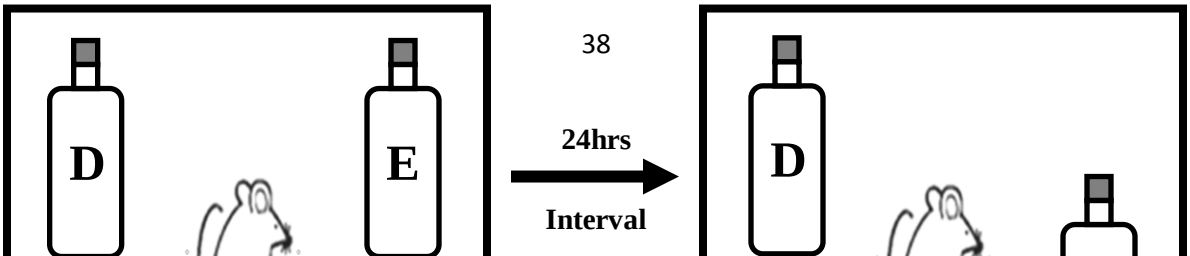


Figure 3.4.3: A schematic representation for object location test by mice (adopted from Wang *et al*., 2009)



Plate IV: A picture of the object location test model used in the study for the retention phase

3.5.2 Evaluation of brain catalase cctivity

Catalase was analyzed using catalase activity assay kit NWLSS^{TM} NWK- CAT01 (Northwest Life Science Specialties, LLC, Vancouver, WA 98662), according to the manufacturer's instruction. This was based on the method of Beers and Sizer (1952) in which catalase enzyme activity was measured by monitoring the consumption of H_2O_2 to

generate H_2O and O_2 at a wavelength of 240nm.The standard assay substrate mixture contained 0.30 mL H_2O_2 in 50 mL 0.05 M sodium phosphate buffer, pH 7.0. The sample aliquot (20 µL) was added to 980 µL substrate mixture. The initial absorbance was recorded after 1 minute and the final absorbance after 6 minutes. A standard curve was established using purified catalase under identical conditions. All samples were diluted with 0.1 mmol/L sodium phosphate buffer (pH 7.0) to provoke a 50% inhibition of the diluent rate (that is, the uninhibited reaction). Results are expressed as IU/mL.

3.5.3 Evaluation of brain glutathione peroxidase activity

Glutathione was analysed using Glutathione Activity Assay Kit NWLSS^{TM} NWK-GPX01 (Northwest Life Science Specialties, LLC, Vancouver, WA 98662), according to the manufacturer's instruction and based on the method described by Paglia and Valentine, (1967). The underlying principle was that glutathione peroxidase catalyzes the reduction of hydrogen peroxide (H_2O_2), oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP+ (resulting in decreased absorbance at 340 nm) and recycling the GSH. Since GPx is limiting, the decrease in absorbance at 340 nm is directly proportional to the GPx concentration. one unit of GPx-1 = the amount of enzyme necessary to catalyze the oxidation (by H_2O_2) of 1.0 µmole GSH to GSSG, per minute at 25 ºC, pH 7.0. 50 µL of the sample was added to wells and 50 µL each of working NADPH and H_2O_2 added. Readings were taken from the microplate reader after a minute at an interval of 30 seconds for 5 minutes at an absorbance of 340 nm. Then, a curve was plotted from which glutathione peroxidase activity was calculated and expressed as mU/mL

3.5.4 Evaluation of brain superoxide dismutase activity

Superoxide dismutase activity was analyzed using the superoxide dismutase activity assay Kit NWLSS^{TM} NWK-SOD02 (Northwest Life Science Specialties, Vancouver, WA 98662) as stated by the manufacturer. This was based on the method of Martin *et al*. (1987). This method is based on monitoring the autoxidation rate of haematoxylin. In the presence of SOD enzyme at specific assay pH, the rate of auto-oxidation was inhibited and the percentage of inhibition was linearly proportional to the amount of SOD present within a specific range. Sample SOD activity was determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample. To each well used for testing, 230 µL of assay buffer was added, and 10 µL is added to both blank well and sample then mixed and incubated for 2 minutes. There after 10 µL of haematoxylin was added, and absorbance was read at a wavelength of 560 nm every 10 seconds for at least 5 minutes. Results are expressed as IU/mL.

3.6 Determination of Gonadal Hormone Levels

3.6.1 Determination of testosterone

Testosterone level was assessed using AccuDiag^{TM} Testosterone ELISA KIT Cat# 2095Z (Diagnostic Automation Inc, Calabasas, CA. 91302 USA) as stated by the manufacturer using the principle of competitive binding between testosterone in the test specimen and testosterone-horseradish peroxidase (HRP) conjugate, for a constant amount of rabbit anti-testosterone. 10 µL of standards, specimen and controls were added into appropriate wells then 100 µL of testosterone-HRP was added. 50 µL of rabbit antitestosterone reagent was added to each well and thoroughly mixed for 30 seconds,

thereafter incubated at 37ºC for 90 minutes (Lamberts. 1997). In the incubation, goat anti-rabbit IgG-coated wells were incubated with testosterone standards, controls, animal samples, testosterone-HRP conjugate reagent and rabbit antitestosterone reagent for 90 minutes. During the incubation, a fixed amount of HRP-labeled testosterone competed with the testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific testosterone antibody. Thus, the amount of testosterone-HRP immunologically bound to the well progressively decreased as the concentration of testosterone in the specimen increased. Unbound testosterone-peroxidase conjugate was then removed and the wells washed, followed by addition of tetremethyl benzidine (TMB) reagent resulting in the development of blue colour. The colour development was stopped and the absorbance was measured spectrophotometrically at the wavelength of 450 nm and within 15 minutes. The intensity of the colour formed was proportional to the amount of enzyme present and was inversely related to the amount of unlabeled testosterone in the sample. A standard curve was obtained by plotting the concentration of the standard versus the absorbance. The testosterone concentration of the specimens and controls run concurrently with the standards can be calculated from the standard curve drawn and results are expressed as ng/mL.

3.6.2 Determination of oestrogen level

Estrogen level was assessed using AccuDiag^{TM} Oestradiol ELISA Kit Cat＃2046Z (Dagnostic Automation Inc. Calabasas, CA. 91302, USA) as stated by the Manufacturer

using the principle of competitive binding between estradiol (E2) in the test specimen and E2-HRP conjugate for a constant amount of rabbit anti-Estradiol (Tsang *et al*., 1980). In the incubation, goat anti-rabbit IgG-coated wells were incubated with 25-ml E2 standards, controls, animal samples, 100 ml Estradiol-HRP Conjugate Reagent and 50 ml rabbit anti-Estradiol reagent at room temperature (18-25°C) for 90 minutes. During the incubation, a fixed amount of HRP-labeled E2 competed with the endogenous E2 in the standard, sample, or quality control serum for a fixed number of binding sites of the specific E2 antibody. Thus, the amount of E2 peroxidase conjugate immunologically bound to the well progressively decreased as the concentration of E2 in the specimen increased. Unbound E2 peroxidase conjugate is then removed and the wells washed. Thereafter, a solution of tetremethyl benzidine (TMB) reagent was added and incubated at room temperature (18-25°C) for 20 minutes, resulting in the development of blue color. The colour development was stopped with the addition of 1N HCl, and the absorbance was measured spectrophotometrically at 450 nm within 15 minutes. The intensity of the colour formed was proportional to the amount of enzyme present and was inversely related to the amount of unlabeled E2 in the sample. A standard curve was obtained by plotting the concentration of the standard versus the absorbance. The E2 concentration of the specimens and controls run concurrently with the standards was calculated from the standard curve and results expressed as pg/mL.

3.7 Statistical Analysis

Statistical analysis was carried out using Statistical Package for Social Scientists (SPSS 20, Chicago, IL, USA). Data obtained were expressed as Mean ± SEM. All analyses were done

using ANOVA and Least Significant Difference (LSD) post-hoc test. Values of $P \leq 0.05$ were considered significant. The relationship between serum level of gonadal hormones and recognitive indices was analyzed using MS-EXCEL Regression (Duncan, 1977).

CHAPTER FOUR

RESULTS

4.0 Neurobehavioural Assessments

4.1 Elevated Plus Maze for Memory

Tables 4.1 and 4.2 show the transfer latency for both acquisition and retention phases of the experimental procedure. Transfer latency was shortest in the soya oil group of both males (31.25 ± 7.60) and females (22.50 ± 4.17). The highest value was observed in the vitamin C- treated group in the males (55.00 ± 12.21) and vitamin E treated group of the females (57.75±14.92). During the acquisition phase, there was significant difference ($P < 0.05$) in transfer latencies between vitamin C, soya oil and the vitamin E-treated of both male and female mice. However, during the retention phase a significant difference ($P < 0.05$) was recorded between the control group and the soya oil and vitamin C-treated group, vitamin C + E treated-groups of male mice, and between vitamin C and E treated-group of female mice.

Table 4.1: Effect of Treatment on Transfer Latencies of Male Mice in the Elevated Plus Maze for memory (Mean ± SEM)

TRANSFER LATENCIES OF MALE MICE		
TREATMENT	ACQUISITION TEST (s)	RETENTION TEST (s)
Distilled water	45.50±15.71^{ns}	53.50±17.10**
Soya oil	31.25±7.60*	22.00±7.01*
Vitamin C	55.00±12.21*	26.75±5.48*
Vitamin E	37.75±14.61**	38.75±11.30^{ns}
Vitamins C+E	18.75±2.75^{ns}	13.50±4.33**

*Means across the columns are statistically significant at $P < 0.05$

^{ns} = no statistically significant difference, n = 4

Table 4.2: Effect of Treatment on Transfer Latencies of Female Mice in the Elevated Plus Maze for Memory (Mean ± SEM)

	TRANSFER LATENCIES OF FEMALE MICE	
TREATMENT	ACQUISITION TEST (s)	RETENTION TEST (s)
Distilled water	37.00 ± 5.61^{ns}	23.00 ± 2.16^{ns}
Soya oil	22.50 ± 4.17*	30.25 ± 16.50^{ns}
Vitamin C	19.25 ± 2.39*	20.50 ± 7.14*
Vitamin E	57.75 ± 14.92**	57.50 ± 17.16*
Vitamins C+E	37.00 ± 17.82^{ns}	41.50 ± 5.83^{ns}

*Means across the columns are statistically significant at $P < 0.05$

^{ns} = no statistically significant difference, n = 4

4.2 Object Recognition Test

Tables 4.2.1 and 4.2.2 show the effect of treatment on the three indices in the ORT model for assessing memory. Statistically significant difference is recorded where $P < 0.05$. In the acquisition phase, for D_1, significant difference was observed between the vitamin C-treated group and soya oil, vitamin E and C + E-treated groups in the male population but in the female significant difference was observed between the control group and the soya oil, vitamins C, E, and C + E-treated groups; and between the vitamins C + E-treated group and the soya oil, vitamins C and E-treated groups. DI showed significant differences between treatment groups and control and in between treatment groups. In the retention phase, the females showed significant difference in all three parameters between the control and the vitamin C + E-treated group only. But in the males, D_1 showed significant difference when control was compared with vitamins C, E and C + E-treated groups. DI and RI showed significant difference for comparisms between the control and treatment groups and between treatment groups.

Table 4.2.1: Effect of Treatment on indices of the Object Recognition Test in the Male Mice (Mean ± SEM)

	ACQUISITION PHASE			RETENTION PHASE		
DRUGS	D_1 (s)	DI	RI (s%)	D_1 (s)	DI	RI (s%)
Distilled water	-4.50±7.63^{ns}	-0.08±0.10***	46.10±5.17*	-8.75±14.83***	0.13±0.09**	46.23±5.70***
Soya oil	7.25±1.16*	0.24±0.06**	53.45±7.24^{ns}	11.75±3.22^{ns}	0.18±0.05*	55.35±1.23*
Vitamin C	-18.75±6.45***	-0.17±0.06***	41.63±2.77**	20.00±7.52*	0.21±0.04*	60.45±1.95**
Vitamin E	13.75±2.30*	0.22±0.02**	61.53±0.88**	24.75±7.39*	0.34±0.05*	63.38±2.30**
Vitamin C+E	17.00±10.63*	0.30±0.15**	59.65±5.70*	33.50±8.26*	0.41±0.06***	70.55±2.83****

D_1 - Difference of time spent with novel and familiar object, DI – Discriminatory Index and RI – Recognitive Index

*Means across columns are considered significant at $P < 0.05$

^{ns} = no statistically significant difference at $P < 0.05$

n = 4

Table 4.2.2: Effect of Treatment on indices of the Object Recognition Test in the Female Mice (Mean ± SEM)

	ACQUISITION PHASE			RETENTION PHASE		
DRUGS	D_1 (s)	DI	RI (s%)	D_1 (s)	DI	RI (s%)
Distilled water	-27.25±2.06****	--0.36±0.02***	32.13±1.18***	11.25±5.60*	-0.02±0.19*	55.76±3.09*
Soya oil	-9.00±1.68*	-0.16±0.04**	46.83±5.60**	20.50±5.80^{ns}	0.27±0.05^{ns}	62.63±2.73^{ns}
Vitamin C	-10.75±1.89**	-0.21±0.04**	39.60±1.90*	20.25±5.63^{ns}	0.24±0.05^{ns}	61.93±5.42^{ns}
Vitamin E	-1.00±9.00**	0.02±0.11**	49.16±5.71*	14.50±4.11^{ns}	0.25±0.08^{ns}	62.58±3.76^{ns}
Vitamins C+E	-6.95±3.53***	0.27±0.03***	60.35±2.99***	28.00±4.24*	0.41±0.05*	70.38±1.88*

D_1 - Difference of time spent with novel and familiar object, DI – Discriminatory Index and RI – Recognitive Index

*Means across columns are considered significant at $P < 0.05$

^{ns} = no statistically significant difference at $P < 0.05$

n = 4

4.3 Object Location Test

Tables 4.3.1 and 4.3.2 show the effect of treatment on the three indices (D_1, DI and RI) of the OLT model for assessing memory. A difference is considered statistically significant when $P < 0.05$. In the males, during the habituation phase, a significant difference for all three indices was recorded between vitamin C-treated group and the control and all treatment groups. In the females, D_1 showed significant difference between all groups with exception of the group treated with vitamin E. DI showed significant difference between the control and vitamins C + E-treated group and C + E all treatment groups with exception of the soya oil-treated group. RI showed significant difference between vitamins C + E-treated group with control and vitamn C-treated group. In the retention phase, the males showed significant difference when control was compared with vitamin C and vitamins C + E-treated groups for D_1 while none was recorded in the females. DI in the males showed significant difference for all groups while for females, significant difference was observed in between vitamin C-treated group and control, soya oil and vitamins C + E-treated groups. RI for the males showed significance between the control group and vitamins C and C + E-treated groups while in the females significant difference was seen between vitamin C and control, soya oil and vitamins C + E-treated groups.

Table 4.3.1: Effect of Treatment on Indices Object Location Test for Male Mice (Mean ± SEM)

	ACQUISITION PHASE			RETENTION PHASE		
DRUGS	D_1 (S)	DI	RI (S%)	D_1 (S)	DI	RI (S%)
Distilled water	17.50±1.55*	0.24±0.02*	61.95±1.25*	1.50±4.57**	0.03±0.04***	51.75±2.14**
Soya oil	22.50±5.40*	0.29±0.08*	65.83±4.55*	14.25 ± 3.33^{ns}	0.17±0.03*	58.63 ± 1.26^{ns}
Vitamin C	-21.75±15.84****	-0.33±0.11****	30.20±3.32****	29.00±9.71*	0.33±0.08*	66.43±3.73*
Vitamin E	11.75±2.40*	0.15±0.03*	57.48±1.56*	18.25 ± 10.02^{ns}	0.25±0.09**	53.78±8.77*
Vitamins C+E	13.50±7.24*	0.18±0.08*	58.92±4.22*	29.00±7.77*	0.43±0.05***	71.63±2.68**

D_1 - Difference of time spent with object on the right and object on the left side, DI – Discriminatory Index and RI – Recognitive Index

*Means across columns are considered significant at $P < 0.05$

^{ns} = no statistically significant difference at $P < 0.05$

n = 4

Table 4.3.2: Effect of Treatment on Indices Object Location Test for Female Mice (Mean ± SEM)

	ACQUISITION PHASE			RETENTION PHASE		
DRUGS	D_1(S)	DI	RI(S%)	D_1(S)	DI	RI(S%)
Distilled water	23.25±7.18*	0.55±0.22**	70.80±6.09*	13.00±5.26^{ns}	0.15±0.05*	57.78±2.80*
Soya oil	12.50±4.29*	0.29±0.08*	64.73±4.00^{ns}	12.25±1.31^{ns}	0.15±0.02*	57.63±0.90*
Vitamin C	25.75±2.95**	0.38±0.08*	72.30±1.34*	24.50±3.77^{ns}	0.38±0.04***	70.43±2.95***
Vitamin E	22.00±3.16^{ns}	0.37±0.06*	68.68±2.77^{ns}	24.75±6.50^{ns}	0.30±0.07^{ns}	65.15±3.61^{ns}
Vitamins C+E	9.75±2.46**	0.18±0.04***	58.78±2.03**	16.25±4.40^{ns}	0.22±0.06*	61.02±2.72*

D_1 - Difference of time spent with object on the right and object on the left side, DI – Discriminatory Index and RI – Recognitive Index

*Means across columns are considered significant at $P < 0.05$

^{ns} not statistically significant at $P < 0.05$

n = 4

4.4 Lipid Peroxidation and Antioxidant Enzyme Assessments

Table 4.4.1 and 4.4.2 show results obtained from biochemical analysis of the sample and serum of the animals. A difference is considered statistically different when $P < 0.05$. In the males, MDA, SOD, GPx showed no significant dfference. For catalase however, comparism between the control group all treatment groups showe significant difference. In the females, catalase showed no significant difference, MDA, showed significant difference when the control was compared to the vitamin C-treated group, SOD, showed significant difference when the control group was compared with vitamins C + E and E-treated groups and between vitamins C + E and vitamin C-treated group. GPx showed a significant difference between the control and soyaoil and vitamins C + E-treated groups.

Table 4.4.1: Effect of Treatment on the Level of Malondialdehyde and Antioxidant Enzymes in Male Mice

		ANTIOXIDANT ENZYMES		
DRUGS	MDA (μmol/ml)	SOD (U/mL)	GPx (mU/ml)	CAT (U/mL)
Distlled water	1.33±0.10^{ns}	2.25±0.17^{ns}	49.00±2.16^{ns}	63.75±1.11****
Soya oil	1.65±0.07^{ns}	1.65±0.13^{ns}	39.25±2.02^{ns}	50.25±1.89*
Vitamin C	1.63±0.21^{ns}	1.90±0.30^{ns}	42.00±2.27^{ns}	51.50±3.75*
Vitamin E	1.88±0.35^{ns}	2.08±0.28^{ns}	46.75±3.22^{ns}	47.75±4.21*
Vitamins C+E	1.67±0.29^{ns}	1.93±0.44^{ns}	45.33±6.94^{ns}	49.67±3.92*

*Means across columns are considered significant at $P < 0.05$

^{ns} not statistically significant at $P < 0.05$

n=4

	ANTIOXIDANT ENZYMES			
DRUGS	MDA (µmol/ml)	SOD (U/mL)	GPx (mU/ml)	CAT (U/mL)
Distilled water	1.65±0.12*	2.35±0.96**	47.75±3.22**	54.50±2.87^{ns}
Soya oil	2.15±0.23^{ns}	1.85±0.10^{ns}	40.00±2.89*	51.50±5.11^{ns}
Vitamin C	2.28±0.19*	2.28±0.14*	46.25±1.03^{ns}	54.75±0.75^{ns}
Vitamin E	2.05±0.17^{ns}	1.78±0.31*	46.25±2.66^{ns}	52.25±4.61^{ns}
Vitamin C+E	1.73±0.22 ^{ns}	1.55±0.21**	39.50±1.20*	48.50±2.18^{ns}

Table 4.4.2: Effect of Treatment on the level of Malondialdehyde and Antioxidant Enzymes of Female Mice

*Means across columns are considered significant at $P < 0.05$

^{ns} not statistically significant at $P < 0.05$

n = 4

4.5 Gonadal Hormone Assay

Table 4.5.1 shows effect of treatment on the serum level of testosterone and oestrogen of animals. A difference is considered statistically significant when $P < 0.05$. Generally there was a decrease across the treatment groups from the control to the vitamins C + E-treated groups of both male and female. In the males however, a significant difference was observed between the control and soya oil and vitamin E-treated groups while the females showed a significant difference between the vitamins C + E-treated group and vitamin C and control groups.

Table 4.5.1: Shows the serum testosterone and oestrogen of mice after 21 day regimen

DRUGS	OESTROGEN (pg/mL)	TESTOSTERONE (ng/mL)
Distilled water	398.00±35.02*	5.33±1.26**
Soya oil	350.00±24.61^{ns}	0.85±0.16*
Vitamin C	368.25±29.57*	3.98±1.71^{ns}
Vitamin E	362.75±51.41^{ns}	0.98±0.41*
Vitamins C+E	267.50±10.97**	3.47±0.64^{ns}

*Means across columns are considered significant at $P < 0.05$

^{ns} = no statistically significant difference at $P < 0.05$

n = 4

4.6 Relationship between Serum Gonadal Hormones and Memory Indices in Mice

Figures 4.6.1 - 4.6.6 show the relationship between the level of serum testosterone and oestrogen of the mice with the memory indices of all three neurobehavioural models for memory. The transfer latencies of the male mice as recorded in the EPM model for memory and represented in figure 4.6.1 shows a positive relationship between transfer latencies and serum testosterone, with a coefficient of determination R^2 of 0.0081 and a regression equation of y = 0.7523x + 29.774. Results from the female mice showed R^2 of 0.0449 and y = 0.0706x + 9.8744 seen in figure 4.6.2.. The results obtained for the ORT in the male show a negative relationship with increasing serum testosterone levels yielding a decreasing valvue of Recognition as seen in Figure 4.6.3 below with R^2 = 0.0393 and y = -0.06976x + 60.442 . The same trend was also seen in the female mice as depicted in Figure 4.6.4 with R^2 = 0.3601 and y = -0.0568x + 82.506. In the OLT model, the results obtained from the male mice showed a positive relationship between serum testosterone and recognitive Index with recognition increasing as serum testosterone increases as seen in figure 4.6.5, R^2 = 0.0003 and y = 0.0701 + 59.971 The same trend was observed in the female mice as shown in figure 4.6.6 with R^2 = 0.0978 and y = 0.0295x + 52.098. .

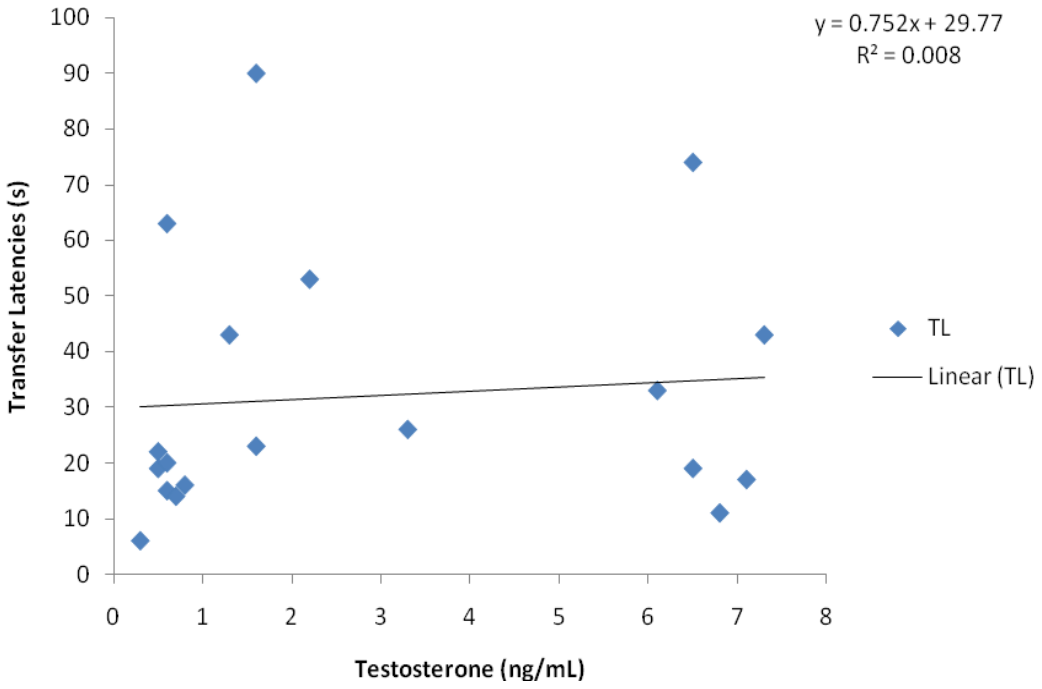


Figure 4.6.1: The relationship between serum testosterone and transfer latencies of mice in the elevated plus maze model for memory

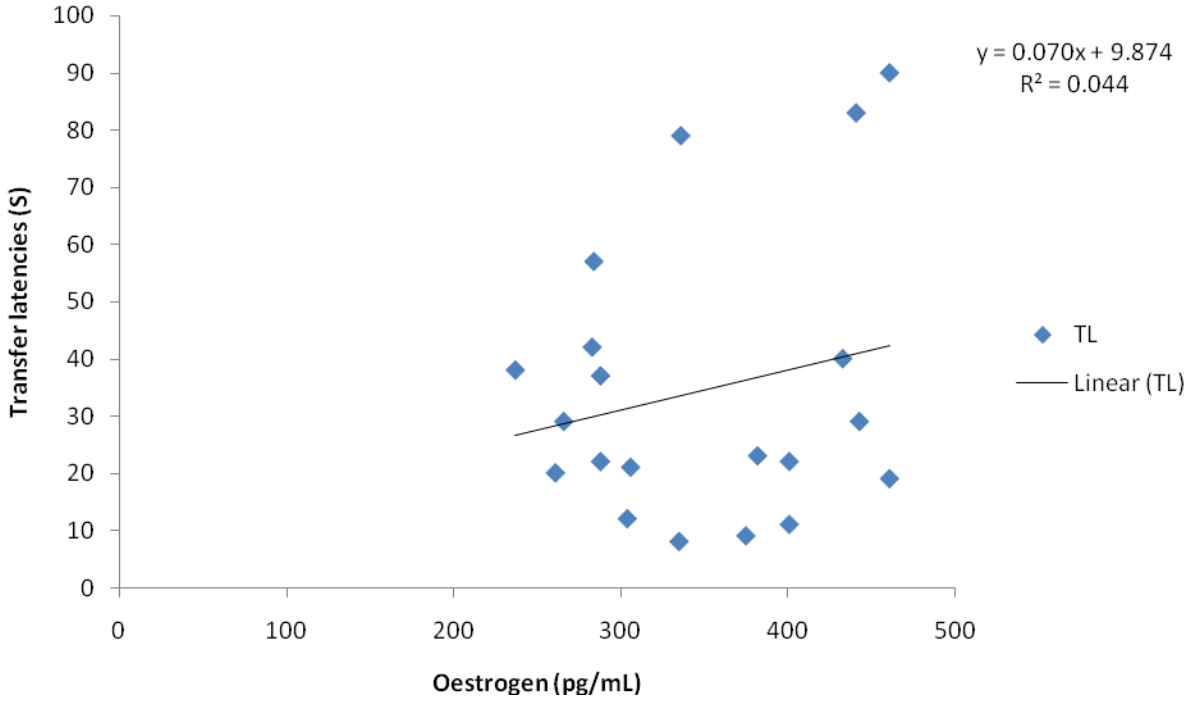


Figure 4.6.2: The relationship between serum oestrogen and transfer latencies of mice in the elevated plus maze model for memory

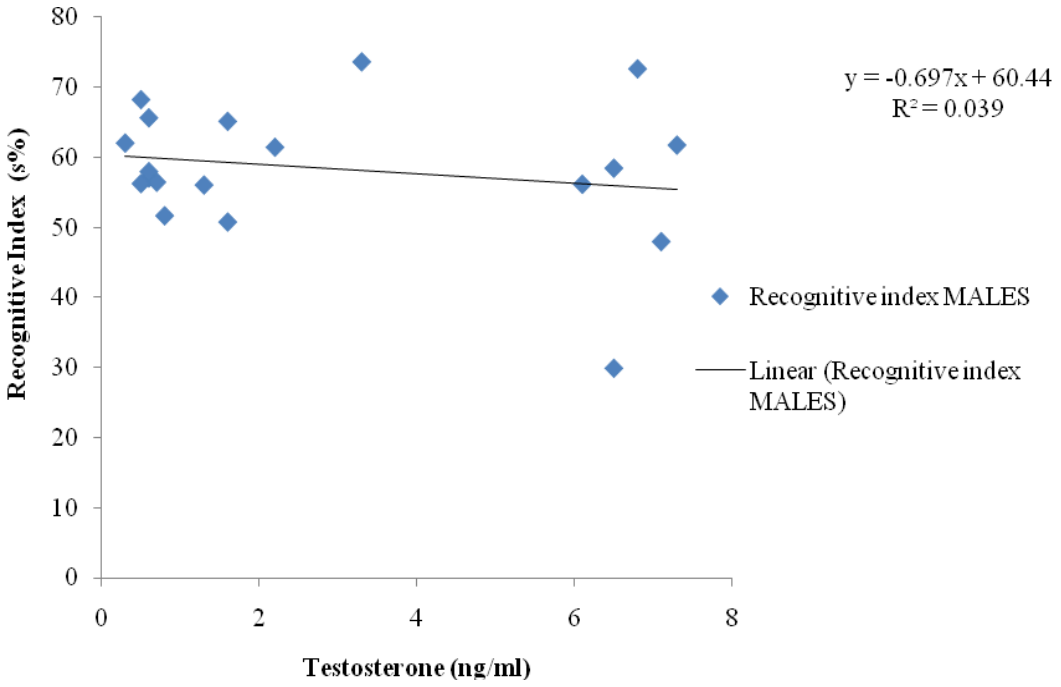


Figure 4.6.3: The relationship between recognitive index and serum testosterone level in the object recognition test

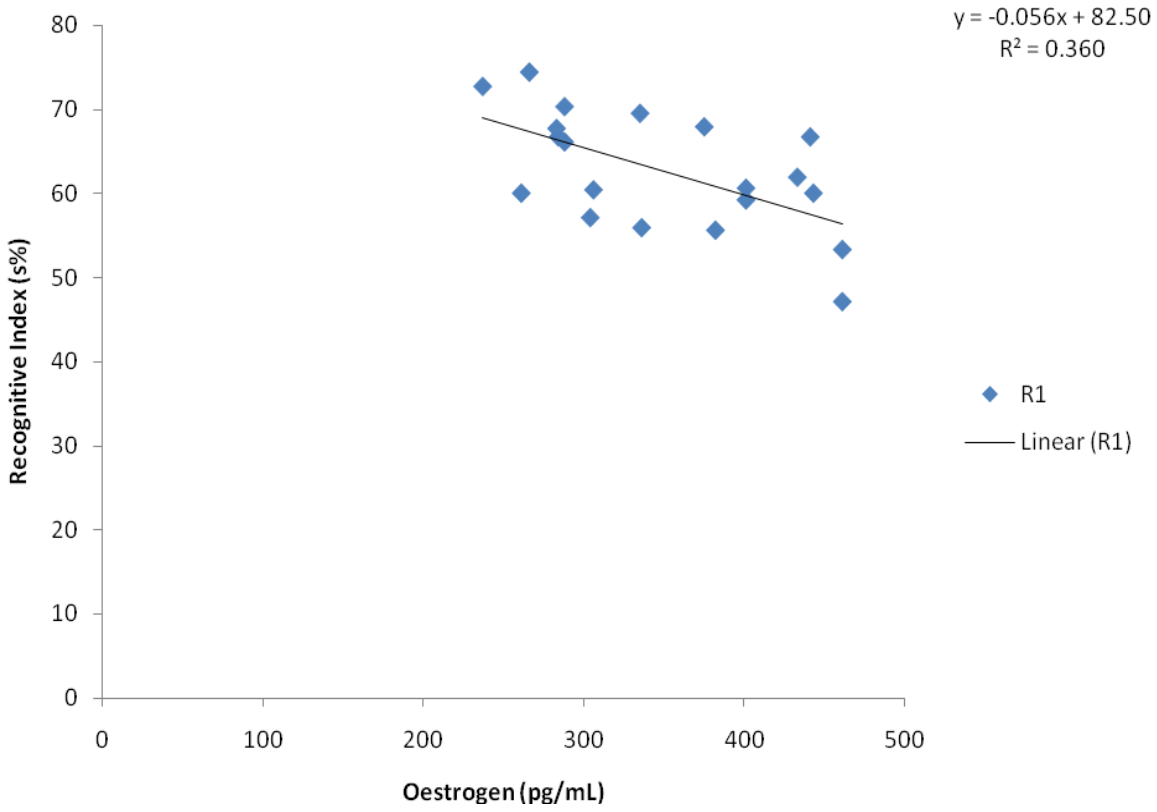


Figure 4.6.4: The relation between recognitive index and serum oestrogen level in the object recognition test

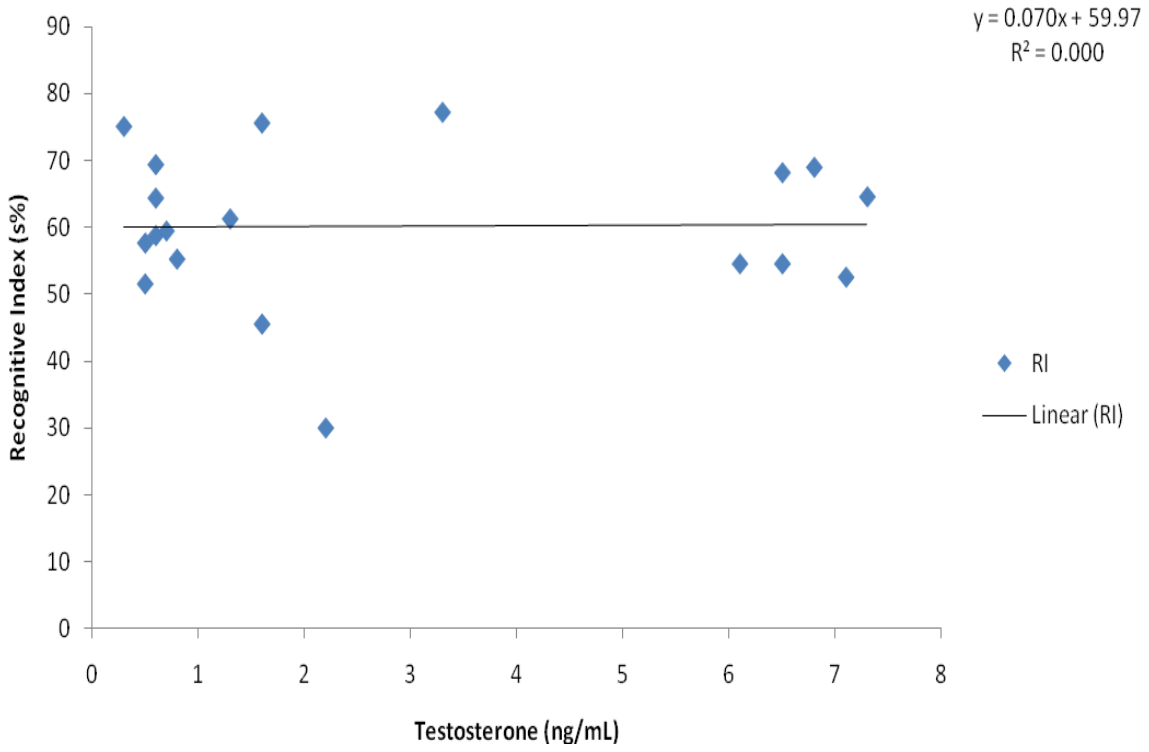


Figure 4.6.5: The relationship between recognitive index and serum testosterone level in the object location test

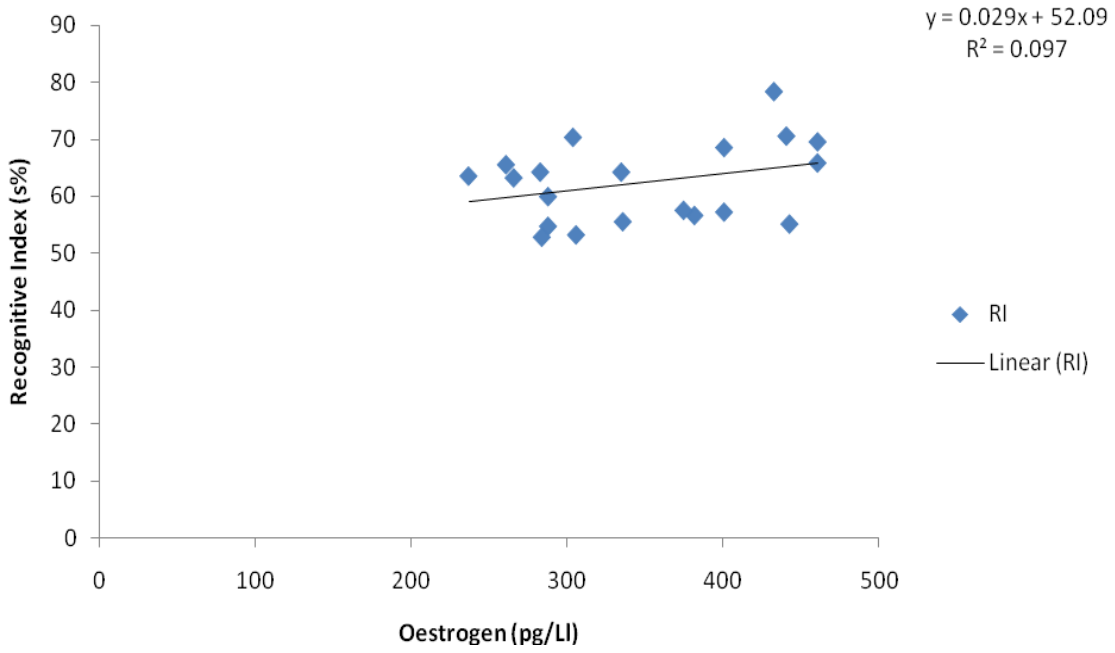


Figure 4.6.6: The relationship between Recognitive Index and serum oestrogen level in the object location test

CHAPTER FIVE

DISCUSSION

The purpose of this study was to study the effect of administration of vitamin C and E singly and in combination affect memory in adult mice using the elevated plus maze, object recognition and location neurobehavioural models for memory and the relationship between serum testosterone and oestrogen with animals' performance in the three neurobehavioural models.

Itoh *et al.* (1990) suggested that changes in the latency of mice and rats to go from an open arm to an enclosed arm of the EPM is an indicator of learning and memory. This is the basis used in this study for using the EPM to test for memory in the mice. The animals were tested for one task-the one way escape from the open arm, a response considered to measure unconditioned fear of the animals as well as retention (Adiga *et al.*, 2012). Results obtained from this study showed a decrease in transfer latency to move from the open arm to the enclosed arm with shorter transfer latencies on the second day of the experiment and across the treatment groups. This indicates improvement in memory (Dhingra and Kumar, 2012) that can be linked to the treatment, there is however the possible influence of fear/anxiety levels as noted by Blatt and Takahashi (1998). They noted that in an experimental condition, there is an aversive experience of height and openess of the maze that may induce escape learning responses in the animals which may not necessarily have to do with treatment as in this experiment.

Animals treated with soya oil, vitamins C and E had improved transfer latency with the most effect seen on the second day of the experiment. The females, however, showed a

completely different trend with an increase in transfer latency from the first day to the second day of the experiment, the only exception being the control group. This difference between the male and female performance in the EPM test for memory may be due to higher influence of anxiety/fear on the males than the females, which induced a greater escape learning in the males than in the females.

In the ORT, female mice in the present study significantly more than the males discriminated the novel object from the familiar object by differential exploration on the first day (acquisition phase) on the seconday however, the males performed better with greater recognitive indices and higher significant differences ($p \leq 0.05$). This finding disagrees with work of Bettis and Jacobs (2009) who studied sex differentiation in spatial orientation in C57BL/6J mice and found female advantage for object recognition at the termination of the study. This finding could not be due to the presence of olfactory cues as the maze was thoroughly cleaned with 70% methylated spirit after each test for each animal male or female. This result, however implies the possibility that the males collected more information during the test phase-day one of the experiment. The source of the male advantage in the ORT may be due to the inherent ability of the male to outperform females on tests of visual memory and spatial ability (Halpern, 1992). From the results obtained, it is obvious that females and males pay attention differently to their surroundings with the males paying more attention than females to novel objects. This is not to say that the female mice were not able to discriminate between novel and familiar objects. In fact, they did, but generally, the males showed larger values in the difference (D_1) in time spent exploring novel and familiar object (Ennaceur and Delacour, 1988; Wang *et al*., 2009). This result agrees with the findings of Frick and Gresack, (2003) who

studied the behavioural responses to spatial and object novelty in adult C57BL/6J mice and found that male mice were able to discriminate between novel and familiar objects better than the females.

Discriminatory index (DI), which quantifies discrimination between the novel object and familiar objects; with a positive value indicating more time spent with novel object, and a negative value, indicating more time spent with familiar object (Ennaceur and Delacour, 1988; Antunnes and Biala, 2012), results from this study showed that in both males and females, treated with vitamin E and vitamins C+E, there was an increase in the value of DI obtained. The values were positive for both sexes for the two days of the experimental procedure. This result agrees with the finding that greater antioxidant effect was exerted when vitamins C and E are combined than when each is acting singly (Hathock et al., 2005).

Recognitive index (RI), which is the measure of novel object recognition, is the main index of retention. It is recorded as a percentage (Wang *et al*., 2009; Antunnes and Biala, 2012). The results from this study showed the male advantage contrary to the findings of Padhorna and Brown, (2002) and Bettis and Jacobs, (2009). This may be due to the significant methodological differences between this study and those of Padhorna (2002) and Bettis (2009). One of such difference is the time lapse between the sample and test phase. In this study the time lapse between these two phases was 24 hours similar to Bettis and Jacobs, (2009); but 15 minutes in the study conducted by Podhorna and Brown, (2002). Another important difference is the time the experiment was conducted. This study was conducted in the light phase (that is, the natural period of inactivity of

rodents) as opposed to the works of both Podhorna (2002) and Bettis (2009) in which the studies were carried out in the dark phase (period of activity for rodents).

Frick and Gresack (2003) did a similar work (same time lapse and phase of animal cycle) to the current study, and obtained similar results showing male advantage in the recognition task. There is however, a difference between this study and those of Padhorna and Brown (2002), Frick and Gresack (2003) and Bettis and Jacobs (2009) which is the strain of animals used. While animals used for this study were albino mice, the animals used by Padhorna and Brown, (2002), Frick and Gresack, (2003) and Bettis and Jacobs, (2009) are all genetically-modified strains of experimental mice. There is also the possibility that males and females vary in their sensitivity to activity phase.

Object location memory was first proposed in rats as a one-trial spatial memory model where animals could learn without reinforcers (food and or electric shocks) shaping the training process (Takahashi *et al*., 2009). In this study, it was used for mice and the results obtained showed no particular advantage to any of the sexes. In some indices, the females were advantaged as was seen in the recognition test (De Goede and Postma, 2008). In some instances within the same index, the males performed better. The margin of difference was not as wide as observed in the object recognition test (ORT). Thus, based on the results of this study, it can be said that sex difference in location memory in the OLT is not wide as opposed to the report of Spiers *et al*. (2008) that demonstrated the female advantage in location memory in humans. Spiers *et al*. (2008) demonstrated that females recalled the location of 16 common grocery store items better than their male counterparts in the Virtual Reality Spatial Object Location Test, which was developed to

determine sex differences in spatial object location memory in a 3D virtual environment that simulates the real world.

Generally, animals of both sexes showed preference for object in new location than the object at the familiar location due to the influence of familiarity and novelty on memory. This influence as stated by Ennaceur (2010) is dependent on the time interval between the test and sample phases (delay) of the experimental procedure. At longer delays, the memory of familiar locations becomes weaker while at shorter delays it becomes almost intact. This is because when the animal is in contact with an object placed in a new location, the intensity of its exploration depends on the amount of residual memory of the familiar stimulus at a particular delay interval, which needs updating and reconsolidation (Antunnes and Biala, 2012). Summing up the performance of individual animals in all three neurobehavioural models for memory (EPM, ORT and OLT), the results showed the male advantage in spatial tasks. This finding is in agreement with the obtained results of previous studies (Frick and Gresack, 2003; Rampon *et al.*, 2000; Tang *et al*., 1999).

Malondialdehyde is one of many low molecular weight end-products of lipid hydroperoxide decomposition and is the most measured index of lipid peroxidation (De Zwart, 1999). It is assayed *in vivo* as a biomarker of oxidative stress (Janero, 1990). Results obtained from this study showed an increase in the level of MDA across the treatment groups with least value in the control group. The results indicate the presence of oxidative stress, occuring during metabolic reactions in the mitochondrion (Schmidley, 1990; Morrisey and O'Brien, 1998). This may be due to stress which could lead to the production of reactive oxygen species (El-Medany *et al*., 2012). The animals used in this

study went through stress in the course of the neurobehavioral tests prior to the termination of the experiment when brain and blood samples were collected for biochemical assessments. It may also be due to a limitation in the experimental procedure which may have led to reduction in bioavailable vitamin C which is fundamental as an antioxidant and co-antioxidant to vitamin E. vitamin C regenerates α-tocopherol and helps prevent α-tocopheryl radical-induced lipid peroxidation. Thus, depletion in antioxidants will lead to lipid peroxidation which is marked by increase in the level of MDA (Bendich et al., 1986; Halliwell and Guteridge, 1986). These increasing levels of MDA across the treatment groups disagree with the result obtained by Ryan *et al*. (2010) and Ambali *et al*. (2010) who conducted a similar study in rats and mice respectively. However, one fundamental difference between these two studies and the present one was that oxidative stress was induced in the animals through exposure to toxic substances and antioxidants was administered following the exposure; while animals in the present study were not given any treatment prior to administration of antioxidants. The antioxidant enzymes considered in this study are found endogenously in animals and are known to metabolize ROS and their by-products, thereby reducing oxidative stress (Da Costa *et al*., 2012).

Research has shown that components of endogenous antioxidant defence system work together and in concert with dietary antioxidants to prevent and reduce oxidative stress (Da Costa *et al*., 2012; El-Medany *et al*., 2012). Results obtained from this study showed a decrease in the serum activities of these enzymes from the control down through the treatment groups of both males and females. This could be as a result of the presence of

exogenous antioxidants, which work in consonance with the endogenous antioxidant enzymes to prevent or reduce oxidative stress.

The highest activity of these enzymes was observed in the controls. There was however, a significant difference in the activity of catalase of the vitamin E-treated group in the males as compared to the control. The activity of superoxide dismutase in the females declined across the treatment groups with significant difference between the control and vitamin E and C + E-treated groups and between vitamin C and C + E-treated group. Glutathione peroxidase in the females also significantly differed between the control, soya oil and the vitamins C + E-treated groups.

The high activity of the antioxidant enzymes observed in the controls could be explained by the fact that, in these groups, animals did not receive any form of treatment with exogenous antioxidants (vitamins C and E). Though the animals were not exposed to any form of stress or disease condition, the level of the enzymes in these groups as compared to treatment group are higher due to metabolic reactions recurring in the animals' body (Schmidley, 1990; Morrisey and O'Brien, 1998). The obtained result agrees with that of El-Medany *et al*. 2012, which showed a decline in the activities of these endogenous enzymes as alpha-tocopherol dosage increased in treated rabbits.

Results obtained from this study showed a decrease in serum testosterone concentrations across the groups from the control to the treatment groups with significant difference ($p \leq 0.05$) observed when the control group is compared to the the soya oil and vitamin E-treated groups. The highest value observed was in the controls and the least in the soya oil-treated group (0.85 ± 0.16 ng/mL). This result disagrees with the findings of Salman

et al.,(2010). A comparison of the relationship between the serum level of testosterone and recognition indices in two of the three neurobehavioural models for memory used in this study showed a positive relationship. Therefore, based on the results from this study, serum testosterone plays a role in the performance of mice in memory tasks. Serum estradiol measurements are a valuable index for circulating estradiol, which is physiologically active. Results obtained from this study showed a decline in the serum levels of estradiol from the control group to the treatment groups with the least value recorded in the vitamins C and E-treated group. A significant dfference was observed between the vitamins C + E-treated groups, control and vitamin C-treated groups. As observed in the males, the serum oestrogen level when compared to recognition, showed a positive relationship except in the EPM model for memory. This result agrees with reports from available literature that demonstrated the contributory role of gonadal hormones to cognitive processes, particularly memory (Sherwin, 2003; Ulubaev *et al*., 2009; Henderson and Popat, 2011; Leonard and Winsauer, 2011). It is known that facilitation of learning and memory by the gonadal hormones is through modulation of the structure and function of the hippocampus (Geinisman *et al*., 2001; Kasai *et al*., 2003; Lang *et al*., 2004).

This can, however, not be proven from the results obtained from this study as whole brain samples were used and a study of the gross structure of the brain was not done. A study conducted by Barrett-Connor *et al*.,(1999) supports an association between endogenous sex hormone levels and cognition in older men as obtained in this study, with the only difference being that the experimental animals are adults and not ageing. Frye and Koonce (2007) concluded, based on the data obtained from their study that cognitive-

enhancing effects of steroids may be mediated through ERα and/or ERβ further confirming the result obtained by of Rissman *et al*. (2002). Since this finding cannot be proven based on the results from this study because mapping of the brain regions for oestrogen receptors subserving memory was not conducted in the course of the experiment,further investigations are required.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

Endogenous gonadal hormones, vitamins C and E improved memory in adult mice as seen in their performance in the three (the elevated plus maze, object recognition and location tests) neurobehavioural tasks carried out in the study. The improvement was obtained across the groups from the control through the treatment groups, with the highest recognitive index recorded in the group administered with both vitamins C and E. The male animals outperformed the females in all three neurobehavioural tests for memory. The relationship between serum gonadal hormones and recognitive index in all three neurobehavioural models for memory showed a positive relationship though weak

6.2 Conclusion

Vitamin C and E supplementation enhanced the improvement of memory performance and the female mice performed better than the male in the spatial memory task. Endogenous gonadal hormones correlate positively with recognition index.

6.3 Recommendations

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

1. A study on the effect of endogenous levels of gonadal hormones on specific brain areas subserving memory.
2. A study should be carried out on the relationship between dorminant hemisphere and the performance of mice in the object location and recognition tests.

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