NEUROBEHAVIOURAL, OXIDATIVE STRESS AND HIPPOCAMPAL HISTOLOGIC CHANGES IN MICE EXPOSED TO MOSQUITO COIL, CIGARETTE AND PETROL-POWERED GENERATOR FUMES

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN HUMAN PHYSIOLOGY

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DECEMBER, 2019

DECLARATION

I declare that the work in the thesis, entitled: “Neurobehavioural, Oxidative Stress and Hippocampal Histologic Changes in Mice Exposed to Mosquito Coil, Cigarette, and Petrol-Powered Generator Fumes” has been performed by me in the Department of Human Physiology, Ahmadu Bello University, Zaria under the supervision of Professor R. A. Magaji, Dr. Y. Tanko, and Dr. M. G. Magaji. The information derived from the literature has been duly acknowledged in the text and the list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other university.

_____ _____

Musa Kurawa IBRAHIM Date

CERTIFICATION

This thesis, entitled 'Neurobehavioural, Oxidative Stress and Hippocampal Histologic Changes in Mice Exposed to Mosquito Coil, Cigarette, and Petrol-Powered Generator Fumes' by Musa Kurawa IBRAHIMmeets the regulations governing the award of the degree of Doctor of Philosophy (Ph.D.) of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To my family.

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In the name of God, The Most Gracious, The Most Merciful. To God, The Almighty is the glory, who taught man that which he knew not; may His peace and blessings be upon His noble prophet Muhammad (S.A.W), his household, companions, and all those who follow his righteous path, (Ameen).

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andAmina), my lovely wife, Maryam Hamisu Garba for her patience, understanding, and commitment.

ABSTRACT

This study was aimed at assessing the toxicity of exposureto smoke from three common sources (generator exhaust, cigarette and mosquito coil smoke), in our community. The structural and biochemical brain changes were assessed using brain tissue histology and oxidative stress markers (malondialdehyde, superoxide dismutase and glutathione), respectively. The % COHb in the plasma and learning and memory impairment were also assessed using both Barnes and Elevated plus mazes. The study involved 64, adult, male, mice, weighing between 17 – 25 g that were randomly assigned in to one of the 8 groups (each group had 8 mice). Four out of the 8 groups underwent either the BM or the EPM and were exposed to smoke either from mosquito coil, cigarette (Aspen), or generator exhaust fumes, except their controls. Digital CO metrewas used to measure the amount of CO inside the gas chamber. The results indicated that generator produced the highest CO (≈ 1000 ppm) that was associated with decreased weight gain and 25% mortality, followed by the cigarette (356 ppm) and MC (304 ppm). There were significant oxidative stress in the brain of mice exposed to cigarette and MC smokes. In general, smoke exposure from either MC or generator fumes significantly impaired both learning and memory; however, exposure to cigarette smoke was associated with enhanced ability to learn and recall possibly because of the central nervous system excitatory effect of nicotine. Exposure to smoke from all the three sources were associated with pyramidal cell death in the hippocampus via either apoptosis or necrosis, and focal areas of inflammation. In conclusion, brief exposure (15 min.) to smoke from the three common sources for 14 days was associated with oxidative stress, structural and functional changes in the brain. People should be aware of the dangers associated with smoke exposure from the three sources and avoid/ reduce exposure or use personal safety measures.

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ABBREVIATIONS

AQS	Air Quality Standard
BBB	Blood-Brain-Barrier
BNB	Blood-Nerve-Barrier
C	Cigarette
CDC	Centre for Disease Control
$CMRO_2$	Cerebral Metabolic Rate for O_2
CNN	Cable Network News
CNS	Central Nervous System
COHb	Carboxy Haemoglobin
CO_2	Carbon dioxide
CO	Carbon monoxide
CPSC	Consumer Product Safety Commission
DWI	Diffusion Weighted Images
^0C	Degree Centigrade
EDTA	Ethylene Diamine Tetra acetic Acid
EPA	Environmental Protection Agency
EPM	Elevated Plus Maze
FEPA	Federal Environmental Protection Agency
g	Gram
Hb	Haemoglobin
H_2O_2	Hydrogen peroxide
IAQ	International Air Quality

ICE	Internal combustion engine
kg	Kilogram
kW	Kilowatt
m	Metre
m^3	Metre cube
mEq/L	Milli-equivalent per litre
MC	Mosquito coil
mg	Milligram
ml	Millilitre
µg	Microgram
µl	Microlitre
MRI	Magnetic Resonance Images
NAAQS	National Ambient Air Quality Standards
NO	Nitrogen Oxide
•NO	Nitric Oxide Free Radical
NO-*Hb*	Nitrosyl-Haemoglobin
NO-Mb	Nitrosyl-Myoglobin
nmol	Nano mole
O_2	Oxygen
ρmol	Pico mole
ppm	Parts per million
Po_2	Partial pressure of oxygen
Pco_2	Partial pressure of carbon dioxide

ρco	Partial Pressure of Carbon Monoxide
PG	Prostaglandins
RBC	Red Blood Cells
SEM	Standard Error of the Mean
STP	Standard Temperature and Pressure
USEPA	United States Environmental Protection Agency
V	Volts
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1Preamble

Human beings live in a common environment together with other living and nonliving things. This natural environment provides the basic needs for normal human survival; however, human activities may alter the natural environment by producing pollutants that may be toxic to human and other animals' health. According to Environmental Protection Agency (EPA) Act of 1990, environment can be defined as "including water, air, land and all plants and human beings and/or animals living therein and the interrelationships which exist among these or any of them."The nature and interactions with their environment may pose a great danger to health. People, especially the urban dwellers, are generally exposed to a multitude of chemical, physical and biological toxins in their environment, some of which are toxic, or apparently harmless. Human exposure to environmental contaminants occurs via various ways (including air, water and food) and routes of entry (inhalation, ingestion and dermal). Inhalational exposure occur both in and outdoors and in different microenvironments such as home, workplace, market, during transportation (Masjedi et al., 2019).

Pollution can occur on the land, in water, or in the air environment. Human beings require food, clean water, and also air for their survival. On average, human beings require about 12 kg of clean air within 24 hours; this is about 12 to 15 times greater than the amount of food consumed in a day (Martin and Grocott, 2015). The air we breathe should normally contain 78.09% nitrogen, 20.95% oxygen, 0.93% argon, 0.04% carbon dioxide, and small amounts of other gases in addition to a variable amount of water vapor (Augustine, 2012).

Pollution can be defined as “The presence in the atmosphere of one or more contaminants such as fumes, dust, gases, mist, odor, smoke, smog or vapors in considerable amount or duration that can be injurious to the health of humans, animals or plant life or unreasonably interferes with the comfortable enjoyment of life and property” (Anjaneyulu, 2005). Pollutants are usually expressed as parts per million (ppm) or $\mu g/m^3$. They can exist in the atmosphere as solid particles, aerosols, or gas. The amount and level of toxicity of these pollutants could be subject to variations, based on the climatic conditions like temperature, humidity andwind (Ghorani-Azam et al., 2016). The severity of any air pollution in an environment may depend also on the population density, human activities, industrialization, energy consumption, and many other factors (Zhou *et al.*, 2018).

Major cities in Nigeria are facing a common problem of rural to urban migration. The ever increasing population density in the urban areas causes a commensurate increase in human activities for their survival. Increased human activities, in turn, lead to increased demand for energy in form of fuel used for cooking, generating electric power, transportation, and other things. Air pollution introduces abnormal gasses like nitrogen oxides (NO_2), sulphur oxides (SO_2), carbon monoxide (CO), particulate matter (PM), and lead (Pb) into the atmosphere at a much higher concentration than normal. Most of these pollutants are emitted from incomplete combustion of hydrocarbons in internal combustion engines(ICE) like gasoline-powered generators.

These fuels are mixtures of hydrocarbon compounds containing hydrogen and carbon atoms. In a "perfect" combustion, oxygen in the air would convert all the hydrogen and carbonatoms in the fuel to water and carbon dioxide, while nitrogen remains unaffected.However,in reality, the combustion process is never "perfect"; and automotive engines may emit significant amount of CO instead of CO_2, oxides of nitrogen (NOx), and unburned hydrocarbons (HC) depending on the availability of oxygen and the constituents of the fuel and other factors during the combustion process. The unburnt carbon particles, as well as engine oils, debris, soot and ash particulates make up the particulate matter (PM) (United States Air and Radiation, 2011).

$$\textbf{Fuel} + \textbf{Air} \rightarrow \textbf{Unburned Hydrocarbons} + \textbf{Nitrogen Oxides} + \textbf{CO2} + \textbf{Water} \pm \textbf{CO}$$

Various factors responsible for vehicular pollution includes but are not limited to poor quality fuel coupled with poor condition of the engine, improper air/fuel ratio composition, changes in driving condition and related traffic laws and its regulation, and lack of implementation of air quality controls (Okunola *et al*., 2012). Nearly 50% of the global hydrocarbons, CO, and NOx emissions from combustion of fossil fuel came from gasoline- and diesel-powered engines (Ayub and Baig, 2013). Levels of CO measured in all the 7 locations in Abuja were found to be lower than 50 ppm when compared to that in Kano which is above 50 ppm in most locations. The likely factor responsible for the differences may not be more than the better town planning and traffic control in Abuja than in other states of Nigeria (Ukpata and Etika, 2012).

Clean air is an important and basic factor for human health and wellbeing. Polluted air was considered a major global health problem (Smith, 2013). Maintaining proper indoor air quality (IAQ) is very important because people spend most of their time indoors (Perez-Padilla et al.,

2010). There is also higher indoor concentration of pollutantsindoors (Semple et al., 2012). A major source of indoor air pollution is the environmental tobacco smoke (ETS) (Stroup *et al*., 2000). It is composed of the exhaled mainstream smoke (MS) and side stream smoke (SS) released from the smoldering tobacco product. According to Oberg *et al*.,2004, almost half of the world's children were regularly exposed to ETS (Oberg *et al*., 2011). About 5000 compounds were identified in cigarette smoke (Marco and Grimalt, 2015), 69of which are known toxins and carcinogens (Hoffmann and Hoffmann, 2001). Exposure to particle pollution (PM10 and PM25) is associated with increased morbidity and mortality from cardiopulmonary diseases (Pope and Kanner, 1993; Atkinson *et al*., 2001; Sunyer and Basagana, 2001; Dominici *et al*., 2006; Ghio and Sint, 2008; Ko and Hui, 2009), cardiovascular diseases (Gehring *et al*., 2006; Brook *et al*., 2010; Vidale *et al*., 2010), exacerbation of asthma (Gavett and Koren, 2001; Dominici *et al*., 2006; Tecer *et al*., 2008) and other conditions (Yeatts *et al*., 2007). Equally, exposure to PM2.5 has been linked to the development of cancer (Pope *et al*., 2002).

Mosquito coils are mostly used by people in the night when they retire to their rooms to sleep. Some people burn it in an enclosed bedroom and later extinguish it and open the room ventilation before they sleep. While some will keep it burning throughout the night as theysleep. In either case, people (pregnant women, children and the elderly) get exposed to the coil smoke for quite a number of hours in the night. Some rooms may be well or poorly ventilated, especially during the cold season when people close most of their windows for warmth. The effectiveness of mosquito coil is usually more when used indoors or areas with limited ventilation; however, it is always advised to maintain good ventilation to avoid toxicity (Hogarth et al., 2018).

According to the World Health Organization (WHO), one coil is sufficient for a room of space of about $3.5m^2$ (Hogarth et al., 2018). After lighting up the free end of the mosquito coil, it will continue to release insecticide for about 7-12 hours which continue to accumulate in the area. The time taken to reach a certain effective concentration, depends on the type of the mosquito coil, the concentration of the active ingredients, the size of the room, and also the wind speed. Long-term exposure to mosquito coils smoke has been shown by many studies to induce asthma and persistent wheeze in children (Kurmi *et al.*, 2012).

1.2 Statement of the Research Problem

Air pollution can either be indoor (IAP) or outdoor air pollution (OAP) (United Nations, 2011). The IAP is more important accounting for approximately 2,200 deaths per million people when compared to the 300 deaths per million due to OAP in the same year (United Nations, 2011; Omole *et al*., 2014). Out of the 1.6 million people who died from cooking stoves fumes around the world, 396,000 deaths occurred in Sub-Saharan Africa, with the highest incidence occurring in Nigeria (Margulis *et al*., 2006). About a third of infant mortality in Africa was thought to be associated with IAP, due to the use of biomass for cooking (Hogarth et al., 2018). The results of a study conducted in Lagos and Ogun States to determine the risk patterns of IAP among the residents indicates that 62.2% lived in buildings associated with some form of commercial activities; 6.4% smokes indoors; 9.2% used generators within the building confines; 35.2% used kerosene stoves for cooking; and 4% cooked in poorly ventilated kitchens (Omole and Ndambuki, 2016).

1.3 Justification of the Study

Although a lot of researches were conducted on the effects of various fumes on human health; however, not much has been done concerning its toxicities to the brain. The use of petrol-powered generator at homes, offices and market areas is very common in Nigeria (Libby, 2019). This can affect the indoor air quality. Burning cigarette or MC produces CO, aside other toxic substances specific to the source. Although burning any carbonaceous material can produce CO, there is paucity of data on chronic exposure studies to ambient levels of CO. Acute CO toxicity is easier to diagnose because of the history of exposure; however, chronic toxicity lack any pathognomonic signs or symptoms.Therefore, high level of suspicion is the only key to making diagnosis. Measurement of CO levels alone is grossly inadequate to make tentative diagnosis because blood levels of COHb might have already dropped below detectable limits by the time a patient reaches the hospital.

Many studies have confirmed the existence of delayed neurologic symptoms many days or weeks after CO poisoning, a condition that is still poorly understood (Pepe *et al*., 2011). Most of the effects of CO toxicity were considered to be due to induction of hypoxia; however a lot of other effects identified now cannot be explained by the hypoxia alone. Therefore, oxidative stress and other mechanisms were also considered as other mechanisms of CO toxicity or beneficial effects. Oxidative stress occurs in all the body organs at a rate based on the metabolic activity of the organ (Halliwell, 2006). The relative high metabolic rate of the brain and reduced capacity to regenerate makes it more susceptible to insult and less likelihood for repair; thus, every insult on the brain tissue may be a permanent neurologic deficit that may lingers on for life.

This study was aimed at evaluating the effects of sub-acute exposure to generator exhaust fumes, cigarette smoke and mosquito coil smoke on the neurobehaviour, oxidative stress, and hippocampal structure in mice.

1.4 Aim and Objectives

1.4.1 Aim

To assess the neurobehavioural, oxidative stress and hippocampal histologic changes in mice exposed tomosquito coil,cigarette and petrol-powered generator fumes.

1.4.2 Objectives

- **i)** To assess the level of carbon monoxide produced from each of the three emission sources (generator exhaust fumes, cigarette smoke and mosquito coil smoke).
- **ii)** To determine the percentage carboxyhemoglobin levels in blood.
- **iii)** To assess the temperature changes in each exposure group.
- **iv)** To determine the effects of the smoke exposure on the body weight of the animals.
- **v)** To screen for any motor coordination deficits in the animals using Beam walking test.
- **vi)** To assess learning and memory using Barnes and Elevated plus mazes.
- **vii)** To establish the oxidative status of the brain, by measuring the malondialdehyde, superoxide dismutase and glutathionelevels.
- **viii)** To determine the structural changes in the CA1-region of the hippocampus.

1.5 Research Hypothesis

Sub-acute exposure to smoke from the three sources (generator exhaust fumes, cigarette smoke and mosquito coil smoke) does not have any significant toxicity in mice.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Common Sources of Indoor Air Pollution in Nigeria

Many machines like generators, pumps, industrial sweepers/scrubbers, material handling equipment (such as conveyors), and portable well-drilling equipment all can be categorized under the ICE. The three primary fuels used for these engines are gasoline, diesel fuel, and natural gas. Gasoline is used primarily for mobile and portable engines. Diesel fuel oil is the most versatile fuel and is used in ICEs of all sizes. Most of the pollutants from ICEs are emitted through the exhaust. Although insignificant, some total organic compounds (TOC) escape from other parts due to evaporation. The primary pollutants from internal combustion engines are oxides of nitrogen (NOx), total organic compounds (TOC), carbon monoxide (CO), and particulates, which include both visible (smoke) and nonvisible emissions (Sher, 1998).

Mosquito coils are slowly burning, coiled substance containing one or more insecticides that emit smoke which is widely used as mosquito repellent (Garba *et al*., 2007). The most common active ingredients of mosquito coils are various pyrethrins (0.3 – 0.4% of the coils mass), organic fillers such as sawdust, binders such as starch gel, and synergist such as piperonyl butoxide (PBO) which enhances the insecticide properties of the active ingredients (Pest Control Advisory Section, 2009). Other additives which are capable of burning well without flame may include combustion regulator such as potassium nitrate, fungicide such as sodium dehydroacetate to prolong the shelf-life, dyes which gives the desired colour and fragrance which gives acceptable smell of the smoke (Lukwa and Chandiwana, 1998). They are effective against many genera of mosquitoes including aedes, anopheles, and mansonia (Krieger *et al*., 2003). Sub-

micrometreparticles and gaseous irritants such as the aldehydes, sulphates and polycyclic aromatic hydrocarbons released when the mosquito coil is burning evaporate together with the insecticide (Liu *et al.,* 2003). This prevents mosquito from entering the room.

Cigarette contain dried tobacco leaves which are mainly smoked. Tobacco is a product prepared from the leaves of tobacco plant, Nicotiana tabacum. The chief ingredient in tobacco is the alkaloid nicotine which is a central nervous system (CNS) stimulant. Dried tobacco leaves are processed in many forms to be smoked as cigarettes, cigars, pipe tobacco, and flavored shisha tobacco (WHO, 2015). They can also be consumed as snuff, chewing tobacco, dipping tobacco, etc. There are many types of tobacco such as Virginia (40 % of world tobacco production), Burley (11 % of world tobacco production), Maryland (small total amount of world tobacco production), Oriental (16 % of world tobacco production), and Rustica. A cigarette seldom contains only one type of tobacco, rather a mixture, or blend, of several types of tobacco from a variety of sources. Casing and flavoring ingredients may be used in the blends, in varying quantities, depending on the style of blend.

Nicotine and CO are the main harmful substances in a cigarette smoke (Rodgman and Perfetti, 2013). Nicotine stimulates the adrenal medulla to secrete adrenaline, resets the aortic and carotid body receptors to maintain a higher blood pressure, and also stimulates the sympathetic system which results in an increase in heart rate, blood pressure and peripheral vascular resistance. Increased heart rate and contractility leads to an increase in oxygen demand and consumption. In smokers, the plasma concentration of nicotine can reach up to 15-50 ng/mL (Russell *et al.,* 1976).

Constituents of ETS comprise the exhaled mainstream smoke, side-stream smoke emitted from smoldering tobacco, contaminants emitted during the puffs and contaminants that diffuse through the cigarette paper and the mouth end of cigarettes between puffs. Emissions contain both particle phase and vapor phase contaminants. Side-stream smoke contributes over half of the particulate matter and nearly all of the vapor phase. The first mainstream smoke, which the smoker pulls through the mouthpiece when inhaling or puffing is considered less dangerous than the side-smoke which goes directly into the air and are inhaled by the non-smokers (Appendix VI). The main differences between the mainstream and side-stream is that the side-stream temperature is lower (600^0C), pH is higher (6.7-7.5) and the content of O_2 is also lower (2%) than the mainstream (US Department of Health and Human Services, 2014).

2.2 Carbon Monoxide

Carbon monoxide (CO) is one of the most common and widely distributed air pollutants in the world. It is a colourless, odorless, tasteless and non-irritating gas that is poorly soluble in water (H_2O) and slightly lower in density than air (United States Air Quality Criteria 2000). It is a quite stable diatomic molecule that exists as a gas in the Earth's atmosphere. It is a significant public health problem in both rural and urban areas of both developed and the under developed countries. It may be responsible for more than one half of all fatal poisonings that were reported worldwide (Smollin and Olson, 2010).

Toxicities of CO were known since ancient times when Greeks and Romans used it to execute criminals (e.g., suicide of Roman author Seneca, 65 AD; Cicero, Rome, 106 to 43 BC). So, over the years scientists considers CO as a waste, pollutant, and toxic to the body. Although its

toxicities were known over 100 years ago, attention were focused on the gas after the pioneer scientific paper presented on the gas by Claude Bernard and John Haldane (Gross, 1998). These two researches then triggered series of investigations towards understanding the nature of its toxicities. Explanations on the attachment of CO to Hb (producing COHb) was evaluated by Douglas *et al.* (1912). Many studies then focus their attention on the earlier known mechanism of action which was *hypoxia* and also uses high concentration of the gas to demonstrate its numerous toxicities (Haldane, 1927).

Occupational hazards of CO poisoning in relation to the clinical presentation were evaluated by Grut, in 1949. Many researches on CO poisoning continued for several years before the beneficial effects begun to surface (Ernst and Zibrak, 1998). Scientists were fascinated by the "Double-natured" gas as the beneficial effects of the gas continue to be revealed. The sources, local concentrations, its interaction with a specific environment, and bioavailability were found to integrally determine which side of the gas will dominate. Carbon monoxide was called "the Silent killer" because of its known toxicity, however it is now considered as the "Double-sided or Double-edged sword" because of the numerous potential benefits been recognized (Blumenthal, 2001). Although many of the beneficial effects are known aside the already known toxicities, most of the mechanisms of action is still not clear; a reason why it is also called "the mysterious gas" (Trichter and Helpern, 1952).

About 75–80% of CO produced in the body is derived from Hb metabolism, and the remaining from metabolism of other haem proteins, such as myoglobin, cytochromes, peroxidases, and catalase (Chiabrando*et al.,* 2014). Therefore, endogenous production alone contributes to a blood COHb concentration of approximately 0.4–0.7% (WHO, AQG, 2000). Carbon monoxide can be

produced from any tissue that express haem oxygenase (HO); however, the major sites of haem metabolism, including liver, spleen, and reticuloendothelial system produce more CO than any other tissue (Khan and Quigley, 2011). Haem proteins are the major source of CO in the body. Non-haem sources contribute a minor percentage and is of no physiological importance. Numerous physiological factors and diseases can affect the rate of endogenous production of CO. The rate of production can also be accelerated in conditions that increase catabolism of Hemoglobin (Hb) or other haem proteins, including hemolysis, hematomas, hemolytic anemia, thalassemia, and Gilbert's syndrome (Hampson, 2008). The endogenous production was found to be associated with most of the beneficial effects of the gas in the body; however, it is not yet clear whether the toxic effects could equally be associated with the physiological levels.

Haem oxygenase-1 (HO-1) is highly inducible and is involved in cellular responses to stress (Maines *et al*., 1997). Haem oxygenase-1 is also responsible for the destruction of haem in senescent red blood cells (RBCs) (Arora and Kapoor, 2012). Expression of HO-1 protein is induced in response to accumulation of haem, oxidative stress, various metals, various cytokines, and also exogenous CO (Wu and Wang, 2005). It is concentrated in peripheral tissues, such as spleen and liver. Haem oxygenase-1 can be induced by its substrate, haem, NO, hydrogen peroxide (H_2O_2), several cytokines, and lipopolysaccharide, inflammation, and oxidative stress. It is also a heat-shock protein and is considered to be an essential antioxidant enzyme that is up regulated in response to cellular stress (Calabrese*et al*., 2011).

On the other hand, haem oxygenase-2 (HO-2) is constitutively expressed and discretely localized to neurons in the brain, enteric system, and interstitial cells of Cajal (ICC) in the mouse intestine.

Haem oxygenase-2 is specifically highly concentrated in certain areas of the brain such as in hippocampal pyramidal and granule cells, in the granule and Purkinje cell layers of the cerebellum, in the piriform cortex, tenia tecta, olfactory epithelium, and in the neuronal and granule cell layers of the olfactory bulb (Mishra and Fomusi Ndisang, 2014). Carbon monoxide like NO, binds to the iron in haem, and stimulates guanylate cyclase to activate cGMP (Ibrahim *et al.,* 2010). Little is known about the third isoform, HO-3. The existence of different isoforms of HO and the specific nature of their distribution will point at their specificity and diversity of functions throughout the body depending on the prevailing circumstances. There is a strong relationship between the expression of HO-1 in astroglial cells in relation to brain development, neurodegeneration, and brain injury associated with inflammation (Li Volti *et al.*, 2004).

The fate of available CO in the body includes either expiration through the lungs, redistribution of the body CO store by scavenging molecules or a small amount that is believed to be oxidized to CO_2 depending on many other factors in the body (Fenn and Cobb, 1932). Carbon monoxide rapidly diffuses across the alveolar–capillary membrane, a process influenced by alveolar gas volume, ventilation, and the concentration of Hb in the pulmonary capillaries (Coburn and Forman, 1987).

2.3 Types of Exposure to Smoke

Typically, acute and chronic forms of CO exposure were recognized; however, a third and mostly unrecognized form of exposure is the acute-on-chronic exposure that occurs in most of the circumstances. Acute CO exposure usually occur in accidental situations where people are acutely exposed to very high doses of the gas, which in most incidences is fatal. The diagnosis of

this kind of poisoning is mostly glaring and lead to immediate medical attention. The health effects however is mostly fatal, leading to the death or severe incapacitation of the patient. Temporary recovery could be attained, however long lasting consequences in form of delayed neurological symptoms or long term psychological symptoms were documented (Chang *et al*., 1992). Carbon monoxide is widely used in suicidal attempts or those who really committed the suicides (Skopek and Perkins, 1998).

Chronic exposure on the other hand is usually associated with varying low to moderate levels of exposure to CO over a long period of time. The diagnosis as well as the symptomatology is difficult to understand and is usually either unnoticed, missed, misdiagnosed, or misinterpreted (Eichhorn et al., 2018). This kind of exposure affects virtually everybody, either knowingly or unknowingly. Unlike the acute exposure, here, diagnosis is difficult and currently no any pathognomonic feature of chronic CO toxicity has been identified (Wright, 2002). Chronic exposure to CO is unlikely to cause sudden death because of its subclinical nature, but can be associated with long term health consequences such as Delayed neurological symptoms (DNS), psychiatric manifestation, or possibility of neurodegenerative disease (Sonmez *et al.*, 2018).

Another form of exposure is the acute-on-chronic. It occurs when a person is acutely exposed to CO in addition to the daily chronic exposure. Since the chronic exposure goes unnoticed, diagnosis is usually made in relation to the acute exposure. There is usually no linkage between the clinical manifestations and the previously ongoing chronic exposure, therefore everything is attributed to the most recent glaring acute exposure (Asani *et al.,* 2014).

Burning either MC or cigarette produces smoke containing substances other than CO. People inhale the smoke either in confined space (indoors) or from the exposed environment (outdoor).People (especially women and children) are unwittingly been exposed to toxic chemicals from burning cigarette, mosquito coil, or exhaust fumes of power generating set. In most occasions, such exposure occurs on daily basis over the lifetime or significant part of a person's life. These insignificant exposures may however build up in the body, leading to serious consequences that may not easily be identified or ascribed to a specific agent. Environmental tobacco smoke in offices, vehicles and homes can raise the 8-hour average CO concentration by 20-40 ppm (U.S. AQC, 1991). Acute, high concentrations of CO up to 100 ppm can be found in the kitchen where women spend most of their life time (Smith *et al.,* 2010). Carbon monoxide emanating from burning wood alone can raise the average indoor level of 5 ppm approximately 1000 times (Raub *et al*., 2000).

Smoking is another global major source of indoor CO exposure that cut across ethnic, racial, gender, age, social class, and even economic variations (Sen *et al.,* 2010). Smoking is a habit that is ubiquitously found all over the world, in all races, gender, social, and economic groups (Mueller *et al.,* 2011). It is a significant and major source of indoor CO pollution worldwide (Obanya *et al.,* 2018). Indoor garages and underground parking lots could also predispose people to unacceptable levels of CO indoors (Emmanuel, 2013).

A particularly regular source of indoor CO exposure is the use of gasoline or diesel-powered generators for the supply of electricity in Nigeria. Nigeria is the second biggest market for generator driven economy in Africa, after Egypt and remain the leading importer of generators in

Africa (Nwachukwu, Jan. 10, 2011). Concentrations in homes and other workplaces have been found to be in excess of 100 ppm; a concentration that can lead to COHb levels of greater than 10% after 8 h of exposure (US AQG, 2000). Automobile exhaust is the largest source of CO outdoors, while combustion of bio-fuel is the main source indoors (Prockop and Chichkova, 2007). In Nigeria, the most important sources are exhaust fumes from motor vehicles and gasoline-powered generators; smoke from kerosene stoves, wood burning and cigarette smoke (Ayodele *et al.,* 2007).

The World Health Organization's (WHO) recommendations on CO exposure were concentrations below 100 mg/m^3 (87 ppm) for 15 min, or 60 mg/ m^3 (52 ppm) for 30 min, or 30 mg/ m^3 (26 ppm) for 1 hour, or 10 mg/ m^3 (9 ppm) for 8 hours (USEPA, 1991). It is important to know that various substances serving as sources of power mentioned above could produce varying degrees of CO depending on the type of fuel, contents of the fuel, nature, make, sophistication and efficiency of the combustion engine, availability of oxygen, ventilation, wind speed and direction and many other climatic conditions and environmental factors.

2.4. Health effects of smoke inhalation

Indoor air pollution was associated with pneumonia in children, asthma, tuberculosis, upper airway cancer and cataract (Omole and Ndambuki, 2016). Higher cases of bronchiolitis and pneumonia in children was found to be associated with high concentrations of carbon monoxide (CO), nitrogen dioxide (NO2), sulphur dioxide (SO2) and bezene (Sofoluwe, 1968). About 2

million premature deaths per year occurred worldwide due to air pollution (Gajalakshmi *et al*., 2003).

For decades, health effects of environmental tobacco smoke (ETS) was well documented. It is found to be associated with premature death, acute respiratory infections, sudden infant death syndrome, more severe asthma and ear problems in the exposed children (DerSimonian and Laird, 1986; Higgins *et al*., 2003). In adults however, it is associated more with acute coronary heart disease (Begg and Mazumdar, 1994; Egger *et al*., 1997; Dong *et al*., 2001) and lung cancer (Mishra *et al*., 1999; Malhotra *et al*., 1996).

Mosquito coils contain small particles (<1 μm) metal fumes, vapors and free radicals that can irritate the upper respiratory tract (Chang and Lin, 1998). Upon ignition, other sub-micrometre particles like dyes and binders/ organic fillers caused carcinogenesis (John and John, 2015). Acetaldehyde and formaldehyde found in the MC are strong irritants to the upper respiratory tract (Koo and Ho, 1994; Chang and Lin, 1998). Pyrethroids depletes the antioxidants levels, therefore causes oxidative stress (El-Demerdash, 2011; Mossa and Refaie, 2013). It also paralyses the insect by causing prolonged opening of sodium channels (Casida, 1980; Soderlund and Bloomquist, 1989; Chang and Lin, 1998; Macan *et al*., 2006). Synthetic pyrethroids can cross the blood brain barrier (BBB) and affect maturation of the BBB and causes some biochemical changes (Gupta *et al*., 1999). Long-term exposure to MC was associated with metaplasia of the trachea (Liu and Sun, 1988; Liu and Wong, 1987); leukocytosis (Garba *et al*., 2007); cellular and organ injuries (Moya-Quiles *et al*., 1995); chromosomal abnormalities (Das

et al., 1994; Moorthy and Murthy, 1994); and increased reactive oxygen species (ROS) (Madhubabu and Yenugu, 2012).

2.5 Special Vulnerability of the Brain to Insult

Oxidative stress occurs in all the body organs at a rate based on the metabolic activity of the organ (Halliwell, 2006). The relative high metabolic rate of the brain and reduced capacity to regenerate makes it more susceptible to insult and less likelihood for repair; thus, making every insult on the brain tissue to be a permanent neurologic deficit (Vaccarino and Ment, 2004). Among the body organs also, brain is particularly susceptible to free radical attack because it has less antioxidants and generates more oxidative by-products per gram tissue than any other organ (Valko *et al.,* 2005). Although the brain contributes only about 2% of the body's weight, it utilizes up to 20% of the oxygen consumed by the body (Clarke *et al*., 2000). There is a very high content of iron in some areas of the brain which favours production of more ROS(Anderson *et al.,* 2002). The brain is also rich in lipids that can act as a potential target for lipid peroxidation (Piantadosi, 1997). Although the CNS is partially protected, the nervous system remains susceptible to toxic injury (Linnea *et al*., 2011). This is because non-polar highly lipid–soluble substances generally have easier access to cross most membranes in the body (Friis *et al.,* 1980).

The brain is known to be rich in lipids that can act as a potential target for lipid peroxidation (Halliwell and Gutteridge 1992). The high iron content in some areas of the brain also favours production of more ROS (Anderson and Root 2004). With this level of vulnerability, brainneeds an efficient antioxidant system in order to avoid oxidative damage. However, the brain contains only low to moderate activities of superoxide dismutase (SOD), catalase (CAT), and glutathione

peroxidase (GPx) when compared to either liver or kidneys (Dringen*et al*., 2000). This, in turn, makes the brain much more vulnerable to oxidative damage. Both exogenous and or endogenously produced CO may lead to oxidative stress through initiation of many signaling pathways related to ROS production. The ROS produced depends on the CO concentration based on the location of haem proteins, and also specific oxidation-reduction (redox) reactions (Piantadosi, 2008).

Evidences also shows connection between increased ROS and loss of neurons during the progression of neurodegenerative diseases like Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) (Anderson and Root, 2004). In another study, chronic oxidative damage was linked to age-related neurodegenerative diseases (Anderson and Root 2004). Increased levels of nitrotyrosine, a permanent marker of $ONOO^-$ attack on proteins, and of 4-HNE (the most cytotoxic product of lipid peroxidation) were demonstrated in AD, PD, ALS, and other neurodegenerative diseases (Pacher *et al.,* 2007). Acute CO poisoning was found to be associated with perivascular oxidative stress (Thom *et al.,* 2006).

Glutathione (GSH) is an important antioxidant, especially in the brain tissue. Because of the "thiol" group, GSH is capable of neutralizing the damaging effects of most ROS such as free radicals, peroxides, lipid peroxides, and heavy metals by reducing the disulfide bonds formed within cytoplasmic proteins to cysteines and serving as an electron donor. In the process, glutathione is converted to its oxidized form, glutathione disulfide (GSSG). Hydroxyl free radicals were usually produced in the brain after CO hypoxia in rats; these free radicals can either be diminished or accelerated after severe CO poisoning depending on the oxygen partial

pressure employed during therapy (Piantadosi *et al.*, 1995). These free radicals produced in the body were believed to be the key to brain ageing (Poon *et al.*, 2004). Metal-induced toxicity through the formation of free radicals causes various modifications to *DNA* bases, enhanced lipid peroxidation, and altered calcium and sulfhydryl homeostasis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment, Drugs and Reagents

Plexiglas gas chamber, sources of smoke [cigarette (Aspen), mosquito coil (wavetide), gasoline-powered generator], apparatus for neurobehavioural studies (Beam walk, Elevated plus maze, and Barnes maze), spectrophotometre (model 721, product ID 201510106), centrifuge machine, Canon camera (PowerShot SX30) and other laboratory equipment.Carbon monoxide metre (PCMM05 Pyle, China, serial number 201503017364). Superoxide dismutase (SOD) assay kit (WST-1 method) with the catalog No: BC0020, 96T from Elabscience Company. Reduced glutathione (GSH) assay kit, with catalog number BC0051 from Elabscience Company.

3.1.2 Animals

Sixty four, male strain of Swiss Albino mice (6-8 weeks) weighing between 17 – 25 g were obtained from the animal house of the Department of Pharmacology, Bayero University Kano for the study. They were housed in the laboratory and preconditioned for a week prior to the start of the study. They were maintained under normal atmospheric conditions of the Savannah region (24 – 30^{o}C). They were fed with laboratory animal feed and tap water ad libitum. The animals were randomly categorized into 8 exposure groups, each containing 8 mice. Groups 1, 2, and 3 were exposed to CO gas from mosquito coil smoke, cigarette smoke, and exhaust fumes of gasoline-powered generator, respectively; while group 4 served as the control (Table 3.1). Ethical approval and clearance was sought from the A.B.U. committee on Animal Use and Care (ABUCAUC). They were housed in groups of 6-8 mice per cage, and standard ethical guideline

was strictly adhered to in handling the animals. Safety precautions were taken in accordance with the standard universal precautions (West and Cohen, 1997).

3.1.3 Gas Chamber

It was an improvised chamber that was made up of Plexiglas. It was an air tight chamber that measures 75 x 50 x 50 cm. There was an inlet where the gas will be introduced into the chamber and an outlet. There is also a small window (12 cm x 10 cm) for ventilation to fresh air (Plate I).

3.1.4 Carbon Monoxide Gas Analyser

The carbon monoxide metre PCMM05 Pyle manufactured in China, with serial number 201503017364 was used to measure the CO gas in the air. The metrehas a range between 1-1000 ppm; with an accuracy of +/- 5% or +/- 10 ppm. The metre displays the readings of the presence of CO on the LCD in ppm and also through a beeper tone. From 35 to 200 ppm, the beeper sounds discontinuously and then continuously above 200 ppm. The metre can be operated within a temperature range of between 0^{o}C to 50^{o}C and0-99% relative humidity (Plate II).

3.1.5 Sources of Exposure to Smoke

Sources of smoke include mosquito coil (Wavetide, Xiaoshan Yunshi, China) (Plate III), cigarette (Aspen) (Plate IV) and exhaust fumes of gasoline-powered generator (TIGER, TG950, 220v/240v) (Plate V). The brand of mosquito coil and cigarettes were obtained from vendors in the market.

Plate 3.1: Exposure Set up Within the Gas Chamber Snapped by the Researcher

Plate 3.2: Carbon Monoxide MetreSnapped by the Researcher

3.2 Methods

The initial body weight of all the animals was taken a day prior to the start of the study. The animals also underwent the beam walk test prior to the start of the study. The exposure was done in the mornings between 8-9 am daily for 14 days (Jiang and Tyssebotn, 1996).

Cigarette and mosquito coil, were burnt inside the partially ventilated gas chamber for the duration of exposure, while the exhaust fume was delivered into the gas chamber through the inlet pipe. Mice in the gas chamber were expected to have inhaled the smokes emanating from the sources for 15 minutes, daily, up to 14 days period. The control groups were however maintained in similar conditions but without CO exposure. Environmental temperature and that inside the chamber were measured during each exposure sessions. The dose of CO exposure was recorded by the digital CO gas analyser placed inside the gas chamber together with the experimental animals. Depending on the source of CO, the peak daily dose attained within the period of exposure were recorded. Neurobehavioural trainings were started on the last 4 days of the exposure depending on the paradigm protocol. On the last day of the study, animals were finally weighed, exposed to CO, and then subjected to the neurobehavioural tests. They were then sacrificed and blood samples taken for biochemical analyses. The organs (liver, kidney, lungs, and brain) were harvested for further histological and biochemical analyses.

Behavioural tests were conducted in the neurobehavioural laboratory between the hours of 9 a.m. and 5 p.m. (Sunyer *et al.*, 2007). Long-term learning and memory deficits were assessed using the Barnes maze (Barnes, 1979) and Elevated plus maze.

Plate 3.3: Brand of Mosquito Coil Used in the Study Snapped by the Researcher

Plate 3.4: Aspen Cigarette Snapped by the Researcher

Plate 3.5: Portable Gasoline-Powered Generator Snapped by the Researcher

Table 3. 1: Animal Grouping

Group	Exposure	Barnes Maze (BM)	Elevated Plus Maze (EPM)
1	Mosquito coil smoke	BM1	EPM1
2	Cigarette smoke	BM2	EPM2
3	Generator exhaust fumes	BM3	EPM3
4	Control (room ambient air)	BM4	EPM4

n = 8

There is however a general requirement for motor strength and coordination before a reliable assessment of cognitive behavior can be made (Carter *et al*., 2001).Beam walk assay was therefore used to assess the locomotor activity of all the animals in order to rule out any gross motor coordination deficit that might ultimately affect the cognitive behaviour (Stanley *et al.*, 2005). Behavior was timed using a stop watch, and events were observed manually and evaluated, however the BM procedure was completely videotaped (Figure 3.1).

3.2.1 Beam Walk Assay

The protocol was based on those of Southwell *et al*. (2009) and Carter *et al*. (2001) with some modifications. It consisted of 100 cm long 12 mm and 6 mm flat beams resting on two poles which are 50 cm above the surface of the table. A black escape box is attached to one end of the beam at the finish point. The box contains nesting material from home cage to attract mice (Plate VI). A lamp (60 watt light bulb) was positioned at the starting end of the beam to serve as an aversive stimulus.

Mice were allowed to cross the 12 mm beam from the starting point end (0 cm) to the finish line (80 cm) three times on the trial days. They were allowed to rest in the black box in between trials. Foam was spread directly under the beam to cushion the effect of any accidental fall. The beam was cleaned with methylated spirit (95% ethanol and 5% methanol) and allowed to air dry after each trial. Gentle push was provided to encourage any mouse that failed to proceed to the completion of the test. On the test day, each mouse was allowed to traverse the 6 mm flat beam to the black box. Time to cross the beam was recorded and also the number of foot slips (left and right).

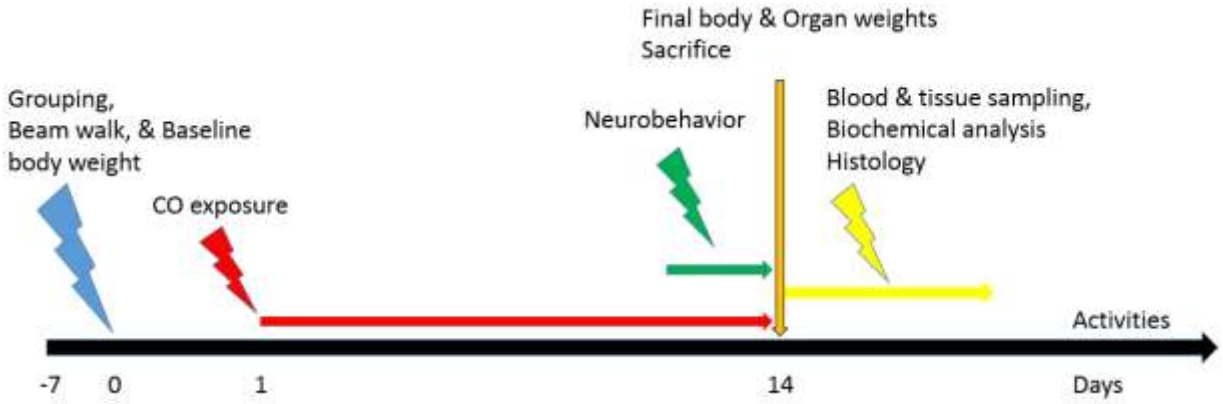

Figure 3.1: Gantt Chart of the Study Design

Plate 3.6: Beam Walk Apparatus for Motor Coordination Snapped by the Researcher

A video camera was set on a tripod stand at the height of 2.5 m to record the performance aside manual observation by the investigator and his team. Compounding factors such as motivation and over trained and uncooperative mice were minimised.

3.2.2 Barnes Maze

It was made up of a circular platform (122 cm in diametre) with 40 equally spaced holes (5 cm diametre; 3.5 cm between holes; 2 cm from the edge) along the perimetre and was elevated 90 cm above the floor. The maze was coloured dark brown to provide enough contrast between mice and the open surface. There was a small dark recessed "goal box" (28 x 14 x 18 cm) located under the platform where mice can escape the aversive stimulus and hide (Plate VII). Visual cues used included a chart which was positioned on the wall, the tripod stand located about a metre away from the BM that carries the video camera positioned about 150 cm above the platform and the two researchers, with one wearing laboratory coat and the other a maroon coloured T-shirt. All the cues were kept permanently in their positions throughout the days of the study (Rosenfeld and Ferguson, 2014).

For the adaptation, mouse was placed in a cylindrical black start chamber in the middle of the maze for 10 seconds, after which the chamber was lifted, the lights were turned on, and the mouse was gently guided to the escape box. Once the mouse was inside the box, the light was turned off and was allowed to stay in the escape box for 2 minutes. The BM was cleaned thoroughly with a solution of 70% ethanol to avoid olfactory cues. Spatial acquisition is similar to adaptation, but here the mouse was allowed to explore the maze for 3 minutes, during which

the number of primary errors, total errors and primary latency were measured by the experimenter.

Plate 3.7: Barnes Maze Apparatus Used for Spatial Learning Snapped by the Researcher

The trial ends when the mouse successfully entered the goal tunnel or after 3 minutes have elapsed. Immediately after the mouse entered the tunnel, the light was turned off and allowed to stay in the box for 1minute. After each trial, the mouse was returned to its home cage to rest for at least 15 minutes before the next trial.

The spatial acquisition procedure was repeated over and over again till each mouse received 4 trials/ day for up to 4 days. Reference memory/ long-term retention/ probe trial was conducted **o**n day 5, which was 24 hours after the last training day. The procedure for spatial acquisition was repeated, however, the mouse was allowed to explore the maze for 90 seconds only and the target hole remained closed. Here, number of pokes/ errors (total number of head deflections into incorrect holes) in each hole, latency (time taken to locate the target hole), and path length (total length of the path to locate the target hole)to reach the virtual target hole were measured.

The search strategies used by the mice can be grouped in to three different categories. Direct (Spatial) search strategy involves moving directly to the target hole or to an adjacent hole before visiting the target. In mixed search strategy, the target hole searches were separated by crossing through the center of the maze or an unorganised form of search. In the serial search method, the first visit to the target hole was preceded by visiting at least two adjacent holes in serial manner; either clockwise or counter-clockwise direction. The strategies were observed by the experimenters during the study and cross-checked with the video recordings at the end of every day's trials.

3.2.3 Elevated Plus Maze

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 (Plate VIII). The plus maze was elevated 50 cm above the floor and the whole apparatus was painted black (Itoh *et al*., 1990). The procedure for the test was described by Itoh *et al*. (1990). On the first day (acquisition test), each mouse was placed at the end of one of the open arms, facing away from the central platform.

Latency for the mouse to enter one of the closed arms was recorded in seconds. Following entry into the arm, the animal was allowed to explore the apparatus for 30 seconds. All the paws were required to be in the arm before being counted as an entry. Twenty four hours later, the second trial (recall test) was performed. After each trial, the plus maze was thoroughly cleansed with alcohol and allowed to dry before the next trial. The time taken for a mouse to move from the starting point of the open arm to any of the closed arms (transfer latency) was measured (in seconds) to indicate learning (first day) and memory (second day) (Walf and Frye, 2007).

3.3 Anaesthesia, Blood Collection, and Harvesting of Brain Tissue

About 15 minutes after the last exposure, mice were anaesthetized using the "Open drop" method. About 0.1 – 0.2 mL of chloroform was applied to a cotton ball that was inserted into an air tight transparent rubber jar. The mouse was then placed inside the jar for some few seconds to achieve anesthesia. About 2.5 ml of blood were collected in test tubes containing potassium ethylene diaminetetraacetic acid (K^+EDTA, potassium salt) through cardiac puncture. The mouse was then humanely decapitated. The skull bone was gently broken to expose the brain tissue which was later extracted out of the cavity and cleaned thoroughly of adhering tissues before it was weighed (Spijker, 2011).

Plate 3.8: Elevated Plus Maze Used for Anxiety-Like Memory Snapped by the Researcher

3.4 Biochemical Analyses

Five mice from each group were used for the biochemical analyses. The brain tissue was minced and grinded in a cold glass mortar and homogenized with ice-cold 100 mM phosphate buffer (pH 7.4; 1 g of tissue/ 9 mL). The homogenates (10% w/v) was then centrifuged at 3,000 rpm for 15 minutes and the supernatant so formed was transferred to a plain sample bottle containers and stored in temperature between $4^{o}C$ – $8^{o}C$ before analyses of oxidative stress markers. The analyses was done at Plasma Diagnostic and Research Laboratory, Kano. Assessment of products of lipid peroxidation, specifically Malondialdehyde (MDA) and measuring the enzyme activities of superoxide dismutase (SOD) and glutathione (GSH) were used to assess the level of oxidative damage. The percentage of blood COHb was measured from the blood samples taken.

3.4.1 Estimation of Malondialdehyde in the Brain

The method of Ohkawa *et al*. (1979), which was used here, measured the concentration of thiobarbituric acid-reactive substances (TBARS) produced during the lipid peroxidation process. Malondialdehyde is a secondary product of lipid peroxidation and is used as an indicator of tissue damage. The MDA form a 1:2 adduct with thiobarbituric acid (TBA) and produces a pink coloured product that can be maximally absorbed at 532 nm. The reagents includes, thiobarbituric acid (100 mg of TBA in 30 mL distilled water and 30 mL of acetic acid); Trichloacetic acid (TCA) 10%; Normal saline solution (0.9% NaCl); and n-butanol solution (90%). The concentrations of the TBARS was expressed as (ɲMol/mL) and the MDA concentration was calculated using the following formula:

$$\text{MDA conc.} = \text{Absorbance}/\ 1.56 \text{ x } 105 \text{ cm} - 1 \text{ m} - 1 \text{ x path length}$$

3.4.2 Assessment of the Activity of Superoxide Dismutase in the Brain

The activity of SOD in brain tissue was tasted using the superoxide dismutase (SOD) assay kit which adopts the xanthine oxidase (hydroxylamine method) to measure SOD activity. Superoxide dismutase is an enzyme that plays a vital role in the body by its ability to balance the redox status of the body. The enzyme can remove the superoxide anion free radical produced, thereby protecting the cells from damage. The activity of SOD was measured by the WST-1 method in this kit. Water-soluble tetrazolium, the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate(WST-1), was used as a detector of superoxide radical generated by xanthine oxidase and hypoxanthine. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD.

3.4.3 Estimation of Glutathione

Reduced glutathione (GSH) assay kit was used for the study. Estimation of GSH was based on the development of a relatively stable (yellow) colour when 5', 5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) was added to sulfhydryl compounds. The chromophoric product was then read at 412 nm in a spectrophotometre (Srivastava and Beutler, 1970).

3.4.4 Determination of the Percentage Blood Carboxyhaemoglobin Level

Blood COHb is the principal biomarker for identifying exposure to CO. The blood samples obtained was used for COHb analyses by means of spectrophotometric method (Ernest and Carol, 1984). This method for estimating carboxyhaemoglobin was based on the production of two-pigment mixture by reducing oxyhaemoglobin and methaemoglobin with sodium

hydrosulfite. Absorbances of the pigment were measured at 420 and 432 nm. Materials required for the analyses included (1) Buffer: KH_2PO_4/K_2HPO_4, 0.1 mol/L, pH 6.85 (2) Haemolyzing solution: Buffer diluted 10-fold with water, (3) CO-Hb diluting solution: prepared by adding about 25 mg of sodium hydrosulphide to 20 mL of buffer just before used.

3.5 Histology of the Hippocampus

Three mice from each group were used for the histology. The brain tissue were kept in Bouin's fluid before further analyses. Selected pieces of the brain slices from the hippocampus were obtained and dehydrated by passing them through ascending grades of alcohol. Tissues were then cleared with toluene, infiltrated with molten paraffin wax and sectioned at 5 microns on a rotator microtome. The sections were later stained with haematoxylin and eosin (HandE) stain for identification of general tissue structure and Toluidine blue for staining nucleic acids (Auwioro, 2010). Slides were examined with Leica icc50 HD brand of microscope and photographed with its camera.

3.6 Statistical Analyses

Data obtained from the study were expressed as means ± standard error of the mean (Mean±SEM), or as medians and interquartile ranges. Tables and graphs were used to present and also summarise the data. Most of the data did not pass the normal parametric assumptions, therefore non-parametric equivalents such as the Mann-Whitney u-test for Independent sample T-test; Wilcoxon Matched-pairs Signed Ranks Test for Paired T-test; Kruskal-Wallis H-Test for One-way ANOVA; and Friedman Test for repeated-measure ANOVA were employed to analyse the results, followed by appropriate posthoc test. For all evaluations, values of $p \leq 0.05$ was

considered to imply statistical significance. Microsoft office Excel version 2013 and statistical package for social scientist (SPSS) version 22.0 software were used in analysing the data.

CHAPTER FOUR

4.0 RESULTS

4.1 Carbon Monoxide

4.1.1 Carbon Monoxide Produced from the Sources

Highest dose of up to 1000 ppm of CO was produced by the generator exhaust fumes, followed by cigarette smoke (356 ppm) and then mosquito coil (304 ppm) (Figure 4.1). There were highly significant differences (P=0.0001) in the doses of CO produced by the different sources. The result of the pairwise comparison indicates differences in-between the groups; however, there was no significant difference between mosquito coil and cigarette groups (P=0.705) after Bonferroni adjustment.

4.1.2 Assessment of the Blood Percentage Carboxyhaemoglobin

Mice that were exposed to the generator exhaust fumes had the highest blood level of % COHb (18.9%), followed by those exposed to cigarette smoke (17.4%), then mosquito coil (16.7%) (Figure 4.2). The blood % COHb was directly proportional to the exposure doses in all the groups (1000 ppm for Generator, 356 ppm for Cigarette, and 304 ppm for Mosquito coil).

4.2 Environmental Temperature Measured in Various Groups

The temperature pattern did not vary much between the inside of the gas chamber and the environment. Generally, the mean temperature was higher in the animals that were exposed to the generator exhaust fumes because of the hot fumes from the generator.

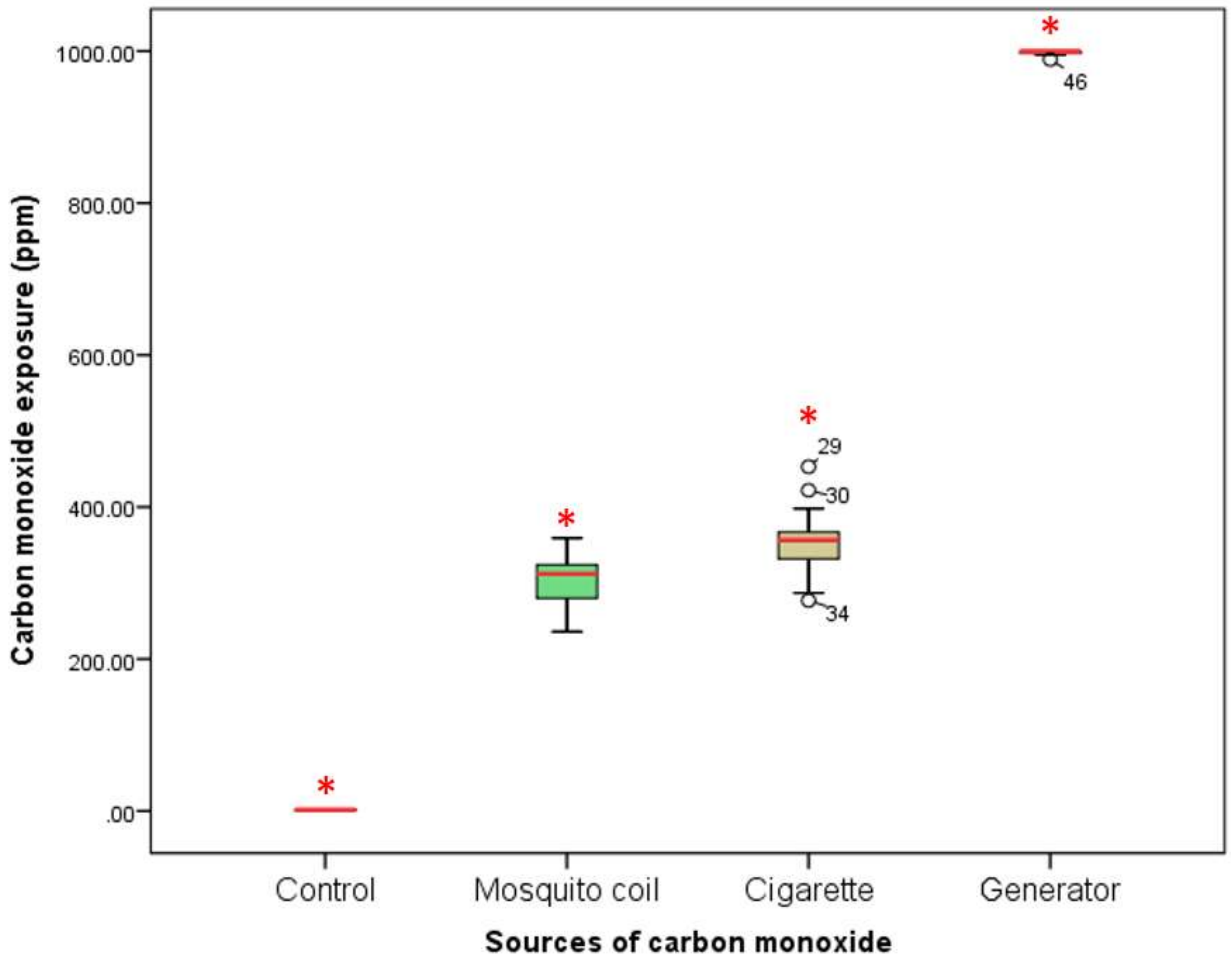

Figure 4.1: Carbon Monoxide Exposure Level in Relation to the Source

Kruskal-Wallis Test indicates significant (χ^2=49.65, P=0.000) differences between all the groups [control and MC (P=0.048), control and cigarette (P=0.000), control and generator (P=0.000), MC and generator (P=0.000), and between cigarette and generator (P=0.048)] after Bonferroni adjustment, except between MC and cigarette groups (P=0.705); n=8, $P \leq 0.05$. * indicates statistical significance, and its absence indicates insignificance.

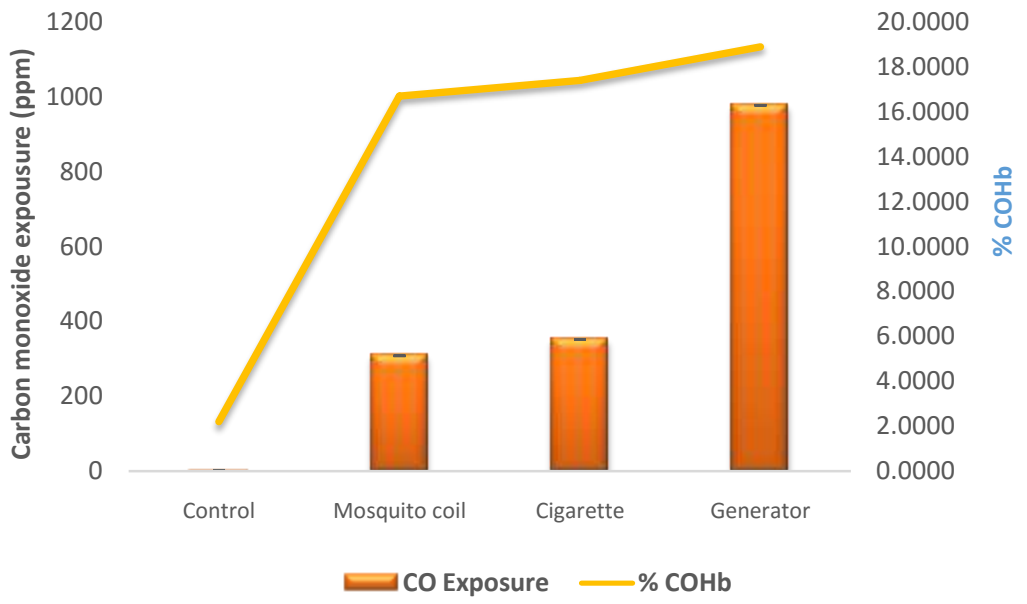

Figure 4.2: Carbon Monoxide Exposure in Relation to the %Carboxyhaemoglobin

Although the ambient temperature was 3% higher in the generator group, the difference with other groups was statistically insignificant (P=0.137) (Figure 4.3).

4.3 Body and Organ Weight Measured in Various Groups

There were significant differences between the initial and final body weights in all the exposure groups (control P=0.12; MC P=0.017; C P=0.017), except in the animals that were exposed to generator exhaust fumes (P=0.123). The absolute weights of the brains, liver and lungs were not significantly different between the groups. However, the absolute weights of the kidneys of mice that were exposed to mosquito coil was significantly (P=0.01) higher than that of the generator group (Figure 4.5).

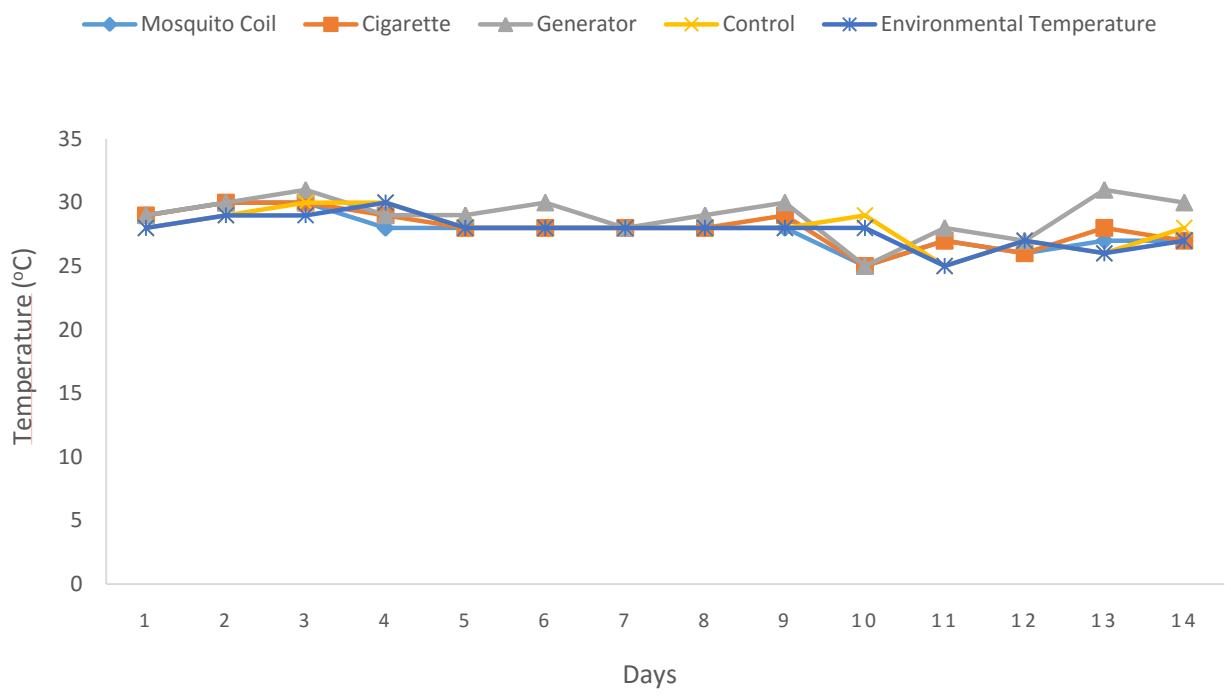

Figure 4.3: Dynamics of the Daily Temperature Fluctuations in the Groups that were Exposed to SmokeInside the Gas Chamber, the Control Group, and that of the Environment.

Repeated measure ANOVA indicates no significant (P=0.137) difference between the groups and environmental temperature, n=8, $P \leq 0.05$.

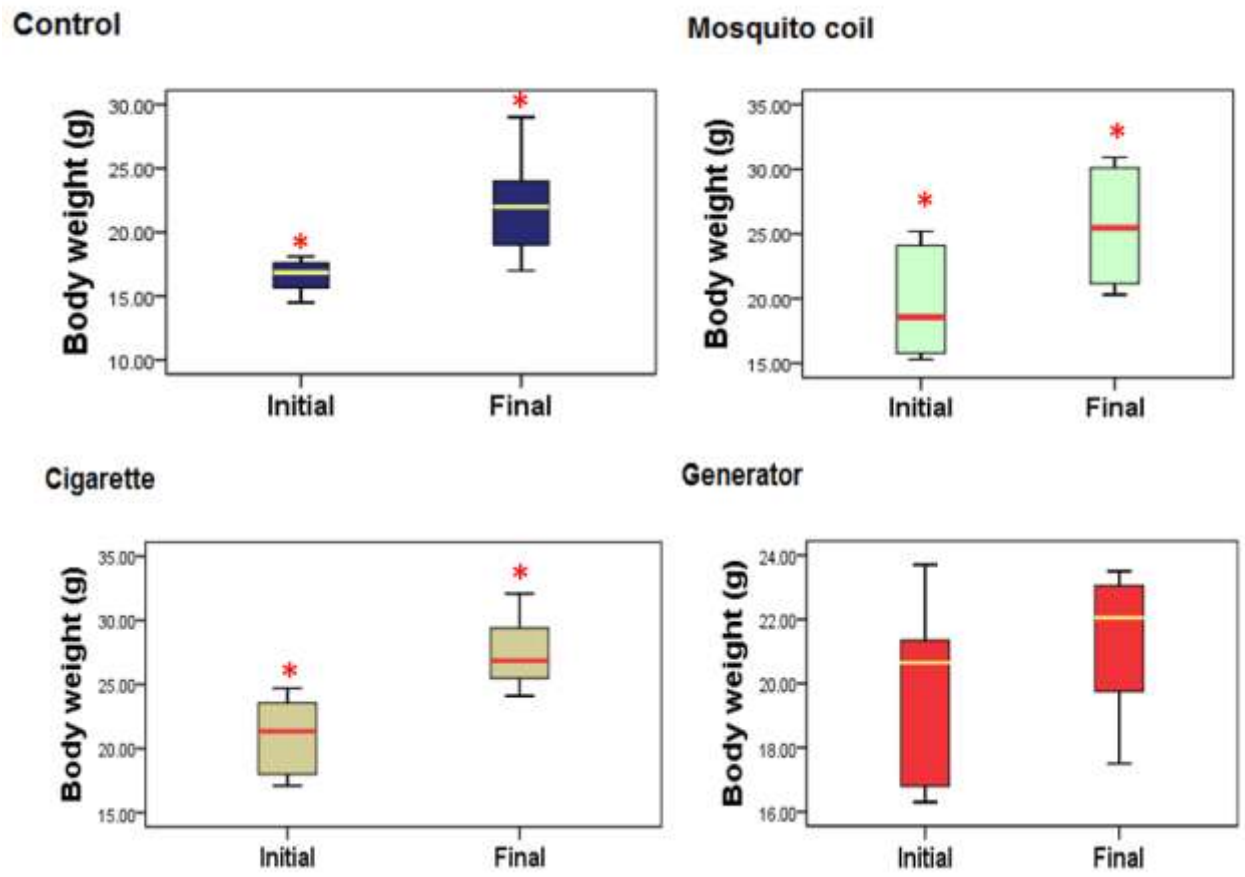

Figure 4. 4: Effects of SmokeExposure From Various Sources on the Body Weight of Mice

Wilcoxon Signed Ranks Test indicates significant differences between the initial and final body weights in the control (Z=-2.521, P=0.012), mosquito coil (Z=-2.383, P=0.017), and cigarette groups (Z=-2.380, P=0.017); however, it is insignificant in the generator group (Z=-1.540, P=0.123), n = 8, $P \leq 0.05$. * indicates significance and its absence indicates insignificance.

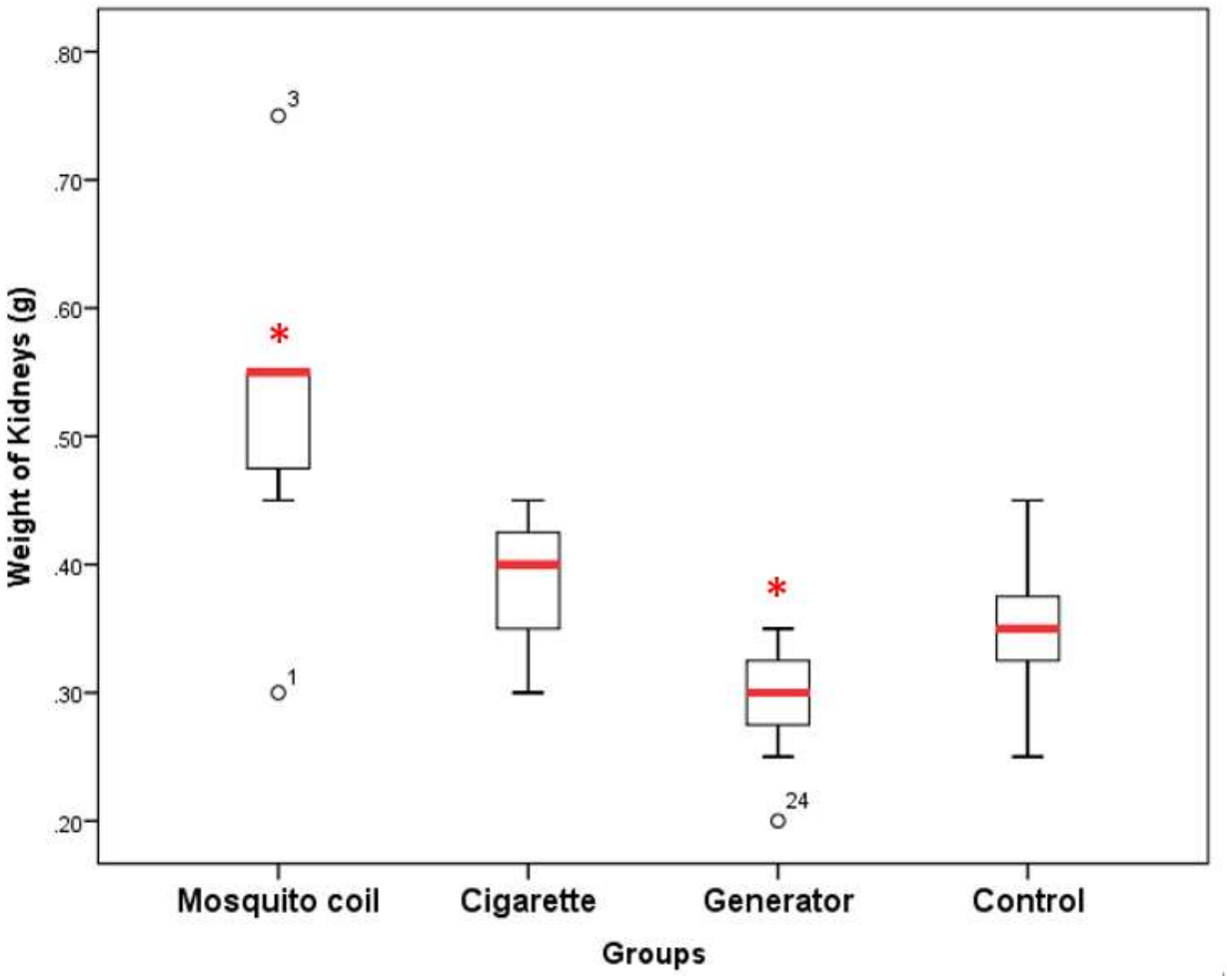

Figure 4.5: Effects of Smoke Exposure From Various Sources on the Absolute Weight of Kidneys

Kruskal-Wallis Test indicates significant (X^2=16.94, P=0.001) difference in the kidney weights between the groups. The differences was between the mosquito coil and the generator group after pairwise comparison (P=0.000), n = 8, $P \leq 0.05$. * indicates significance and its absence indicates insignificance.

4.4 Foot Slips and Latencies of Mice Exposed to Various Sources of Smoke

4.4.1 Foot Slips and Latencies of Mice in the Barnes Maze Category

All the animals that undertook BM test were free of any gross motor coordination and balance deficits as observed from the results. There were no significant differences in terms of the left foot slip (LFS, P=0.312), right foot slip (RFS, P= 0.394), and latencies (P=0.117) between the exposure groups (mosquito coil, cigarette, and generator) and the controls (Table 4.1).

4.4.2 Foot Slips and Latencies of Mice in the Elevated Plus Maze Category

All the animals that underwent EPM paradigms were free of any gross motor coordination and balance deficits as observed from the results. There were no significant differences in terms of the LFS (P=0.102), RFS (P=1.000), and latencies (P=0.814) between the exposure groups (mosquito coil, cigarette, and generator) and the controls (Table 4.2).

4.5 Assessment of Long-Term Spatial Memory

4.5.1 Barnes Maze Paradigm

There was gradual but significant [X^2=8.58, P=0.035] decrease in the primary latency (PL) over the acquisition period in the control group. Lowest PL level was achieved on the fifth day (D5) in the control group. However, the PL increased, then gradually decreased towards D5 in the animals exposed to the mosquito coil smoke and the generator exhaust fumes (Figure 4.6). Although the pattern of the decrease in the PL in the cigarette smoke exposed group was similar to that of the controls, it was much higher and the difference was significant.

Table 4. 1: Foot Slips and Latencies of Mice in the Barnes Maze Category

Groups	Left Foot Slip (LFS)	Right Foot Slip (RFS)	Latency (Sec)
Mosquito coil	1.88	0.94	55.31
Cigarette	1.00	0.81	45.25
Generator	0.63	0.94	40.82
Control	1.50	0.50	57.06

Kruskal-Wallis Test indicates no significant difference between all the groups [X^2=3.57 (LFS), 2.99 (RFS), and 5.90 (L), and P=0.312 (LFS), 0.394 (RFS), 0.117 (L)], n=8, $P \leq 0.05$. * indicates statistical significance, and its absence indicates insignificance.

Table 4. 2: Foot Slips and Latencies of Mice in the Elevated Plus Maze Paradigm

Groups	Left Foot Slip (LFS)	Right Foot Slip (RFS)	Latency
Mosquito coil	0.00	0.00	50.69
Cigarette	0.13	0.00	57.44
Generator	0.00	0.00	58.75
Control	0.00	0.00	55.94

Kruskal-Wallis Test indicates no significant difference between all the groups [X^2=6.20 (LFS), 0.00 (RFS), and 0.946 (L); and P=0.102 (LFS), 1.000 (RFS), 0.814 (L)], n=8, $P \leq 0.05$. * indicates statistical significance, and its absence indicates insignificance.

The behaviors observed in the smoke exposed groups is indicative of poor learning when compared to the control (Figure 4.6).

There were sudden and significant decreases in the primary errors committed by mice before locating the target hole in the mosquito coil (X^2=15.435, P=0.001) and cigarette (X^2=10.975, P=0.012) smoke exposed groups, similar to that of the control (X^2=19.74, P=0.000). However, the decrease was not significant in the generator fumes exposed group (X^2=7.303, P=0.063) (Figure 4.7). The total errors however, decreases significantly in the control group (X^2(3) =18.577, P=0.000) and the mosquito coil group (X^2(4) =14.516, P=0.006),but the decrease was not significant in all the other exposure groups (cigarette $\chi 2$ (3) = 4.443, P = 0.217; and generator $\chi 2$ (3) = 8.316, P = 0.040) (Figure 4.8).

The primary path length (PPL) covered by mice before reaching the target hole for the first time increases in all the exposure groups [mosquito coil (X^2=5.80, P=0.215); cigarette (X^2=1.60, P=0.809); and generator (X^2=6.343, P=0.175)], before it suddenly declines on D5. However, mice in the control group had a significant (X^2=11.190, P=0.025) and gradual decrease in the PPL, which suggest normal learning process (Figure 4.9). The total path length (TPL) also increased gradually throughout the acquisition period in all the exposure groups [mosquito coil (χ^2 (3) = 8.760, P = 0.033); cigarette (χ^2 (3) = 1.080, P = 0.782); and generator (χ^2 (3) = 1.080, P = 0.782)]. This clearly indicates impaired learning abilities when compared to that of the controls (Figure 4.10). Significant decrease in the TPL was observed in the control group (χ^2 (3) = 10.846, P = 0.013).

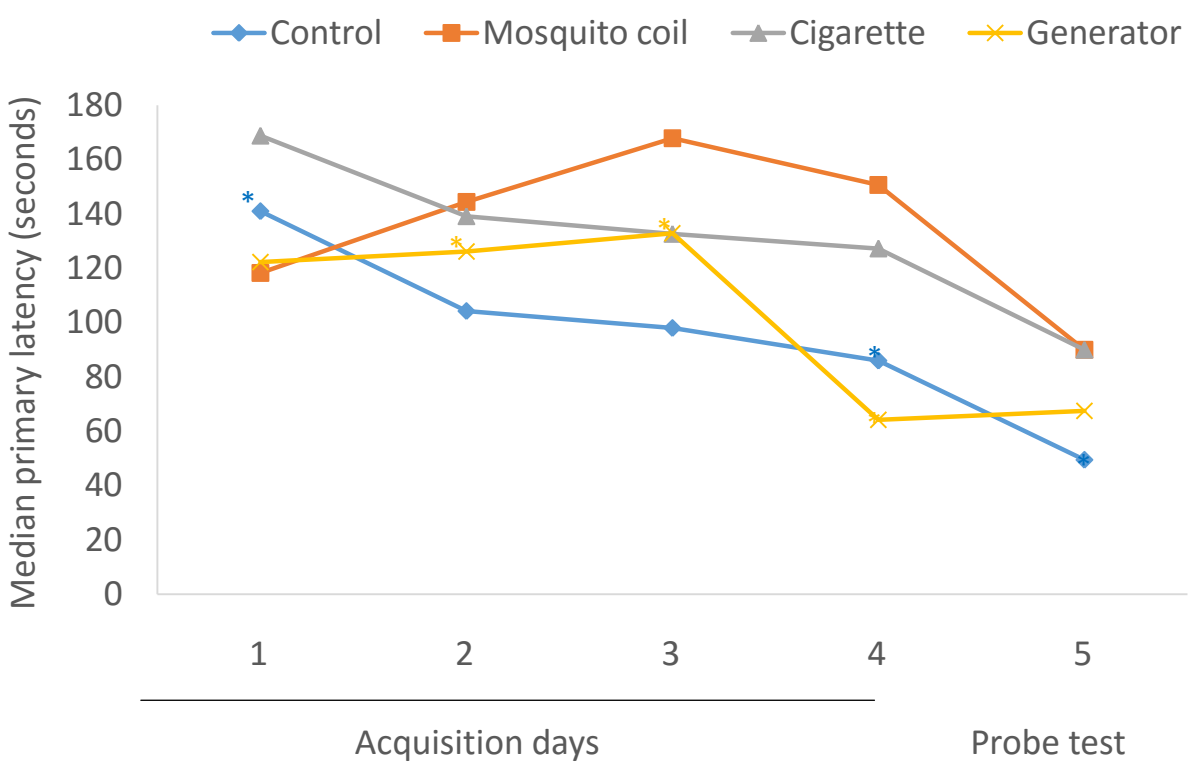

Figure 4. 6: Effect of Exposure to Smoke on the Primary Latency

Friedman Test indicates significant [X^2=8.58, P=0.035] difference between D1 and D4 (P=0.015) latencies in the control group. No significant differences were observed between the latencies in the mosquito coil (X^2=1.880, P=0.598) and cigarette (X^2=1.080, P=0.782) groups. There was however significant (X^2=11.40, P=0.012) decrease in the latencies that occurred between D4 and D3 (P=0.040), and D4 and D2 (P=0.012) after pairwise comparison, in the generator group n=8, P≤0.05.

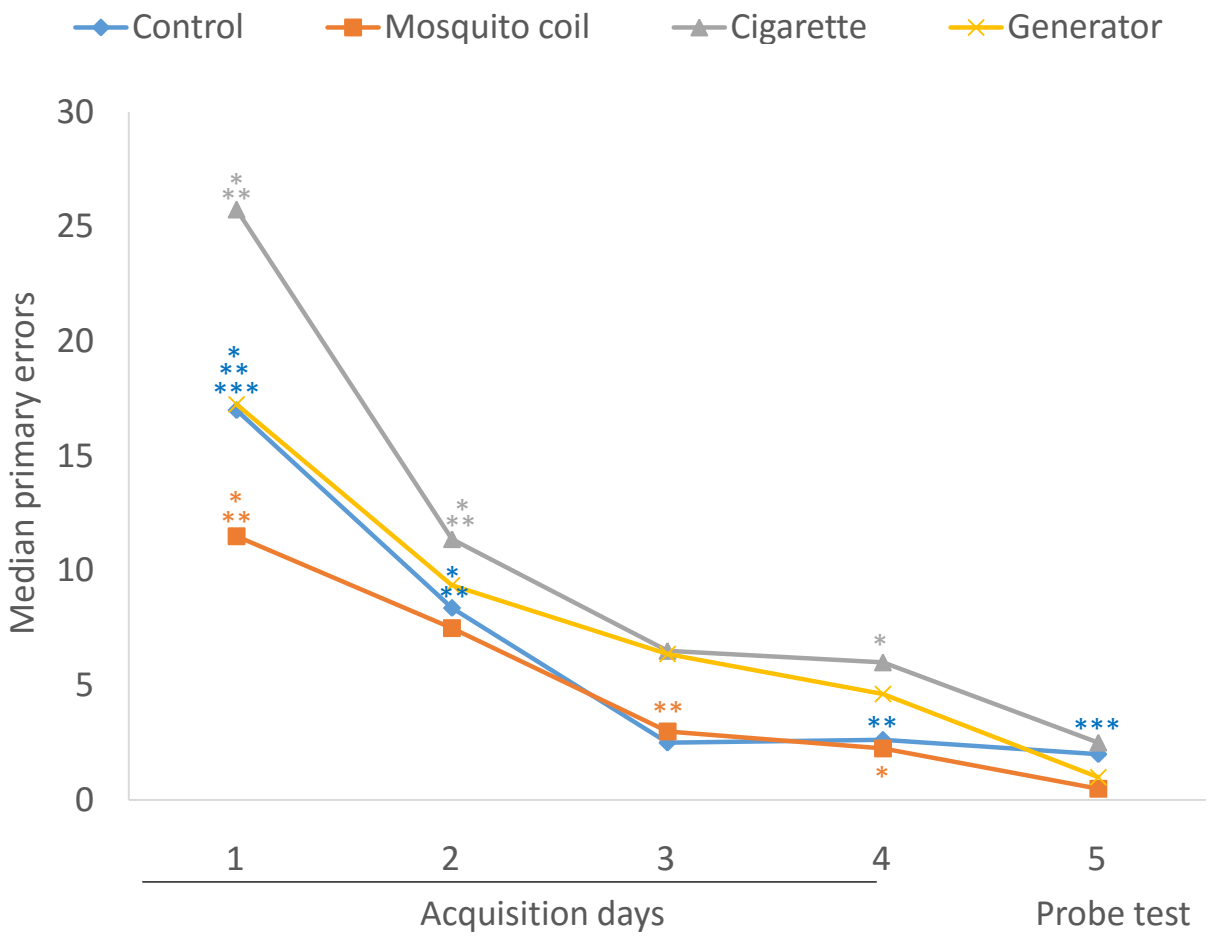

Figure 4. 7: Effect of Exposure to Smoke on the Primary Errors

Control: Friedman Test indicates significant decrease in errors [X^2=19.74, P=0.000] between D4 and D2 (P=0.030); D4 and D1 (P=0.006); D3 and D2 (P=0.040); and D3 and D1 (P=0.008). **Mosquito coil: S**ignificant (X^2=15.435, P=0.001) decrease in errors that occurred between D4 and D1 (P=0.008), and D3 and D1 (P=0.012). **Cigarette:** Significant (X^2=10.975, P=0.012) decrease in errors that occurred between D3 and D1 (P=0.026), D3 and D2 (P=0.015), D4 and D1 (P=0.026), and D4 and D2 (P =0.015) after pairwise comparison. **Generator**: no significant (X^2=7.303, P=0.063) decrease in the primary errors, n=8, P≤0.05.

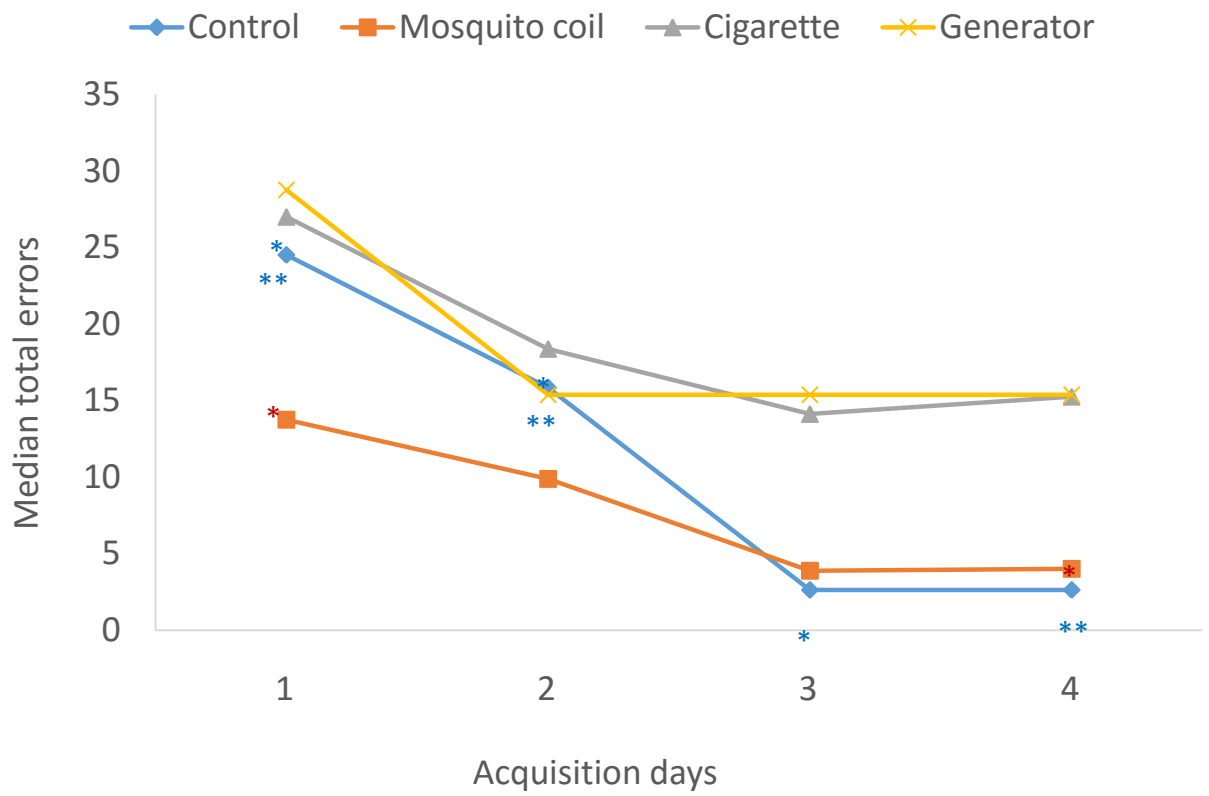

Figure 4. 8: Effect of Exposure to Smoke on the Total Errors

Control: Friedman test indicates significant decrease in the total errors [$X^2(3) = 18.577$, P=0.000] between D2 and D3 (P=0.022), D1 and D3 (P=0.008), D2 and D4 (P=0.030), and between D1 and D4 (0.012) after posthoc comparison. **Mosquito coil:** there was significant change in the total error [$X^2(4) = 14.516$, P=0.006] that occurred between D5 and D1 after pairwise comparison (P=0.004).**Cigarette**: no significant change in the total errors [$\chi 2\ (3) = 4.443$, P = 0.217]. **Generator**: also, the significant decrease in the total errors [$\chi 2\ (3) = 8.316$, P = 0.040] observed was lost after posthoc comparison. P≤0.05, n=8.

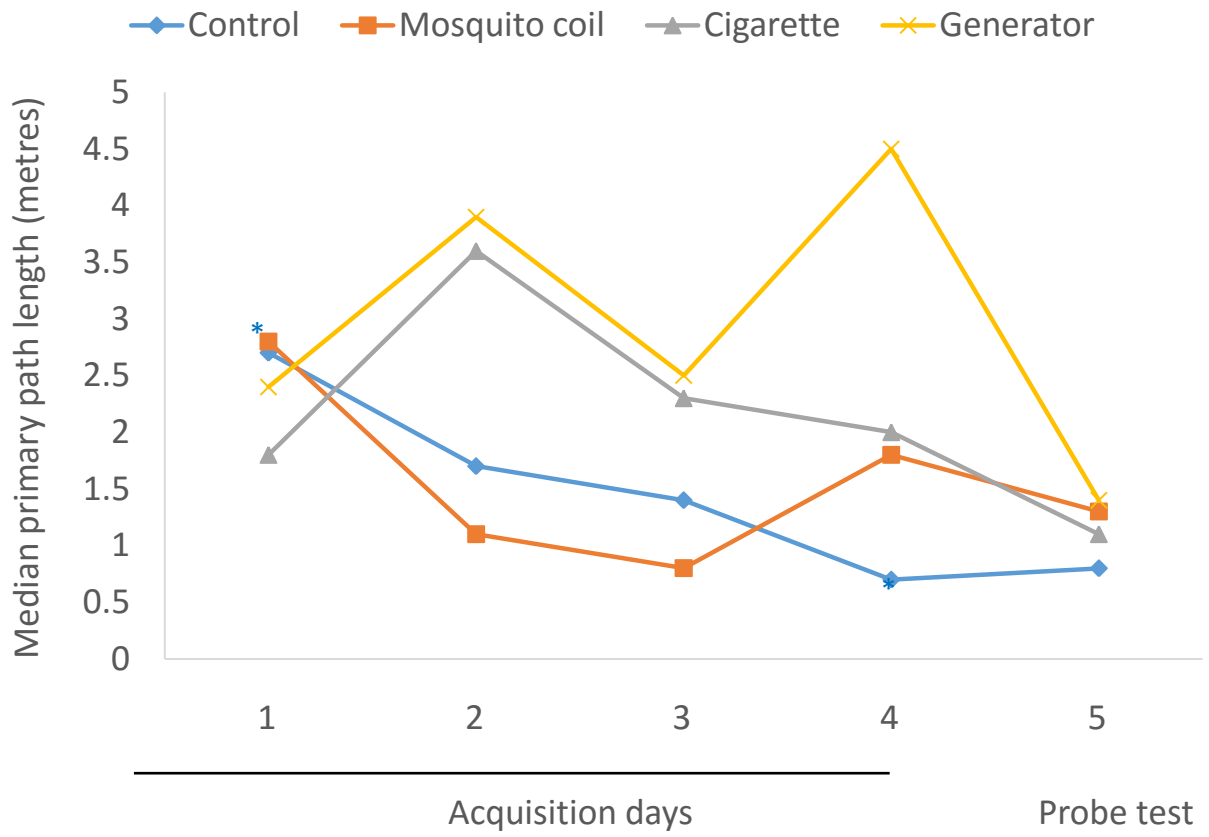

Figure 4. 9: Effect of Exposure to Smokeon the Primary Path Length

Friedman Test indicates significant (X^2=11.190, P=0.025) difference in the path lengths between D4 and D1 (P=0.05) in the control. No significant difference between the path lengths was observed in the mosquito coil (X^2=5.80, P=0.215), cigarette (X^2=1.60, P=0.809), or the generator groups (X^2=6.343, P=0.175), n=8, P≤0.05.

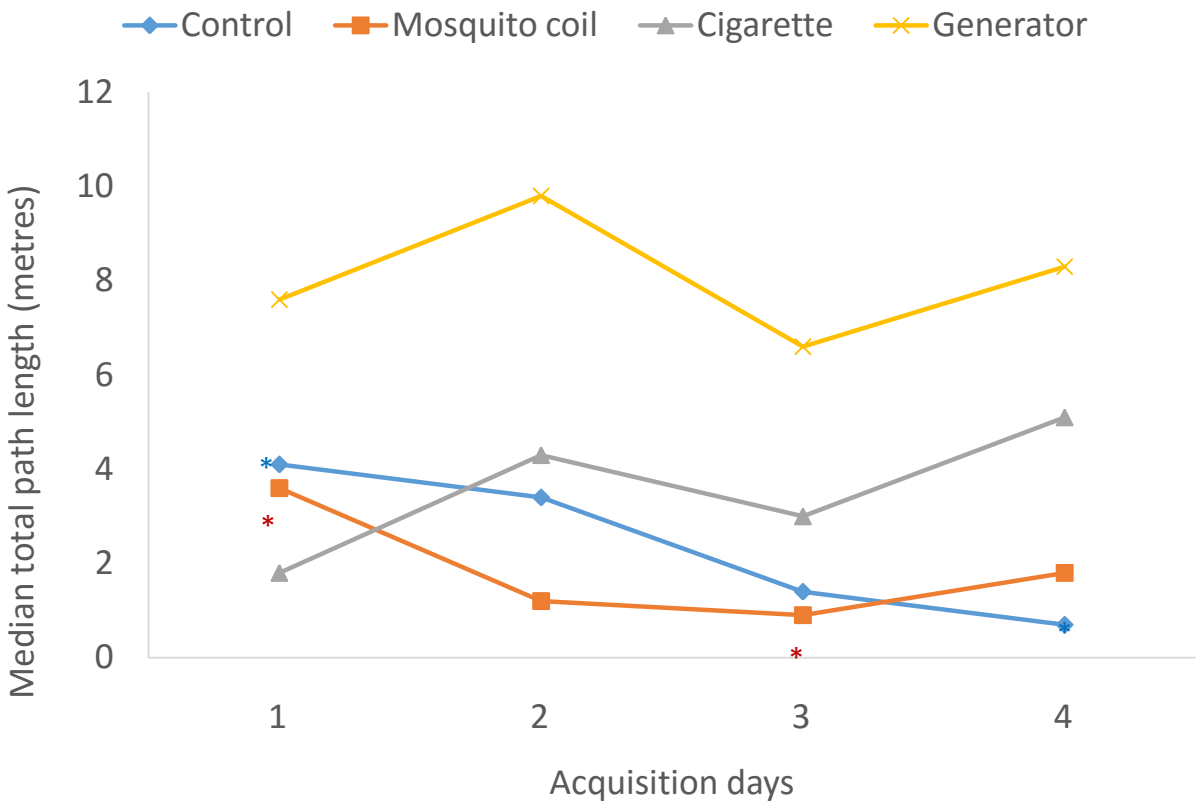

Figure 4. 10: Effect of Exposure to Smoke on the Total Path Length

Control: Friedman test indicates significant decrease in the total path length [χ^2 (3) = 10.846, P = 0.013] that occurred between D1 and D4 (P = 0.024). Significant decrease in TPL [χ^2 (3) = 8.760, P = 0.033] was also observed in the mosquito coil group that occurred between D3 and D1 (P=0.020) after pairwise comparison. No significant change in TPL was observed in the cigarette [χ^2 (3) = 1.080, P = 0.782], or the generators group [χ^2 (3) = 1.080, P = 0.782], P≤0.05, n=8.

Generally, mice in all the groups preferred the virtual target hole on D5 than any other hole, as shown by the highest number of pokes recorded from the target hole, followed by the neighboring holes (Figure 4.11). However, the control group had significantly higher preference to the virtual target hole when compared to the exposure groups.

In the control group, the animals first tried the serial strategy, then followed by the mixed strategy before they finally learn the location of the target hole. On D5, the "direct strategy" was used the most in locating the virtual target hole. This shows that the control animals were able to learn the location of the virtual target in order to escape from the aversive stimuli and go into hiding. However, mixed form of strategy was more preferred by mice in all the exposure groups on D5 as opposed to the direct (Figure 4.12).

4.5.2 Elevated plus maze paradigm

There was significant (P=0.046) decrease in the TL on the second day (recall) when compared to the first day (acquisition) in the control group. This means that the animals in the control group, as expected, were able to recall the closed arms of the maze and immediately avoid the open, exposed arm. There was also a significant (P=0.043) decrease in the TL on the second day when compared to the first day in the cigarette smoke's group (Figure 4.13).

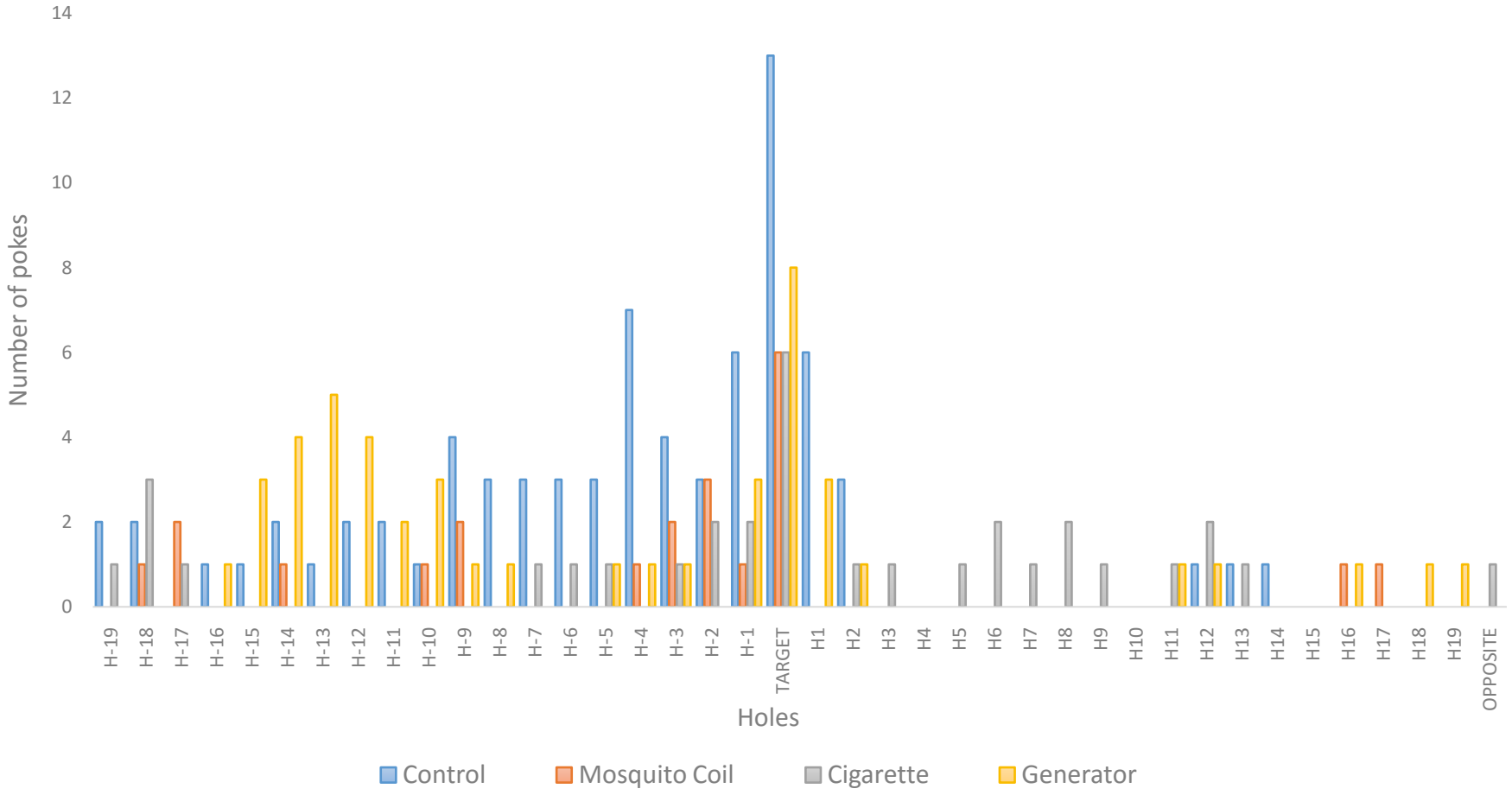

Figure 4. 11: Preferencesof Mice to the Virtual Target Hole on the Probe Test Day

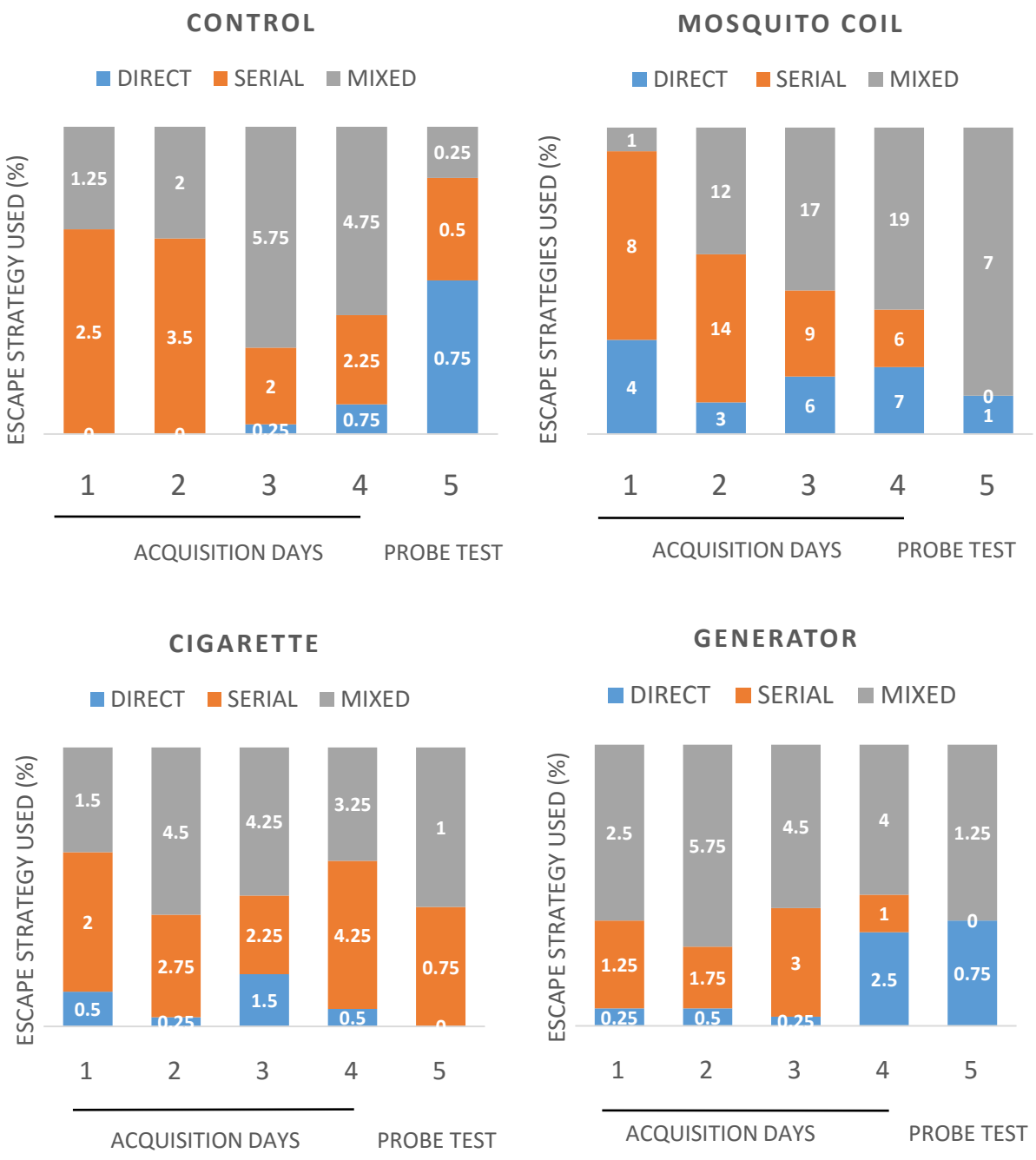

Figure 4. 12: Effect of Exposure to Smoke on the Choice of Strategy

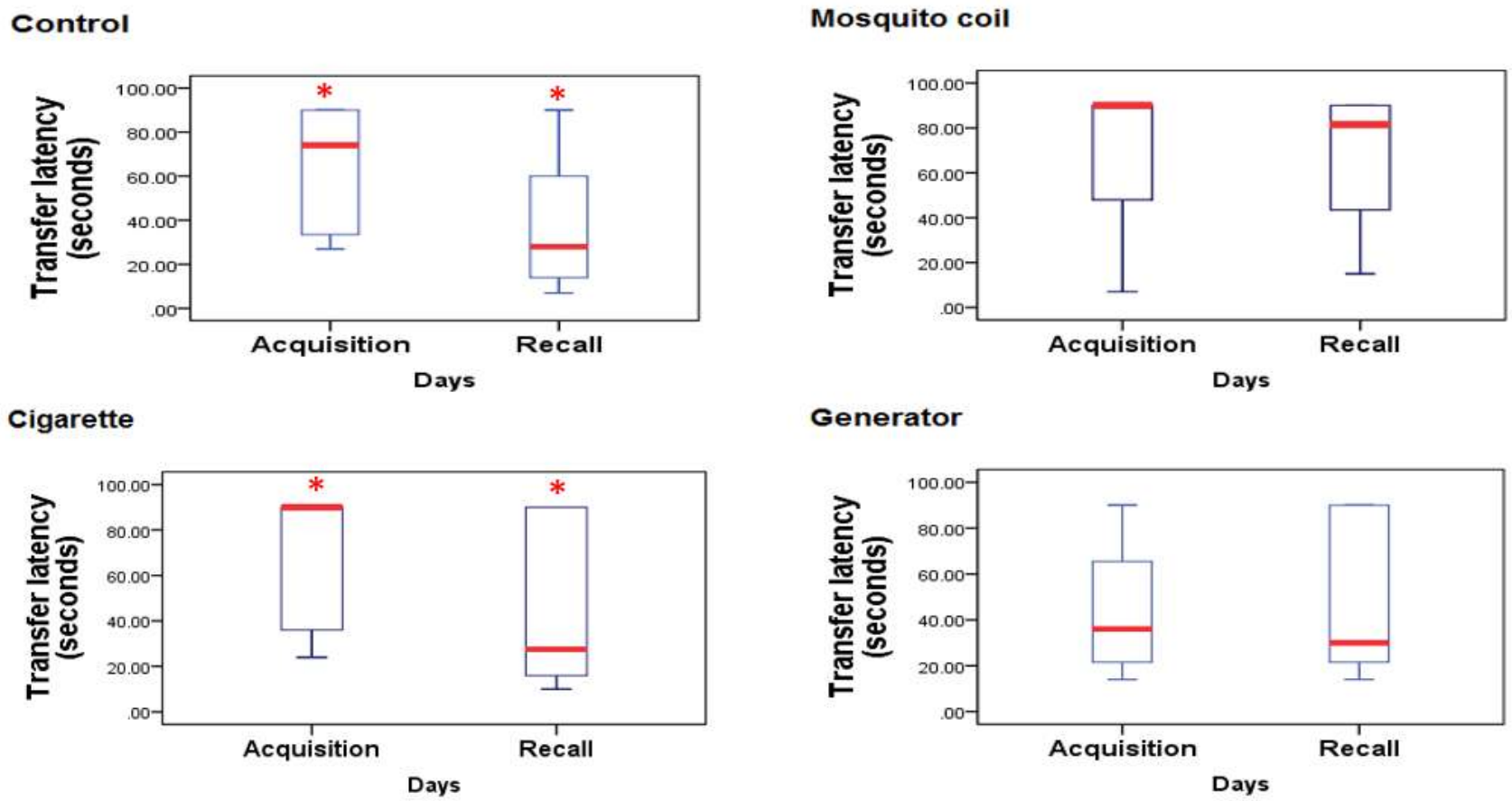

Figure 4. 13: Effect of Exposure to Smoke on Learning and Memory in the Elevated Plus Maze Task

Wilcoxon signed-rank test indicates significant difference between day1 and day2 in the control (Z=-1.99, P=0.046) and cigarette (Z=-2.02, P=0.043) exposed groups as opposed to the generator (Z=-0.31, P=0.753) and mosquito coil groups (Z=-0.00, P=1.00), n=8, $P \leq 0.05$. * indicates statistical significance, and its absence indicates insignificance.

4.6 Analyses of the Oxidative Status in the Brain

4.6.1 Glutathione

Higher GSH concentration was found in the control (251.44± o.82 mg GSH/ gprotein) group than any other group (MC=132.34, C=145.58, and Gen. =149.98 mg GSH/ gprotein) (Figure 4.15). The difference was however, found to be significant (P=0.036) only between the animals exposed to mosquito coil smoke and control group after posthoc comparison. Lowest level of GSH found in the mosquito coil group indicates oxidative stress, because it is normally consumed during neutralization of ROS produced. The oxidative stress could be due to some specific constituents of mosquito coil other than CO.

4.6.2 Superoxide Dismutase

Higher SOD activity was found in the animals exposed to the cigarette smoke (2.36± 0.12 µ/mgprotein), followed by the generator (2.31± 1.22 µ/mgprotein), mosquito coil (2.28 µ/mgprotein), and then control (1.62 ± 0.31 µ/mgprotein) group (Figure 4.16). The difference between the cigarette exposed mice and the control was found to be significant (0.022) after the pair-wise comparison.

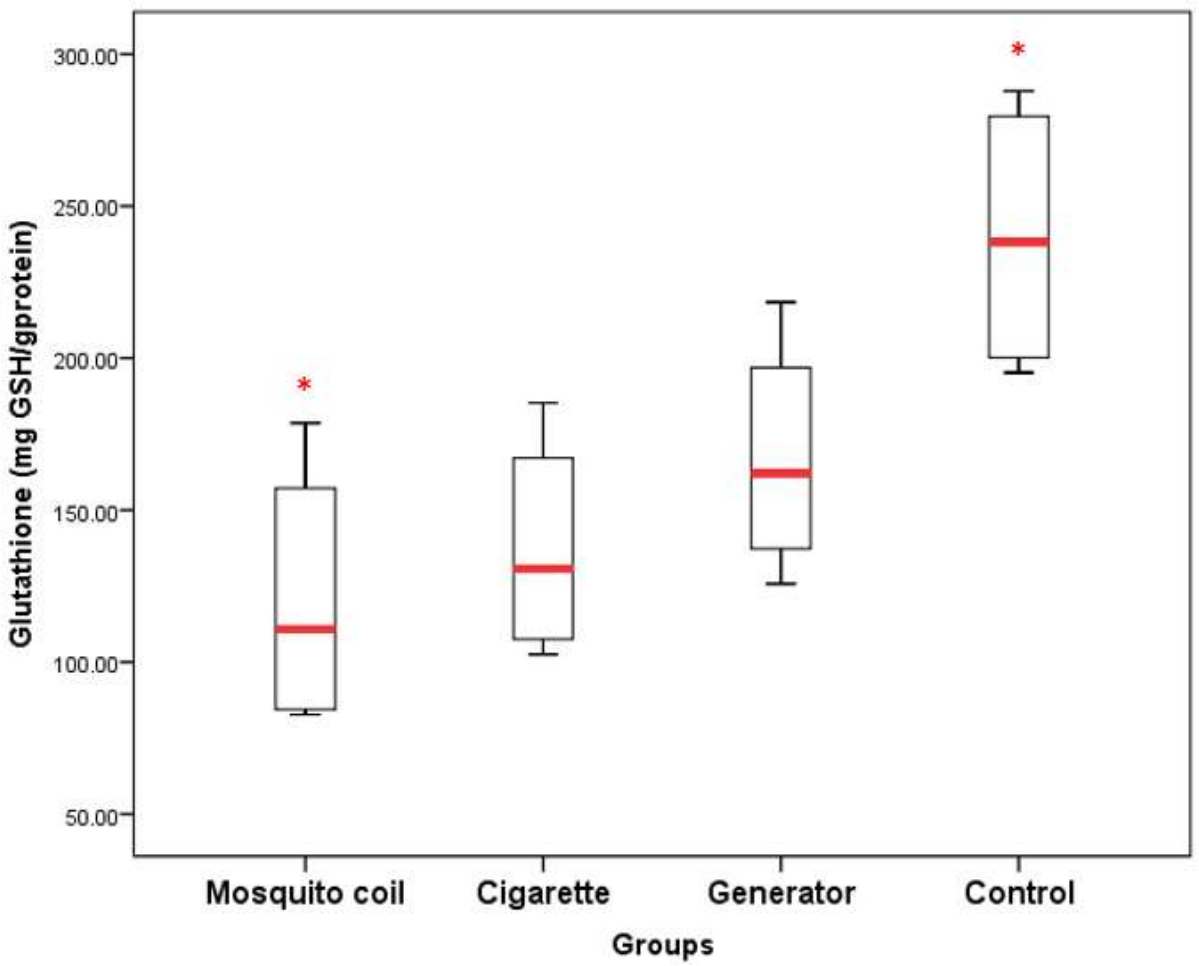

Figure 4. 14: Effects of Smoke Exposure on the Levels of Glutathione in the Brain Homogenate of Mice

Kruskal-Wallis Test indicate significant difference (χ^2=8.54, P=0.036), observed between mosquito coil and control group after Bonferroni correction (0.036). * indicates statistical significance, and its absence indicates insignificance, n = 8, P≤0.05.

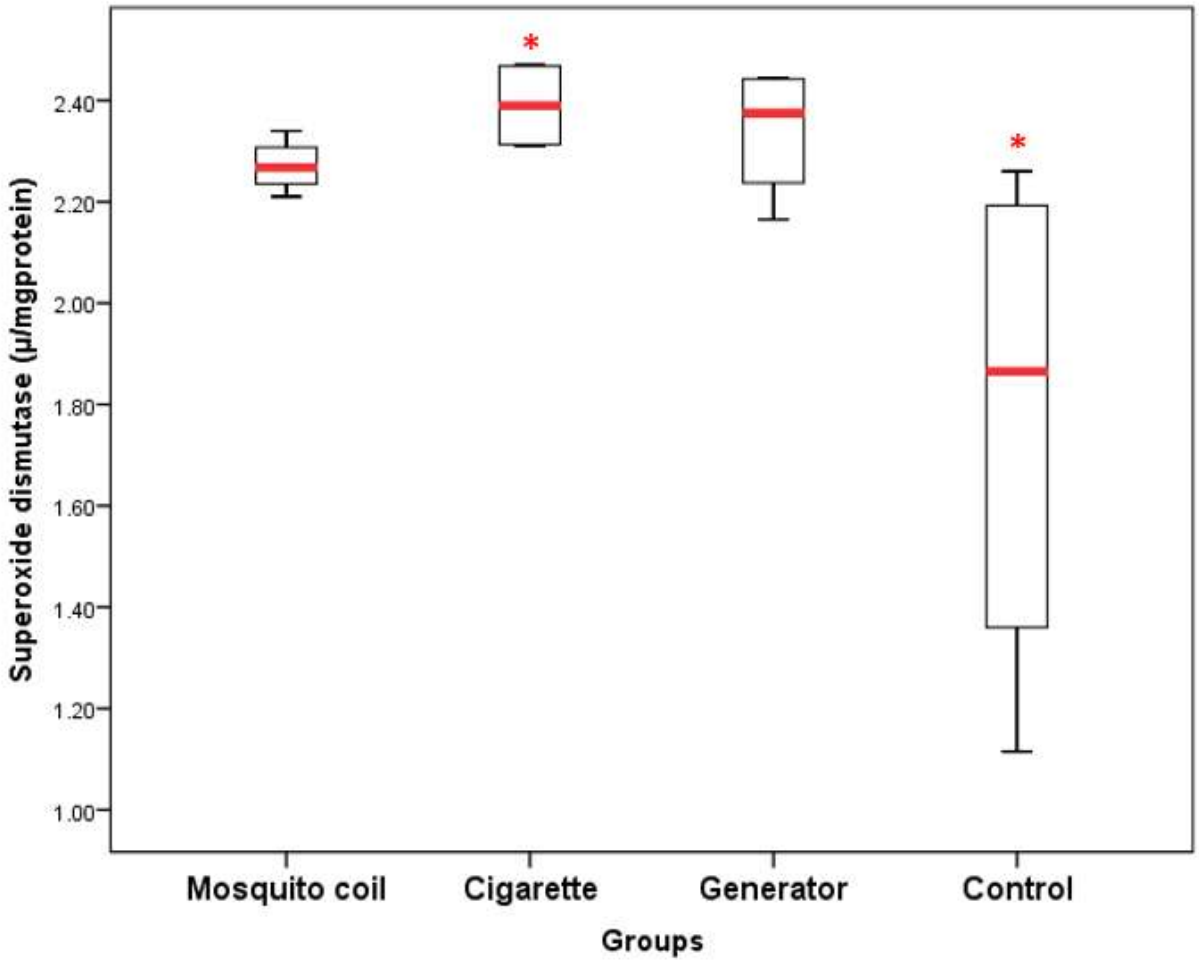

Figure 4. 15: Effects of Smoke Exposure on the Levels of Superoxide Dismutase in the Brain Homogenate of Mice

Kruskal-Wallis Test indicates significant difference (X^2=9.04, P=0.029), observed between the cigarette and control group after Bonferroni correction (P=0.022). * indicates statistical significance, and its absence indicates insignificance, n = 8, P≤0.05.

4.6.3 Malondialdehyde

Higher MDA concentration was found in the generator (0.04 ± 1.63 μMol/mL) group, followed by the control (0.03 ± 1.05 μMol/mL) group, and then mosquito coil and cigarette groups (Figure 4.17).

4.7 Histology of the CA1 Region of the Hippocampus

Fewer pyramidal cells in the CA1 region of the hippocampus were observed in all the animals exposed to smoke from the three sources (mosquito coil, cigarette, and generator) when compared to the control group (Plate IX). Focal areas of inflammation were observed in the CA1 region of the hippocampus in mice exposed to cigarette smoke (Plate X). Focal areas of inflammation that were surrounded by microglial cells were also observed in the animals exposed to the generator exhaust fumes (Plate XI).

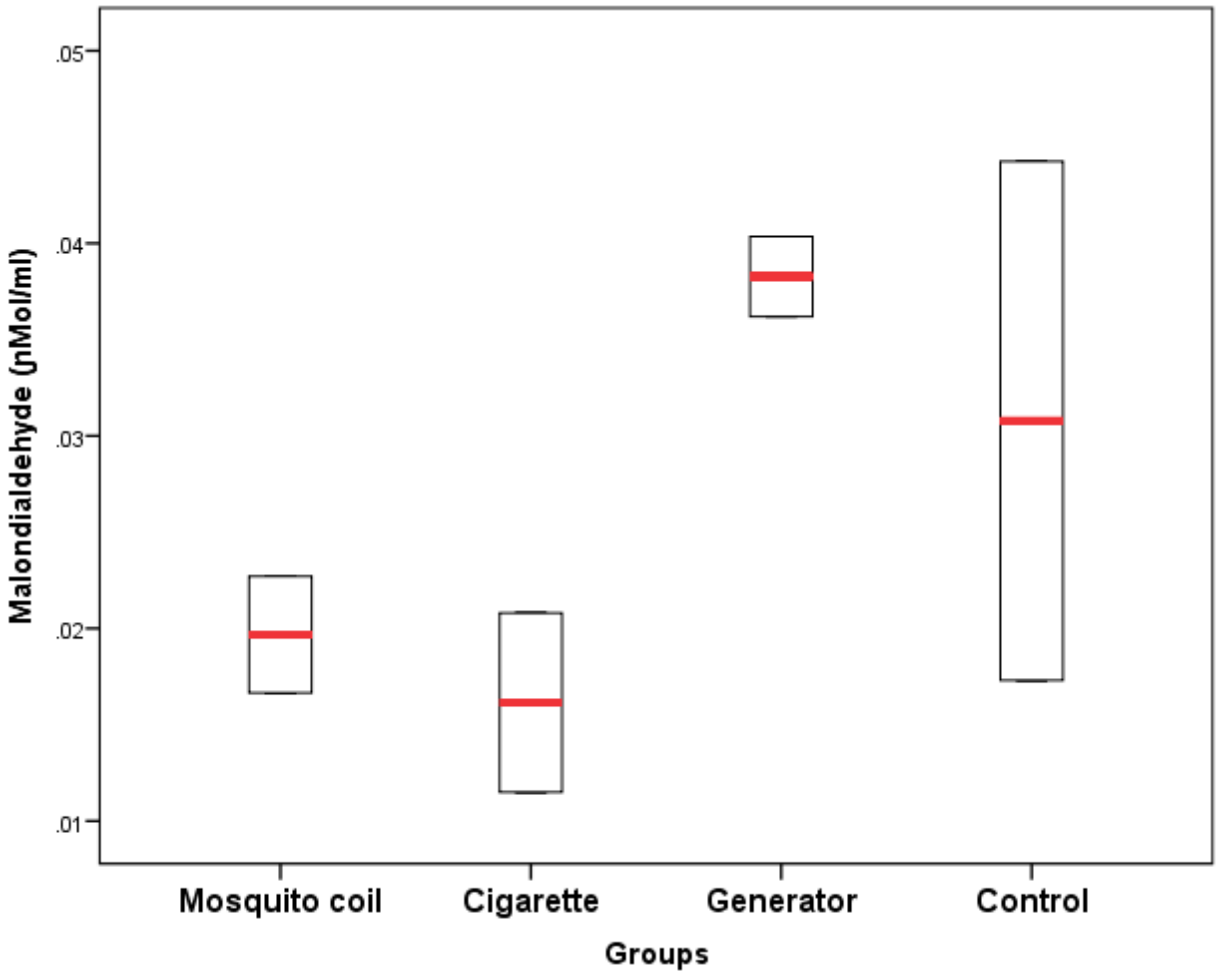

Figure 4. 16: Effect of Smoke Exposure on the Levels of Malondialdehyde in the Brain Homogenate of Mice

Kruskal-Wallis Test indicates no significant difference (χ^2=3.33, P=0.343) between all the groups. * indicates statistical significance, and its absence indicates insignificance, n = 8, P≤0.05.

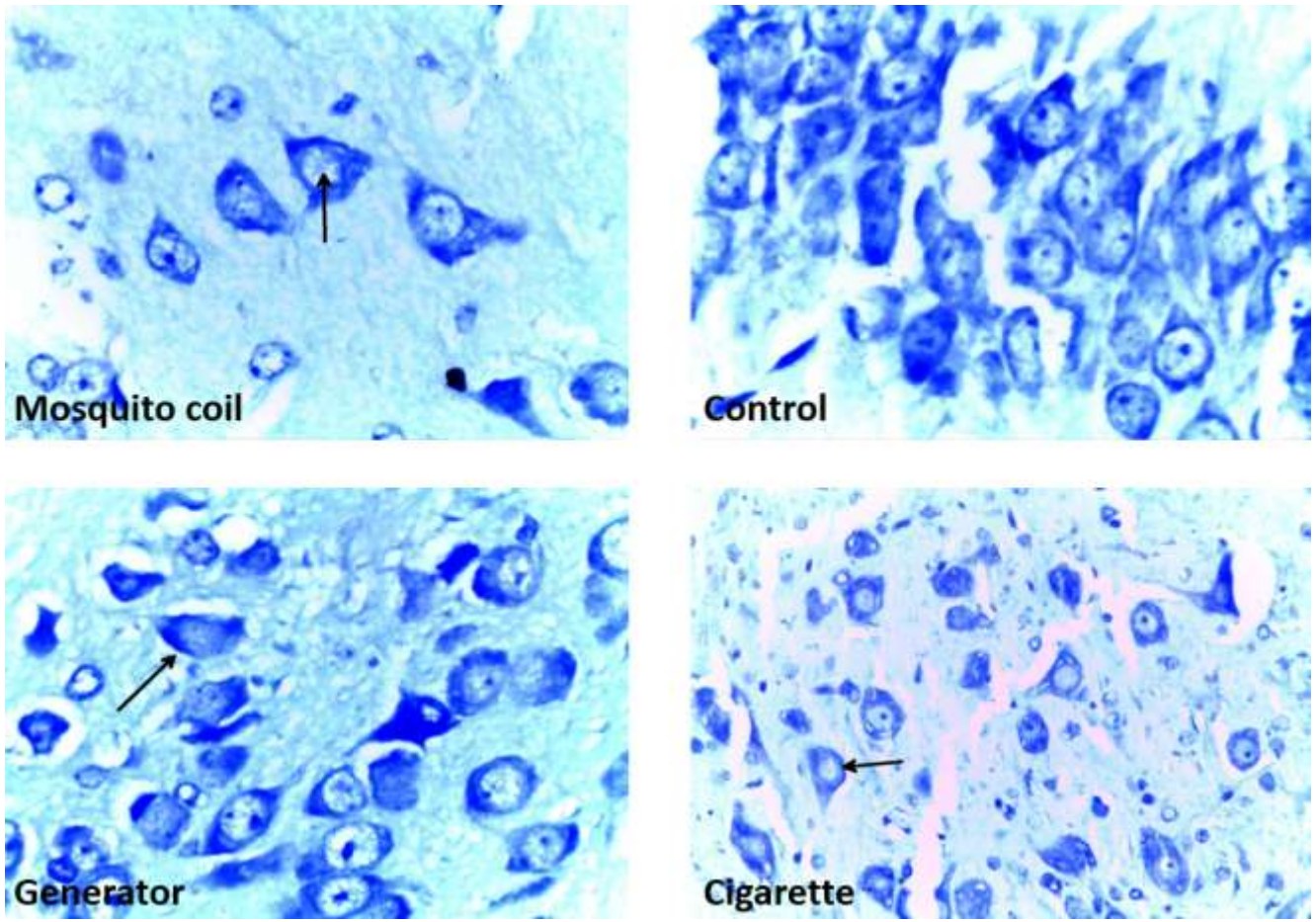

Plate 3.9: Effects of Smoke Exposure on the Structure of the CA1 Region of the Hippocampus in Mice

Fewer pyramidal cells were observed in the smoke-exposed groups when compared to the controls. Arrows indicates pyramidal cells without nuclei. Mag X1000. Toluidine Blue stain.

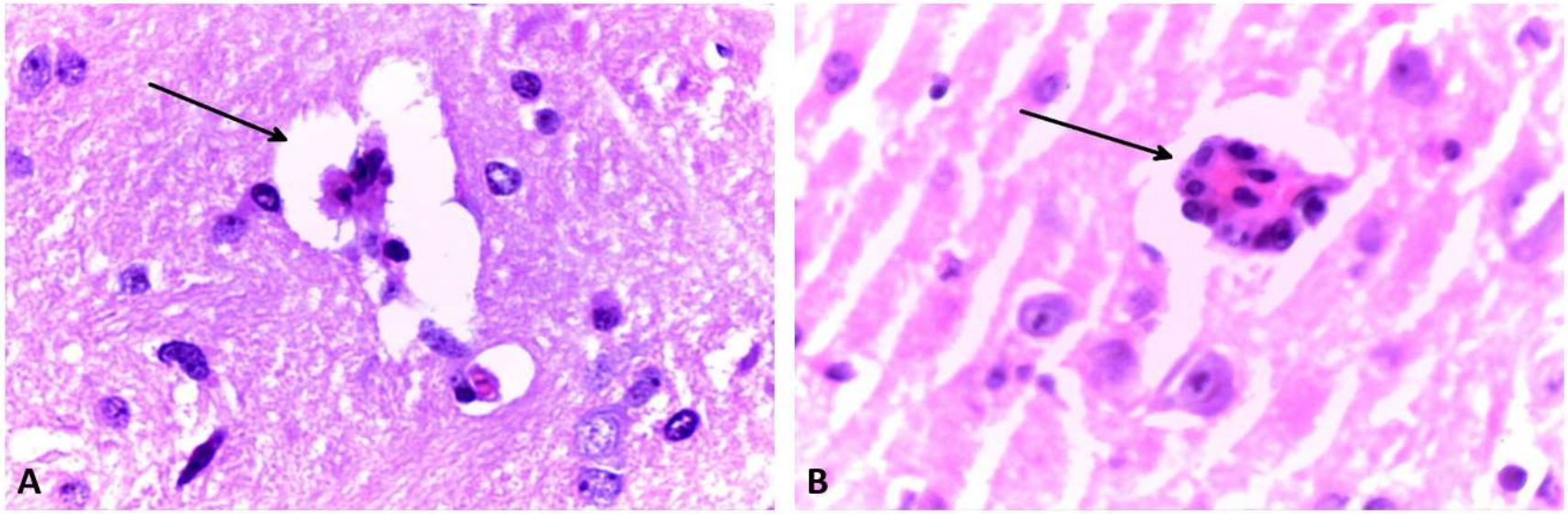

Plate 3.10: Effects of Smoke Exposure on the Structure of the CA1 Region of the Hippocampus in Mice

Focal areas of brain damage with microglia in the center and gliosiswas observed in the cigarette smoke-exposed group.

[Mag. X1000, HandE]

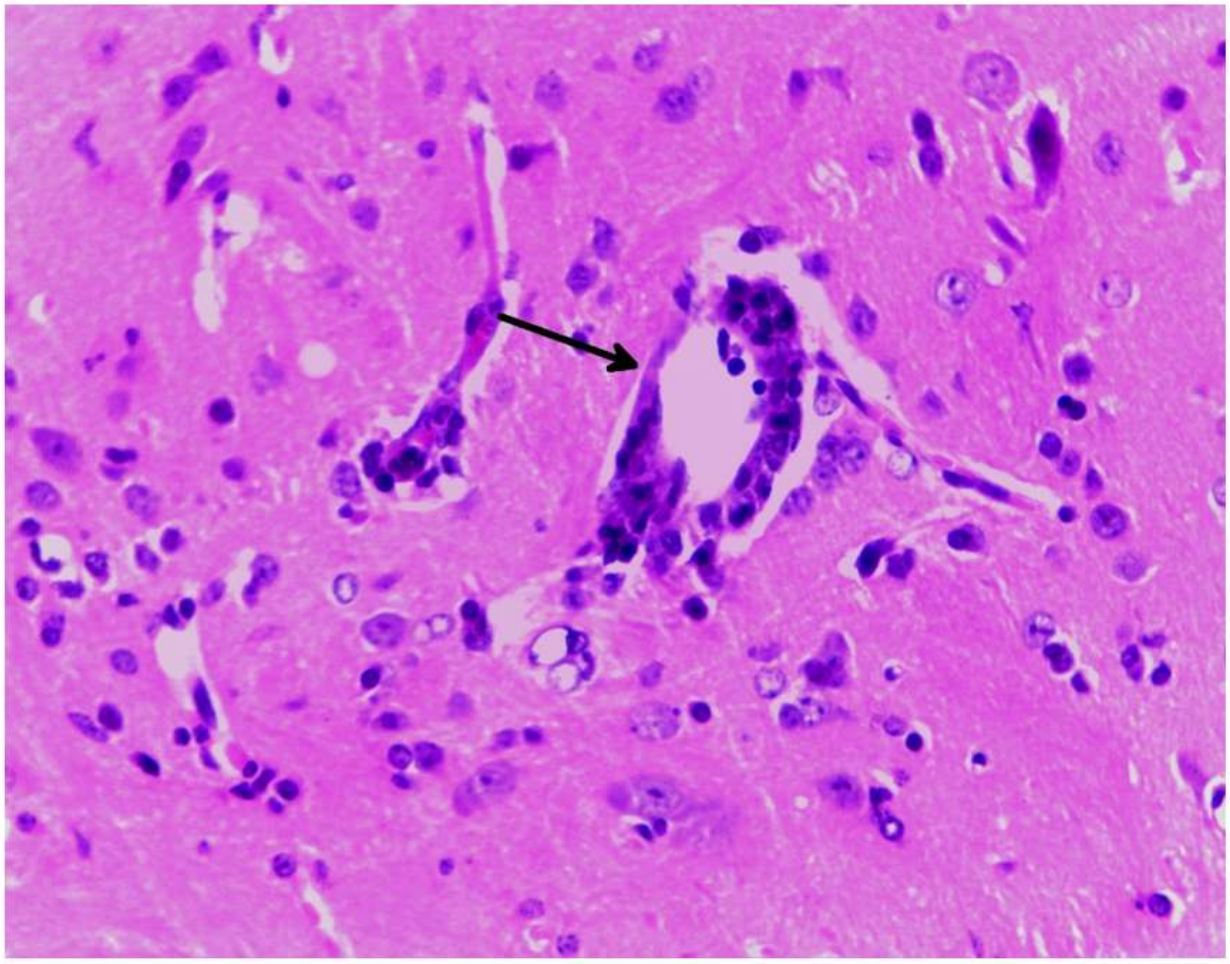

Plate 3.11: Effects of Smoke Exposure on the Structure of the CA1 Region of the Hippocampus in Mice

Focal areas of brain damage surrounded by microglial cells was observed in the generator exhaust fumes-exposed group. [Mag. X1000, H&E]

CHAPTER FIVE

5.0 DISCUSSION

The aim of the study was to assess the toxicity of exposure to smoke from three common sources (mosquito coil, cigarette and generator exhaust fumes). Functional, structural and biochemical changes were assessed in order to assess the magnitude of the toxicity. A total of 64 adult, male, albino, mice were randomly grouped in to eight groups, each having eight mice. The eight groups were divided in to two broad categories to undergo separate neurobehavioural paradigms (BM and EPM) for learning and memory. Each group had animals that were exposed to either MC smoke, cigarette smoke, or generator exhaust fumes. Beam walk test was used to screen all the animals for possible gross motor coordination and balance deficits before the start of the study, and were all found to be free from any deficit.

During the study period, an initial and final body weights of all the animals were measured to provide an objective measure of the growth status. Normal weight gain was observed with significant differences between the initial and final body weights. With the exception of mice in the generator group, all other animals had significant weight gain over the period of the study. Hang *et al*., 2017 observed significant reduction in the body weights of both male and female mice after exposure to thirdhand smoke (THS). In terms of CO emission, mice in the generator groups were exposed to 3 times the amount ofCO (≈ 1000 ppm) than other sources. Hypoxia, rather than economic status was found to be associated with a low birth weight (Giussani *et al.*, 2001).

When the absolute weights of the organs (brain, liver, kidney, and lungs) were compared between the four groups; only the kidney's weight in the generator group was significantly different from that of the mosquito coil group. Enlarged kidneys observed in the MC group could be as a result of specific content of the MC rather than CO; because CO exposure was highest in the generator group. The enlarged kidney could be as a result of severe inflammation, hypertrophy or hyperplasia. From the literature, both pyrethrins (Siddique *et al.,* 2015), and hydrotreated heavy naphtha (IACR, 1989), are all found to be nephrotoxic. Aside from the possible toxicity of the above components of MC on the kidneys, CO also has pro-inflammatory effects (Li and Moore, 2011).Animals exposed to MC similar to that of humans for just 60 days, had a lower body weight and an elevated levels of liver enzyme activities (Ayorinde *et al.,* 2014). Nephrotoxicity (Garba *et al.,* 2007), other organs damage (Taiwo *et al.,* 2008) and neurobehavioural changes (Patel *et al.,* 2012) have all documented after MC smoke exposure.

Twenty five percent of mice in the generator group died during the study. Rose *et al*., also showed that exposure to generator exhaust fumes was lethal to animals (Igwo-Ezikpe *et al.,*2014). Exposure to indoor air pollution from solid fuel use has been linked to approximately 1.5 million annual deaths (WHO Report, 2010). Tragic incidences of the death of the entire family through overnight exposure (Lyness and Crane, 2011), or children that were transported behind an enclosed pickup van, or even soldiers in training camps (Henz *et al.,* 2005), have all documented.

It is evident that heat, hypoxia and cold affects human cognitive functions due to a variety of psychological and or biological processes (Tylor *et al*., 2016). That is why the environmental

temperature of all the groups were monitored throughout the period of the study. The results showed that temperature inside the gas chamber during the exposure did not differ significantly with the temperature outside the gas chamber, nor was it different from that of the control group's environment (Figure 4.3), throughout the study period. Although there was slightly higher temperature in the generator group than in the other groups; the difference was not significant throughout the period of the study. Therefore, all the groups were maintained under similar values of ambient temperature throughout the period of the study; and any observed behavior couldn't have been caused by the variations in the temperature.

All the mice were screened for possible motor coordination deficits prior to the start of the study. In the BM category, parametres like the primary latency (PL), primary error (PE), primary path-length (PPL), number of pokes per hole on the probe trial day, and the pattern of strategies used by mice to locate either real or virtual target holes (VTH) were considered in order to assess learning and memory behavior.

The pattern of the strategies exhibited by mice during the acquisition period indicated learning as the number of trainings increases over the days. The use of the "serial" strategy initially, followed by a switch to the "direct" form of strategy in order to locate the target hole was the most appropriate pattern that indicated gradual and progressive learning. The use or preference of the "mixed" strategy however, indicated impaired learning (during the acquisition period) or impaired recall (on the probe test day/ D5). The earlier the animals switch from the use of other strategies to the use of the "direct" strategy indicated faster/ enhanced learning abilities. On day 5, the "direct" strategy should be more preferred/ favoured than any other strategy if at all they

have learned and were able to perfectly recall the actual location of the target hole. It appears that neurobehavioural tests can be relied upon in the assessments of subtle neurologic dysfunctions resulting from CO poisoning (Rajiah and Mathew, 2011).

The controls had significantly progressive decline in the PL, PE and also PPL during the acquisition period. They were able to achieve perfection as early as D3 of the acquisition period; this indicates how fast they were able to learn the location of the target hole. In D5, the virtual target hole had the highest number of pokes, followed by the adjacent targets. To show actual recall, some mice sat by the side of the closed virtual target hole. The sharp switch from the use of serial and mixed strategies, to the use of direct strategy on the third day of acquisition period indicated the faster learning ability of the animals in that group.Although the % COHb recorded in the control was up to 2%, it was within the non-smoker range and did not pose much toxicity risk. Such mild exposures were even found to enhance learning (Kurawa and Magaji, 2016). From the literature, patients who were mildly exposed to CO (<1 %COHb) usually had intact memory functions, equivalent to that of the control group, and were even better in some areas like learning, word recall, and quality of learning by Buschke's verbal memory testing. Attention was also found to be better in the patients, in whom visual reaction time was shorter than their controls (Deschamps *et al.,* 2003).

Contrary to what was obtained in the control group, mice in the MC group had impaired learning ability throughout the acquisition period, as shown by the almost horizontal line graph of the PL. They however, had significant decrease in PE over the acquisition days. The pattern of the primary path length exhibited by the MC group also clearly shows the gross impairment in

memory. Although there was more preference to the virtual target hole (VTH) on D5, the pattern of the choice of strategy during the acquisition period and on D5 clearly indicates impaired memory. In both acquisition period and probe trial, mice in this group, non-specifically tried locating the TH, with total disregard to the use of visual cues. Patients with CO poisoning usually exhibit impaired memory, attention, and executive functions (Penney, 2007).

As for the cigarette group, there was a non-significant decrease in the PL, and significant drop in the PE over the acquisition days. The animals also preferred the VTH on D5, and the use of the "serial" followed by the mixed search strategy to locate the TH throughout the study period. Increased activity observed in the animals could be related to the stimulatory action of nicotine, and could explain the significant enhancement in learning and memory observed in the EPM paradigm.

In the generator group however, the learning process was a bit delayed, as shown by the increase in the PL, till the third day when it was followed by a significant decrease in the PL. Although there was some form of learning, the process was delayed compared to their control counterparts. The nature of the PE and number of pokes on D5 were similar to that of the MC group. The mice however were confused in the choice of the search strategies. The acquisition period was dominated by the use of the mixed strategy throughout the study period. Interestingly, there was a sharp rise in the use of the "direct" form of strategy on D3 which corresponded to the same day (D3 of the acquisition period) when they actually started learning after the 3 days delay when compared to the controls. The delayed learning process could explain the delayed choice of the

appropriate search strategy. This clearly indicated poor learning process and ultimately poor recall.

Exposure to CO from combustion engines like gasoline-powered electric generators, automobile exhaust, etc. has been and is still an uncontrollable menace even in the developed world (Chamberlain, 2016). While the incidences of CO poisoning in the developed countries occurs during recreational events (silver and Hampson, 1995), suicidal attempts (Ruszkiewicz *et al.*, 1997), and accidental power failures (Daley *et al.*, 2000) which were unusual conditions and rare; in Nigeria and other developing countries, incidences of CO poisoning from these sources is common and occurs daily.

As for the EPM category, transfer latencies (TLs) in day 1 (acquisition) were compared with that of day 2 (recall) in order to assess learning and memory. Significant decrease in the TL on D2 when compared to TL in D1 indicated enhanced learning and memory abilities. Here, it clearly shows impaired learning and memory in both the MC and generator groups. The enhanced learning and memory ability observed in the cigarette group could be as a result of the nicotine acting as a CNS stimulant (Milhorn, 1990).

Here, close observation of the pattern of the PL, PPL, and the choice of strategy of the cigarette exposed animals indicated hyperactivity caused by the nicotine which likely gave rise to the enhanced learning and memory. On the average, a cigarette can yield up to 2 mg of nicotine, a dose that is stimulatory, however higher doses (50 – 100 mg) could be harmful. The nicotine in blood passes immediately to the brain (within seconds) where it stimulates nicotinic

acetylcholine receptors; this indirectly promotes the release of many chemical messengers such as acetylcholine (Ach), norepinephrine (NE), serotonin, dopamine and beta-endorphins (Benowitz, 2010).

Highest GSH concentration was found in the control group with significant difference between the control and mice exposed to mosquito coil smoke. Higher GSH concentration was expected because of the minimal amount of ROS due to low level of oxidative stress in the control group. However, because of higher ROS as a result of the oxidative stress in the mosquito coil group, the level of GSH was low, because most of it was used up in neutralizing the ROS. The severity of oxidative stress was also higher in the mosquito coil group followed by cigarette and generator group. Severe level of oxidative stress observed in the mosquito coil group couldn't have been solely due to CO; likely, some constituents of the mosquito coil aggravate the condition. Least level of oxidative stress was observed in the generator group, although they were exposed to the highest dose of CO. This is likely because CO alone may not be responsible for the oxidative stress observed. Also, CO poisoning could be associated with either an increase or decrease in oxidative stress (Piantadosi, 2008; Akyol *et al.*, 2014).

Superoxide dismutase is an enzyme that alternately catalyzes the dismutation (or separation) of the superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Thus,it acts as an important antioxidant defence, especially in the brain. Highest SOD level was found in the animals exposed to cigarette smoke, and was statistically significant when compared to that of the control group. High level of SOD activity observed here indicates high level of oxidative stress in the cigarette smoke exposed group. The high level of oxidative stress

in the cigarette group may be as a result of other constituents of cigarette that apparently aggravated the condition. Chronic exposure to as low as 25 ppm of CO was found to cause a significant increase in both SOD-1 and SOD-2 in the cerebellar cortex of the CO poisoned pups (Lopez *et al*., 2009). Total oxidant status (TOS) and COHb levels were found to be significantly increased in CO-poisoned patients;However, TOS, oxidative stress index (OSI) and COHb levels were reduced immediately after the treatment. Measurements of the TOS, total antioxidant status (TAS) and OSI levels were then proposed by Kavakli (2011) to be useful markers of severity of CO poisoning.

Malondialdehyde is a product of lipid peroxidation of polyunsaturated fatty acids found in abundance in the plasma membranes. The degree of lipid peroxidation can be estimated by the amount of MDA produced in the tissue. Reactive oxygen species can easily degrade polyunsaturated lipids, forming MDA. The MDA produced is equally reactive, and causes toxic stress in cells by the formation of covalent protein adducts. When MDA reacts with deoxyadenosine and deoxyguanosine in DNA, it forms DNA adducts. It was conclusively agreed that the unifying factor in determining toxicity and carcinogenicity for most metals such as iron (Fe), is the generation of ROS and nitrogen species (Valko *et al.*, 2005). In this case, both Fe and NO are strongly associated with CO; this may lead to a vicious cycles of CO toxicity in the brain.

Although highest MDA levels were recorded in the generator group, the differences between all the four groups (MC, cigarette, generator, and control) were statistically insignificant. One possible explanation for the observed result here could be because the ROS produced was

already neutralized by the GSH and SOD. Brief exposure to CO (15 minutes here) might not be enough to produce high amount of CO in the body to cause any significant oxidative stress. This can be confirmed from the Coburn-Forster-Kane (CFK) estimates where the %COHb observed in the generator group that was exposed to ≈ 1000 ppm was just 18% instead of the expected 61% that was predicted by Coburn (1965) in his CFK model (Appendix III).

Since mice have a high respiratory rate (80-230 breaths/ min.), excess CO in the blood circulation can easily be washed out of the body before it even reaches the brain in toxic levels to cause any significant oxidative stress in the neurons. However, in their conclusion on the role of NO-derived oxidants in vascular injury from CO poisoning, Thom *et al*. (1999) revealed that exposure to relatively low CO concentrations is enough to alter vascular status by several mechanisms and that many changes were linked to the NO-derived oxidants. Products of lipid peroxidation were also found to increase by 75% over the baseline values 90 minutes after CO exposure at a concentration sufficient to cause unconsciousness (Thom, 1990). In this case, the anti-oxidant system in the brain effectively neutralizes the insignificant oxidation caused by the smoke exposure.

In general, all the smoke exposed groups had fewer number of pyramidal cells in the CA1 region of the hippocampus. The severity of the neuronal death was however more in the generator group than in the other groups. In contrast with the control groups, numerous pyramidal cells were found in similar locations of the hippocampus. Neuronal cell death was widespread in the brain and was not restricted to only pyramidal cells in the hippocampus alone. Previous data suggested

specific toxicity of CO on memory functions in animals and also delayed neuronal death in areas involved in memory process (Busl and Greer, 2010).

Aside the general features which were common to all the threesmoke-exposed groups; mice in the cigarette group had several focal areas of brain damage associated with microglial infiltration and many areas of gliosis. Similar focal areas of brain damage (as observed in the cigarette group) that were surrounded with extensive microglial cells were also found in the generator group.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The results indicate significant growth retardation in the generator group who received the highest exposure to CO (≈ 1000 ppm). There was 25% mortality in the generator group. When the organs (brain, liver, kidneys, and lungs) were finally weighed at the end of the study; the absolute kidney weight of the MC group was significantly heavier than in the generator group. Exposure to each of the different sources of smoke lasted for only 15 minutes, daily, for up to 14 days period. A plexiglas gas chamber (75 x 50 x 50 cm) that had inlet and outlet holes with a small ventilation window (12 x 10 cm) was used for the exposure. Exposure to smoke from the three sources were associated with ventilation to fresh air as occurs in our daily lives.It can be concluded that, since the dose of CO exposure cannot explain this observation; likely, pyrethrins and hydrotreated heavy naptha, which were known to be nephrotoxic, could be responsible for this. Mice in all the groups were maintained under similar study conditions such as feeding, housing, shelter, and temperature, except for the different sources of smoke.

The CO exposure dose in all the groups didn't correlate well with the %COHb as predicted in the CFK model; however, it was significantly higher in the exposure groups than in the controls. This can be explained by numerous factors that determine bioavailability as well as the elimination of the gas as shown in the literature. Neurotoxicity to the brain can be both structural and/ functional. The functional impairment was assessed by the neurobehavioural paradigms used (BM and EPM) and the structural damage by the histological assessment of the brain tissue. Smoke exposure impaired learning and memory in the mosquito coil and generator groups.

However, mice exposed to cigarette smoke in the EPM category, exhibited a normal learning and memory ability similar to that of the control group. In general, exposure to the generator exhaust fumes and mosquito coil smoke were associated with impaired learning and memory, neuronal cell death and growth retardation in the generator group.

Assessment of oxidative markers in the brain, specifically GSH, MDA, and SOD, indicates significant oxidative stress in the cigarette and mosquito groups, and minimal oxidative stress in the generator group. Although CO causes oxidative stress, the brief nature (15 minutes) of the exposure, may not have raised the CO level in the body to cause any significant oxidative damage in the brain.

Histology of the CA1 region of the hippocampus indicates loss of pyramidal cells in the smoke-exposed groups likely due to either apoptosis, necrosis or both. The cigarette and generator groups specifically exhibited focal areas of inflammation, associated with microglial cells infiltration and gliosis which was more severe in the cigarette smoke exposed group.

6.2 Conclusion

Exposure to smoke from any of the three sources (mosquito coil, cigarette, and generator) was associated withoxidative stress in the brain, neuronal cell death, and impairedspatial learning and memory and also anxiety-like memory in adult mice.

6.3 Recommendations

i. People should be aware of the dangers associated with smoke exposure from the three sources and avoid or reduce exposure.

ii. Personal safety measures can be taken to reduce/ prevent exposure, like avoiding passive smoke, use of mosquito nets instead of MC, opening windows and doors when MC is being used, stationing of generators away from the living area when in use, and avoiding ignition of vehicles in closed garages.

iii. The use of personal and home gas monitors will be very helpful towards preventing accidental poisoning.

6.4 Contributions to Knowledge

1. Impaired anxiety-like memory andspatial learning and memory abilities in the mosquito coil and generator groups.
2. Increased oxidative stress in the mosquito coil and cigarette groups.
3. Neuronal cell death in the CA1 region of the hippocampus in all the smoke-exposed groups.
4. Exposure to generator exhaust fumes and cigarette smoke were associated with focal areas of brain damage and inflammation.
5. Exposure to generator exhaust fumes was associated withdecreased weight gain and mortality.

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APPENDICES

Appendix I: Nigerian Ambient Air Quality Standards

Pollutants	Time Of Average	Limits
Particulates	Daily average of daily values	250 μgm^3
	1 hour	600 μgm^3
Sulphur dioxide (SO2)	Daily average of hourly values	0.01 ppm (26 μgm^3)
	1 hour	0.1 ppm (260 μgm^3)
Non-Methane Hydrocarbons	Daily average of 3 hourly values	160 μgm^3
Carbon Monoxide	Daily average of hourly values	10 ppm (11.4 μgm^3)
	8 hourly values	20 ppm (22.8 μgm^3)
Nitrogen Oxide	Daily average of hourly values (range)	0.04 ppm – 0.06 ppm
		(75 μgm^3 – 113 μgm^3)
Photochemical Oxidants	Hourly values	0.06 ppm

(Igile *et al*., 2015)

Appendix II: Nigerian Ambient Air Tolerance Limits, Guidelines for Convectional Pollutants

Pollutants	Long-Term mg/m^3	Limits +(Hours)	Short-Term mg/m^3	Limits +(Minutes)
Carbon monoxide	1.0	24	5	30
Chlorine	0.03	24	0.1	30
Chloroform	10	24	50	30
Fluorides	.012	24	0.055	30
Hydrocarbons (Total)	0.01	24	-	-
Hydrogen sulphide	0.008	24	0.008	30
Lead	0.005	24	0.02	30
Lead sulphide	0.001	24	-	-
Nitric acid	0.008	24	0.008	30
Nitrogen Dioxide	0.085	24	0.085	30
Nitrogen Monoxide	0.4	24	0.8	30
Nitrogen Oxide	0.004	24	0.1	30
Oxidants	0.08	24	0.1	30
Ozone	0.1	24	0.2	30
Soot	0.05	24	0.1	30
Sulphur Dioxide	0.05	24	0.5	30
Sulphur Acid	0.1	24	0.5	30
Suspended Particulate	0.15	24	0.5	30

(Ede and Edokpa, 2015)

Appendix III: Blood Carboxyhaemoglobin Levels as Predicted by Coburn-Forster-Kane model

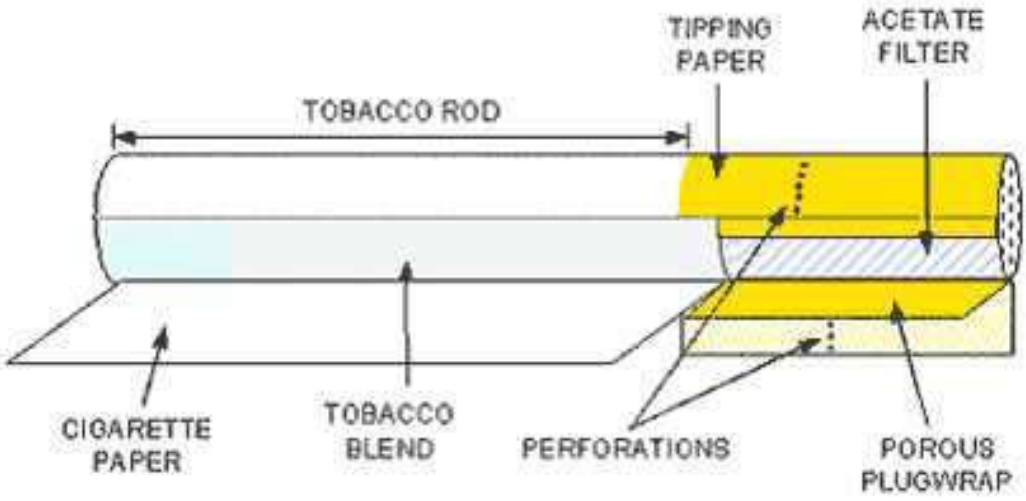
Carbon monoxide exposure concentration (ppm)	**Steady-state blood Carboxyhemoglobin (%)**
0.1	0.25
0.5	0.32
1	0.39
2	0.5
5	1
10	1.8
15	2.5
20	3.2
40	6.1
60	8.7
80	11
100	14
200	24
400	38
600	48
800	56
1,000	61

(Coburn, 1965)

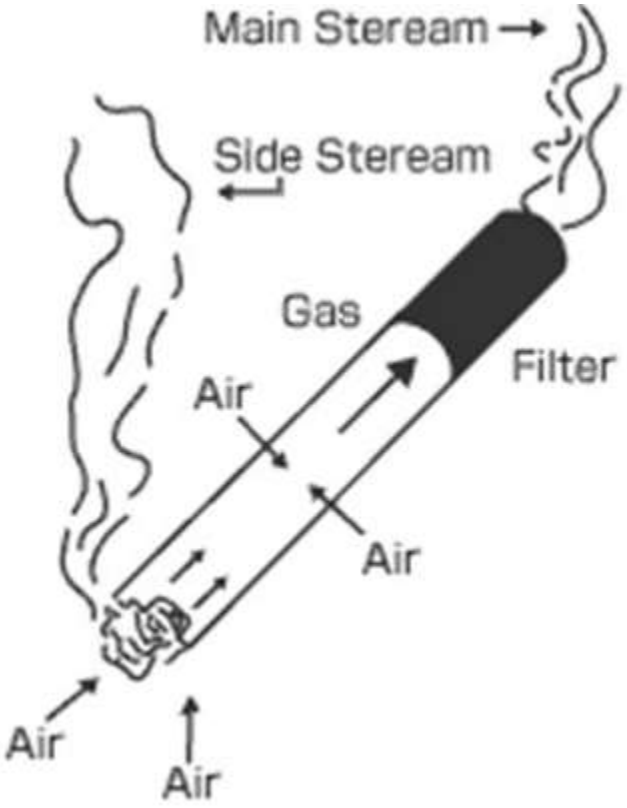
Appendix IV: Additives in Tobacco

Substance	Place of use	Function
Glycerol Triacetate	Filter	Filter bonding agent
Ink	Paper	Monograms
Cellulose Acetate	Filter	Filter material
Titanium Dioxide	Filter	Whitening agent
Calcium Carbonate	Paper	
Sodium Potassium Citrate	Paper	
Guar Gum	Paper	Binder
Polyvinyl Acetate	Paper	Adhesive
Sugar	Paper	
Phenolic Resin	Paper	
Glycerol	Tobacco	Humectant
Hexanal	Tobacco	Flavor
Lemon Oil	Tobacco	Flavor
Linalool	Tobacco	Flavor
Potassium Sorbate	Tobacco	Preservative
Propyl-PHB	Tobacco	Preservative
Sugars-Invert Sugar	Tobacco	Humectant, flavor
BHT	Paper	Antioxidant
Cocoa and Theobromine	Tobacco	Encourages expansion of airways and facilitates increased smoke and nicotine intake
Glycyrrhizin	Tobacco	Ingredients of liquorice. Acts as bronchodilator

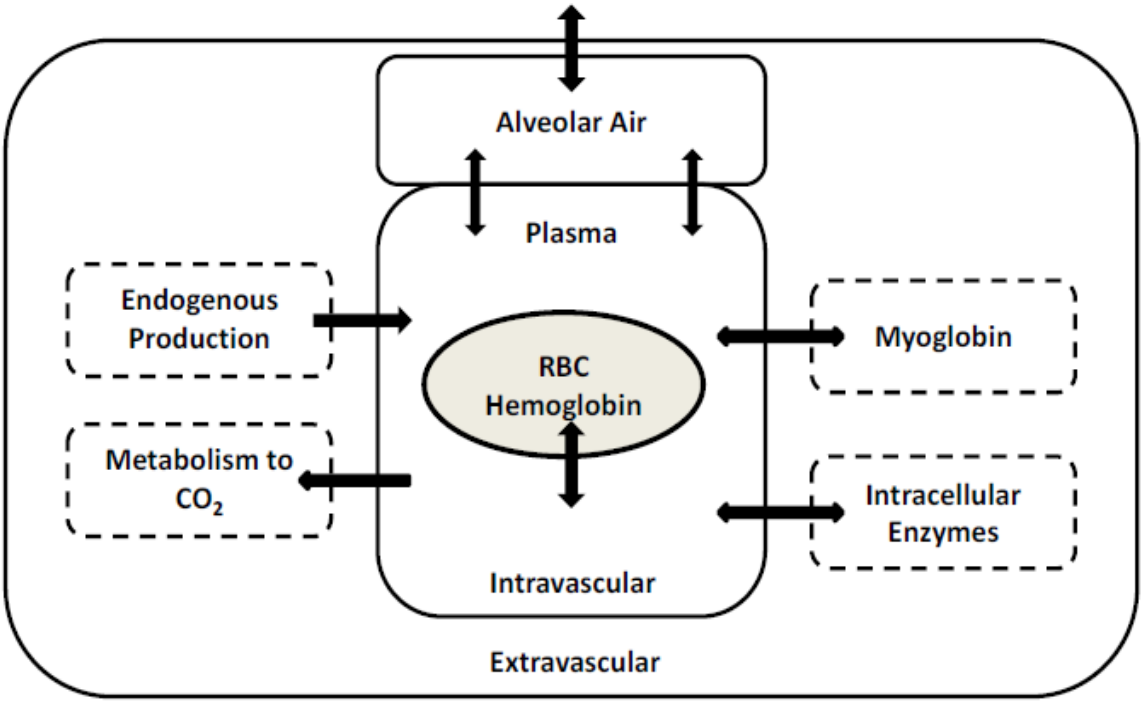
(WHO, 2014)



Appendix V: Non-Tobacco Components of a Cigarette(White and Parfetti, 1995)



Appendix VI: Components of Cigarette Smoke(Harris, 1996)



Appendix VII: Summary of Carbon Monoxide Uptake, Distribution and Elimination Pathways (EPA, 2000)