MODULATORY ROLE OF VITAMINS A AND E ON LEARNING, MEMORY, MOTOR STRENGTH AND COORDINATION OF CYANIDE INDUCED NEUROTOXICITY IN ALBINO MICE

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ZARIA, NIGERIA

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ZARIA, NIGERIA

JANUARY, 2017

DECLARATION

I declare that the work in this dissertation entitled "MODULATORY ROLE OF VITAMINS A AND E ON LEARNING, MEMORY,MOTORSTRENGTH AND COORDINATION OF CYANIDE INDUCED NEUROTOXICITY IN ALBINO MICE" was carried out by me in the Department of Human Physiology ABU, Zaria under the supervisions of Prof.Aliyu Mohammed 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 other university.

AishatuAbubakar ISHAKU _____ _____

Signature Date

CERTIFICATION

This dissertation entitled "MODULATORY ROLE OF VITAMINS A AND E ON LEARNING, MEMORY, MOTOR STRENGTH AND COORDINATION OF CYANIDE INDUCED NEUROTOXICITY IN ALBINO MICE" by Aisha Abubakar ISHAKU meets the regulation governing the award of the degree of M.Sc Human Physiology of the Ahmadu Bello University Zaria, and it is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this work to Almighty God for His Blessings, Mercy, Kindness and Guidance, who made life worth living; my dear parents, for their unconditional love, and my late sister, Maryam (we still miss you dearly).

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All praise belongs to the creator of the Heavens and Earth who sees me through this work and more.

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LIST OF ABBREVIATIONS

ACTA	-	Amino thiazoline carboxylic acid
AChE	-	Acetylcholinestrase
ACh	-	Acetylcholine
ALP	-	Alkaline phosphatase
ALT	-	Alanine amino transferase
ANOVA	-	Analysis of variance
AOVs	-	Antioxidant vitamins
AST	-	Aspartate amino transferase
ATP	-	Adenosine Triphosphate
BHA	-	Butylhydroxyanisole
CAT	-	Catalase
CCO	-	Cytochrome C Oxidase
COHb	-	CarboxyHemoglobin
CO	-	Carbon monoxide
CO_2	-	Carbon dioxide
CN^-	-	Cyanide ion
CNS	-	Central Nervous System
CPSC	-	Consumer Product Safety Commission
DNA	-	Deoxyribonucleic acid
EDTA	-	Ethylene DiamineTetraacetic Acid
EPA	-	Environmental Protection Agency
EPM	-	Elevated plus Maze

Fe^{3+}	-	Iron III ions
Fe^{2+}	-	Iron II ions
g	-	Gram
GPx	-	Glutathione peroxidase
Hb	-	Haemoglobin
HCN	-	Hydrocyanic acid
HClO	-	Hypochlorous acid
Hr	-	Hour
H_2O	-	Water
H_2O_2	-	Hydrogen Peroxide
kg	-	Kilogram
KCN	-	Potassium Cyanide
KW	-	Kilowatt
LD	-	Lethal dose
m	-	Metre
MDA	-	Malondialdehyde
mEq/L	-	Milli-equivalent per litre
mg	-	Milligram
ml	-	Millilitre
Min	-	Minute
Mpt	-	Melting point
m^3	-	Metre cube
NADH	-	Nicotinamide Adenine Dehydrogenase

NADPH	-	Nicotinamide adenine phosphate dehydrogenase
NMDA	-	N-methyl-D-aspartate
NH_3	-	Ammonia
nMol	-	Nanomole
NO	-	Nitrogen oxide
NO-Hb	-	Nitrosyl-Haemoglobin
•NO	-	Nitric oxide free radical
OCN^-	-	Cyanate
OH	-	Hydroxyl radical
O_2	-	Oxygen
O_2^-	-	Superoxide anion
Pa	-	Partial Pressure
ppm	-	Parts per million
RAWM	-	Radial arm water maze
RBC	-	Red Blood Cells
RA	-	Retinoic Acid
RAR	-	Retinoic acid Receptor
ROS	-	Reactive Oxygen Species
SAA	-	Sulphur amino acid
SCN^-	-	Thiocyanate
SEM	-	Standard Error of mean
SOD	-	Superoxide dismutase
SPSS	-	Statistical package for social scientist

STP	-	Standard Temperature and Pressure
TAN	-	Tropical ataxic neuropathy
TBA	-	Thiobarbituric acid
TLs	-	Transfer latencies
USEPA	-	United States Environmental Protection Agency
UV	-	Ultraviolet
VAD	-	Vitamin A Deficiency
Vs	-	Versus
WHO	-	World Health Organization
μg	-	Microgram
μl	-	Microliter
α	-	Alpha
^0C	-	Degree Centigrade
%	-	Percentage
>	-	Greater than
<	-	Less than
±	-	Plus or minus
w/v	-	Weight per volume
n	-	Nanometre
cm	-	Centimetre
v/v	-	Volume per volume
nMol/mg	-	Nano molecule per milligram

ABSTRACT

Background: cyanide is a potent neurotoxic substance that can initiate series of intracellular reactions leading to oxidative stress. **Objective:** To evaluate effect of sublethal administration of KCN on motor functions, visuo-spatial learning and memory. Some biochemical parameters which include brain malondialdehyde concentration, superoxide dismutase, catalase and acetylcholinesterase activities in adult swiss albino mice and possible ameliorative roles of vitamins A and E. **Methods:** Thirty five adult swiss albino mice weighing between 18-22g were used. In each group served as subjects for this study. An acute toxicity study was carried out and LD_{50} was calculated as 15mg/kg. The animals were randomly divided into five groups (n = 7) and exposed to sublethal concentration of potassium cyanide (10% LD_{50}; 1.5 mg/kg). The groupings were as follows; group I (control received 10 ml deionised water), group II (1.5mg/kg KCN), group III (1.5mg/kg KCN + 25mg/kg vitamin A), group IV (1.5mg/kg KCN + 50mg/kg vitamin E) and group V (1.5mg/kg KCN + 25mg/kg vitamin A + 50mg/kg). The animals were fed for 28 days during which neurobehavioral tests using different paradigms was carried out prior to sacrifice and isolation of tissues for biochemical assays. During the study period, Mice were examined for signs of toxicity. **Results:** From the results obtained from this study, it suggests that motor strength was considerably reduced in the KCN treated group as compared to the vitamins groups. Group2 displayed hypolocomotion on the stationary beam in week 4 (15.8±0.58) while group III recorded decrease in transfer latencies to traverse the beam in week 4 (3.20±0.58). In acquisition and retention, using EPM, group II recorded an increase in transfer latencies (50.40±1.72) and (57.60±0.93) as compared to group IV (29.40±0.68; 5.60±0.60), mice spent more time in the open arms than in the closed arms. Increase in MDA concentration induced by KCN (3.35±0.19) which is an index of lipid peroxidation was mitigated when administered with vitamins A and E (1.82±0.21). Brain SOD activity was significantly ($p<0.05$) decreased in group2 (1.30±0.27) as compared to group V (2.78±0.10). Brain acetylcholinesterase activity as a biomarker in cyanide toxicity was increased in group II (32.10±0.90) as compared to group III (14.80±0.86). **Conclusion:**the study suggests that motor deficits and cognitive impairments were correlated with cyanide induced neurotoxicity with increased MDA concentration levels and decreased antioxidant enzymes while the ameliorative effects of vitamins A and E may be due to the inhibition of free radicals overproduction and maintenance of antioxidant defence mechanisms.

CHAPTER ONE

1.0 Introduction

Cyanide is a rapidly acting, potentially deadly chemical that can exist in various forms (Jones *et al.,* 2003). It is a chemical substance that exists freely in nature either as free cyanide (CN-) or hydrocyanic acid (HCN) or cyanogen chloride (CNCl), both of which can be in the gaseous state or aqueous state (Abdel *et al*., 2014). As crystal it exists as sodium cyanide (NaCN) or potassium cyanide (KCN). It is described as having a "bitter almond" smell and odorless, not everyone can detect this odor.

1.1 Sources of Cyanide

Many substances are potential sources of cyanide exposure, including edible and non-edible plants (e.g., cassava), industrial operations (e.g., plastics processing), fires, and cigarette smoke (Ogundele and Olu, 2012). Although the primary natural source of cyanide poisoning is from plants (Osuntokun, 1981), other natural sources include volcanoes, bacteria, and fungi. Anthropogenic sources include malfunctioning catalytic converters, fires involving the burning of plastics, cigarette smoke, and illicit uses of cyanide (e.g. terrorist activities). Additionally, over one million tons of cyanides are manufactured annually worldwide for industrial uses, including chemical syntheses, electroplating, plastics processing, paint manufacturing, gold and silver extraction, tanning, and metallurgy (Pauluhn, 1992).

1.2 Mechanism of Toxicity

1) The toxic effect of cyanide is attributed predominantly to the production of anoxia following inhibition of cytochrome oxidase, a terminal mitochondrial respiratory chain enzyme (Borowitz*et al.,* 1995). This enzyme contains two heme A and two copper ions. Cyanide has a

special affinity for the heme molecule and the reaction of cyanide with the multi-meric iron enzyme complex facilitated by first penetration of cyanide to protein crevices, with initial binding of cyanide to the protein followed by binding of cyanide to heme ion (Ogundele and Olu-Bolaji, 2011). There by, a cyanide-heme cytochrome oxidase complex is formed which renders the enzyme incapable of utilizing the oxygen.

The resultant oxygen saturation of the blood imparts a cherry red colour, which aids the diagnosis in most instances of cyanide poisoning (Alarie, 2002). Inhibition of cytochrome oxidase results in interruption of electron transport chain and the oxidative phosphorylation. Resultant anaerobic metabolism with severely decreased ATP generation and concomitant increase in lactic acid production eventually leads to tissue hypoxia and metabolic acidosis.

The inhibitory properties of cyanide may be ascribed to its ability to complex with metals. Besides iron containing cytochrome oxidase, there are other metallo-enzymes containing molybdenum, zinc or copper which are equally sensitive to cyanide (Barone*et al.*, 2003).

2) Other mechanism for cyanide inhibition may be attributed to its affinity to Schiff base intermediates, e.g. ribulosediphosphate impairs the cellular respiration leading to a cascade of events culminating in cell death (Astier*et al*., 2003).

3) The cyanide has the ability to bind to iron ions. However, cyanide binds to erythrocytes but does not affect the oxygen transfer. CN affects the mitochondria by binding to the enzyme cytochrome-*c* oxidase (CCO), the primary effect of is blocking of the mitochondrial respiration chain and the formation of intracellular adenosine triphosphate (ATP) (Hyldegaard*et al*., 2011). The result is cytotoxic hypoxia caused by the inhibition of CCO by the high affinity of CN to

heme aa_3 of the enzyme. The effect is a structural change and a reduced activity of the enzyme and an increase in lactate production resulting in metabolic acidosis.

1.3 Absorption and Distribution

Absorption of cyanide occurs rapidly, whether absorption by ingestion of cyanide salts of HCN through the lungs or by dermal exposure to HCN gas or aqueous solutions of HCN (Baud *et al*., 1991). Others include potassium cyanide or sodium cyanide. This rapid absorption of cyanide occurs in both animals and humans (Anand*et al*., 2009). However, we know empirically from the extremely rapid onset of symptoms that cyanide is readily absorbedinto the blood stream and distributed throughout the body. Once cyanide is in the bloodstream, it is distributed to other body tissues, and after the initial exposure to cyanide, the tissues show increased cyanide concentrations relative to blood (Hebert, 1993).

The distribution of cyanide among the various body tissues is somewhat uniform, although the highest levels are generally found within the liver, lungs, blood and brain. Cyanide does not accumulate in blood and tissues following chronic exposure (Bhattacharya, 2000).

1.4 Kinetics and Metabolism

The cyanide ion (CN^-) is the toxic moiety in hydrogen cyanide. The toxicity of simple cyanidesalts, such as potassium and sodium cyanide is, therefore, similar to that of hydrogencyanide (Jillian, 2011).The small size and moderate lipid solubility of hydrogen cyanide favors rapid absorption across mucous membranes and uptake by the alveolar epithelium before distributionthroughout the whole body. Absorption of hydrogen cyanide from the stomach is alsorapid, with the acid environment favoring the non-ionized form and facilitating absorption (Philbrick, 1979).Cyanide absorbed from the gastrointestinal tract undergoes first pass

metabolism in the liver. Ingestion of simple cyanide salts rapidly results in formation of hydrogen cyanide in thestomach as free cyanide ion is bound to hydrogen ion in the acidic stomach environment (Howard and Hanzel, 1995).

Cyanide in solution is absorbed across intact skin due to its lipid solubility.Cyanide ion toxicity is mediated primarily by its high affinity for the ferric moiety ofcytochrome c oxidase in mitochondria (Isom*et al.,* 1975), a key component in oxidative respiration. This stablebut reversible interaction blocks the last stage in the electron transfer chain, resulting incellular hypoxia and a shift of aerobic to anaerobic cellular respiration, leading to cellular ATPdepletion, lactic acidosis and cell and tissue death (Felner*et al.,* 2002).

Metabolism of cyanide ion primarily involves its conversion to soluble thiocyanate (SCN-) bythe enzyme rhodanese, with about 80% of cyanide detoxified by this route (Baskin *et al*., 2010). This requiressulphane-sulphur as a co-factor i.e. one sulphur atom bonded to another sulphur atom suchas in a thiosulphate salt (e.g. sodium thiosulphate). This conversion is irreversible and thethiocyanate ion may then be readily excreted in the urine. While the majority of absorbed cyanide is excreted in the urine as thiocyanate, smallamounts of free cyanide may also be excreted via the lungs, sweat, urine and saliva. It has a plasma half life of 20 min to 1 hour has been estimated for cyanides in humans.

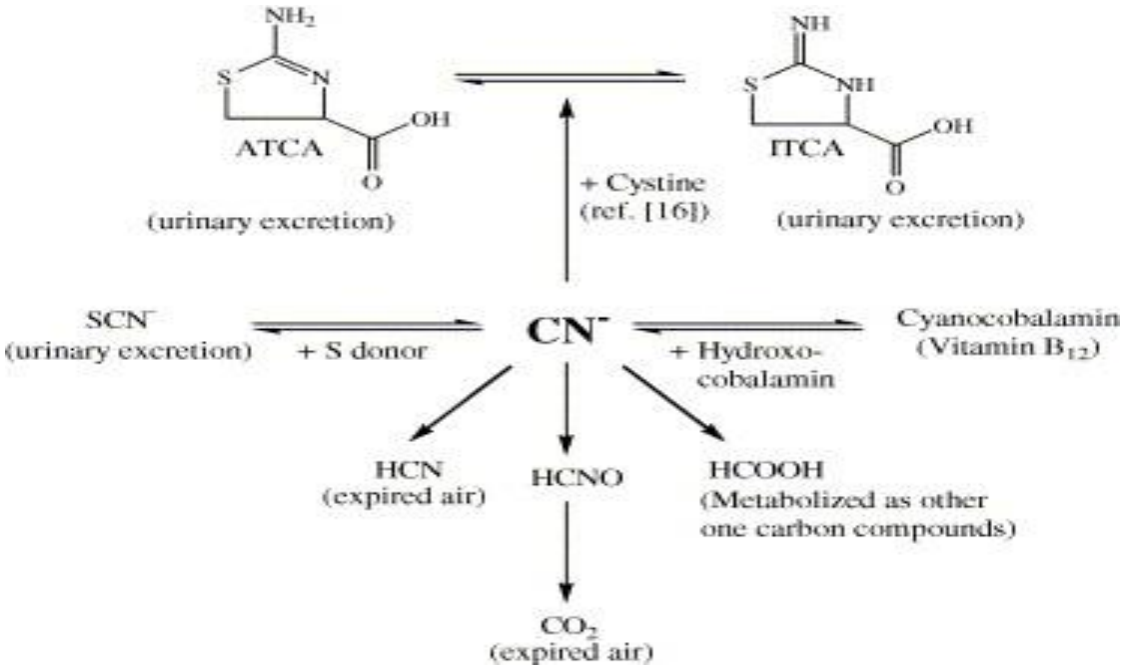


Fig. 1.1:Human metabolism of cyanide (Baskin *et al*., 2010).

1.5 Neurotoxicity of CN

1.5.1 Acute toxicity

Cyanide inhibits cytochrome oxidase preventing oxygen utilization leading to cytotoxic anoxia. Acute effects depend on the degree of histotoxic-hypoxia (Barone*et al.,* 2003). Death results from CNS depression. Signs of acute intoxication by cyanide include rapid breathing, gasping, headache, salivation, nausea, anxiety, vertigo, cardiac arrhythmias, tremors, hypotension, respiratory failure, convulsions and death. Venous blood may remain oxygenated and thus, victims may appear pink. The Mean lethal oral dose of cyanide in human adults is thought to be in the range of 50 to 200 mg and death is rarely delayed more than one hour (Hyldegaard*et al.*, 2011).

1.5.2 Chronic toxicity

Exposure to low levels of cyanide over a prolonged period produces symptoms which differ from acute exposures. Chronic cyanide intoxication has been associated with such human diseases as

retro-tubular neuritis in pernicious anaemia, Leber's optic atrophy and Nigerian nutritional neuropathy (Asonye and Okolie, 2004). Chronic exposure to tobacco smoke has been implicated in tobacco amblyopia, an eye disorder common to people who smoke (Baud *et al*., 1991). Electroplaters and workers chronically exposed to cyanide solutions commonly suffer from dermatitis.

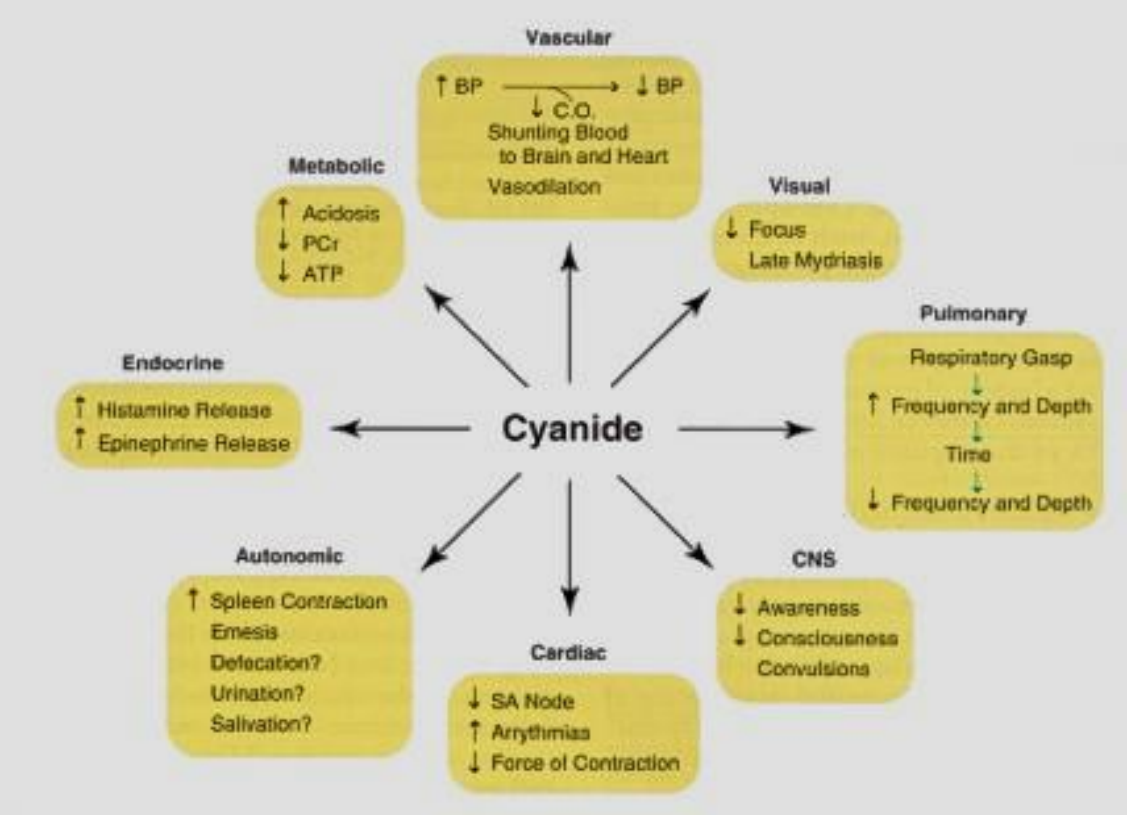


Fig.1.2: Clinical manifestations of cyanide toxicity (Baskin et al., 2010).

1.6 Statement of Research Problem

The neurotoxicity of cyanide is a known problem globally with awide range incidence of neurodegenerative diseases in casava endemic parts of Nigeria, Niger, Zaire and Mozambique leading to motor and cognitive impairments (Ayuba, 2014). With increase industrialisation, man

is continuously disturbing the ecological balance, among the different sources which cause environmental deterioration, HCN is the most important one (Leuschner*et al.,* 1991).Well documented complex toxic manifestations of cyanide toxicity in almost all the body systems (Caxton *et al., 2010*).

Some of the major effects of cyanideposisoning are neurobehavioralimpairements (Boivin, 2013), alterations in brain structure learning and cognitive deficits in children have been observed. Clinical symptoms showed the patients suffered from upper motor neuron disease leading to movement disorders resembling those observed in parkinsonism, tropical ataxic neuropathy (Link *et al.,* 2000, systemic endemic paraparesis (konzo) and gradual loss of vision due to degeneration in the visual cortex or as a result or retrobulbar neuritis (Solar-martin *et al.,* 2010).The toxicity of cyanide arises from inhibition of cytochrome oxidase, the terminal electron acceptor in the respiratory chain. Thus, cyanide blocks electron transport, mitochondrial oxygen utilisation and cellular respiration (David *et al.,* 2007).

The antioxidant vitamins (A and E) function through blocking initial production of, scavenging the oxidants, converting the oxidants to less toxic compounds, repairing the molecular injury induced by or enhancing the endogenous antioxidant defense system of the target (Charkrabort*yet al.,* 2014). These defense mechanism acts cooperatively to protect the body from oxidative stress.Furthermore, there is uncertainty on safety, efficacy and correct indication for use on the current antidote in use (Bhatcharya *et al.,* 2000). Therefore, there is a need to find easier alternative means in alleviating the sufferings of these endemic problemsin our environment.But the question is whether antioxidant vitamins (A and E) play a role in mitigating cognitive and motor functions in cyanide induced neurotoxicity?

1.7 Justification

Antidotes in cyanide poisoning are based on induction of methaemoglobin, which temporarily removes cyanide ions from solution. Cyanide detoxification occurs in vivo mainly by conversion to thiocyanate (Dietrich, 1983). This reaction is catalysed by the enzyme rhodanese. In the presence of excess cyanide, the rate-limiting step is an adequate supply of sulphanesulphur (a divalent sulphur atom bonded to another sulphur atom), which can be supplied by thiosulphate (Beasley and glass 1998). The release of cyanide from various industries has been estimated to be more than 14 million kg/year (Rajesh et al., 2009). Being extensively used, the compounds of cyanide as industrial effluents are the major sources of toxic pollutants in an environment whichis of global concern because of their well-documented negative effects on human and ecosystem (Mataka, *et al.*, 2006). This study intends to determine themitigative role of antioxidant vitamins (A and E) in cognitive and motor deficits of chronic cyanide induced neurotoxicity that lead to oxidative stress which plays an important role in cyanide inducedneurodegenerationand this may be overcome byenhancement of antioxidant status viasupplementation with AOVs.This will solve the problems of lack of compliance, acceptability and adverse reactions of the existing antidotes.

1.8 Aim

The aim of this study is to evaluate the mitigative role ofvitamins A &on learning, memory, motor strength and coordination at sublethal level of potassium cyanide induced cognitive and motor deficitsin mice.

1.9 Specific Objectives

The specific objectives are:

i. To assess motor strengthin mice using wire grid test.

ii. To assess motor coordination of cyanide induced neurotoxicity in mice using coat hanger test and stationary beam walk test.

iii. To evaluate learning and memory of cyanide induced neurotoxicity in mice using Elevated PlusMase.

iv. To evaluate biochemical assays: malondialdehyde concentration, superoxide dismutase, catalase and acetylcholinestrase.

1.10 Research Hypothesis

Administration of antioxidant vitamins ameliorates potassium cyanide-induced neurotoxicity.

CHAPTER TWO

2.0 Literature Review

2.1 Antioxidant Systems

Antioxidant is a molecule that inhibits the oxidation of other molecules. They are man-made or natural supplements that prevent or delay cell damage in the body. Antioxidants terminate these chain reactions by removing radical intermediates and inhibiting other oxidation reactions by being oxidized themselves (Moreira *et al*., 2008). The antioxidant defense systems function through blocking initial production of, scavenging the oxidants, converting the oxidants to less toxic compounds, blocking the secondary production of toxic metabolites or inflammatory mediators, blocking the chain propagation of the secondary oxidants, repairing the molecular injury induced by or enhancing the endogenous antioxidant defense system of the target (Bosch *et al*., 2000). These defense mechanism acts cooperatively to protect the body from oxidative stress. The antioxidant defense system consists of powerful enzymatic and non enzymatic antioxidants.

2.1.1 Enzymatic Antioxidants

All cells in the body contain powerful antioxidant enzymes. The three major classes of antioxidant enzymes are the superoxide dismutases, catalases and glutathione (GSH) peroxidises (Kapur*et al*., 2004). In addition there are numerous specialized antioxidant enzymes reacting with and detoxifying oxidants.

2.1.1.1 Superoxide dismutases (SODs)

They are a class of closely related enzymes that catalyse the breakdown of the anion into oxygen and hydrogen peroxide (Asonye and Okolie, 2004). They are present in almost all aerobic cells

and in the extracellular fluids. They contain metal ions copper, zinc and manganese dismutases. SOD removes oxygen by catalysing a dismutation reaction. SOD catalyzes the conversion of the highly reactive superoxide anion to $O_2$and to H_2O_2 (Mates *et al.,* 1999). This less damaging molecule can be converted spontaneously to highly reactive hydroxyl radicals.

2.1.1.2 Catalase (CAT)

Catalase decompose hydrogen peroxide (H_2O_2) by two different modes of enzymatic activity, the catalatic mode of activity and the peroxidatic mode of activity which has one of the highest turnover rates of all enzyme. It is present in all prokaryotes and eukaryotes with the exception of erythrocytes. It is predominantly located in peroxisomes of all types of mammalian cells where $H_2O_2$is generated by various oxidases from the metabolism of long-chain fatty acids (Atli and Canli, 2007; David et al.,2008).

2.1.1.3 ThioredoxinAnd Glutathione Systems

The system includes glutathione reductase and glutathione peroxidase. These enzymes are at high levels in the liver and also help in metabolism (Bentler*et al.,* 1963). They are the most significant component which directly quenches ROS such as lipid peroxides and plays major role in xenobiotic metabolism. GR belongs to the defense system protecting the body against chemical and oxidative stress (Anyanwu*et al*., 2010).

2.2 Non-Enzymatic Antioxidants

The most widely studied dietary antioxidants are vitamin C, vitamin E, and beta-carotene.

2.2.1 Vitamin C

Vitamin C or ascorbic acid is considered the most important water-soluble antioxidant in extracellular fluids, as it is capable of neutralizing ROS in the aqueous phase before lipid peroxidation is initiated (Cepada*et al*., 2006).

2.2.2 Vitamin E

Tocopherols and tocotrienols (vitamin E) is a major lipid-soluble antioxidant, and is the most effective chain-breaking antioxidant within the cell membrane where it protects membrane fatty acids from lipid peroxidation (Abu-Rashid *et al.,* 2011). It protects cell membrane from against oxidation by reacting with lipid radicals produced in the cell membrane and remving the the free radical intermediates. Tocotrienols have a spscialised role in neuroprotection (Blumberg *et al.,* 1998).

2.2.3 Vitamin A

Retinoids,beta-caroteneand other vitamin A derivatives are compounds with lipophilic propertiesthat have antioxidant functions in lipid bases (Carta*et al.,* 2002). Beta-carotene besides being a precursor to vitamin A has potent antioxidant properties as it removes single oxygen thus protects against free radical attack.

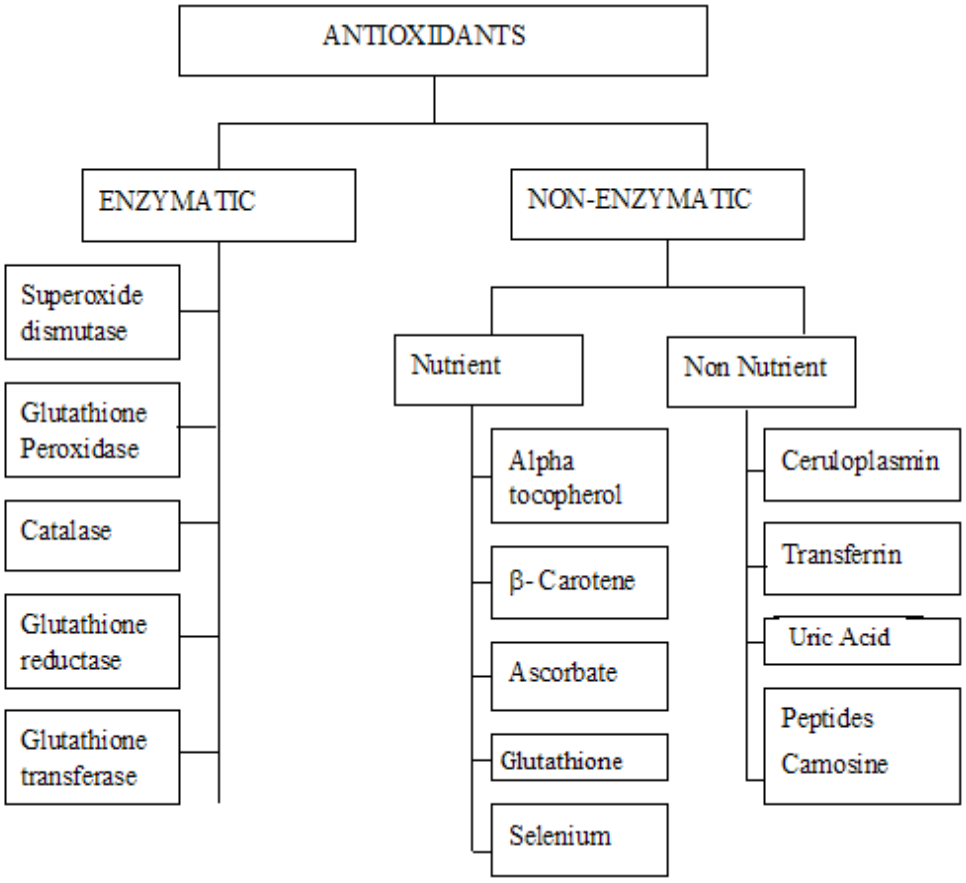


Fig.2.1: Classification of Antioxidants (Patil and Shetti, 2011).

2.3 Antioxidant Mechanisms of Vitamin A

Vitamin A has three active forms (retinal, retinol and retinoic acid) and a storage form retinyl ester. The three active forms of vitamin A each serve different overlapping functions (Deuster, 2002). For instance, retinal is required for rhodopsin formation and vision, while retinoic acid is the principal hormonal metabolite required for proper growth and differentiation of epithelial cells. Their action at molecular level is mediated by several nuclear receptors belonging to the steroid/thyroid receptor super family. Three retinoic receptors (RAR alpha, -beta and -gamma) all-trans retinoic acid (t-RA) and 9-cis RA, while three retinoid x receptors (RXR alpha, -beta and –gamma) bind 9-cis RAbut not t-RA (De Oleivera*et al.*, 2007). As a class, these receptors

are ligand-inducible trans-acting transcription factors, which can modulate the expression of specific target genes by interaction with cis-acting DNA sequences termed retinoic response elementsBraubacher and Weisler, 1985). RA exerts its molecular action mainly through RAR and RXR nuclear receptors. In addition to the nuclear receptors based mechanism of RA action, covalent binding of RA to cell macromolecules has been reported (Collins and Mao, 1999). RA derives from retinol by oxidation through retinol and retinal dehydrogenases and several cytochrome P450s (CYPs).

Circulating retinol is primarily bound to retinol-binding protein RBP), and can enter and leave the liver several times per day in a process known as retinol recycling, which acts to relate the amount of retinol in circulation and protects cells from the damaging effects of free retinol or retinoic acid (Deuster, 2002). Retinol bound to a cellular RBP can be esterified by the enzyme lecithin:retinolaceyltransferase (LCAT), the resulting retinyl ester being stored primarily in liver stellate cells (Bailey and Lane, 2005). LCAT provides a readily retrievable storage form of vitamin A, as well as regulating its bioavailability for other pathways.

2.4 Antioxidant Mechanisms of Vitamin E

Vitamin E compounds (tocopherols and tocotrienols) are well recognized for their effective inhibition of lipid peroxidation in foods and living cells (Allart*et al.,* 2009). Antioxidative activity of the tocopherols is related to scavenging the free radicals of unsaturated lipids. Alpha-Tocopherol, the most biologically active and abundant form of vitamin E in vivo, efficiently transfers a hydrogen atom to a lipid free radical, such as peroxyl, alkoxyl, and carbon-centered radicals, giving the corresponding non-radical product of the lipid and an alpha-tocopheroxyl radicals, once formed, react with a second free radical or each other to form a non-radical

product (Forte *et al*., 2008). Each molecule of alpha-tocopherol consumes thus two lipid free radicals and terminates the free-radical chain reaction.

2.4.1 Alpha - Tocopherol as free radical scavenger

Lipid peroxidation (or autoxidation) is a chain reaction that proceeds in three stages: initiation, propagation, and termination (Jishage*et al*., 2006). In the initiation step, a carbon-centred lipid radical (an alkyl radical) is produced by the abstraction from a polyunsaturated fatty acid moiety. The initiation reaction can be catalysed by heat, light and transition metals. In the propagation step, the alkyl radical reacts with molecular oxygen at a very high rate, giving a peroxyl radical.

The peroxyl radical, a chain carrying radical, is able to attack another polyunsaturated lipid molecule. Although the initial peroxyl radical is converted to a hydro-peroxide, this process produces a new alkyl radical, which is rapidly converted into another peroxyl radical (Forte *et al*., 2008). The chain reaction does not stop until the chain carrying peroxyl radical meets and combines with another radical to form inactive products (termination step).

Alpha-Tocopherol is a chain-breaking antioxidant to inhibit the propagation step. Alpha-Tocopherol donates its phenolic hydrogen atom to a peroxyl radical and converts it to a hydroperoxide. The tocopheroxyl radical that is formed is sufficiently stable to be unable to continue the chain and, instead, is removed from the cycle by reaction with another peroxyl radical to form an inactive, non-radical product (Chow, 1991; Bosh *et al.,* 2000).

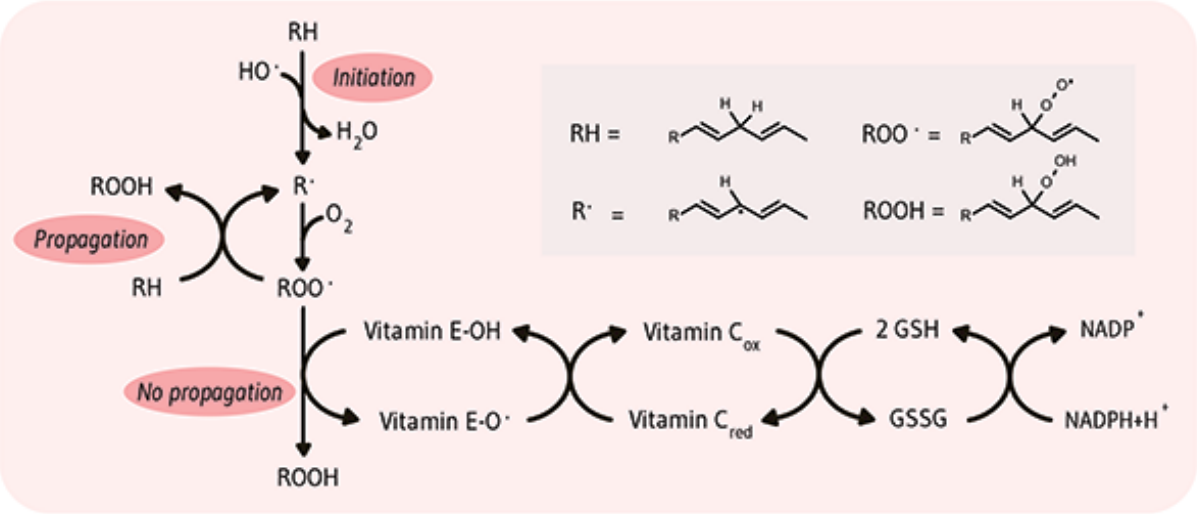


Fig. 2.2: Reaction of α-tocopherol during the autoxidation of unsaturated lipids. LH, polyunsaturated lipid; L•, carbon-centred lipid radical; LO•, lipid-alkoxyl radical; LOOH, lipid hydroperoxide; LOO• lipid-peroxyl radical; TO•, α-tocopheroxyl radical; k, rate constant in M^{-1} s^{-1}

2.5 Reactive Oxygen Species (Free Radicals)

The brain is a metabolically active organ exhibiting high oxygen consumption and robust production of reactive oxygen species (Aliyu*et al*., 2012). The large amounts of ROS are kept in check by an elaborate network of antioxidants, which sometimes fails and leads to neuronal oxidative stress (Kamendulis*et al.*, 2002). Thus, ROS are typically categorized as neurotoxic molecules and typically exert their detrimental effects via oxidation of essential macromolecules such as enzymes and cytoskeletal proteins. Most importantly, excessive ROS are associated with decreased performance in cognitive function (Cynthia and Eric, 2010).

However, at physiological levels, ROS are involved in functional changes necessary for synaptic plasticity and hence, for normal cognitive function (Cassol*et al*., 2010). The inability to restrain either age or pathology-related increases in ROS levels leads to opposite, detrimental effects that are involved in impairments in synaptic plasticity and memory function (Gozalez*et al*., 1991).

In the recent years the term “Reactive Oxygen Species” or “Reactive Oxygen Intermediates” is a collective term which has been adopted to include molecules such as the:

- Hydroxyl radical (OH)
- Superoxide anion (O_2^-)
- Hydrogen peroxide (H_2O_2)
- Hypochlorous acid (HClO)
- Nitric Oxide (NO)

Free radicals are highly reactive molecules or chemical species capable of independent existence. Generation of highly Reactive Oxygen Species (ROS) is an integral feature of normal cellular function like mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism, ovulation, and fertilization (Dixit *et al*., 2011). Their production however, multiplies several folds during pathological conditions.

2.5.1 Major Sources of ROS

1) Endogenous sources: these are internal sites of reactions which produce reactive oxygen species (Hussain et al.,1999).
    - Mitochondria
    - Peroxisome
    - Plasma membrane
    - Cytoplasm
2) Exogenous sources: these are external causes of reactive oxygen species
    - UV light
    - Inflammation

- Cytokines
- Pathogens
- Pollution
- Smoking

2.5.2 Physiological Effects of ROS

Positive effects of ROS mediate the following functions (Avery, 2011);

- Mobilization of ion transport systems, and roles in redox signaling.
- Kill microorganism
- Second messenger (H_2O_2)
- Cellular differentiation & proliferation
- Regulate signal transduction & transcription

2.5.3 Pathological Effects of ROS

Some negative effects of over production of ROS are implicated in many cardiovascular, inflammatory and neurological diseases(Finkel and Holbrook, 2000). Generally, harmful effects of reactive oxygen species on the cell are most often.

- DNA mutation
- Damage of RNA
- Oxidations of polydesaturated fatty acids in lipids (lipid peroxidation).
- Oxidations of amino acids in proteins.
- Oxidatively inactivate specific enzymes by oxidation of co-factors.

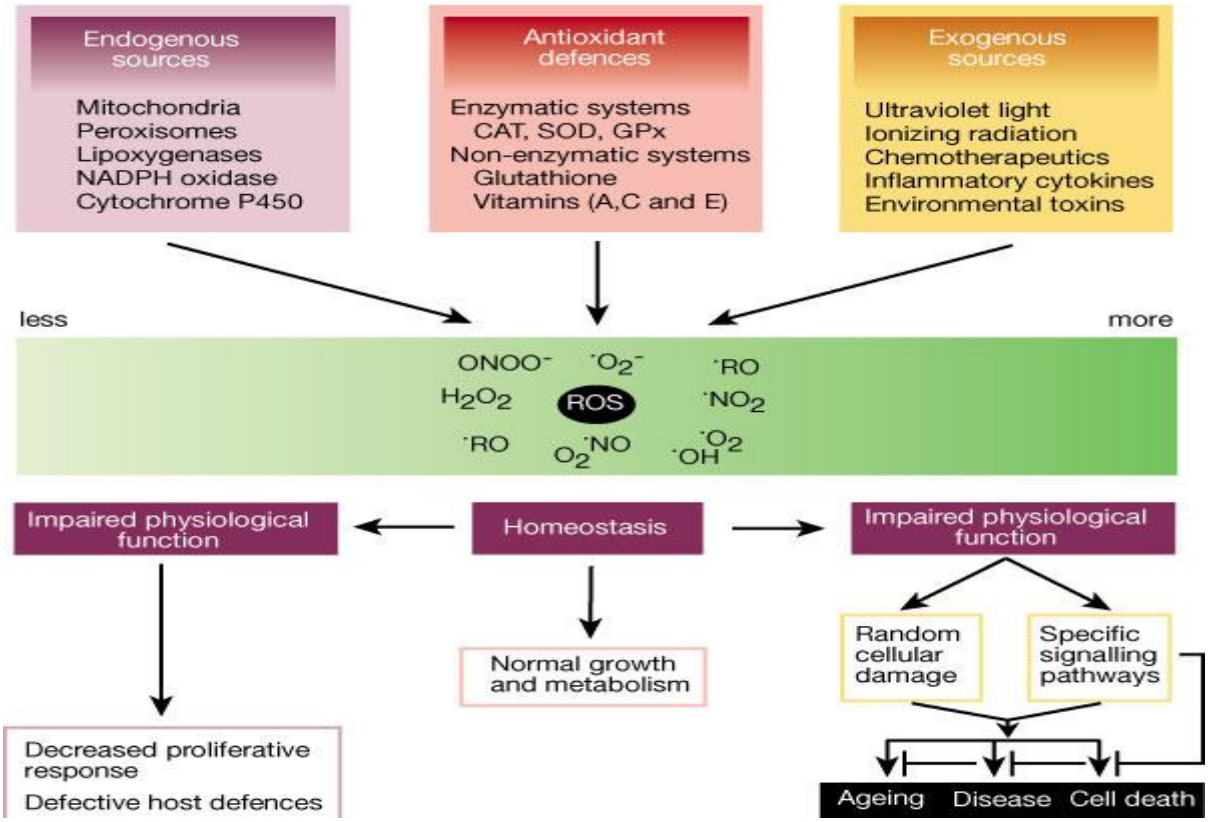


Fig. 2.3: Sources and cellular responses to reactive oxygen species (ROS) (Finkel and Holbrook, 2000).

2.6 Cyanide and Oxidative Stress

Oxidative stress arises from an imbalance between the production of free radicals and physiological antioxidant capabilities in the body (Andersen, 2004). Although the body system is equipped with antioxidant machineries to combat the danger posed by reactive oxygen species (ROS) under normal circumstance (Kamendulis*et al*., 2011). However, oxidative stress as previously reported in cyanide poisoning, the body's antioxidant systems are overwhelmed by the oxidants (Doherty *et al*., 1982). The oxidative stress resulting from either over production of prooxidants or deficiency of antioxidants has been suggested as an important factor in many degenerative diseases and also believed to be a primary factor in cyanide neurotoxicity (Abdel,

2014). Some of the key events that manifest cyanide intoxication are lipid peroxidation, inhibition of antioxidant enzymes, and generation of reactive oxygen species (ROS).

Oxidative stress is a common symptom of cyanide toxicity (Dube*et al.,* 2013); this leads to loss in cell functions and eventually to cell death, and contributes to loss in organ function. This process leads to the development of most chronic diseases and exacerbates existing conditions (Caxton*et al*., 2012).Resulting from oxidative stress, cyanide induces lipid peroxidation *in vitro* that is reversible by co-incubation with N-acetyl cysteine and curcumin (Bhattacharya *et al*., 2010). The induction of oxidative stress by cyanide may involve increases in reactive oxygen species and

Cyanide is a neurotoxin that stimulates intracellular generation of Reactive Oxygen Species (ROS), some attack of generated reactive oxygen species on some targeted organs and cells such as the liver, kidney, and mitochondria. Also exposure to high levels of ROS leads to depletion in antioxidant levels in animals (Anyanwu*et al.,* 2010).The damage caused by free radicals leads to several damaging effects as they attack lipids, protein, enzymes, carbohydrates, and DNA in cells and tissues. They induce undesirable oxidation, causing membrane damage, protein modification, DNA damage and cell death induced by DNA fragmentation and lipid peroxidation (Deacon and Rawlins, 2005).

The oxidative damage or stress associated with cyanide is believed to be involved not only in the toxicity but also in the pathophysiological diseases like heart disease, atherosclerosis (Gupte*et al*.,2013), cataract (Asonye and Okolie, 2004), cognitive dysfunction (Mathangi and Namasivayan, 2000), cancer (neoplastic diseases), diabetic retinopathy, critical illness such as sepsis (Cassol*et al.,* 2010), and adult or acute respiratory distress syndrome, shock, organ

dysfunction, disseminated intravascular coagulation and spastic paraplegia associated with neurodegenerative diseases (Boivin., 2013).

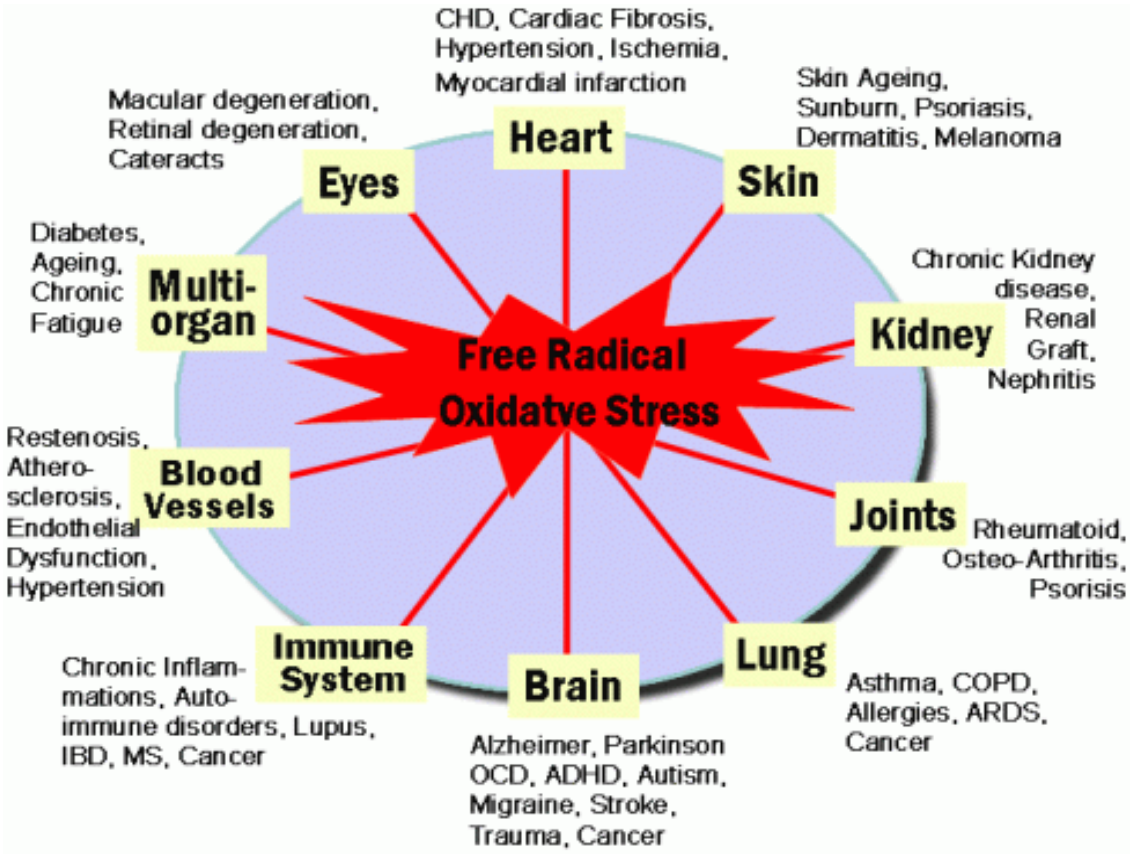


Fig. 2.4:Harmful effects of over production of free radicals due to oxidative stress (Alvandiand Hosetti, 2014).

2.7 Learningand Memory

Learning is the process by which we acquire knowledge about the world, while memory involves encoding, storage and retrieval of such information for future use (Sharma *et al*., 2010). Memory can be classified according to its duration, into short term memory and long term memory. Short term memory is a type of memory that last for seconds or at most minutes, it involves processing of information in the hippocampus and the medial temporal lobe, to create memory traces that later can be converted to the long term memory. Long term memory can store larger quantities of

memory that last for years and sometimes for a lifetime (Sharma *et al.*, 2010). Memory is broadly divided into three viz: sensory, short-term and long-term memories. Sensory memory holds sensory information for a few seconds or less after an item is perceived. The ability to look at an item, and remember what it looked like with just a second of observation, or memorization, is an example of sensory memory. The brain stores sensory information for very short periods of time in a working memory, to be able to use it later (Arash*et al.*, 2014). Brain areas involved in the neuroanatomy of memory include the hippocampus, the amygdala, the striatum, or the mammillary bodies which are thought to be involved in specific types of memory. For example, the hippocampus is believed to be involved in spatial learning and declarative learning, while the amygdala is thought to be involved in emotional memory (Labark and Cabeza, 2006).

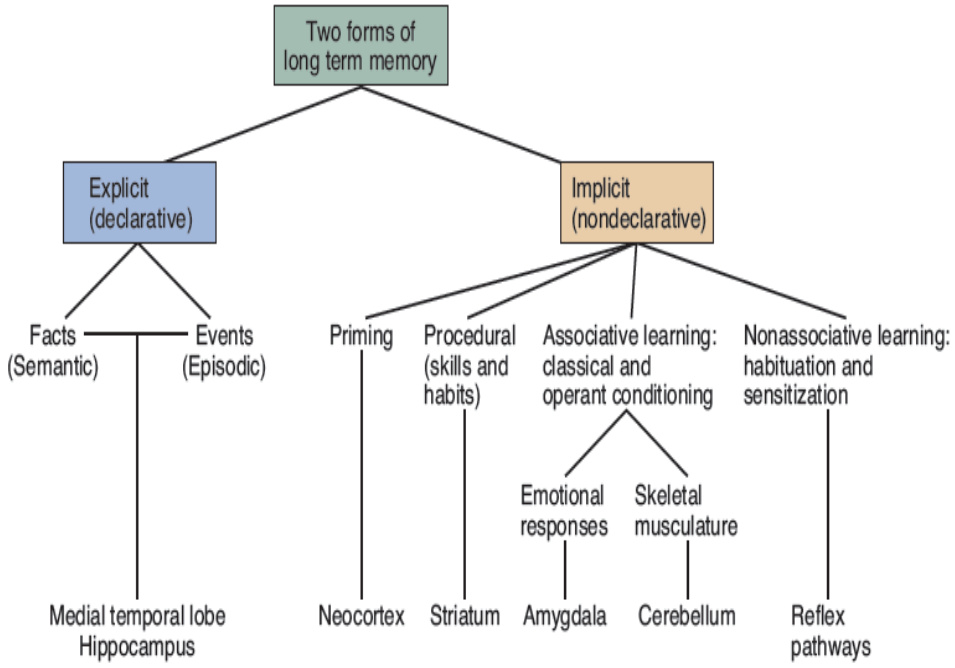


Fig. 2.5:Types of Memory (Adopted from Hershel and Michael, 2011).

2.8 Effects of Cyanide on Learning and Memory

The toxic effect of cyanide ion on brain is attributed mainly to its combination with the oxidized form of cytochrome oxidase and consequent interference with oxygen utilization. At molecular level it is well known that cyanide inhibits cytochrome oxidase and these results in histotoxic hypoxia. It could be anticipated that these profound metabolic changes will affect large number of enzyme systems and also those involved in the metabolism of neurotransmitters in the CNS (AbdulBasit*et al*., 2014). Functional role of neurochemicals in the maintenance of behaviour is exemplified in catecholaminergicsystemand behaviour. Chronic cyanide intoxication decreasesmemory along with reduction in the levels of dopamine and 5-hydroxy tryptaminein the hippocampus(Bhatcharya*et al.,* 2010). Their study showed that the major target site of cyanide is the brain in which hypoxia leads to alteration in memory and behavioural profile.

Furthermore, reports have shown that cyanide exposure causes lag of response as evidenced by prolongation of reaction time. Since reaction time is the time taken by memory to recall information at present, time testing may be the most reasonable approach for assessing neurotoxicity resulting from environmental exposure to cyanide.The exposure to cyanide has toxic effects on the memory through neurotransmitter especially acetylcholine (ACh) which plays a major part in memory (Ogundele and Olu, 2012).

Due to effective detoxification of cyanidecummulative effects of cyanide are rarely seen, but even at sublethal dosesmay at times alter the physiological parameters when exposed over a period of time. But Osuntokun and co-workers 1981 described several degenerative changes in population exposed to cassava based diet; leading to loss of cognition. Similarly studies showed that poorly processed bitter cassava can cause memory loss, affect problem solving abilities and disable other cognitive functions (Boivin, 2013).

2.9 Role of Vitamin A on Learning and Memory

Vitamin A is an important fat soluble vitamin that carries out physiological functions via its main derivative retinoic acid (RA).The hippocampus and its surrounding meninges synthesize and metabolize RA and express retinol-binding protein. RA stimulates retinoic acid receptor (RAR) a nuclear receptor that regulates genes controlling neuronal differenciation and proliferation, neurite outgrowth, patterning of the anterior-posterior axis of neural tube, synaptogenesis and other functions (Carta*et al*., 2002; Bailey and Lane, 2003; Mark *et al.,* 2005). However, excessive vitamin A exposure has been linked to cognitive impairments and may induce congenital defects, including neuronal malformations. Recently, vitamin A supplementation is able to alter behavioural parameters and induce a pro-oxidant state in hippocampus and striatum of adult male rat (Behr *et al.*, 2011).

Vitamin A remains to be important to other central nervous system (CNS) related functions in learning and memory (Carta*et al.*, 2002). Vitamin A and its related retinoids easily penetrate into blood-brain-barrier while CNS contains the molecular apparatus responsible for the production and maintenance of all-trans-retinoic acid in neurons, through retinal dehydrogenases and cellular retinoid proteins action (Deuster, 2000).It is widely believed that synaptic plasticity is the basis for learning and memory, and long-term potentiation (LTP). It refers to the complex multi-faceted physiological processes that mediate the ongoing structural and functional modifications occurring, at various time and scales in the ever changing immature and adult brain and that represent the basis for a role in the pathophysiology and neuropsychiatric dysfunctions.

Furthermore, vitamin A is also a redox-active molecule and has been demonstrated to play a potential pro-oxidant effect in concentrations slightly above the physiologic levels in different in-

vitro experimental models (Moreira *et al.*, 2008). Pro-oxidant effects of vitamin A treatment include increase lipid peroxidation, protein carbonylation, DNA damage and modified activity of antioxidant enzymes in cultured cells (Cassol*et al.*, 2010). Vitamin A supplementation at clinical doses induced a pro-oxidant state in different rat brain regions like the hippocampus, striatum, and frontal cortex (De-oliveira*et al.*, 2008).

2.10 Role of Vitamin E on Learning and Memory

The brain is particularly susceptible to oxidative stress due to its large oxygen turnover, which leads to the generation of oxygen related free radicals (Allard *et al.*, 2009). It is very rich in polyunsaturated fatty acids and thus relies heavily on the antioxidant properties of the lipid-soluble vitamin E (α-tocopherol), which can cross the blood–brain barrier and accumulate in brain tissues. The chain breaking antioxidant activity of this fat soluble vitamin is essential to protect polyunsaturated lipids of cell membranes, lipid bodies and lipoproteins from the peroxidatic activity of free radicals. However, besides antioxidant action, vitamin E exerts a wide range of non-antioxidant activities, mainly by affecting signalling pathways and gene expression (Ambrogini*et al*., 2016).

The hippocampal formation, an important brain structure involved in learning and memory and in emotions, appears to be particularly affected by oxidative stress leading to cognitive impairment which is characterized by gradual and progressive loss of function overtime, with downstream effects on overall health and well-being. Vitamin E slows or prevents memory impairments that accompany several conditions such as mental stress, diabetes, cerebral ischemic injury, Alzheimer's disease, stroke and aging (Abdul-El-Fattah *et al*., 2010). Vitamin E also influences neuroplasticity by exerting effects on many plasticity-related processes in both

physiological and pathological brain. Besides structural plasticity, the hippocampus is able to exhibit the form of synaptic plasticity in the form of long-term potentiation which is one of the main neural mechanism by which memory is stored in the brain. Studies have shown that high maternal intake of vitamin E protect offspring development in rodent, reduce brain atrophy and DNA damage, suppressing neuronal degeneration and hemorrhagic damage in foetal brain exposed in utero to neurotoxicants.

2.11 Effects of Cyanide on Motor Disturbances

The brain stem and spinal cord are regions of the central nervous system that contain interneuronal and motoneuronal networks responsible for the production of reflexive and rhythmic motor behaviours (Borowitz*et al*., 2000). These networks are regulated by descending modulation from cerebral cortex to produce voluntary movements or from sensory afferents from the peripheral nerves to produce reflexes (Gonzalez *et al.,* 2001). The basal ganglia and the cerebellum are important regions of CNS that play crucial role in the coordination of movement by exerting a modulatory influence on most of the cerebral cortex (Ayuba*et al.,* 2014). These areas of the brain are involved in initiating and modifying commands more adaptive, accurate and perform the following functions: maintenance of balance and posture, coordination of voluntary movements, motor learning and cognitive functions (Cardosso*et al*., 2002). Furthermore, the body maintains equilibrium from sensory inputs from the eyes, the vestibular system in the ears, the proprioceptive sytem (joints, bones and muscles) regarding the position of the body with respect to the external environment (Abdulbasit*et al*., 2014).

Neurological disturbances have been reported from parts of Africa with protein-deficient populations and attributed to cyanide (CN^-) exposure from prolonged dietary use of cassava, a cyanophoric plant(Osuntokun, 1981). Cyanide is normally metabolized to thiocyanate (SCN^-) by

the sulphur-dependent enzyme rhodanese. However, in protein deficient subjects where sulphur amino acids (SAA) are low, CN may conceivably be converted to cyanate (OCN), which is known to cause neurodegenerative disease in humans and animals(Anand*et al.,* 2009). One form of cassava related disease is a slowly developing ataxic myeloneuropathy. It is an upper-motor-neuron disease characterized by irreversible and non-progressive symmetric spastic paraparesis that has an abrupt onset. The development of these syndromes is hypothesized to depend on:

- Amount and duration of exposure to dietary cyanide.
- The ability of the body to detoxify cyanide, a function which varies with nutritional status.

It mostly affects children and women of child bearing age. Severe cases have a spastic toe-scissor. High urinary thiocyanate concentrations and presence of ankle clonus were also observed (Cliff and Nicala, 1997).

Another effect of cyanide on motor disturbances is tropical ataxic neuropathy (TAN). It describes several neurological syndromes attributed to toxico-nutritional causes and has occurred mainly in Africa, particularly Nigeria.

The main clinical features of some of the syndromes included;

- Sore-tongue,
- Angular stomatitis,
- Skin desquamations,
- Optical atrophy,
- Neurosensory deafness and
- Sensory gait ataxia (Link *et al*., 2010).

This is attributed to dietary cyanide exposure from chronic-monotonous consumption of foods processed from cassava. The onset of TAN is usually slow over months or years and the mean age of people affected is greater than 40 years. It affects males and females in all age groups equally.

2.12 Mitigative Roles ofAntioxidants in Motor Disturbances.

Various kinds of antioxidants particularly from natural sources such as enzymes, tocopherols, retinoids, carotenoids, ascorbic acids, polyphenols etc inhibits the cellular damage mainly through free radical scavenging property (Karpe*et al.,* 2014). Chronic neurodegenerative diseasesare associated with high levels of oxidative damage and are known to play major role in the destruction of neurons to enable combat the negative impact of free radical scavenging activity by antioxidants through its normal physiological process. The imbalance between the production of free radicals and the ability of cells to counteract or detoxify free radicals that eventually leads to DNA oxidation, protein nitration and lipid peroxidation culminating in cellular oxidative stress.

The antioxidant defense system biologically scavenged or quenched by converting them into metabolically non-destructive cellular molecules. This mechanism prevents free radical mediated damage cells. They involve both enzymatic and non-enzyamatic strategies. Specific quenching and chelating redox metals by antioxidants influence the gene expression profile of tissue

A series of orally bio available antioxidants including mitoQ, mitovitE, and mito TEMPOL are known to by-pass the biological membranes, accumulate within mitochondria and effectively protect against mitochondrial oxidative damage and they are useful in treating neurodegenerative disease with motor disturbance.

Antioxidant benefits have clearly been recognized in improving cognition and motor performance through protecting degenerating neurons, by enhancing existing neuronal function or stimulating neuronal regenerating. Neurogenerative potential of antioxidants has recently evolved due to the neuronal plastic roles of adult stem cells of the brain as a result a number of naturally occurring dietary antioxidants have been identified with properties that support neurogenesis.

CHAPTER THREE

3.0 Materials and Methods

3.1 Animals and their Management

Thirty five (35) apparently healthy mice (18-22g) aged 6-8 weeks were purchased from the animal house of the Faculty of Pharmaceutical Sciences Ahmad Bello University (A.B.U.) Zaria. The mice were housed in cages containing dust-free sawdust beddings. They were fed with pellets made from grower's mash and water *ad libitum*.

3.2 Materials

Potassium cyanide was obtained fromsigma Aldrich, USA.It was dissolved in di-ionised water. Vitamins A and E were also purchased fromPatterson zoochonis Ltd, Nigeria.

Other materials include:

- 35 1ml syringes
- Cannula
- Methylated spirit
- Cotton wool
- Soya oil (vehicle)
- Dissecting kits
- Phosphate Buffers
- Chloroform
- Formalin
- Gloves
- EDTA bottles

3.3 Acute Toxicity Study

3.3.1 Median lethal dose (LD_{50}) of potassium cyanide (KCN)

The LD_{50} of potassium cyanide was determining using the method as described by Lorke (1983) in mice. This test was carried out in two phases; in the first phase, three are three groups of threeanimals, the animals were treated with potassium cyanide at the doses of 10, 100 and 1000 mg/kg body weight per os and observed for signs of toxicity (e.g salivation, convulsion,

asphyxia, piloerection, soft faecal bolus etc) and death within 48 hours. In the second phase; three (3) groups with each containing one mouse were administered with three more specific doses of KCN based on the results of the first phase (2, 4 and 9 mg/kg respectively). The LD_{50} value was determined by calculating the geometric mean of the lowest dose that cause death and the highest dose at which all the animals survived.

$$LD_{50} = \sqrt{MLD \times MTD}$$

Where LD_{50} = median lethal dose

MLD = minimum lethal dose

MTD = maximum tolerated dose

3.4 Treatment Protocol

The mice were divided at random into five groups (n = 7 mice per group) and the treatments administered to the different groups were as follows:

The animals were divided into five groups of 7 animals each

- Group 1: Distilled water (10ml/kg)
- Group 2: 1.5mg/kg KCN.
- Group 3: 1.5mg/kg KCN + 25mg/kg vitamin A.
- Group 4: 1.5mg/kg KCN + 50mg/kg vitamin E.
- Group 5: 1.5mg/kg KCN + 25mg/kg vitamin A + 50mg/kg vitamin E.

KCN was administered orally while vitamins were administered intra-peritonially (IP) once daily for 28 days. The animals were observed for clinical signs and possible deaths throughout the study period.

The experiment was conducted as specified by the Ahmadu Bello University Animal Research Ethic Committee and in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996).

3.5 Neurobehavioral Assays

The different neurobehavioral paradigms were carried out using different models. All behavioural parameters were assessed by four trained observers blind to the animals' treatment status in order to eliminate bias.

3.5.1 Assessment of motor strength using wire grid

Motor strength was measured with the wire grid test. Strength suspension (four paws) was assessed on a grid bordered with masking tape to prevent the mouse from walking off the edge (Delatour*et al.,* 2008). The mouse was placed on the centre of the grid that was slowly turned upside down at a 20 cm height above the floor. Latency to fall was recorded (cut off: 1 min).

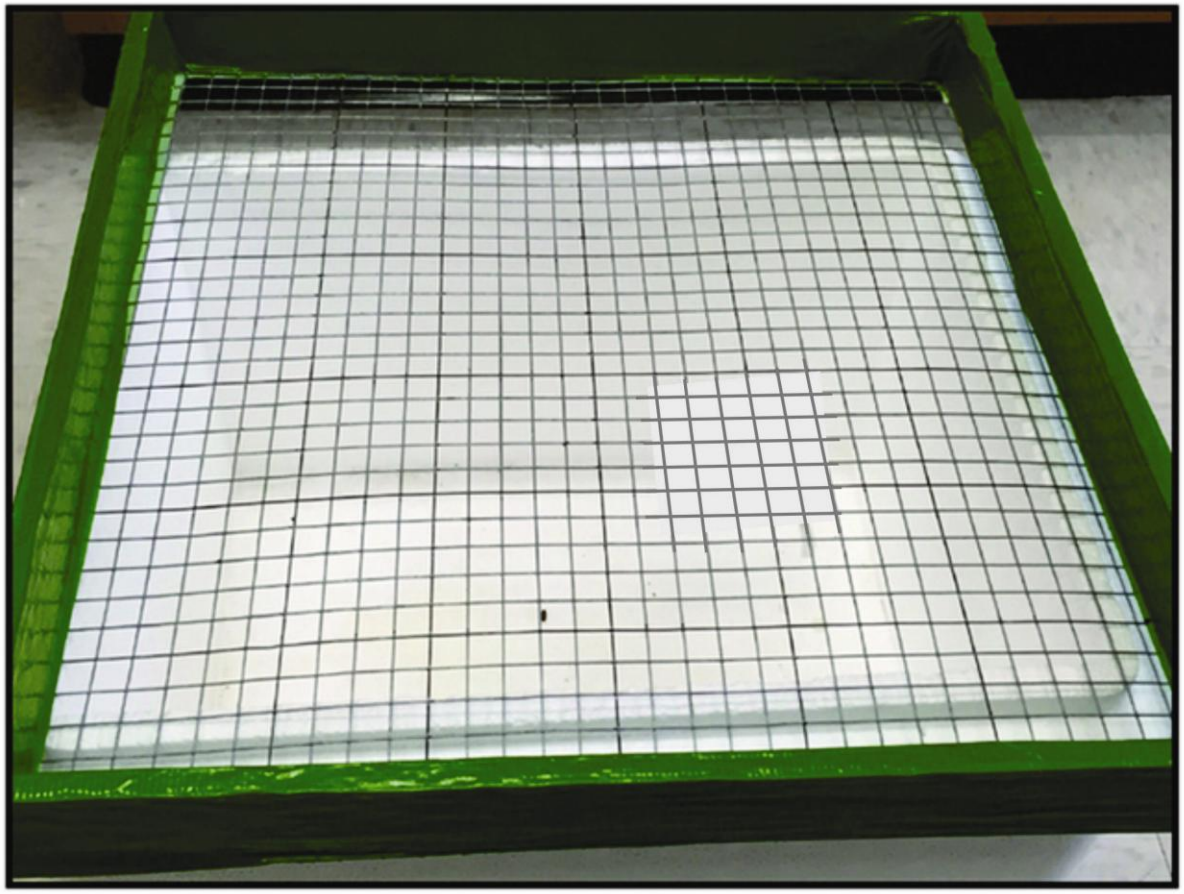


Fig. 3.1:Wire Grid Test model (Snapped by the candidate 10/02/2016)

3.5.2 Assessment of motor coordination using coat hanger

The coat hanger test was used to evaluate motor coordination. An iron horizontal bar (1.3 mm diameter) was placed at a height of 30 cm from the floor; a cardboard wall was inserted at each end of the bar in order to prevent mice from escaping sideways. Mice were placed in the middle part of the horizontal bar. Each trial began only when the forepaws gripped the bar at the moment of release (Delatour*et al*., 2008).

Three trials were recorded (inter-trial interval: 5 min), and after each trial, mice were re-isolated. The latencies of the traction reflex (i.e. time taken by the animal to catch the bar by one of its hind paws) and alternatively the latencies before falling (cut off: 30 s) were measured.



Fig. 3.2:Coat Hanger Test model (Snapped by the candidate 10/02/2016)

3.5.3 Assessment of Motor Coordination Using Beam Walk

Beam walk was conducted in order to detect any neurological deficits in sensory, balance, or motor performance.The materials used were methylated spirit, cotton wool, and the Beam walk apparatus which is made up of 75 cm strips of two smooth woods. One is flat like a ruler of 25

mm width by 10 mm thickness. The other is round and a diameter of 20 mm. Narrow supports stand to hold up the start section of the raised beam (15 mm cross-section, 40 cm high) was also used, together with the Goal box (20 cm x 15 cm, with a 4 × 5 cm entrance hole) secured on top of a support stand.Mice were trained to traverse an elevated, narrow ruler beam to reach an enclosed escape platform. The protocol described here measures foot-slips and latency to traverse the beam. The latency and foot-slip measurements were taken as mice traverse the beam. A 60W lamp was also hanging by the ceiling to provide lightening and also create an aversive stimulus (bright light) to encourage mice to traverse the beam to the dark enclosed goal box.

The animals in their home cages were transferred, from the holding room to the Neurobehavioral laboratory. They were allowed to habituate to the laboratory for 15 minutes. One investigator noted and recorded the latency to traverse each beam to the Goal box and the other placed mice on the beam at the start of each trial, remove them after, and also record foot slips from behind the animals. A mouse was placed at the start of training beam and the latency to traverse the beam (up to 60 sec) was timed.Each mouse was given few consecutive trials in order to avoid the possible influence of learning and returned to its home cage. The ruler like beam was used for training mice, while the round-beam for the actual test.

Methylated spirit was used to clean the apparatus and allowed to evaporate fully after each trial before reusing the apparatus. This was done in order to avoid bias and also wash off the stained urine which may serve as a guide to most mice and therefore alter the accuracy of data. Each mouse was allowed up to a maximum of 60 seconds to traverse the beam.



Fig.3.3:Stationary beam walk test model (Snapped by the candidate 10/02/2016)

3.5.4 Assessment Of Learning Retention Behavior Using Elevated Plus Maze

The experimental animal in each group undergoes elevated plus maze (EPM) test one by one.

The EPM apparatus was made of plywood and consisted of two open arms (25 x 5 cm) and two closed arms (25 x 5 x 15 cm) which extended from a central 5 x 5 cm platform. The plus maze was elevated 50 cm above the floor and the whole apparatus was painted black.

The procedure for the test as described by Itoh*et al.,* (1990) was used. On the first day (training) a mouse was placed at the end of one open arm, facing away from the central platform. Latency for the mouse to enter one of the enclosed arms was recorded in seconds. Following entry into the arm, the animal was allowed to explore the apparatus for 30 seconds. All four of the mouse's

paws were required to be in the arm to be counted as an entry. Twenty four hours later, the second trial (retention test) was performed. After each trial, the plus maze was thoroughly cleaned with methylated spirit and allowed to dry before the next trial.

The latency for mice to go from the "start" end of the open, to any of the enclosed arms were measured (in seconds) to indicate learning and memory (Itoh*et al.*, 1990). Mice appear unwilling to venture onto the open arms of the maze because of a general aversion to open spaces and to the elevation of the maze; this induces learning responses in the animal (Lister, 1990).



Fig. 3.4:Elevated plus maze test model (Snapped by the candidate 10/02/2016)

3.6 Biochemical Parameters

3.6.1 Lipid peroxidation

The level of thiobarbituric acid reactive substance, malondialdehyde (MDA), as an index of lipid peroxidation was evaluated in brain samples using the method of Draper and Hadley (1990) as modified by Freitas*et al.*, (2005). The principle of the method was based on spectrophotometric measurement of the colour developed during the reaction of thiobarbituric acid (TBA) and malondialdehyde (MDA).

Brain samples were collected from each animal immediately after decapitation, weighed and then homogenized in a known volume of ice-cold phosphate buffer to obtain a 10% (w/v) homogenate which was centrifuged at 600g for 10 min to obtain the supernatant. The supernatant was mixed with 1 ml 10% trichloroacetic acid and 1 ml 0.67% thiobarbituric acid (TBA). The mixture was then heated in a boiling water bath for 15 min and butan-2-ol (2:1, v/v) added to the solution. After centrifugation (800g, 5 min), the MDA concentration was determined from the absorbance at 532 nm.

The MDA concentration in each sample was calculated by the absorbance coefficient of MDA-TBA complex 1.56×10^5/cm/M and expressed as nMol/mg of tissue protein. The concentration of protein in the brain homogenates was evaluated using the Lowry method (Lowry *et al.*, 1951).

3.6.2 Superoxide Dismutase

The assessment of superoxide dismutase activity was used to determine antioxidant enzymes activity using the method described by Freitas*et al*., (2005). The 10% (w/v) homogenate, as

obtained above, was centrifuged at 800g for 20 min and the supernatant obtained was used to assay superoxide dismutase.

S1uperoxide dismutase activity was assayed using xanthine and xanthine oxidase to generate superoxide radicals. They react with 2,4-iodophenyl- 3, 4-nitrophenol-5-phenyltetrazolium chloride to form a red formazan dye. The degree of inhibition of this reaction was used to measure superoxide dismutase activity. The standard assay substrate mixture contained 3 mL xanthine (500 μm), 7.44 mg cytochrome c, 3.0 ml KCN (200 μm), and 3.0 ml EDTA (1 mm) in 18.0 ml 0.05 m sodium phosphate buffer, pH 7.0. The sample aliquot (20 μl) was added to 975 μl of the substrate mixture plus 5 μl xanthine oxidase.

After 1 min, the initial absorbance was recorded and the final absorbance was recorded after 6 min. The reaction was followed at 550 nm. Superoxide dismutase was used to obtain a calibration curve showing the correlation of the inhibition percentage of formazan dye formation and superoxide dismutase activity. Superoxide dismutase activity in the samples was determined from this curve and the results expressed as U/mg of tissue protein.

3.6.3 Catalase

The activity was assessed by the method that uses H_2O_2 to generate H_2O and O_2. The activity was measured by the degree of this reaction. 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 min and the final absorbance after 6 min.

The reaction was read at 230 nm. 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 diluents rate (i.e. the uninhibited reaction). Results were expressed as mmol/min/μg of tissue protein. The concentration of protein in the brain homogenates, in both cases, was evaluated using the Lowry method as above.

3.6.4 Acetylcholinestrase activity

Acetylcholinestrase activity was evaluated using the method of Ellman*et al.,* (1961), using acetylcholine iodide as a substrate. Briefly, the whole brain of each animal was homogenized in a cold (0-4^{o}C) 20mM phosphate buffer saline(PBS) incubated with 0.1M 5,5-dithio-bis (2 nitrobenzoic acid) in 0.1M PBS, pH 7.0. Incubations were made to proceed at room temperature for 10min. Then acetylcholine iodide (0.075M in 0.1M PBS, pH 8.0) was added to each tube, and absorbance at 412nM was measured. Acetylcholinestrase activity was expressed as IU/g.

3.7 Statistical Analyses

Results were expressed as the Mean ± Standard Error of Mean (SEM). Data for multiple variable comparisons were analyzed by one-way analysis of variance (ANOVA). For the comparison of significance between groups, Duncan's test was used as a *post hoc* test according to the statistical package program (SPSS version 17.0). Allvalues $p< 0.05$ was considered as significant for all statistical analyses in this study.

CHAPTER FOUR

4.0 Results

4.1 Acute Toxicity Study of Potassium Cyanide inAlbino Mice

Signs of toxicity were observed in all the animals in the groups during the two phases of the toxicity study prior to commencement of the treated groups.

Table 4.1: Results of acute toxicity study showing the different doses of potassium cyanide administered orally to mice, number of deaths and the percentage mortality.

Phase I

Group (n=3)	Dose (mg/kg)	Number of Death	% Mortality
1	10	1/1	100
2	100	1/1	100
3	1000	1/1	100

Table 4.2: Results of acute toxicity study showing the different doses of potassium cyanide administered orally to mice, number of deaths and the percentage mortality.

Phase II

	No of animals in each group				
Doses (mg/kg)	1	2	3	4	Total
2	0/1	0/2	0/3	0/5	0/11
4	0/1	0/2	1/3	1/5	2/11
9	0/1	1/2	3/3	5/5	10/11

The LD_{50} was estimated in the following manner: The Geometric mean of the doses for which 0/1 and 1/1 were found.

$LD_{50}$of KCN = $\sqrt{16}$ X 14

$=\sqrt{224}$

$=15mg/kg$

Therefore LD_{50} of KCN = 15mg/kg.

But for 10% LD_{50} of KCN = 10/100 x 15mg/kg

= 1.5mg/kg

Vitamin A

Each ampule = 25000 IU

But 1 IU = 0.3µg

1IU → mg

= 0.3/100

= 0.003mg

Concentration 1IU = 0.003mg

25000 = xmg

Therefore xmg = 25000 X 0.003/1

= 75mg/ml

Vitamin E

Each ampule = 1000 IU

1mg = 1000 IU

Amount in ml = Dose (mg/kg) X weight in kg / Concentration (in mg/ml)

Hence, the doses administered include;

KCN → 1.5mg/kg

Vitamin A → 25mg/kg

Vitamin E → 50mg/kg

4.2 Effect of Treatments on Clinical Signs

Toxic signs were observed in the potassium cyanide treated group includes huddling, asphyxiation, dyspnoea, mild tremors, piloerection and soft faecal bolus (diarrhoea). With progressive weeks, one death was recorded in group II. The vitamin pre-treated groups (III, IV and V) showed milder toxic signs compared to the KCN group and the followings was observed; salivation, huddling, tremor, piloerection and dyspnoea.

4.3 Effect of Treatments on Wire Grid Test

4.3.1 Time Taken (Latency) To Fall Off the Grid

Analysis of neurological performance in the WGT showed a significant decrease ($p<0.05$) of motor strength in the KCN treated group as compared with control and vitamins pretreated group. A global decline of performance was noted with progressive weeks 2 and 4 respectively (12.60±0.40) and (9.20±0.58) as compared to group V (27.60±0.93) and (38.00±1.05; $p<0.05$) from table1. This shows that motor deficits can be corrected when treated combined but not separated.

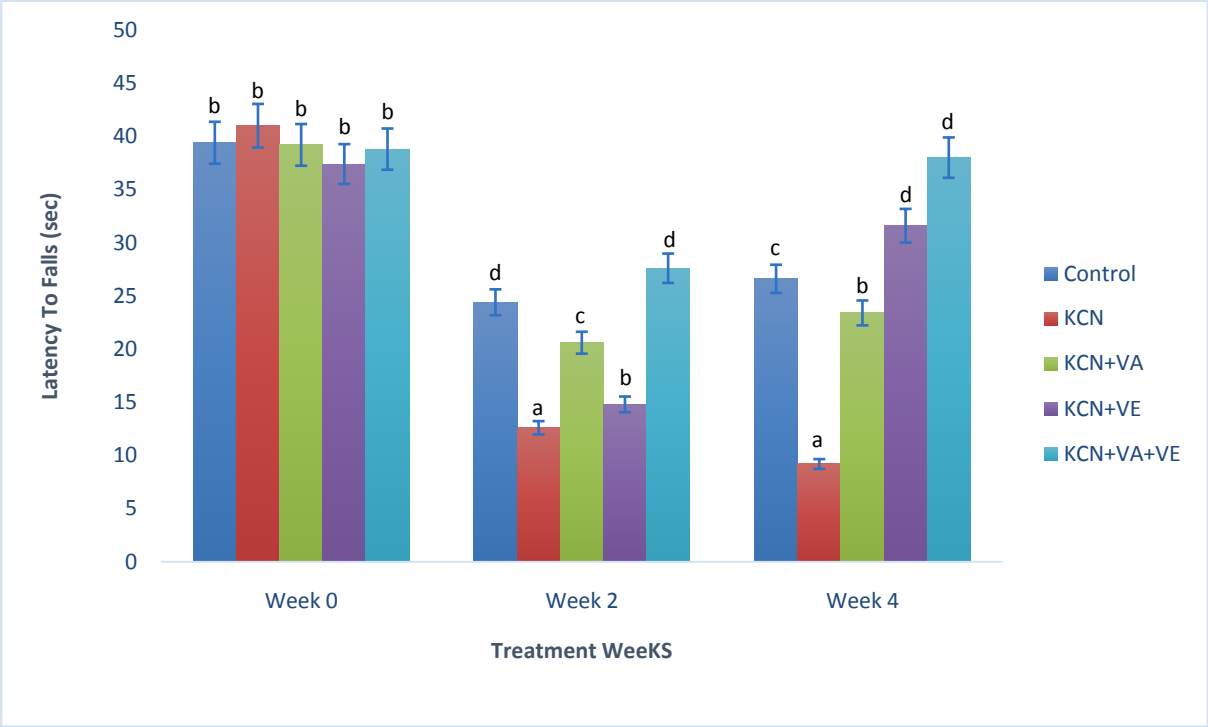


Fig. 4.1: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on wire grid Test in Albino Mice.

a-most significant at $p<0.05$

b-highly significantat $p<0.05$

c-more significantat $p<0.05$

d-significantat $p<0.05$

4.4 Effect Of Treatments On Coat Hanger Test

4.4.1 Time Taken (Latency) To Fall Off the Hanger

The latency to fall from the coat hanger was also measured as an alternative index for motor strength (table 2). Analysis of variance of this variable indicated a global decline of performance in KCN treated group with progressive increase in weeks 2 and 4 respectively (11.00±0.45; 8.20±0.58) when compared to control (24.40±0.68; 26.60±0.51). Pre-treatment with vitamins

showed increase in motor performance as seen in group IV in contrast to group II (8.20±0.58 and 24.60±0.68) $p<0.05$. Motor strength decline can be corrected when treated with vitamin E but not when combined (i.e. vitamin A or E). Group comparison indicated difference amongst the treated groups in rows.

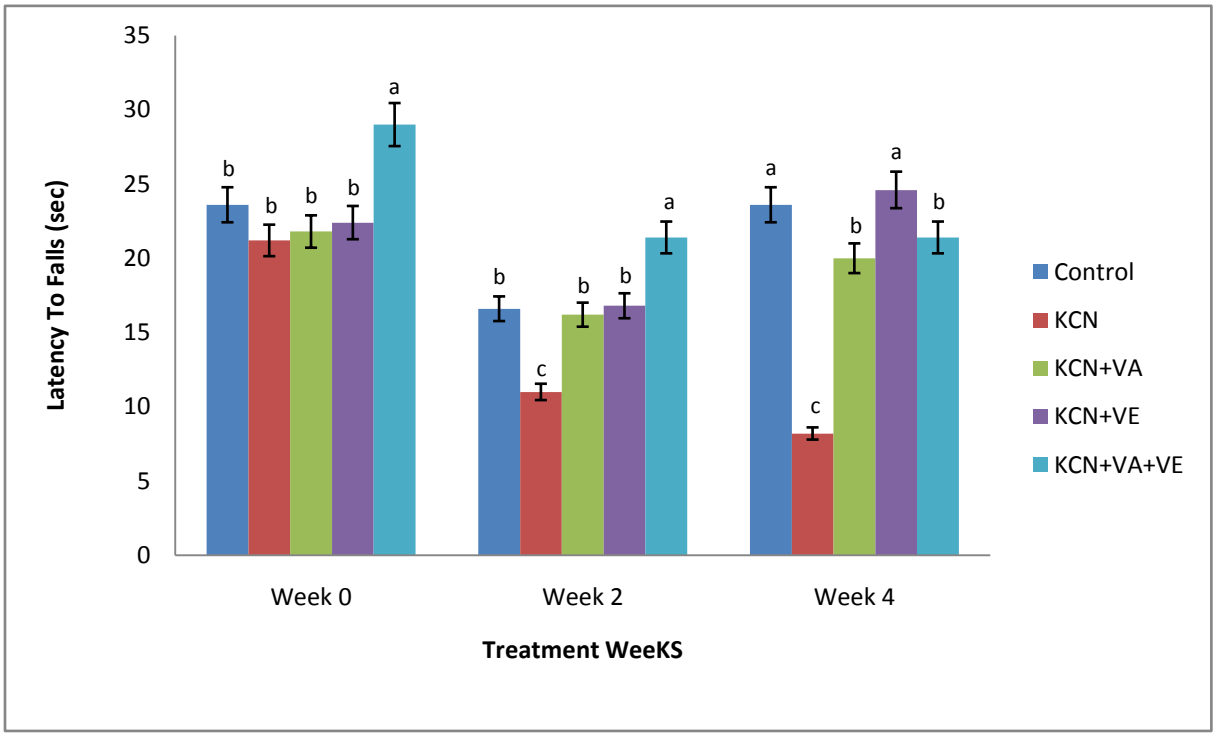


Fig. 4.2: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Dynamics of Coat Hanger's Test in Albino Mice.

a-most significant at $p<0.05$

b- moresignificantat $p<0.05$

c- significantat $p<0.05$

4.5 Effect Of Treatments On Stationary Beam Test

4.5.1 Time Taken (Latency) ToTransverse the Beam

Motor coordination was evaluated with a stationary beam test. Analysis of the number of crossed segments in group II showed decreased activity in KCN treated group (19.60±0.51; 15.80±0.58) when compared with group IV (6.40±0.51; 4.60±0.68), $p<0.05$; table 3. A decline in motor performance as the week progresses as it was recorded in their transfer latencies. Motor performance can be corrected when treated with vitamin A but not when combined (i.e vitamin A or E).

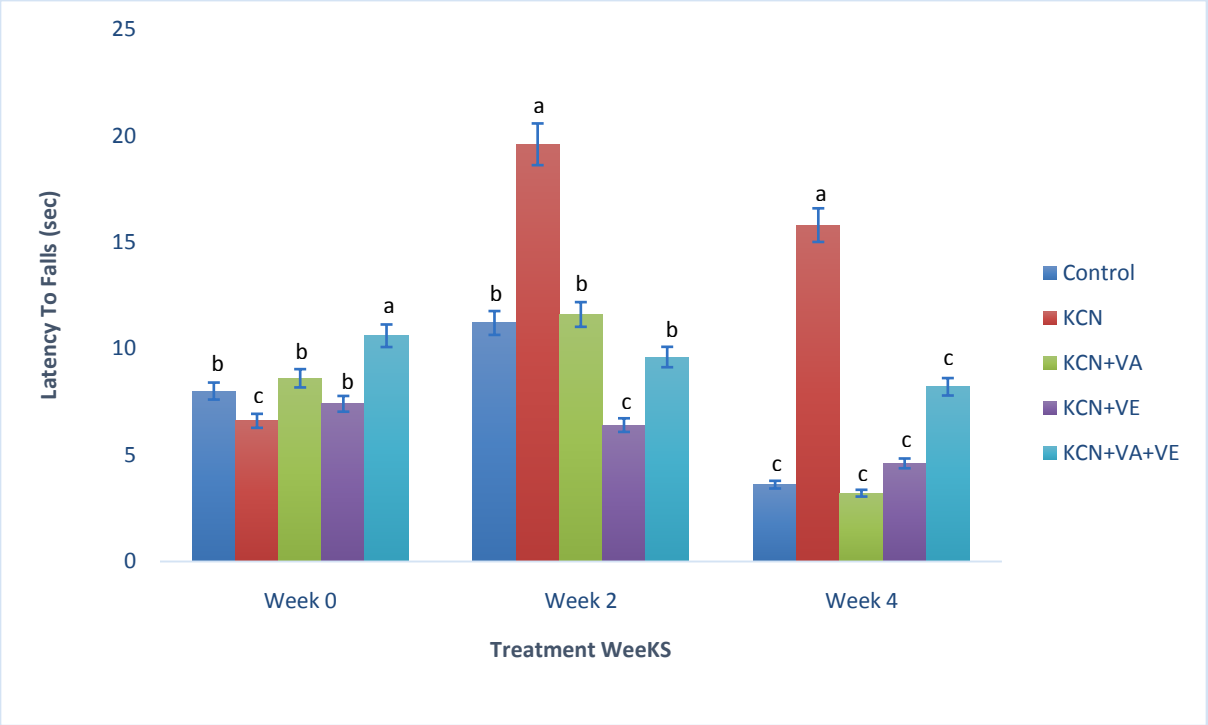


Fig. 4.3: **Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Dynamics of Beam Walk Performance Test in Albino Mice.**

a-most significant at $p<0.05$

b-more significantat $p<0.05$

c-significantat $p<0.05$

4.5.2 Assessment of right foot slips using beam walk

The effects on right foot slip tests of weeks 0, 2 and 4 were compared (Fig. 4.4). The results showed no significant difference ($p>0.05$) amongst the groups in week 0 for groups I, II, III and IV; KCN, V_A+KCN, V_E+KCN and V_A+V_E+KCN respectively. There was no significant difference in the number of slips while crossing to the goal box with groups pre-treated with KCN, V_A+KCN, V_E+KCN and V_A+V_E+KCN in weeks 0 and 2. There was a significant difference ($p<0.05$) in week 4 for all the groups, much slip in KCN treated group. The latency time recorded to cross to the goal box was reduced, less slipping and quite energetic for groups treated with V_A+KCN, V_E+KCN and V_A+V_E+KCN were observed.

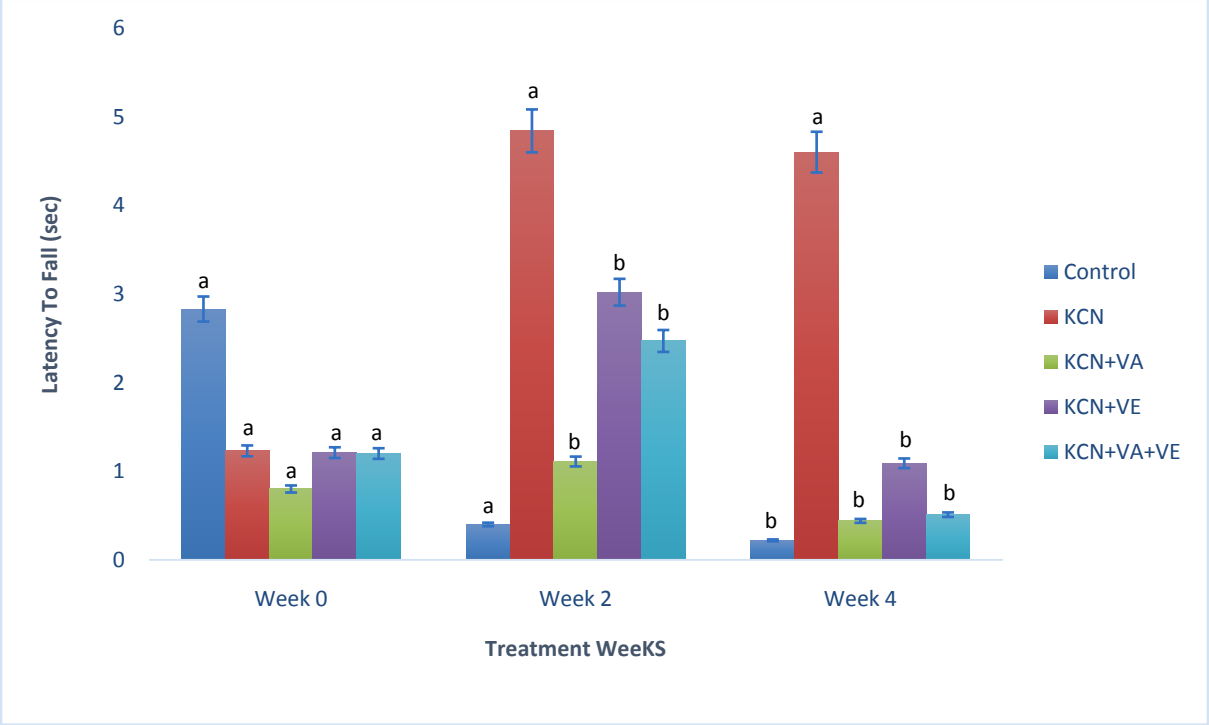


Fig.4.4: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on right foot slips in Albino Mice.

a-most significant at $p<0.05$

b-more significantat $p<0.05$

c-significantat $p<0.05$

4.5.3 Assessment of left foot slips using beam walk

The effects of left foot slip tests of week 0, week 2 and week 4 were compared (Fig. 4.5). There was no significant difference ($p > 0.05$) amongst the groups in week0 in the treatment groups KCN, V_A+KCN, V_E+KCN and V_A+V_E+KCN. As the weeks progresses a significant difference ($p<0.05$)exists amongst the groups in week2 and week4.The number of slips while crossing to the goal box increased and much pronounced in week4. There was lots of slipping in KCN treated group with staggering in movements as compared to vitamin pre-treated groups.

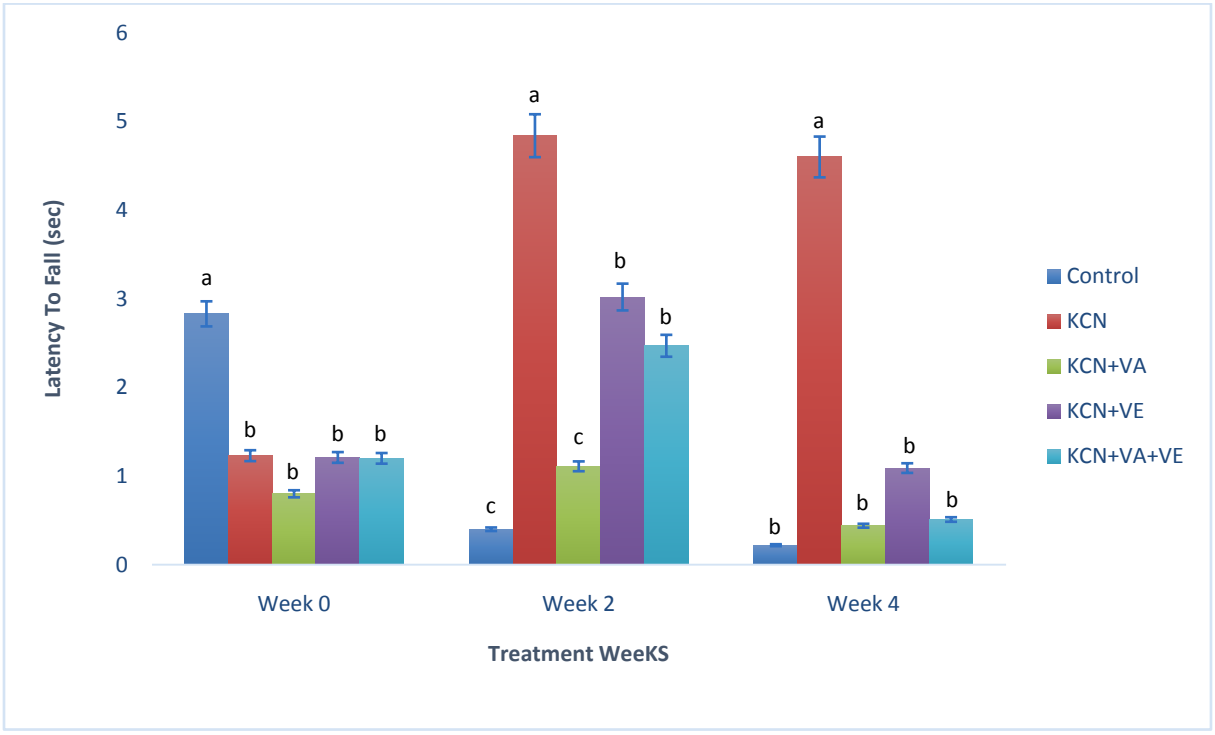


Fig.4.5: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on left foot slips in Albino Mice.

a-most significant at $p<0.05$

b-more significantat $p<0.05$

c-significantat $p<0.05$

4.6 Effect of Treatments on Elevated Plus-Maze Test

4.6.1 Time Taken (Latency) To Find Enclosed Arm

In the EPM test, this was assayed for long-term visuo-spatial memory acquisition and retention latencies. Acquisition latencies (seconds) for KCN treated group and control (50.40±1.72 and 29.60±1.43) while retention transfer latencies were (57.60±0.93 and 10.60±0.51) for both groups. In vitamins pretreated groups III and V acquisition latencies (28.40±1.81and 32.40±0.93) while retention transfer latencies were (11.00±0.71and 14.00±0.70), table 4. The KCN treated group mice spent more time in open arms than in the closed arms in contrast to the vitamin groups underlining avoidance of the anxiogenic area of the apparatus. Acquisition decline can be corrected when treated with vitamin A alone while retention flaws can be improved when treated with vitamin E alone but not when combined.

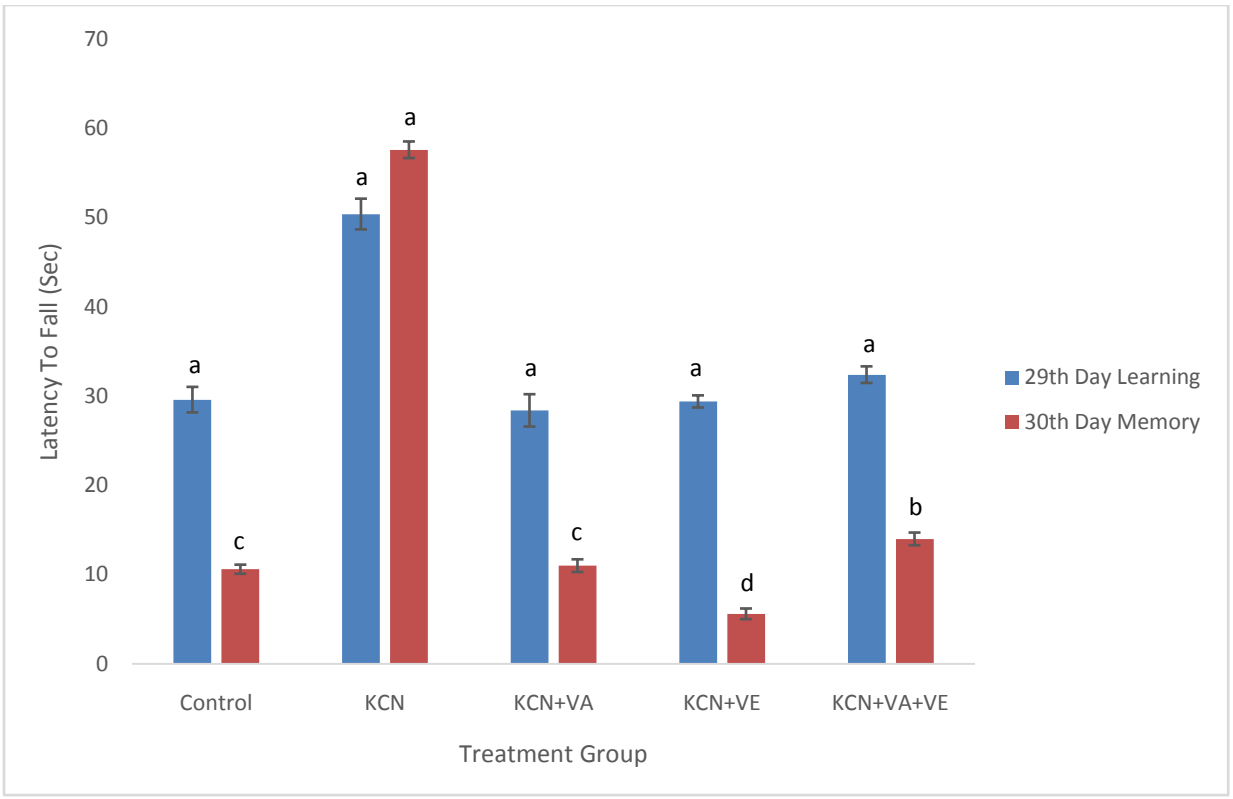


Fig.4.6: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Learning and Memory in Albino Mice.

a-most significant at $p<0.05$

b-highly significantat $p<0.05$

c-more significantat $p<0.05$

d-significantat $p<0.05$

4.7 Effect Of Treatments On Brain Malondialdehyde Concentration

The cyanide induced oxidative stress is consisted with the result. A significant increase in MDA concentration ($p<0.05$) was recorded in the KCN treated group (3.35±0.19) as compared to the control (1.76±0.22). Vitamin pretreated group recorded a significant decrease ($p<0.05$) in MDA concentration with group III recording the least when compared to group II (1.24±0.06). There was no significant change ($p>0.05$) in the brain MDA concentration for group comparison between control and group III nor group IV and V.

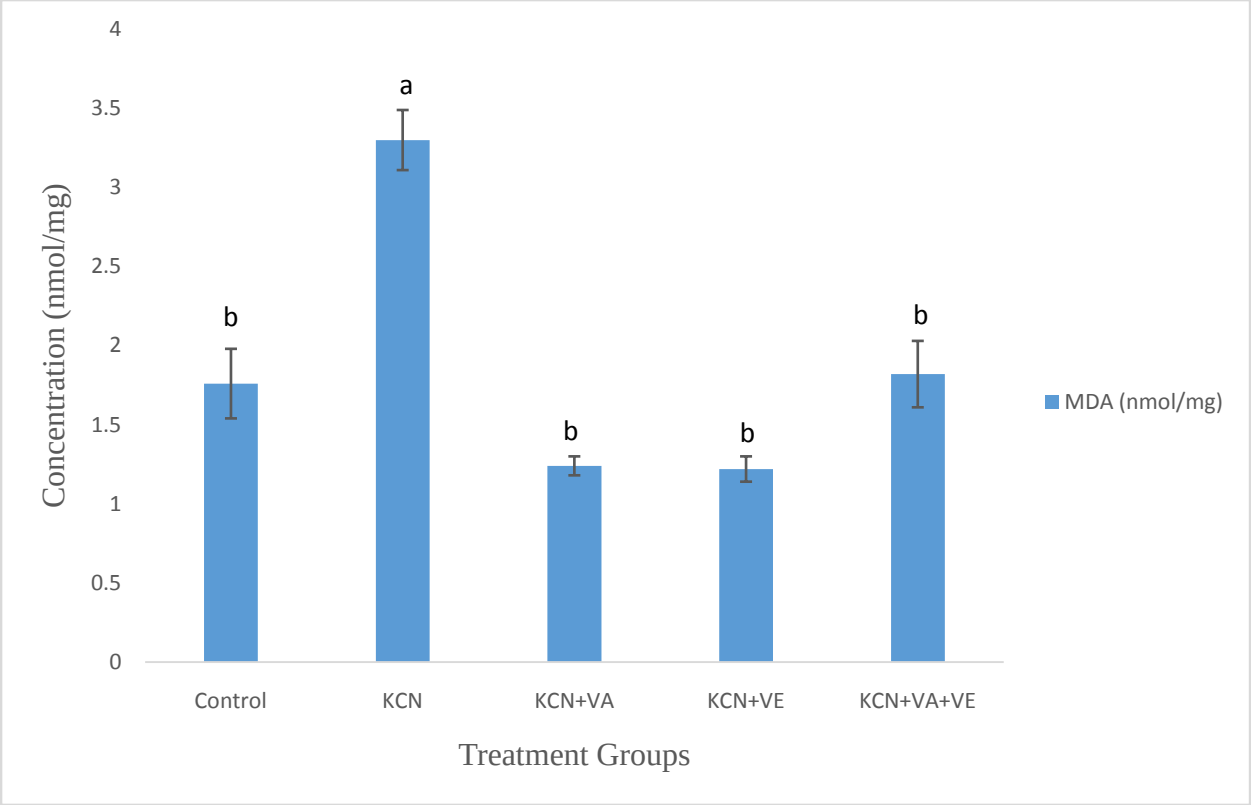


Fig. 4.7: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on brain MDA concentration in albino mice.

a-most significant at $p<0.05$

b-significantat $p<0.05$

4.8 Effect Of Treatments On Brain Superoxide Dismutase Activity

Sublethal exposure of potassium cyanide to albino mice exhibited significant decrease ($p<0.05$) in percentage inhibition in the activity of SOD enzyme during exposure period when compared to control and vitamin pretreated groups. Vitamin treated recorded an increase level in SOD activity ($p<0.05$) with group V having the least inhibition (2.78±0.10) in contrast to KCN group (1.30±0.07). In group comparison, there was no significant change ($p>0.05$) in the brain SOD activity between control or group II nor group V.

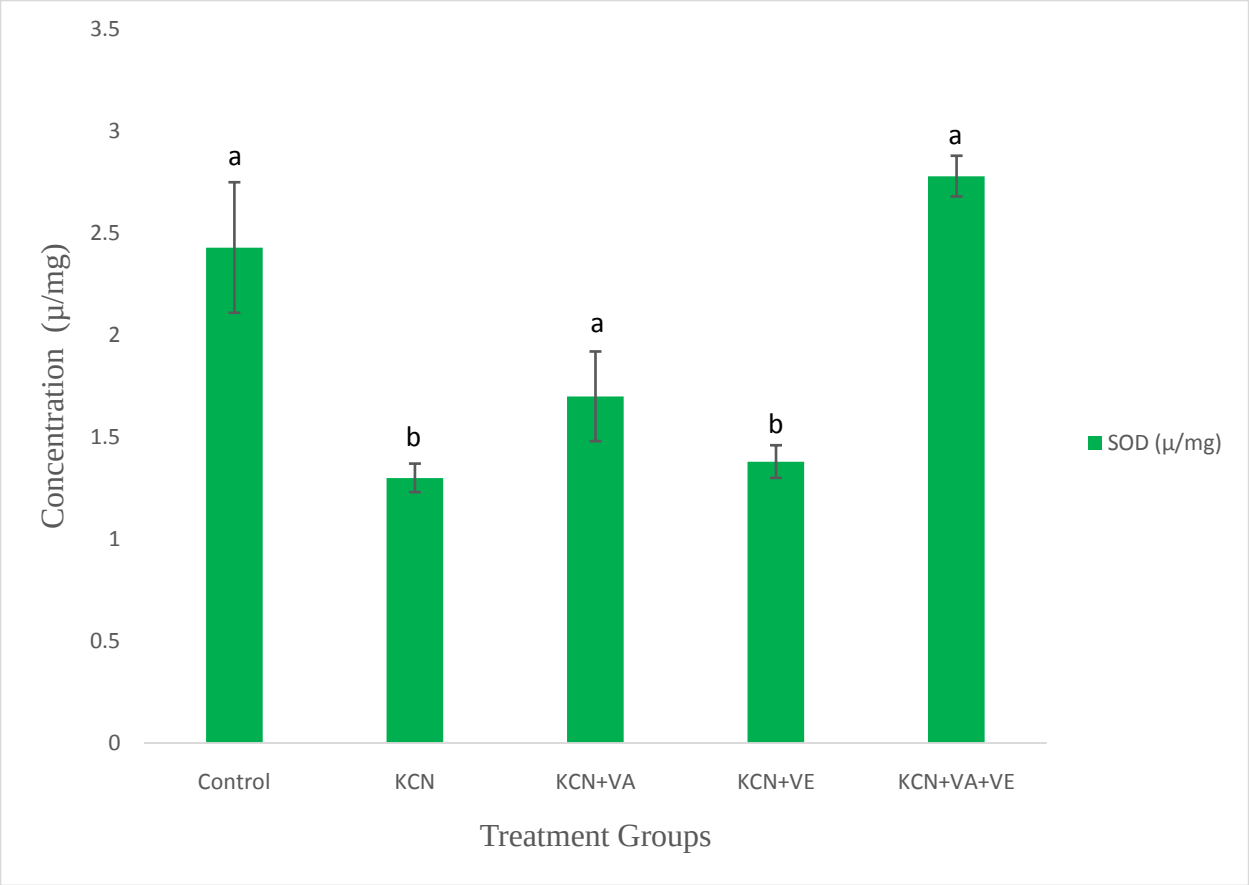


Fig. 4.8: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on brain SOD activity in albino mice.

a-most significant at $p<0.05$

b-significantat $p<0.05$

4.9 Effect Of Treatments On Brain Catalase Activity

A significant decrease ($p<0.05$) level of brain catalase activity was recorded in group II (KCN) when compared to control (30.81±1.43; 42.60±2.10). A less percentage inhibition was recorded in groups that were treated with vitamin (A and E). Catalase activity was increased in both groups with group V having the highest recorded value (48.20±1.57). There was no significant change ($p>0.05$) in the brain catalase activity for group comparison between I and II relative to group III and IV.

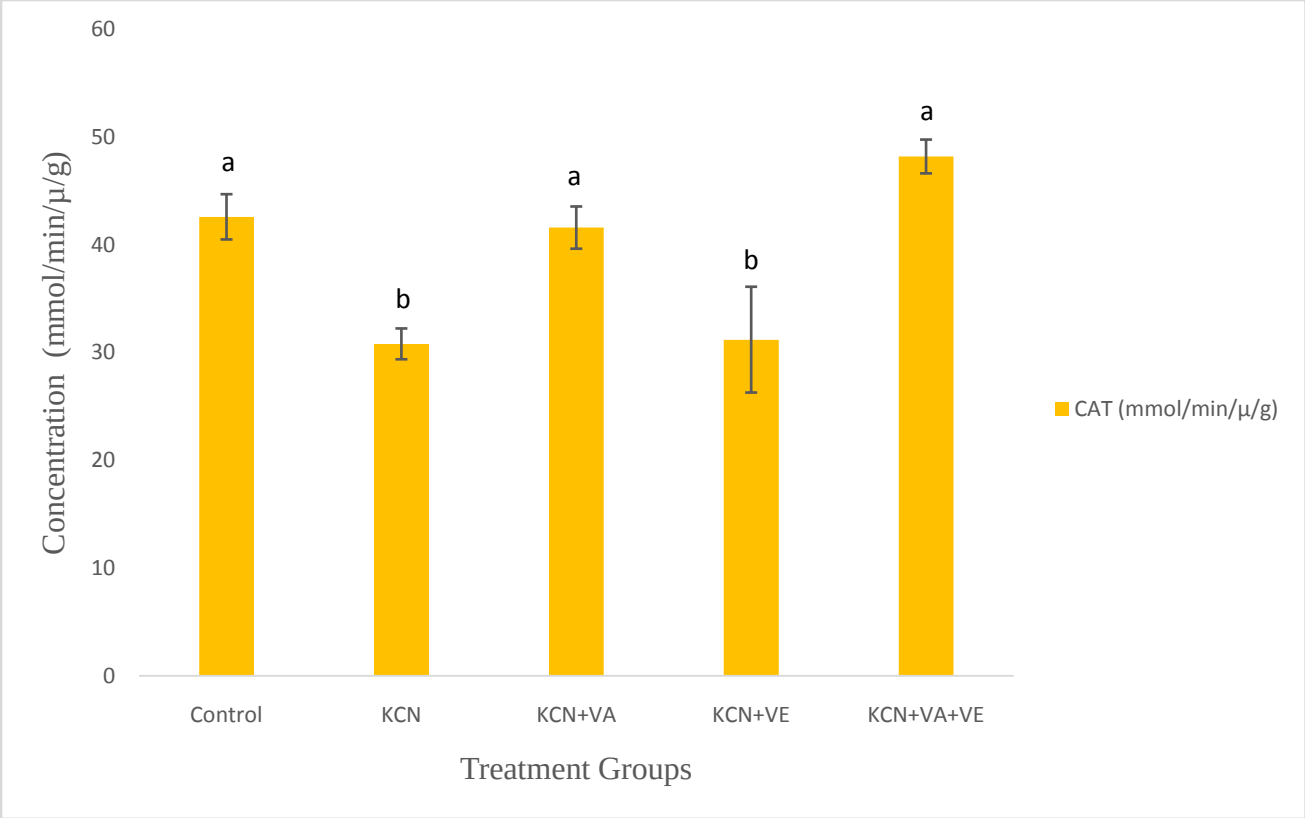


Fig. 4.9: Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on brain CAT activity in albino mice.

a-most significant at $p<0.05$

b-significantat $p<0.05$

4.10 Effect Of Treatments On Brain Acetylcholinesterase Activity

Sublethal exposure of potassium cyanide to albino mice exhibited significant increase ($p<0.05$) in percentage inhibition in the activity of brain acetylcholinesterase during exposure period when compared to control (12.20±0.80; 32.10±0.90). Groups pretreated with vitamin recorded an increase in enzyme activity (AchE) when compared to KCN treated group. Values were statistically significant ($p<0.05$) with decrease inhibition as was seen in group V (16.20±0.90). There was no significant change ($p>0.05$) in the brain acetylcholinesterase activity for group comparison between I and III relative to group IV and V.

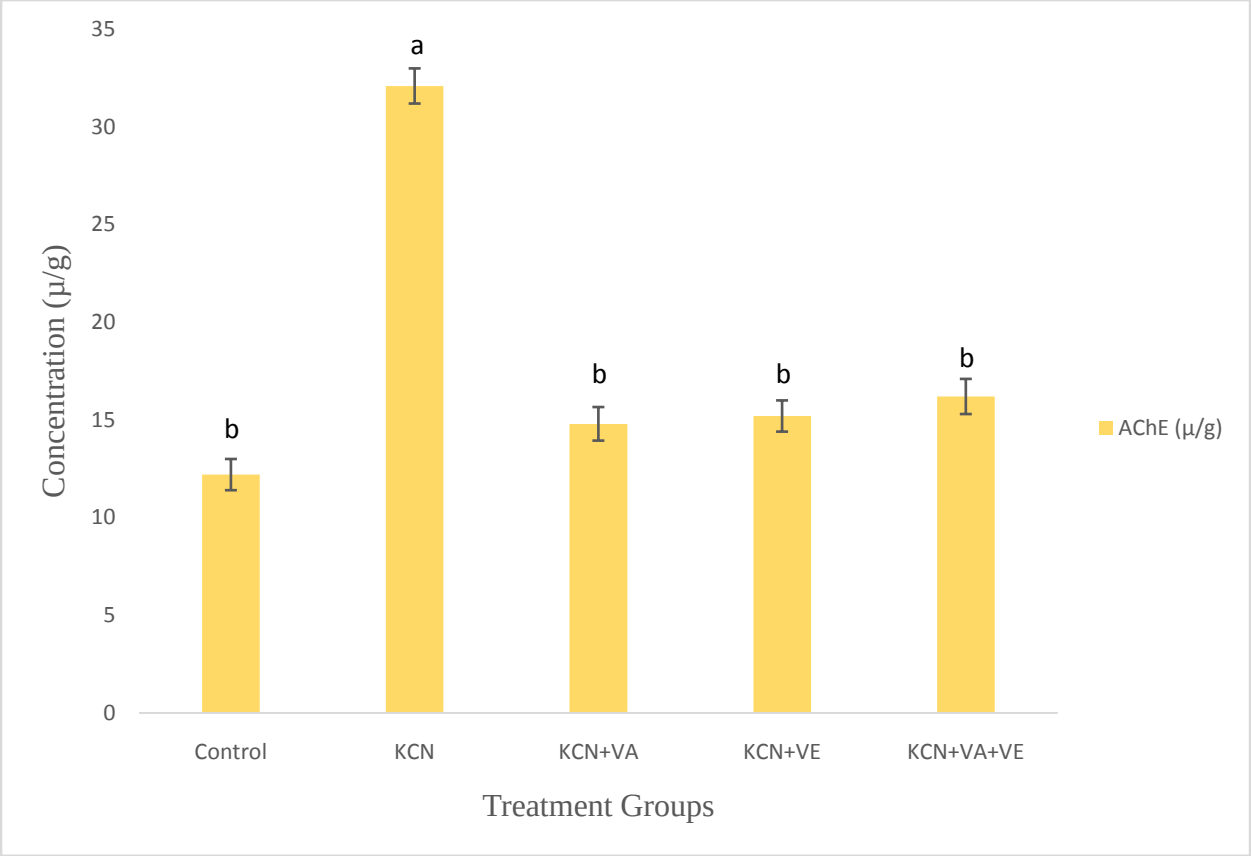


Fig. 4.10: Effect of Subethal Administration of Potassium Cyanide and Administration of Vitamins A and E on brain acetylcholinesterase activity in albino mice.

a-most significant at $p<0.05$

b-significantat $p<0.05$

CHAPTER FIVE

5.0 Discussion

The clinical signs observed in the KCN treated group which include tremors, convulsion and salivation were consistent with cholinergic symptoms observed in cyanide poisoning. These symptoms resulted from the AChE inhibition caused by cyanide leading to accumulation of acetylcholine in the cholinergic receptors. This shows the role of oxidative stress in toxic signs evoked by cyanide. The mitigation of clinical signs and mortality in mice pretreated with vitamins A and E demonstrated the protective effect of vitamins on cyanide induced toxic signs and death.

Analysis of neurological performance in the wire grid test showed a significant reduction in all paws grip time, reflecting deficits in paws motor strength following chronic exposure to cyanide treated group in albino mice. The impairment can either be as a result of cyanide induced lesion in cerebellar cortex, motor area or maybe explained by the triggering of region specific toxic pathways in which oxidative stress maybe a common activator (Borowitz*et al.*, 1999; Hahm*et al.*, 2010; Ayuba, 2015). This may be due to partly reduced brain and perhaps muscle oxidative damage (Aliyu*et al*., 2012).This was observed in the behavior of the animals grip in the fourth week, mice were weak and showed decrease paws strength from their transfer latencies. This can further be explained by inefficient detoxification mechanism since the doses administered were 10% LD_{50} given at sublethal level and it has been demonstrated that cyanide leads to chronic hypoxia and resultant lesion producing decreased motor activity (Boivin, 2013).

The antioxidant pre-treated groups motor deficits was significantly ($p<0.05$) improved as compared to potassium cyanide treated groups. The group that received vitamins A and E had better grip and longer latency in comparison to group III and IV. The combined effects of

vitamins are better and effective in ameliorating the deficits as compared to single vitamin administration in mitigating the effects of cyanide induced oxidative damage via their antioxidant properties.

The latency to fall from the coat hanger was also measured as an index of motor coordination. Analysis of this variable indicates a decrease of performance as week progresses, reflecting deficits in forepaws motor strength following sublethal exposure to KCN in mice. The impairment of motor strength by potassium cyanide may have been due to oxidative damage to muscles resulting in muscular rigidity (Calabressi*et al.,* 2008). Similarly, reports have also shown that reduced strength and loss of muscle strength following acute cyanide poisoning (Messing, 1991). This study demonstrated that after inducing chronic cyanide toxicity, the administration of vitamins caused a significant changein motor forepaw strength and most significant in group IV. Vitamin E administration was more effective from the results, longer time spent on the coat hanger from their transfer latencies as compared to vitamin A or when combined. This could be attributed to the mice less stressful and high exploratory behaviour of mice which make them hold on to the hanger with increase transfer latencies and modulated the effects of cyanide induced neurotoxicity on the muscle mass.

Motor coordination was evaluated using the stationary beam walk. It assayed for muscular coordination and balance. From this present study, the loss of equilibrium followed by erratic movements was observed together with imbalanced body activities and loss of muscular coordination was seen as the mice transverse the beam in group pretreated with KCN. This correlates with the work of Abdulbasit*et al.,* 2014,who carried out work on Motor and memory function in rat models of cyanide toxicity and vascular occlusion induced ischemic injury. The progressive increase in which the mice slipped off the beam indicates impairment of motor

coordination.This might have been responsible for the deficits in mice motor functions as they transverse to reach the goal box. This impairment can also be as a result of either due to cortical or cerebellar damage (Ayuba, 2014), and or pyramidal motor system damage, which controls all of the voluntary movements (Hamakawa*et al*., 2011).It is well documented in the literature that the brain is a critical site for anoxia induced by cyanide, which may cause damage to the regions of the brain associated with the maintenance of equilibrium (De-Latour*et al*., 2008, 2012) as seen in this present study. However groups pretreated with antioxidant vitamins had improved motor performance and decreased number of slipping as compared to group II. However,vitamin A treated group recorded optimum performance as compared to combined vitamins (A and E). The result suggests that antioxidant defense action of vitamin A to play major role in the destruction of cyanide induced neurotoxicity by combating the negative impact of free radical scavenging activity through its normal physiological process by converting them into metabolically non-destructive cellular molecules when administered alone than combined.

Deficits in behaviour and performance in visuo-spatial memory and learning tasks was observed in KCN treated mice. The underlying thread is that cyanide impairs performance in memory and learning. Mice spent more time in the open arms, less explorative to find the enclosed arms, showing less aversion to open space and exhibiting avoidance behavior.The present study also corroborates with the work of Bukachi*et al.,* 2014, which showed memory deficits is associated with sublethal cyanide poisoning relative to cyanate toxicity in rodents.The impairment can be asa result of different hippocampal neurotransmitters (Ayuba, 2014) or cholinergic deficiency; leading to reduced acetylcholine synthesis, or combination of both parameters; more so, from this present study brainacetylcholinesterse activity was increased and this can lead to depletion of acetylcholine and decrease brain performance relative to learning and acquisition.

On the other hand, antioxidant vitaminsadministered was able to ameliorate these acquisition and retention decline through their antioxidant properties. Vitamin E administration from the results showed better performance as compared to vitamin A, or co-administered vitamins.This is in agreement with the work of Abe *et al.,* 2005 that oxidative stress induced by hyperoxia significantly impaired cognitive performance of 3 month old mice, effects that were partially attenuated by dietary vitamin E supplementation. Furthermore,the brain is rich in polyunsaturated fatty acids and relies heavily on the antioxidant properties of the lipid-soluble vitamin E (α-tocopherol) Rashid *et al*., 2011.

5.1 Biochemical Assays

Malondialdehyde

The increase level of MDA concentration in the KCN treated group is an indicator that cyanide is a neurotoxin that evoked lipoperoxidative changes to brain tissues through free radical induction. These findings agreed with results obtained in the works of Baskin *et al*., 2010. After 28 days of administration, we observed the MDA levels significantly ($p<0.05$) increased in KCN treatedas compared to control mice. This increase demonstrates that oxidative damage occurs in brain tissue as a result of lipid peroxidation attributed to cyanide induced toxicitysince membrane lipids, which are rich in polyunsaturated fatty acids, are vulnerable to peroxidation by oxidant (Hyldegard*et al.,* 2011). In agreement with our findings, potassium cyanide leads to higher MDA content and an increase in lipid peroxidation

The low MDA levels in vitaminspre treated groups (III, IV and V) showed their ability to significantly ($p<0.05$) reduce cyanide induced tissue lipoperoxidative damage which is in agreement with the work of Anorou*et al.,* 2008.It has also been reported that antioxidant vitamins administration leads to a decrease in MDA levels in rat liver (Asonye and Okolie, 2004)

and brain (Behr *et al.,* 2011). The present findings study showedthat antioxidant vitamin E treated group caused further decrease in the MDA levels in the brain as compared to co-administration. This suggests single administration is more potent in overcoming lipid peroxidation on the membrane cells.This may have been responsible for the mitigative role of cyanide-provoked clinical and biochemical deficits. Moreover, studies have shown that free radical mediated lipid peroxidation in cigarette smokers can be mitigated by antioxidant vitamins supplementation (Asonye and Okolie, 2004). These vitamins act ssynergistically as antioxidants by neutralizing oxidizing free radicals, including the superoxide and hydroxyl radicals. Their redox properties also allow them to act as reducing agents and are excellent scavengers of superoxide, hydroxyl ion and peroxyl radicals there by inhibiting lipid peroxidation (Kapur*et al.,* 2004).

Superoxide dismutase

Cyanide is a known potent inhibitor of SOD activity as it was seen in previous studies (Jones *et al.,*2003). Thus some of the numerous biochemical activities of cyanide either from dietary source or otherwise could be mediated through depletion in the antioxidant status of the body (Hussain*et al.,* 1999). From the results obtained in this present study, the decrease level of brain SOD activity by cyanide strongly suggests that cyanide imposes oxidative stress on the brain tissue.Reports have shown that cyanide toxicity caused by increased generation of superoxide anion and lipid peroxidation (Daya*et al.,* 2002) with inhibition of antioxidants enzymes (Ardelt*et al.,* 1994). Hariharakrishnan*et al*., (2009) showed marked increase in total ROS and reactive nitrogen species levels and elevated lipid peroxidation after treatment with 1.25mM KCN. Similarly, in the present study, superoxide dismutaseas intracellular antioxidant was depleted after exposure to 15mg/kgof KCN, making the cell environment less reducing and vulnerable to

oxidative stress. So the changes in brain tissues may be induced by cyanide due to the imbalance in the antioxidant enzymes and oxidative stress.Reactive oxygen species generated in the brain tissues induced by cyanide were significantly scavenged by enzymatic antioxidant system SOD as was seen in their increased activity through administration ofnon enzymatic antioxidants such as vitamins A and Ewhich may be responsible for the restoration of SOD activities.These findingsare considered vital, for theimportance of AOV supplements especiallyamongst populations routinely exposed tocyanide through habits, diets andoccupations.

Catalase

Catalase is a prospective biomarker of cyanide toxicity; this indicates that sublethal concentration of cyanide can cause changes in activity of key enzyme, the catalase (Alvandi and Hosetti, 2014). From the results,Exposures to sublethal levels of cyanide significantly ($p<0.05$) decreased catalase brain activities, indicating a typical response to stress in group II pre-treated with KCN as compared to control mice, and this suggested that cyanide is a potential toxicant and induces oxidative stressand may bringserious potential health risk. Works of Bhattacharya*et al.*, (2009) also reported thatthe brainis a highly sensitive organ and is one of the most importanttargets under cyanide toxicity.

The antioxidative enzymes are important in controlling the oxidative stress in animals. The activity of one or more of these enzymes is generally increased or decreased in animals exposed to stressful conditions (Al-ghanimangMahboob, 2011). In the present study, catalase activity in the brain tissues of mice was increased by vitaminspre-treated groups (III, IV and V).Vitamins A and E treated group recorded optimum performance as compared to single administered groups. The result suggests that antioxidant defense system biologically scavenged these toxins by converting them into metabolically non-destructive cellular molecules when administered co-

administeredthan singleadministration and this showed protective roles of AOVs as seen in the present study which corroborates with the works of Asonye and Okolie, 2004.

Acetylcholinesterase

Acetylcholinesterase is an enzyme that regulates neurotransmitter acetylcholine at neurone junctions (Ahmed *et al.,* 2015). Cyanide treated mice showed significant ($p<0.05$) increase in brain acetylcholinesterase activity as compared to control. This finding suggests an involvement of brain AChE in chronic cyanide toxicity. The increaseAChE activity can be attributed to the stress induced by cyanide. Furthermore, it has been reported by many workersAChE activity was regarded as a significant parameter in assessing complex toxicogenic effects of cyanide.The ability of cyanide to phosphorylate the enzyme results in impairment of its activity (Caxton *et al*., 2012).Various studies in mice have successfully reported that increaseacetylcholinesterase enzymes activity leads to excessive decrease of Ach at synapses and neuromuscular junctions and this leads to consequent loss of muscular and nervous coordination which is associated with cyanide induced neurotoxicity (Aliyu*et al.,* 2012) and this corroborates with the present study carried out using wire grid and beam walk test models.

Groups pre-treated with vitamins (A and E) showed significantly decrease activity of the enzyme. This treatment prevented the cyanide-induced rise in AChE activity which corroborates with the work of Jaques*et al*., 2012. This finding suggests the neuroprotective potential of antioxidant vitamins and is involved in cholinergic system modulation. The sublethal concentrations of potassium cyanide for the period of 28 days adversely affected AChE activity in the brain tissue.

CHAPTER SIX

6.0 Summary, Conclusion and Recommendations

6.1 Summary

The oxidative stress resulting from either over production of prooxidants or deficiency of antioxidants has been suggested as an important factor in many degenerative diseases and also believed to be a primary factor in cyanide neurotoxicity (Abdel, 2014). Some of the key events that manifest cyanide intoxication are lipid peroxidation, inhibition of antioxidant enzymes, and generation of reactive oxygen species (ROS).

Superoxide dismutase, catalase, and glutathione peroxidase are three primary enzymes involved in direct elimination of active oxygen species, whereas, glutathione reductase, glucose-6-phosphate dehydrogenase, and cytosolic GST are secondary enzymes, which help in the detoxification of reactive oxygen species by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione and NADPH necessary for optimum functioning of the primary antioxidant enzymes (Kapur*et al*., 2004).

Vitamins A and E are the major lipid-soluble antioxidant vitamins, and are the most effective chain-breaking antioxidant within the cell membrane where they protect membrane fatty acids from lipid peroxidation and oxidative stress (Foley *et al.*, 2000).

6.2 Conclusion

This study has demonstrated thatsublethal administrationof potassium cyanideinduced oxidative stress, impaired sensorimotor and cognitive functions with inhibition of some antioxidant enzymes.While antioxidant vitamins administration (A and E) mitigated these deficits poised by cyanide due to their antioxidant and neuroprotective properties.

6.3 Recommendations

Based on the present study, the following recommendations were made;

1) If animal-to-man extrapolation is permissible, these findings are considered vital, for they underscore the importance of antioxidant vitamins supplements especially amongst population routinely exposed to cyanide through habits, diets and occupations.
2) Further neurobehavioral tests should be carried out using other models to evaluate the effect of cyanide on sensorimotor and cognitive functions.

6.4 Contributions to Knowledge

i. Administration of vitamins A at 25mg/kg and E at 50mg/kg improved motor strength in cyanide induced neurotoxicity in adult swiss albino mice in wire grid test.
ii. Co administration of antioxidant vitamins A at 25mg/kg and E at 50mg/kg improved learning and memory in cyanide induced neurotoxicity in adult swiss albino mice in elevated plus maze.
iii. Administration of antioxidant vitamins A at 25mg/kg and E at 50mg/kg improves MDA, brain SOD, catalase, and acetylcholinestraseactivities in cyanide induced neurotoxicity in adult swiss albino mice in biochemical assays.

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APPENDIXES

Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Dynamics of Wire Grid Test in Albino Mice.

S/N	Treatment Group	Exposure Period (Weeks)		
		Week 0	Week 2	Week 4
1	Control	39.40±1.29	$24.40±0.68^{d}$	$26.60±0.51^{c}$
2	KCN	41.00±1.70	$12.60±0.40^{a}$	$9.20±0.58^{a}$
3	KCN+V_A	39.20±1.07	$20.60±0.40^{c}$	$23.40±0.93^{b}$
4	KCN+V_E	37.40±0.93	$14.80±0.86^{b}$	$31.60±0.93^{d}$
5	KCN+V_A+V_E	38.80±1.16	$27.60±0.93^{d}$	$38.00±1.05^{d}$

Values are expressed as mean ± Standard Error of Mean of five replicates. Mean Value Having Different Superscript Letters in rows are significantly different (p<0.05) (One-way ANOVA followed by Tukey's, multiple comparison test).

Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Coat Hanger Test in Albino Mice.

S/N	Treatment Group	Exposure Period (Weeks)		
		Week 0	Week 2	Week 4
1	Control	23.60 ± 0.68^{b}	16.60 ± 0.81^{b}	23.60 ± 0.51^{a}
2	KCN	21.20 ± 0.58^{b}	11.00 ± 0.45^{c}	8.20 ± 0.58^{c}
3	KCN+V_A	21.80 ± 0.66^{b}	16.20 ± 0.73^{b}	20.00 ± 0.55^{b}
4	KCN+V_E	22.40 ± 0.51^{b}	16.80 ± 0.86^{b}	24.60 ± 0.68^{a}
5	KCN+V_A+V_E	29.00 ± 0.71^{a}	21.40 ± 0.51^{a}	21.40 ± 0.51^{b}

Values are expressed as mean ± Standard Error of Mean of five replicates. Mean Value Having Different Superscript Letters in rows are significantly different ($p<0.05$) (One-way ANOVA followed by Tukey's, multiple comparison test).

Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Dynamics of Beam Walk Performance Test in Albino Mice.

S/N	Treatment Group	Exposure Period (Weeks)		
		Week 0	Week 2	Week 4
1	Control	8.00 ± 0.32^{b}	11.2 ± 0.66^{b}	3.60 ± 0.40^{c}
2	KCN	6.60 ± 0.51^{c}	19.60 ± 0.51^{a}	15.80 ± 0.58^{a}
3	KCN+V_A	8.60 ± 0.51^{b}	11.60 ± 0.51^{b}	3.20 ± 0.58^{c}
4	KCN+V_E	7.40 ± 0.40^{b}	6.40 ± 0.51^{c}	4.60 ± 0.68^{c}
5	KCN+V_A+V_E	10.60 ± 0.51^{a}	9.60 ± 0.51^{b}	8.20 ± 0.37^{b}

Values are expressed as mean ± Standard Error of Mean of five replicates. Mean Value Having Different Superscript Letters in rows are significantly different ($p<0.05$) (One-way ANOVA followed by Tukey's, multiple comparison test).

Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamin A and E on right foot slips in Albino Mice.

S/N	Treatment Group	Exposure Period (Weeks)		
		Week 0	Week 2	Week 4
1	Control	1.40 ± 0.40^{a}	0.80 ± 0.37^{a}	0.60 ± 0.40^{b}
2	KCN	0.60 ± 0.40^{a}	4.60 ± 0.24^{a}	6.60 ± 0.51^{a}
3	KCN+V_A	0.80 ± 0.51^{a}	1.60 ± 0.51^{b}	1.00 ± 0.32^{b}
4	KCN+V_E	0.60 ± 0.40^{a}	2.60 ± 0.40^{b}	1.61 ± 0.24^{b}
5	KCN+V_A+V_E	1.60 ± 0.51^{a}	2.20 ± 0.58^{b}	1.43 ± 0.40^{b}

Values are expressed as mean ± Standard Error of Mean of five replicates. Mean Value Having Different Superscript Letters in rows are significantly different ($p<0.05$) (One-way ANOVA followed by Tukey's, multiple comparison test).

Effect of Sublethal Administration of Potassium Cyanide and Co-administration of Vitamin A and E on left foot slips in Albino Mice.

S/N	Treatment Group	Exposure Period (Weeks)		
		Week 0	Week 2	Week 4
1	Control	2.83 ± 0.37^{a}	0.40 ± 0.24^{c}	0.22 ± 0.02^{b}
2	KCN	1.23 ± 0.37^{b}	4.84 ± 0.37^{a}	4.60 ± 0.40^{a}
3	KCN+V_A	0.80 ± 0.38^{b}	1.11 ± 0.44^{c}	0.44 ± 0.20^{b}
4	KCN+V_E	1.21 ± 0.40^{b}	3.02 ± 0.45^{b}	1.09 ± 0.50^{b}
5	KCN+V_A+V_E	1.20 ± 0.58^{b}	2.47 ± 0.40^{b}	0.51 ± 0.40^{b}

Values are expressed as mean ± Standard Error of Mean of five replicates. Mean Value Having Different Superscript Letters in rows are significantly different ($p<0.05$) (One-way ANOVA followed by Tukey's, multiple comparison test).

Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Dynamics of Learning and Memory in Albino Mice

S/N	Treatment Group	29^{th} Day Learning	30^{th} Day Memory
1	Control	29.60±1.43	$10.60±0.51^{c}$
2	KCN	50.40±1.72	$57.60±0.93^{a}$
3	$KCN+V_A$	28.40±1.81	$11.00±0.71^{c}$
4	$KCN+V_E$	29.40±0.68	$5.60±0.60^{d}$
5	$KCN+V_A+V_E$	32.40±0.93	$14.00±0.70^{b}$

Values are expressed as mean ± Standard Error of Mean of five replicates. Mean Value Having Different Superscript Letters in rows are significantly different ($p<0.05$) (One-way ANOVA followed by Tukey's, multiple comparison test).

Effect of Sublethal Administration of Potassium Cyanide and Administration of Vitamins A and E on Biochemical Parameters.

S/N	Treatment Group	Biochemical parameters			
		MDA (nmol/mg)	SOD (μ/mg)	CAT (mmol/min/ μ/mg)	AChE (μ/g)
1	Control	1.76 ± 0.22^{b}	2.43 ± 0.32^{a}	42.60 ± 2.10^{a}	12.20 ± 0.80^{b}
2	KCN	3.35 ± 0.19^{a}	1.30 ± 0.07^{b}	30.81 ± 1.43^{b}	32.10 ± 0.90^{a}
3	KCN+V_A	1.24 ± 0.06^{b}	1.70 ± 0.22^{a}	41.60 ± 1.96^{a}	14.80 ± 0.86^{b}
4	KCN+V_E	1.22 ± 0.08^{b}	1.38 ± 0.08^{b}	31.20 ± 4.91^{b}	15.20 ± 0.80^{b}
5	KCN+V_A+V_E	1.82 ± 0.21^{b}	2.78 ± 0.10^{a}	48.20 ± 1.57^{a}	16.20 ± 0.90^{b}

Values are expressed as mean ± Standard Error of Mean of five replicates. Mean Value Having Different Superscript Letters in rows are significantly different ($p<0.05$) (One-way ANOVA followed by Tukey's, multiple comparison test).

Results of acute toxicity study showing the different doses of potassium cyanideadministered orally to mice, number of deaths and mortality.

Phase I

A

Weight (kg)	Dose (mg/kg)	Mortality
0.22	10	1
0.20	10	1
0.18	10	1

Total: 3/3 mortality

B

Weight (kg)	Dose (mg/kg)	Mortality
0.25	100	1
0.23	100	1
0.20	100	1

Total: 3/3 mortality

C

Weight (kg)	Dose (mg/kg)	Mortality
0.25	1000	1
0.20	1000	1
0.23	1000	1

Total: 3/3 mortality

Phase II

Dose (mg/kg)	No of animals in each group				
	1	2	3	4	Total
2	0/1	0/2	0/3	0/5	0/11
4	0/1	0/2	1/3	1/5	2/11
9	0/1	1/2	3/3	5/5	10/11