HISTOSTEREOLOGICAL TERATOGENIC EFFECTS OF PRENATAL EXPOSURE TO CARBAMAZEPINE ON THE FETAL BRAIN IN ALBINO RATS (*RATTUS NORVEGICUS*)

ANN WAIRIMU MWANGI

MASTER OF SCIENCE

(Human Anatomy)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

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Histostereological Teratogenic Effects of Prenatal Exposure to Carbamazepine on the Fetal Brain of Albino Rats (*Rattus Norvegicus*)

Ann Wairimu Mwangi

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DECLARATION

This research thesis is my original work and has not been presented for a degree in any other university.

Signature…… Date……

Ann Wairimu Mwangi

This thesis has been submitted for examination with our approval as University Supervisors

Signature …… Date…….

Dr. Joseph Kariuki Kweri, PhD
JKUAT, Kenya

Signature…… Date……

Dr. Reuben Thuo, Mmed Surg
JKUAT, Kenya

Signature…… Date……..

Dr. George Kibe Kafaya, BDS, MSc
JKUAT, Kenya

DEDICATION

I dedicate this thesis to my daughters Gael and Victorine, who provided me with moral support throughout this study period.

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ABBREVIATIONS AND ACRONYMS

AED	Antiepileptic drug
AED	Animal equivalent dose
ANOVA	Analysis of variance
et al	Is a Latin phrase which means "and others"
BD	Bi-parietal Diameter
C	Control
CBZ	Carbamazepine
CNS	Central nervous system
COHES	College of Health Sciences
CRL	Crown rump length
(CYP3A4)	Cytochrome P450 3A4 is an important enzyme in the body, mainly found in the liver and in the intestine.
DMSO	Dimethyl sulfoxide
GD	Gestational period by dates
Gt	Gluconyl transferase
HCG	High carbamazepine group
HED	Human equivalent dose
JKUAT	Jomo Kenyatta University of Agriculture and Technology

Keq	Constant equilibrium -is a characteristic numerical value
Km	Factor/constant used to convert the mg/kg in to mg/m^2 (body weight in to surface area).
Kg	Kilogram
LCG	Low carbamazepine group
L	Litre
MCG	Medium carbamazepine group
Mg	Milligrams
NIH	National Institute of Health
SAFARI	Small Animal Facility for Research and Innovation
SEM	Standard Error of the Mean
SPSS	Statistical Package of Social Scientist
TM_1	Trimester one
TM_2	Trimester two
TM_3	Trimester three
WHO	World health organization
WIM	Water immersion method

DEFINITION OF TERMS

Anticonvulsants (ACs): Are a diverse group of pharmacological agents used in treatment of various conditions like epileptic seizures, bipolar disorders (mood stabilizers), neuropathic pains, borderline personality disorder among others though inhibiting excessive rapid firing of neurons. They are also known as antiepileptic,.or antiseizure medicines.

Carbamazepine: An anticonvulsant medicine also known as tegretol that work by decreasing nerve impulses that cause seizures and nerve pain, such as trigeminal neuralgia and diabetic neuropathy. Also used in treatment of bi- polar disorders among other uses.

Embryolethality: Spontaneous abortion, or stillbirth, due to adverse drug or chemical toxic effects using a microscope.

Histostereology: This is a three-dimensional measurement of microscopic structures important to obtain reliable quantitative data that enables calculation of volumes and volume ratio, the area of samples, the number of particles per unit volume, particle size, unit volume, length and weight.

ABSTRACT

The in-utero exposure to carbamazepine has been shown to perturb the normal morphogenesis of cortical and sub-cortical structurers of the fetal brain. However, the anatomical histostereological effects of prenatal exposure to carbamazepine when exposed at different gestation periods and at different doses is not well elucidated. The broad objective of this study was therefore to evaluate the histological teratogenic effects of prenatal exposure to varied doses of carbamazepine in different gestation periods. In conducting the study, a static group experimental study design was adopted. The animal experimentation was carried out at Small Animal Facility for Research and Innovation (SAFARI) animal house while tissue processing for histology and stereological analysis was done in the department of human anatomy. A Sample size of 30 albino rat dams (*Rattus norvegicus*) weighing between 200-250grams were used in the study as determined by use of the resource equation method. These 30 Albino rats were divided into two broad study groups of 3 rats control and 27 rats experimental. To evaluate the teratogenic histological effects of carbamazepine on differing doses, the 27 rats in the experimental group were further subdivided into three study groups of 9 rats as follows; (i) Low carbamazepine group[LCG-20.7mg/kg/bw] (ii) medium carbamazepine [MCG-72.3mg/kg/bw], (iii) High carbamazepine group[HCG-124mg/kg/bw].To further evaluate the teratogenic effects of carbamazepine on differing gestation periods, the 9 rats in each of the three dose categories were further sub-divided into three groups of 3 rats according to trimesters as follows; (i) Trimester I-(3rats); (ii) trimester II-(3rats) and (iii) trimester III-(3rats) respectively. The findings of the study showed that there is a statistical significant reduction ($P<0.05$) in cortical thickness of the cerebral and cerebellar cortices. It also caused a statistical reduction in cellular densities, disaggregation of corpus callosal fibres and ventricular hypertrophy. The most teratogenic effects were observed when exposed at trimester one and two across all the dose groups. In conclusion, carbamazepine is teratogenic to the developing fetal brains and its teratogenicity is time and dose dependent. The study recommends that carbamazepine should not be used during pregnancy and particularly during 1st and 2nd trimesters.

CHAPTER ONE

INTRODUCTION

1.1 Brief description of carbamazepine

Carbamazepine, an anticonvulsant commonly sold under the trade names of tegretol, temporol, neurotol and others is primarily used in the treatment of epilepsy, bipolar disorders, and neuropathic pains as well as in management of trigeminal neuralgia (Gierbolini *et al.,* 2016; Chen *et al.*, 2012). It has a chemical formula; $C_{15}H_{12}N_2$ with a molecular weight of 236.269g/mol (Tolou-Ghamari *et al.,* 2013). It has an elimination half-life of 36hrs single dose, 16-24hrs repeated dosing and a 100% bioavailability. When administered orally, the Initial half-life clearance values range from 25-65 hours, then decreases to 12-17 hours on repeated oral doses, with 72% of the dose administered excreted in urine and 28% in the feces. It is metabolized in the liver with the enzyme Cytochrome P450 3A4 as the major isoform responsible for the formation of its principal metabolite, carbamazepine-10, 11-epoxide (Solon *et al.*, 2010).

When carbamazepine and its principal metabolite known as *carbamazepine-10,11-epoxide* accumulates in maternal blood, they create a negative osmotic gradient between the maternal and fetal tissue (Etemad *et al.,* 2012) .This negative osmotic gradient coupled with low molecular weight of carbamazepine that is 236.269g/mol Ahmed, (2017), enables them to readily cross the maternal placental barrier accumulating in the fetal tissues, causing morphogenetic disturbances to the developing fetal tissues (Ikonomidou *et al*., 2010).

The morphogenetic perturbations to the developing neuronal tissues in the fetus are believed to be caused by its inhibitory mode of action to the neuro-developmental events that includes; neurogenesis, cell proliferation, migration, synaptogenesis, axonal sprouting, gliogenesis, myelination among others (Würtz *et al*., 2017; Zhao *et al.*, 2019). This leads to physiological apoptotic cell death of the fetal brain tissue and oxidative stress (Elshama *et al.,* 2015; Hemandez -Diaz & Levin, 2014). As

such, these intrauterine disturbances to the developing neuronal tissues in the fetus may cause permanent structural damage to the brain that may manifest in form of some of the behavioural mental conditions seen in adulthood like mild mental retardation, cyclic maniac depressive disorders, suicidal ideation among others, whose causes are yet to be established (Bath & Scharfman, 2013; Fujimura *et al.,* 2017).

1.2 Background information

The comparative patterns of the fetal brain development in both rats and in human depict similar correlational stages of development starting right from the neuro-tube formation that appears in mid-gestation at day 10.5-11 in rats that corresponds to 24th 28th gestation weeks in humans (Buddy *et al.*, 2015; Karten *et al.,* 2015). Subsequently, further brain growth phases that includes; neurogenesis, synaptogenesis, gliogenesis, oligodendrocyte maturation also show similar developmental sequence in both rats and in humans (Heide *et al.,* 2018; Puelles, 2016). For instance, the neurogenic phase, that occurs from 10th week of prenatal development in humans and continues throughout fetal life both prenatally and postnatally is mimicked in rat fetus at the beginning of day 9.5 and is completed by 15th day postnatally *(*De Sesso *et al*., 2014; Semple *et al.,* 2013).

Similarly, other developmental stages like the oligodendrocyte cyto-differentiation and maturation that occurs day 1-3 postnatally in rats occurs from 23rd week and ends 8 weeks postnatally in humans (Chen *et al.,* 2017). Further, gliogenesis that occurs on day 7-10 prenatally in rats occurs on 36-40th weeks in humans (Kawasaki *et al.,* 2017). The brain reaches 90-95% of adult weight in 20-21 day postnatally in rats that correspond to 2-3 years of age in humans (Khelimskii *et al.,* 2013).

On the histo-morphological and stereological effects of anticonvulsants on the fetal brain development, previous studies have shown that anti-epileptic medicines with similar mode of action to carbamazepine including phenytoin, valproate, and phenobabitone among others have yielded variable and inconsistent outcomes on the developing fetal brain structures. For instance, studies by Bath & Scharfman (2013);

Belguis e*t al.,* (2017) on the teratogenic effects of phenobabitone and valproic acid reported ventricular atrophy, overall reduction in total brain volume, disaggregated and reduced cellular densities across the cortical layers of both the cerebral and cerebellar cortices that was occasioned by pronounced neuronal death in the developing fetal brain tissues. This was contrasted by another study by Mohanty *et al.,* 2011 who reported that lamotrigine administered during organogenesis at a dose of 50-200mg/kg per day, resulted to ventricular dilatation, hypertrophy in some cellular brain component with overall increase in brain weight, total brain volume alteration of some cortical layers.

Similarly, another stereological and histo-morphological study done on the cerebellum reported that *in-utero* exposure to phenytoin had a significant reduction in thickness and cellular densities of the cerebellar cortical layers (Plouhinec *et al.,* 2017). This was attributed to delayed cell maturation in the external granular layer, molecular layer and purkinje layer. The cells depicted poor immature arbors with partial irregular arrangement when further examined with immuno-histochemical staining methods. Further, a study by Imosemi & Osinubi, (2011) using phenytoin showed a dispersal of internal granular cell layer and white matter with presence of vacuoles, dilated capillaries and extracellular edema.

The patterns of fetal brain teratogenicity in terms of whether or not the drug being studied are both dose and time dependent following *in-utero* exposure to anticonvulsants has not been very well studied. Most of the studies have reported a one or two dose exposures. Consequently this part is variably reported and also riddled with controversies, with some studies showing that the fetal brain teratogenic effects exposed in-utero are time dependent and others not. This study therefore aims to evaluate the histomorphological and the stereological effects of carbamazepine on the development of the fetal brain when prenatally exposed in varied doses and at different gestation periods in albino rats.

1.3 Statement of the problem

The global WHO mental health reports shows that there is a steady increase in a wide range of mental and behavioral conditions like anxiety disorders, suicidal ideation, cyclic maniac depressive disorders, mild mental retardation, among others (WHO 2019). Majority of these conditions, the cause is yet to be established. On the other hand, studies have shown that there could be an association between intra-uterine exposures to anticonvulsant medicines with some of the observed mental health conditions seen in childhood and adulthood (Charles *et al.,* 2018; Jentink, 2010). Though data exists on teratogenic effects on anticonvulsants, there is no data on the histoquantitive and histomorphological effects on the *in-utero* exposure to carbamazepine on the development of the fetal brain. At the same time, data on whether or not the observed histoquantitive changes on the fetal brain are dose and time dependent also remains unclear.

1.4 Justification of the study

The availability of data repository on the intrauterine teratogenic perturbations following use of carbamazepine will serve as an important predictor into some of the structural alterations on developing fetal brain. This would subsequently inform the causes of some of the mental and behavioral disorders seen in the adulthood today and whose causes are yet to be known. Further, there is paucity of data that shows histomorphological and stereological teratogenic effects of carbamazepine when it is prenatally exposed in varied doses and at different gestation period.

The study findings sought to unravel the controversy surrounding use or nonuse of carbamazepine during pregnancy. In addition, lack of carbamazepine teratogenic data that depicts the most vulnerable teratogenic periods as well as the most critical doses of carbamazepine teratogenicity will also keep on denying mothers the benefits that would accrue in use of safe doses of carbamazepine in management of some maternal condition like epileptic seizures trigeminal neuralgia, among others that would effectively be managed with carbamazepine that is relatively a cheap medicine and readily available. The benefits accruing in either utilization or lack of utilization

of carbamazepine on the side of the mothers will continue being lost if this data is not made available. At the same time lack of histo-quantitative teratogenic data on the side of the fetus will continue to pose a teratogenic risk to the developing fetal brain that would lead to development of some probable mental and behavioral conditions in adulthood.

1.5 Significance of the study.

Data obtained from this study is useful in guiding the clinicians and future researches on the rational use of carbamazepine during pregnancy to confer the maternal benefits in its usage while safe guarding the fetus from the associated teratogenic effects on the developing brain. In addition, the morphological perturbations observed in the developing fetal brain emanating from in-utero exposure to different doses of carbamazepine at different gestation periods would help in elucidating some of the causes of increasing adult conditions like acute mania, suicide ideation among others which are on the increase worldwide. The data obtained from this study therefore is useful in redirecting future teratogenic studies on carbamazepine as well as in redirecting the adjustment of the treatment algorithms in terms of dosages and the appropriate gestational periods when to apply it in managing some maternal conditions during pregnancy.

1.6 Research Questions, Objectives and Hypothesis

1.6.1 Research Question

What are the histo-morphological and stereological teratogenic effects on the developing fetal brain, following in utero exposure to varied doses carbamazepine at different gestational periods in albino rats?

1.6.2 Broad objective

To evaluate the histo-morphological and stereological teratogenic effects of *in-utero* exposure to varied doses carbamazepine when exposed at different gestation periods on the development of the fetal brain in albino rats.

1.6.3 Specific Objectives

1. To establish the pregnancy outcomes following *in-utero* exposure to varied doses of carbamazepine at different gestation periods.
2. To evaluate the histo-morphological outcomes that occur to the developing fetal brain following *in-utero* exposure to varied doses of carbamazepine at different gestation periods
3. To evaluate the histo-stereological changes that occur to the developing fetal brain following *in-utero* exposure to varied doses of carbamazepine at different gestation periods on development of fetal brain structures
4. To determine whether the teratogenic histo-stereological effects of carbamazepine on the developing fetal brain structures are time and dose dependent.

1.7 Hypothesis (H_0)

Prenatal exposure to carbamazepine is not associated with teratogenic effects to the developing fetal brain in albino rats and the effects are not time and dose dependent.

1.8 The study assumptions

In carrying out this study it was assumed that the albino rat (*Rattus Norvegicus)* model would replicate the actual teratogenic induction scenario that would occur in humans due to the known close association of this kind of rat species with human biological and functional outcomes when exposed.

1.9 Study limitations

Some of the anticipated study limitations includes;

1. Failure of some dams becoming pregnant at the same time following the introduction of the males in the cages, hence other attempt for copulation were allowed.
2. Death of the animals along the experimental process following mishaps in drug administration while administering carbamazepine using the gastric gavage needle, hence they were replaced with other rat dams of the same pure colony breed

CHAPTER TWO

LITERATURE REVIEW

2.1 Carbamazepine class, structure and mode of action

Carbamazepine, an anticonvulsant medicine under the class of sodium and potassium channel inhibitor is commonly known by its trade names as tegretol, equetro, among others. It belongs to the class of first generation anti-epileptic drugs and functions by inhibiting and normalizing the nerve cell impulses in the brain as well as in the entire central nervous system, hence smoothening the coordination of body functions, (Elshama *et al.,* 2015). It is mainly used in treatment of epileptic seizures, pain associated with trigeminal neuralgia and diabetic neuropathy, bipolar disorders, attention deficit hyperactivity disorder, schizophrenia, phantom limb syndrome among others (Hill *et al.,* 2010).

Carbamazepine's chemical formula is $C_{15}H_{12}N_2$ and has a molecular weight of 236.27 g/mol. It is metabolized in the liver via the Cytochrome P450 3A4 as the major isoform responsible for the formation of the principal metabolite, *carbamazepine-10, 11epoxide*. Initial half-life values range from 25-65 hours, decreasing to 12-17 hours on repeated oral doses, with 72% of the dose administered excreted in urine and 28% in feces (Aberg *et al.,* 2013). Carbamazepine acts by stabilizing hyper flexed nerve membranes and inhibits repetitive neuronal discharges, hence reducing synaptic propagation of excitatory impulses. Its antiepileptic effect is however enhanced by the reduction of glutamate release and stabilizing of the neuronal membranes (El-gaafarawi &Abouel-magd, 2015).

2.2 The teratogenic induction mechanism of carbamazepine on fetal tissues.

The mode of teratogenic action of carbamazepine on the developing fetal tissue has been elucidated. Study findings by Valin, (2006), showed that the injurious effects of carbamazepine to the developing fetal tissues mainly occurs in two ways. First, it involves the activation of its metabolites in its oxidative pathway that gives rise to

the formation of an injurious active metabolite known as the *carbamazepine 10-11 epoxide* that induces the inhibition activity in the developing fetal tissues (Saddler, 2005). This metabolite acts by the blockade of voltage sodium gated channels and by its action on synaptic transmission hence reducing the frequency of repetitive firing of action potentials in depolarized neuoroblast of the developing fetal tissues (Ghamari *et al.,* 2013). The second mode of teratogenic action is the combination of the carbamazepine itself with its principal metabolite (*carbamazepine-10,11-epoxide*) that accumulates in maternal blood hence creating a negative osmotic gradient between the maternal and fetal tissue (Etemad *et al.,* 2012) .This negative osmotic gradient coupled with low molecular weight of carbamazepine that is 236.27mg/mol enables them to readily cross the maternal placental barrier accumulating in the fetal tissues causing morphogenetic disturbances to the developing fetal tissues (Ikonomidou *et al*., 2010)

2.3 The comparative fetal brain morphogenesis process between rats and humans

The comparative fetal brain morphogenesis between rats in humans depict many similar morphogenetic features across their entire gestational stages despite their differences in terms of their species and gestation periods (Clancy et *al.,* 2001). In both rats and humans, the first major event (precursor) of central nervous system (CNS) development in both human and rats fetuses is the formation of a specialized fold of ectodermal tissue called the neural plate, from which the spinal cord and brain subsequently differentiate, that is later transformed into the neural tube by invagination (Allen *et al.,* 2010). The neural tube then detaches from the ectoderm and thickens to form a massive rostral part within the skull (the brain) and a small caudal part within the vertebral column, the spinal cord (Cina *et al.,* 2007). The rostro part then acquires three swellings; the forebrain, and midbrain and hindbrain vesicle (Bras *et al.,* 2005).

Neural tube formation occurs approximately mid-gestation in rodents, on gestational day (GD) 10.5–11, with birth typically occurring on (GD) 20–21, Finlay *et al.,*

(2001), as compared to humans fetuses where it occurs on 24^{th} -28^{th} gestation weeks (Foley &Stern, 2001). Further, in both fetuses, they depict similar brain growth phases that also includes; neurogenesis, synaptogenesis, gliogenesis, oligodendrocyte maturation that occurs at different gestation dates (Heide *et al.,* 2018; Vliet, 2004). For instance, neurogenesis phase, which begins from 10^{th} week prenatally in humans and continues throughout fetal life occurs in fetal rat at the beginning of day 9.5 and is completed by day 15postnatally (Clancy *et al.*, 2007).The oligodendrocyte cyto-differentiation and maturation occurs day 1-3 postnatally in rats while this occurs between 23-32 weeks in humans (Vliet, 2004). Further, gliogenesis that occurs on day 710 prenatally in rats where it occurs on 36-40^{th} weeks in humans. The brain reaches 90-95% of adult weight in 20-21 day postnatally in rats that correspond to 2-3 years old in humans (Brickler *et al*., 2016.)

2.4 Comparative morphological features of fetal brain between rats and humans.

The comparative gross features of a fetal brain in humans and rats depict major similarities. The three primary vesicles, the procencephalon or forebrain lies closest to the rostrum, the mesencephalon or midbrain lies behind the procencephalon and the rhombencephalon or hindbrain lies most caudal (Gooday *et al.,* 1997). It has distinct right and left cerebral hemispheres derived from the endbrain vesicles and the cerebellum which is continuous across the midline, derived from the dorsal pontine vesicle (Zappaterra *et al.,* 2007).The cerebral and cerebellum hemispheres are attached to a much smaller core of brainstem that is derived from the interbrain, midbrain and hindbrain vesicles that extend caudally as the spinal cord. The cerebral cortex is large with in-folds forming gyri and sulcus (Mohanty *et al.,* 2011).

The brain of a rat on the other hand is similarly divided into three major divisions, the hindbrain, midbrain, and forebrain with the structure and function of the hindbrain and midbrain being very similar to that in humans (Clancy *et al.,* 2001).The forebrain is however more developed in the human fetuses as compared

to the rat fetuses, with the cerebral cortical layer being smaller without gyri and sulcus (Saddler, 2005).

Comparative microscopic features also depict similarities in that the cerebral cortex of both human and rat fetuses forms the largest part of the brain and is composed of an outer grey matter and a subcortical white matter (Dem & Dem, 1998).In both, the motor area is composed of 6 distinguished layers which includes ; The 1st layer, molecular or plexiform layer has fibres coursing parallel to the surface and contains mostly glial cells axons of neurons from other layers and very few neurons (Dehay & Kennedy, 2007). The 2nd layer is external granular layer with small pyramidal cells, 3rd layer is pyramidal cell layer with large pyramidal cells, 4th layer is the internal granular layer with stellate and granule cells which receives input to the cortex from the thalamocortical fibres, association and commissural fibres. 5th layer is the ganglionic layer with the largest pyramidal cells called the Giant cells or Betz cells, and the 6th layer is the multiform layer of polymorphic cells of martinotti (Zecevic & Rakic, 2001).

In both fetuses, the cerebellum located at the lower back of the brain lying under the intraparietal bone and posterior to the cerebral hemispheres has three distinct lobes: two lateral hemispheres and a medial vermis. It controls coordination and equilibrium, act more rapidly than any other part of the brain and is involved in the skilled motor performances and also in various sensory functions including sensory acquisition, tracking, discrimination, and prediction among others (Altman & Shirley, 1985). On the other hand, the hippocampus region in both human and rat fetuses lies under the medial temporal lobe on each side of the brain and is part of limbic system that plays an important role in formation of long term memory (Danglot *et al.,* 2006).

2.5 The histo-morphological effects of anticonvulsants on developing fetal brain

Studies have revealed that different anticonvulsant medicine have different teratogenic effects in the fetal rat brain. A dose of 108 mg/kg of oxcarbazepine administered from 7th to 20th day of gestation caused severe degenerative changes

characterized by marked neuronal cell degeneration, disorganization of the brain tissue, numerous pyknotised cells and vacuolization of the neuropil (Hamdi *et al.*, 2017). A similar study on effects of phenytoin on cerebellum of the fetal brain reported reduction in thickness of layers of the cerebellum as a result of delayed cell maturation in the external granular layer, reduction of molecular layer and purkinje cells had poor immature arbors with partial irregular arrangement (Imosemi & Osinubi, 2011).

A study on histological effects of gabapentin on fetal brain development, revealed statistical significant increase ($p<0.001$) in neurons and degeneration of the hippocampus and striatum, for drug treated groups as compared to normal (Olaibi *et al.,* 2014). Similarly a study on a rat fetal brain prenatally to lamotrigine depicted widespread degeneration of the brain, shrinkage and degeneration of pyramidal and glial cells, pycknotic nuclei with increased size of vacuoles. Hippocampus showed atrophy of granular layer, decrease in size of molecular layer cells and hilus, huge number of vacuoles and fragmented nuclei of granular layer cells (Malchi *et al.,* 2001).

2.6 The histo- stereological effects of anticonvulsants on developing fetal brain of albino rats

Previous histo-stereological studies have indicated that quantitative effects to the fetal brain upon administration of carbamazepine related drugs shows various injuries effects induced to the developing brain. Mohanty *et al.,* (2011) showed significant reduction in numerical density of neurons in the hippocampus, neocortex, and piriform cortex, dilatation of lateral ventricles and less differentiated cerebral cortical layers upon administration of lamotrigine.

Another study on comparative histo-quantitative effects of phenytoin, valproate and phenobabitone, medicines with similar mode of action with carbamazepine showed variable outcomes on the developing fetal brain following in-utero exposure (Bath & Scharfman, 2013; Elgndy *et al*., 2016). These effects includes pronounced neuronal death that leads to reduction of the total brain volume, volume densities and brain

length of cortical and subcortical layers (Torbj. T & Battino, 2009). A comparative study on quantitative effects of lamotrigine and levetiracetum on fetal brain depicted reduced mean values as regards to body weight, crown rump length, bi-parietal diameter and head length in treatment groups as compared with the control (Elgndy *et al.,* 2013).

2.7 The patterns of fetal brain tetogenicity in terms of dose and time of exposure on albino rats

Previous studies done in other anti-epileptic drugs with similar effects to carbamazepine demonstrates that fetal brain teratogenic effects upon in-utero administration depends on the time of exposure. Study findings by Etemad *et al.,* (2012) indicated that antiepileptic drugs have a potential to affect fetal development throughout the gestation period. Another study conducted by Hejazi& Taghdis, (2019) on effects of in-utero exposure to lamotrigine, reported that the pattern of exposure in causing these fetal organs like brain anomalies varies, with most of the drugs causing major structural malformations during the first trimester, a period that corresponds to the embryonic stage during which major organs develops.

Other previous studies went further and indicated that anti-epileptic drugs issued as monotherapy are relatively safe as compared with polytherapy, with some of the antiepileptic drugs having more teratogenic effects as compared with others (kuluga *et al.,* 2011; Holmes *et al.,* 2011. Also Weston *et al.,* (2016) and Hill *et al.,* (2011) reported that monotherapy doubles the risk of malformations while polytherapy triples it. Tomson *et al.,* (2011) reported that the risk of major congenital malformations is influenced not only by type of antiepileptic drug, but also by dose and other variables, which should be taken into account in the management of epilepsy in women of childbearing potential. Similarly, a study to find out the antiepileptic drugs (AED) associated with high risk of teratogenicity, a study by Hernández-Diaz *et al.,* (2011), indicated that older medicines such as valproate and phenobarbital are associated with a higher risk of major malformations than newer AED such as lamotrigine and levetiracetum.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study location/ area

All experiments including breeding, handling, weighing, carbamazepine administration and measurements of fetal parameters were done at the Small Animal Facility for Research and Innovation (SAFARI), situated in Jomo-kenyatta University of Agriculture and Technology (JKUAT) main campus, Kiambu county, Kenya.

3.2 Study design

A static-group controlled experimental study design was adopted.

3.3 Study sample/ subject

Female albino rat dams of the species *Rattus norvegicus* derived from colony of a pure breed of the 3rd series were used as the animal experimental model in this study. These rats were sourced from SAFARI animal house situated in Jomo Kenyatta University of Agriculture and Technology (JKUAT).The use of these albino rat dams was guided by the following known facts; (i) They have a large litter size with an average of 6-12 fetuses, (ii) they have low incidence of spontaneously occurring congenital defects, (iii) they have a relatively short gestational span, making it easier to get study subjects or a pure bleed colony (iv)Low cost of maintaining the animals, (v) they are plentiful, (vi) considerable amount of the reproductive data on the rat is already available, vi) they are relatively small and easy to care for and handle during an experiment (vii) they are relatively resilient in terms of withstanding a wide range of study medicines (Bailey *et al.,* 2014). By appearance, both the male and female albino rats have red eyes and white fur resembling the 'Japanese hooded rats', hence

essentially genetically identical from a common ancestor, (Pritchett & Corning, 2016). They were the first mammalian species domesticated for scientific research. They live about 2-3.5 years (average 3 years). They develop rapidly during infancy and become sexually mature at about 4-5 weeks in females and at around 45-48 postnatal dates in males. This is defined by vaginal opening (females) or balanopreputial separation (males). Reproductive senescence in female rats occurs between 15 and 20 months of age (Pallav Sengupta, 2013**).** Their gestation period is roughly estimated at from 21 to 23 days during which the fetuses are viable. Their gestation period has 3 trimesters, with trimester one being the first 7 days after conception, second trimester from day 7-14 and third trimester from day 14 to day 21. Pregnancy is detectable at about 2 weeks by palpating the abdomen, noticing weight gain or mammary (breast) development and pregnant females making a nest. The usual litter size is 6 to 12 pups. When baby rats are born, they are deaf and blind. Weaning occurs about 21 days after birth. Adult female and male rats typically weigh 12 to 16 ounces (350 to 450 grams) and 16 to 23 ounces (450 to 650 grams), respectively. Male rats are usually larger than females and are about 9 to 11 inches long. Since each male was allocated two females for mating purposes in the current study, 15 sexually mature male albino rats of the same breed as the females were used.

3.4 Sampling method

Sample size was determined at two levels;

a) Level one Sample size calculation for the 30 experimental rat dams

The 30 dams were calculated using the resource equation method as determined by (Arifin *et al*., 2007), since the standard deviation from previous studies was not available as well as the effect size. The Resource equation states that the measured value 'E' which is the degree of freedom of analysis of variance (ANOVA) based on a decided sample size value ('E') should lie between 10 and 20 animals according to this equation. Therefore, a value less than 10 necessitates adding more animals which increases the chance of getting significant results while a value more than 20 has

been shown to increase the cost of the study without increasing the significance of the results (Charan & Kantharia, 2013).

Total number of groups=10 while the total number of animal is 30. E=Total number of Animals-Total number of groups. E is therefore 30-10 which is 20

b) Level two Sample size calculation was for the fetuses used for histo morphology and stereology

The average litter size for both the control and experimental animals in this study was found to be six (6) fetuses per rat. To ensure objectivity of the finding and to eliminate the observer or the process bias, all the fetuses per each dam were weighed and organized in an ordinal sequence. Then, three fetuses from each rat that had the highest, median and lowest weights were selected for stereology and histo-morphological evaluation making a total sample size of 90 fetuses (i.e. 3 fetuses from each of the 30 study equals 90 fetuses). Their brains were harvested for stereology and for histomophology. The rest of the fetuses were preserved in formaldehyde solution for future repository should a problem arise during tissue processing.

Grouping of dams

The 30 dams used in the study were randomly assigned to either 3 rats as the control and 27 in the experimental category. To determine whether carbamazepine is dose dependent, the 27 rats in the experimental category were further divided into three broad study groups of 9 rats each based on the doses applied as follows: 9 rats for the low carbamazepine group (LCG); 9 rats for the medium carbamazepine group (MCG); and 9 dams for the high carbamazepine group (HCG). To determine whether the carbamazepine teratogenicity is time dependent, the 9 rats in each of the three study categories of the low, medium and high carbamazepine groups were further subdivided into three subgroups of three rats each based on the trimester of exposure as follows three (3) rats for trimester one (TM_1), 3 rats for trimester two (TM_2) and 3 rats for trimester three (TM_3), (**Figure 3.1)**.

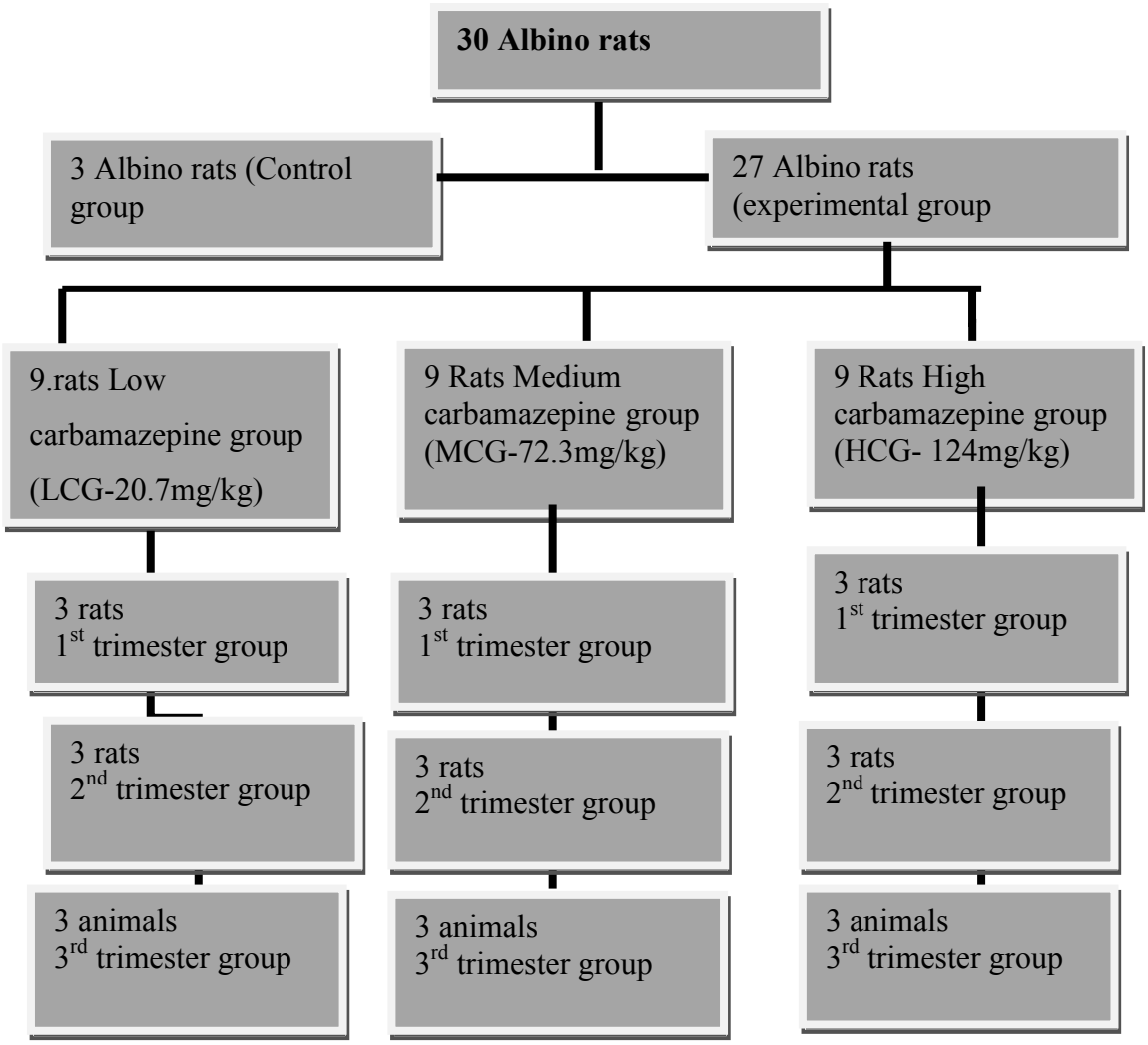


Figure 3.1: Grouping of the 30 dams in both the control and experimental groups according to doses of exposure as LCG, MCG and HCG and according to trimester of exposure as TM_1, TM_2 and TM_3.

3.5 Selection criteria

3.5.1 Inclusion criteria

- All rats that were healthy and shown that conceived in the first day after being introduced to a male overnight
- All fetuses that were alive at point of sacrificing the animals and opening the uterus along the anti-mesomentrial boarder

3.5.2 Exclusion criteria

- All animals that did not have a positive pregnancy test following the introduction of a male were excluded
- All animals that later shown signs of sickness following treatment with carbamazepine
- All fetuses in which mother had an underlying disease state during pregnancy were also excluded

3.6 The rats feeding process

All rats were fed on a standard rodent pellets obtained from Unga feeds limited situated in Thika town, plus water *ad libitum*. Feeding with pellets was done every morning at 0800 hrs, within their spacious polycarbonate plastic cages as outlined by (Allen *et al.,* 2016)**.** All animals were allowed to stay in their cages for seven days to acclimatize before the experimentation began. The animals in the control and in the experimental categories were fed as follows:-

(i) **The control group;** received standard diet as determined by the academy of nutrition and dietetics containing by weight (100g):-68% starch,4% cellulose, 5% lipid (corn oil) and 20% protein) and by calories:-20%proteins, 72%carbohydrates, 12 %lipids, and 54mg/kg zinc and water *ad libitum* for the whole of the gestation period day 1-20. The mothers were then sacrificed on 20^{th} day of gestation

(ii) **The experimental groups:** The animals in the experimental were similarly fed on standard rodent pallets as above in the control and water *ad-libitum* but in addition received carbamazepine treatment based on their doses of low, medium and High (LCG, MCG, HCG) as well as according to the trimester of exposure (TM_1, TM_2 and TM_3) as follows:

(a) The **low dose carbamazepine group-(LCG)**

All rats in this group received a standard diet, water *ad-libitum* and a constant daily dose of carbamazepine **(20.7mg/kg/bw)** administered as a single bolus

through gastric gavage gauge 16 at 0900hrs. The 3 rats in trimester one (TM_1) received carbamazepine treatment daily from day one (GD_1) today 20 (GD_{20}); those in trimester two (TM_2) received carbamazepine treatment starting daily from gestational day 7 (GD_7) all trough to gestation day 20 (GD_{20}), while those in trimester three (TM_3) received daily carbamazepine treatment daily from gestational day 14 (GD_{14}) all through to- gestational day 20 (GD_{20}) i.e. the last day of gestation.

(b) The **medium carbamazepine group-(MCG)**

All rats in this group received a standard diet, water *ad-libitum* and a constant daily dose of carbamazepine **(72.3mg/kg)** administered as a single bolus through gastric gavage gauge 16 at 0900hrs. The 3 rats in trimester one (TM_1) received carbamazepine treatment from day one (GD_1) to day 20 (GD_{20}); those in trimester two (TM_2) received daily carbamazepine treatment starting gestational day 7 (GD_7) all trough gestation day 20 (GD_{20}), while those in trimester three (TM3) received daily carbamazepine treatment from gestational day 14 (GD_{14}) all through to- gestational day 20 (GD_{20}) i.e. the last day of gestation.

(c) The high dose carbamazepine group-(HCG

All rats in this group received a standard diet, water *ad-libitum* and a constant daily dose of carbamazepine **(124mg/kg)** administered as a single bolus through gastric gavage gauge 16 at 0900hrs. The 3 rats in trimester one (TM_1) received carbamazepine treatment from day one (GD_1) to day 20 (GD_{20}); those in trimester two (TM_2) received carbamazepine treatment starting gestational day 7 (GD_7) all trough gestation day 20 (GD_{20}), while those in trimester three (TM_3) received carbamazepine treatment from gestational day 14 (GD_{14}) all through to- gestational day 20 (GD_{20}) i.e. the last day of gestation.

3.7 Handling of Rats

The rats were handled only by the investigator for the purpose of obtaining daily weights between 0800 am and 0900hrs, then feeding with pellets was always done at 0930hrs. All procedures were performed according to the guidelines for care of laboratory animals by the National Institute of Animal Research as outlined by Gomez *et al.,* (2010); and National Research Council, report of 2011. (**Figure 3.2)** shows the procedure for daily weight taking.

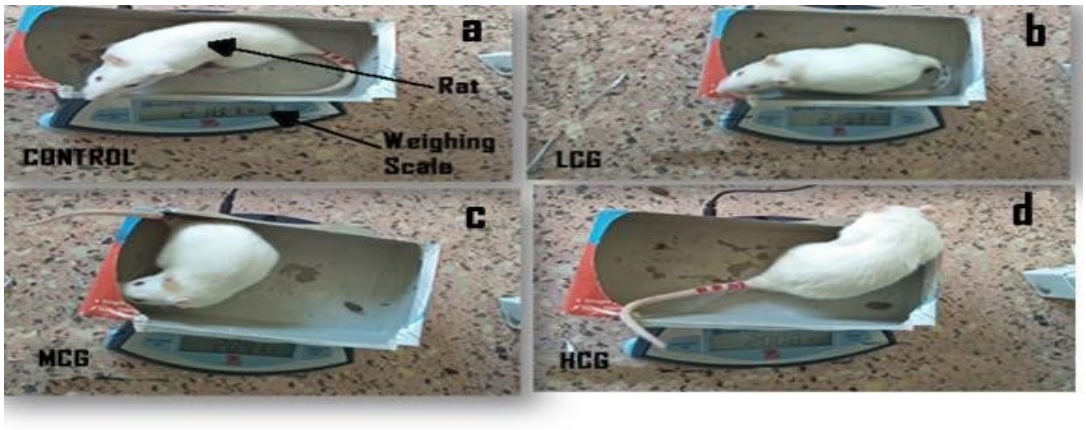


Figure 3.1: Daily weights measurements in; (a) Control, (b) LCG,) (c) MCG (d) HCG (measurements were taken using Scout pro model SPU4001*)*

3.8 Breeding of animals

One male albino rat from a pure colony of the 3rd series and sexually mature (from postnatal dates 50 and above) was introduced into a standard cage with two female rats at 1430HRS (+/- 30 minutes) and at 0900 HRS (+/- 30 minutes) the following morning, the males were removed and returned to their separate cages. The selection of the males was as per the inclusion and exclusion criteria as per the females.

3.9 Pregnancy determination

Pregnancy determination was done at two levels;

Level 1: confirmation of mating; Vaginal smears were taken from the mated dams and the presence of spermatozoon on the smear when observed under the microscope was a confirmation that fertilization or coitus had taken place.

Level 2: confirmation of pregnancy or the first day of gestation; vaginal smears were taken from the 30 mated females the next morning and pregnancy was determined by doing a vaginal wash 24 hours later the presence of polyhedral epithelial cells on the swab was used to determine estrous changes, which was denoted as the first day of gestation (GD_1), (Telendo *et al*., 2019; Hamid &Zakaria, 2013).

a) Materials used in determination of pregnancy

i) Cotton tipped swab
ii) 0.85%phosphate buffered saline
iii) Microscope slides
iv) Ethanol (95%)
v) Absolute alcohol
vi) 10mls blunt tipped disposable pipettes
vii) Giemsa stain

b) The Procedure that was followed in the determination of pregnancy

1. The animal was restrained with a gauze holder against the body
2. One ml of saline was introduced into the vaginal cavity using a blunt tipped disposable pipette (vaginal wash)
3. Cotton tipped swab moistened with phosphate buffered saline was then gently inserted into the vaginal cavity
4. The swab was slightly rolled before withdrawing

5. The moist swab was withdrawn and rolled onto a clean glass microscope
6. The specimen was then spray fixed using 95% ethanol
7. The slides were subsequently air dried and others by dipping in 100% alcohol
8. The slide was then stained with giemsa stain
9. Observations of the slide followed, and was done under the BP Olympus microscope

Observations made: Large polyhedral cornified epithelial cells were observed, many neutrophils on the smear and scattered epithelial cells. This served as an indicator that fertilization had taken place and this was counted as the first day of pregnancy (gestation day one). Majority of the rats tested positive for the pregnancy (98%), with the 2% that did not conceive were allowed for another 24hours with the males after which the test was done again to confirm their pregnancy

3.10 Determination, calculation and administration of the doses

The adult carbamazepine dosages in human ranges between 200mg-1200mg per day with maximum dose of 1600mg per day, depending on the patients need. It is administered in divided doses of two or three per day (Maan *et al.,* 2019). Carbamazepine tablets from Novartis Pharma Pharmaceuticals Company with a batch number of TL787 were obtained from Thika chemist and reconstituted using 5% DMSO.

- All trimester ones (**TM_1**) rats: - (LCG, MCG, HCG) categories received carbamazepine from (GD_1-GD_{20})
- All trimester twos (**TM_2**) rats: - (LCG, MCG, HCG) categories received Carbamazepine doses from (GD_7-GD_{20})
- All trimester three (**TM_3**) rats: - (LCG,MCG,HCG) categories received carbamazepine doses from (GD_{14}-GD_{20})

3.10.1 The procedure for determination of the carbamazepine doses

A simple guide for conversion of animal dosages from human dosages was applied Nair & Jacob, (2016), which states that, dose is equally related to body weight although it is not the lone factor which influences the scaling for dose calculation. The correction factor (Km) is estimated by dividing the average body weight (kg) of species to its body surface area (m^2). For example, the average human body weight is 60 kg, and the body surface area is 1.62 m2. Therefore, the Km factor for human is calculated by dividing 60 by 1.6^2, which is 37. The Km factor values of various animal species is used to estimate the human equivalent dose (HED) as: HED mg / kg = Animal dose mg / kg Animal K /Human K Eq. As the Km factor for each species is constant, the Km ratio is used to simplify calculations. Hence, Equation 2 is modified as: HED mg / kg = Animal dose mg / kg Km ratio Eq. The Km ratio values are already provided and are obtained by dividing human Km factor by animal Km factor or vice versa.

3.10.2 Calculation of the doses

The maximum carbamazepine dose in humans is 1200mg, medium dose is 700mg and minimum dose is 200mg, while the average weight of an adult human is approximately 60kg, (Walpole *et al.,* 2012).

a) Determination of high dose carbamazepine group

Highest dose carbamazepine-1200mg

Average weight of a man-60kg

1200mg = 60kg

X=1kg

X=1x1200/60 =**20mg/kg**

AED = HED X Km factor

Therefore, 20mg/kg x 6.2 =**124mg/kg**

b) Determination of Medium dose Carbamazepine group

Medium dose carbamazepine-700mg

Average weight of a man-60kg

700mg = 60kg

X=1kg

X=1x700/60 =**11.7mg/kg**

AED = HED X Km factor

Therefore 11.66mg/kg x 6.2 =**72.3mg/kg**

c) Determination of Low Dose Carbamazepine group

Low dose carbamazepine-200mg

Average weight of a man-60kg

200mg = 60kg

X=1kg

X=1x200/60 =**3.3mg/kg**

AED = HED X Km factor

Therefore 3.3mg/kg x 6.2 =**20.7mg/kg**

3.10.3 Administration of carbamazepine

Carbamazepine was administered by the researcher daily at 0900hrs.

a) Materials required for administration of carbamazepine

i. Pregnant dams (30)
ii. carbamazepine ©
iii. Gavages' needle gauge 16
iv. 20 ml beaker for dilution
v. Syringes-2ml and 5m

vi. Deionized water (500mls)
vii. A table cloth

b) The procedure for in administering various doses of carbamazepine using gastric gavage

1. The animal was held carefully from the neck region using the left hand
2. The animal was wrapped with the table cloth to avoid the animal from soiling the Investigators clothing's
3. It was then rested against the body with the animal mouth facing the investigator
4. The gavage needle 16 was gentry inserted into the mouth of the animal turning it gentry to pass the esophageal constrictions and the cardiac sphincter
5. The carbamazepine bolus was put in the stomach of the animal
6. The gavage needle was gentry removed

3.11 Determination of the critical dose and most vulnerable carbamazepine on the fetal brain

The critical teratogenic dose of carbamazepine, was determined as follows**: -**

Animal groupings was done as described in (**figure 3.1)** and administration of carbamazepine in each of the groups was done as follows:

In each of the groups (LCG, MCG, HCG), the 9 dams were randomly sub divided in three sub-groups the Trimester 1(TM_1) = 3dams, Trimester 2 (TM_2) = 3dams and trimester 3 TM_3=3 dams. The gestation period of a rat is 21 days, therefore trimester one was between gestational day (GD_1 to GD_7), while trimester 2 was between (GD_7-GD_{14}) and third trimester (GD_{14}- GD $_{20}$).Carbamazepine was administered as follows: -

- Trimester ones (**TM_1**) rats: - (LCG,MCG,HCG) categories received carbamazepine from gestation day (**GD_1-GD_{20}**)
- Trimester twos (**TM_2**) rats: - (LCG, MCG,HCG) categories received carbamazepine doses from gestation day (**GD_7-GD_{20}**)
- Trimester three (**TM_3**) rats: - (LCG, MCG,HCG) categories received carbamazepine doses from gestation day (**GD_{14}- GD_{20}**)

3.12 Determining vulnerable periods of carbamazepine teratogenicity on the fetal brain

To determine the vulnerable periods of carbamazepine teratogenesis, the treatment was administered daily throughout the gestation period starting on day 1(GD_1) for the TM_1 groups, day 7 (GD_7) for the trimester II (TM_2) and day 14 (GD_{14}) for trimester three subgroup, (Freeman *et al.,* 2013).

3.13 Humane sacrificing of the pregnant albino rats, harvesting of the fetuses and Harvesting of fetal brains

3.13.1 Humane sacrificing of the pregnant albino rats

All rats were humanly sacrificed on day 20th just before delivery to avoid devouring the fetuses by the application of concentrated carbon dioxide

Materials

i) The pregnant rat GD_{20}
ii) Carbon dioxide
iii) Cotton gauze or cotton wool
iv) Bell or dissector jar
v) Physiological saline 0.85% concentration
vi) Mounting board
vii) Mounting pins
viii) A pair of scissors

ix) A pair of forceps(toothed)
x) Scalpel blade
xi) Scalpel blade handle
xii) Fixatives- 10% Formaldehyde for light microscopy
xiii) Drip set 2 in number
xiv) Hypodermic needle gauge 20
xv) Gloves (surgical)
xvi) Magnifying glass
xvii) Ruler
xviii) Electronic weighing machine
xix) Specimen collection bottle

b) Procedure for anaesthetizing rats

1. Concentrated carbon dioxide was introduced into a bell jar
2. A tight fitting lid was then put into the bell jar
3. The pregnant rat was then put into the bell jar
4. The rat was anaesthetized for 10-15 minutes
5. The rat was removed from the bell jar and mounted onto the board using mounting pins with dorsal side on the board
6. Using a pair of scissors and forceps the rat was cut along the linear alba from the symphysis pubis to the xiphoid process (**figure 3.2**).

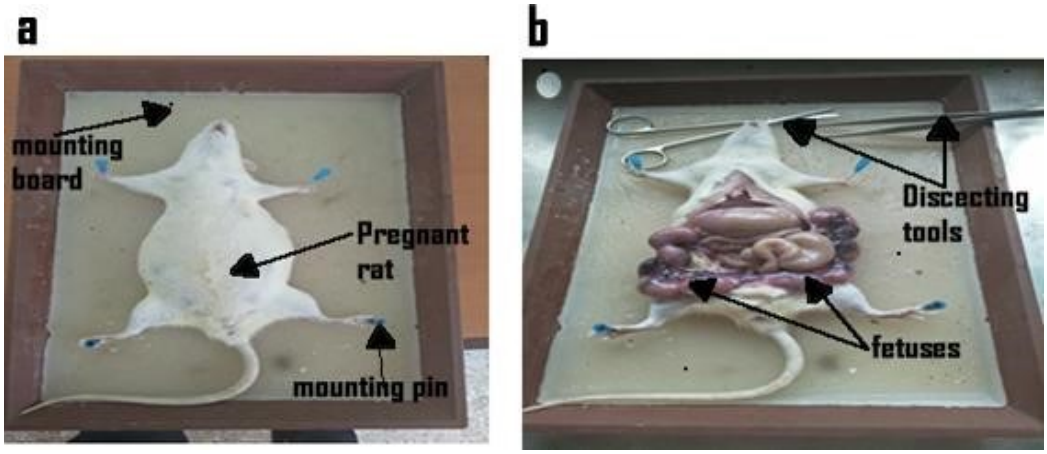


Figure 3.2: (A) A pregnant rat mounted on a dissection board on the 20th day of gestation, (B) Fetuses in the uterine horns after opening the abdomen.

3.13.2 Harvesting of fetuses

All pregnant rats were humanely sacrificed by inhalation of carbon dioxide between 0900HRS and 1100HRS at gestational day 20 to avoid devouring dead fetuses. This was done to prevent the mothers from devouring any damaged or congenitally deformed fetus. Twenty minutes after anesthesia with carbon dioxide, the abdominal wall of the mother was opened from the xiphisternal joint to the symphysis pubis along the linear alba and the full extent of both uterine horns exposed promptly.

Before opening either horn, fetal positions within the horns, as well as the number of live and dead fetuses, as was indicated by their movement following a gentle prodding with a probe was recorded (litter size). Also, the number of the "devoured endometrial glands", characterized by yellowish nodules found along the anti-mesometrial margin of the uterine horns that marks any original implantation site was counted and recoded. Thus, the endometrial glands unoccupied by living or recently dead fetuses represented as the number of prior resorptions (**figure 3.3)**.

The uterine horns were excised along the anti-mesometrial border to expose the fetuses, embryonic membranes and placentas. They were gently removed in totality from the uterus utilizing the blunt end of a pair of forceps. An incision along the dorsal surface of the membranes revealed the fetuses, then each fetus and its placenta were removed, weighed and the general fetal morphology examined and recorded immediately. Fetus size were determined by measuring the anal-nasal length. General examination was done before and after fixation in 10% formaldehyde solution.

For each litter in each rat, three brains from three fetuses with the lowest, median and highest weights were resected for both histological and morphometric analysis.

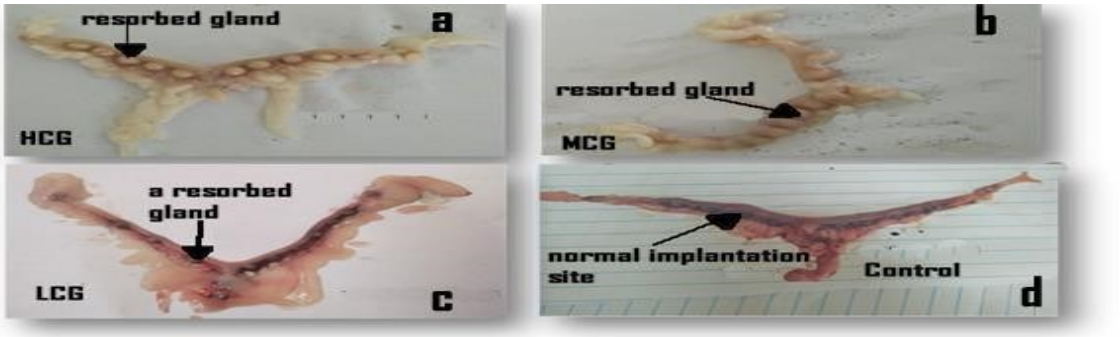


Figure 3.3: Resorbed endometrial glands in (a) HCG, (b) MCG, (c) LCG, (d) Control (photomicrograph taken using a 32 megapixel camera Nickel japan 2018 series)

Procedure followed in harvesting of fetuses

i) The uterine horns were excised along the anti-mesometrial borders using a pair of scissors

ii) The number of present fetuses and the resorbed sites counted and recorded

iii) The fetuses were removed to continue being fixed in situ for hours with the same fixative used during perfusion fixation

iv) The CRL, Bi-parietal diameters, head-lengths for each fetus were taken by use of a Vernier caliper to assess the effects of carbamazepine on overall fetal development

v) Other congenital anomalies were assessed and recoded

vi) Fetal weights in grams were taken with electronic weighing balance and recorded (**figure 3.4**)

vii) Other fetal growth parameters including fetal lengths, crown rump lengths and Bi-parietal diameters were taken and recorded (**figures 3.5, 3.6, 3.7 and 3.8)** respectively

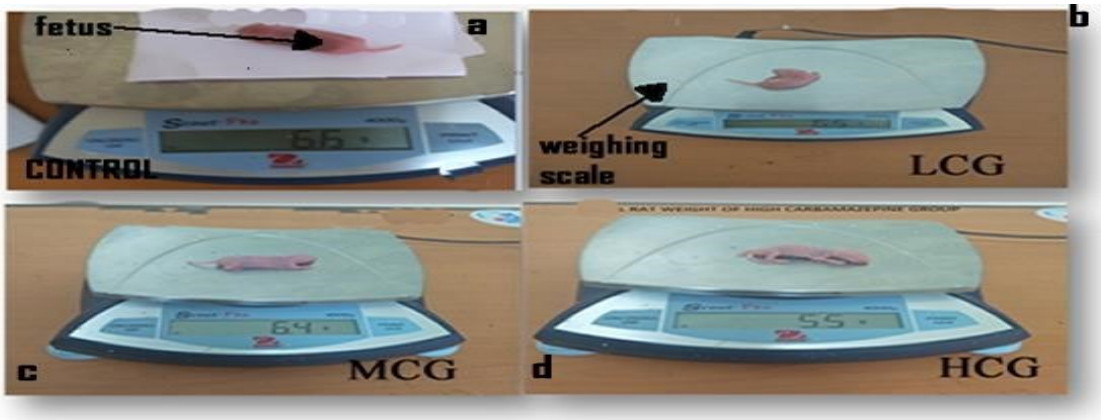


Figure 3.4: Fetal weight measurements in (a) control, (b) LDG,c) MDG, (d) HDG, (Using scout pro model SPU4001 S/N B519923500 from Uhaus Corporation, USA*).*

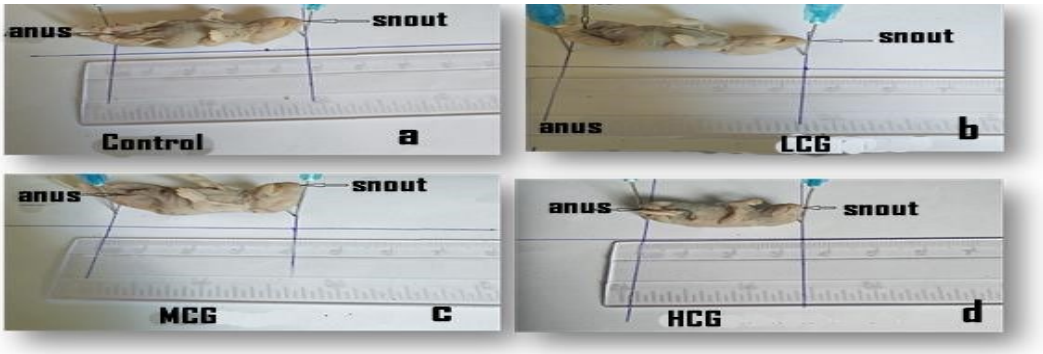


Figure 3.5: CRL measurements for the fetuses using a ruler starting from the tip of the nose (snout) to the root of tail.

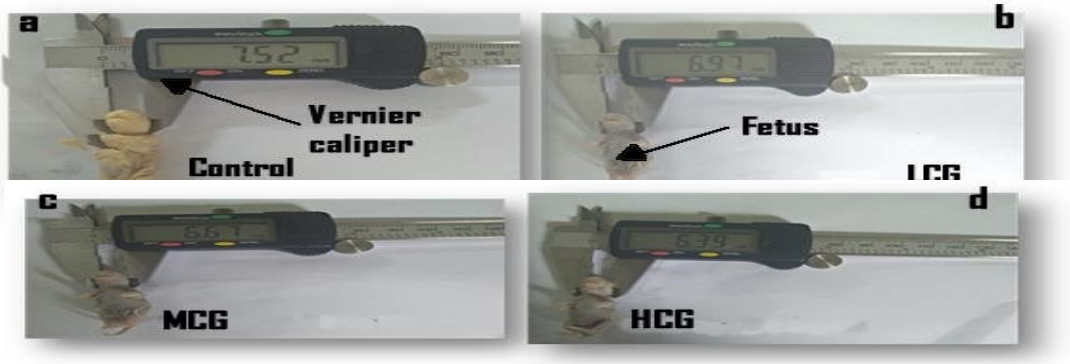


Figure 3.6: Bi-parietal diameter measurements taken using a digital Vernier caliper (from Hercules sealing products-japan model 1.13.2017

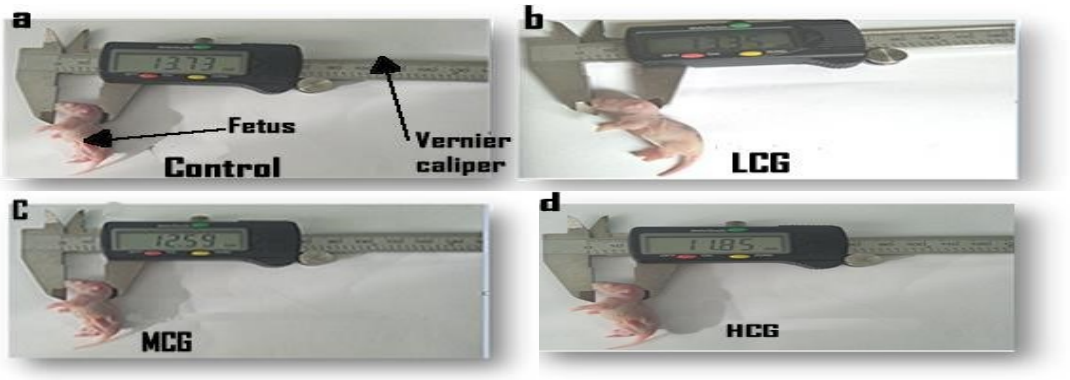


Figure 3.7: Head length measurements were taken; from back of the skull to the extremity of the nose in (a) control (b), LCG), (c) MCG, (d) HCG (using a digital Vernier caliper from Hercules from sealing product-japan model 1.13.2017).

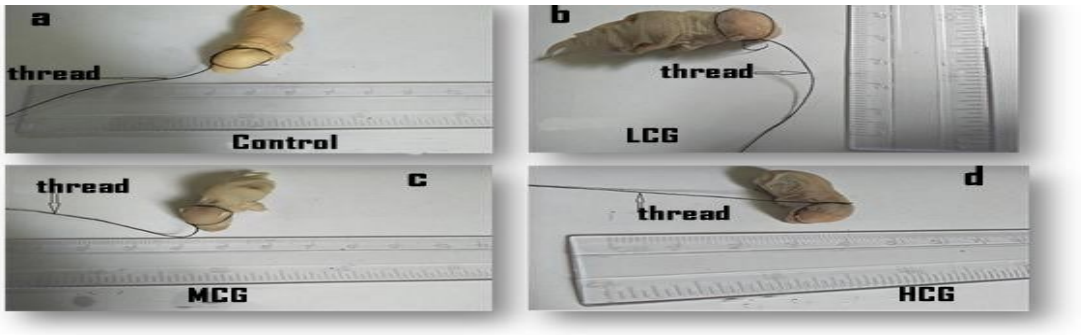


Figure 3.8: Head circumference measurements were taken around the head: (a) control (b), (LCG), (c) MCG, (d) HCG

3.13.3: Procedure for harvesting the fetal brains

Fetal brains for histomorphological and stereological analysis were subsequently harvested using the following procedure:-

a) Fetuses were mounted onto the dissection board using mounting pins -dorsal side facing the board.

b) Using a pair of scissors and forceps the lateral bonders along the lower margin of the temporal bone was opened and the skull cap removed

c). Using a magnifying glass, the whole fetal brain was identified.

d). To avoid damaging the fetal brain, the meninges was opened along the superior sagittal sinus retracted up carefully since the brain lies within the meninges

e).The entire brain was excised/ scooped at the level of foramen magnum

f).Each brain was examined for general external features and obvious congenital malformations

g). Brain weights were taken by use of a digital weighing scale **(Figure 3.9)** and their weights to body weight ratio was calculated.

h).The brain length, width and thickness were assessed using a ruler **(Figure 3.10)**

i).The brains were immersed in the formaldehyde, to proceed with processing either for light or histostreology for 12 hours

Figure 3.9: Showing how the fetal brain weights were taken: (a) control (b), LCG, MCG, (d) HCG), using scout pro model SPU4001 S/N B519923500 from Uhaus Corp- USA)

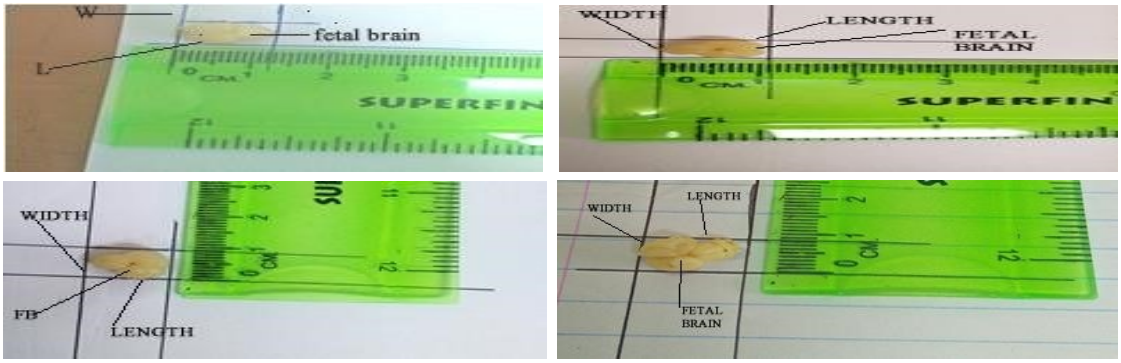


Figure 3.10: Showing how the fetal brain length and width measurements were taken (using a ruler) in; (a) control, (b) LCG, (c) MCG, (d) HCG).

The fetal brains were then processed for light microscopy and for stereological analysis using the following materials enumerated as **3.14 (a)** and the procedure enumerated as in **3.14 (b) :-**

3.14 Processing for light microscopy

a) Materials used for staining

i) The specimens (the fetal brains)

ii) Zenkers solution (1 litre)

- Mercuric chloride 5gms
- -Potassium dichromate 2.5g
- -Sodium sulphate 1gram
- -Distilled water
- -Acetic acid 5mls

iii) Dibutylphthalate Polystyrene Xylene (DPX) moutant

iv) Glass slides and cover slips

v) Hematoxylin and eosin

vi) Glass staining square jars

vii) Paraffin wax

viii) Microtome knives

ix) Rotary microtome

x) Heater and water bath container

xi) Specimen bottles

xii) Slide holders

xiii) Distilled water

xiv) Formaldehyd40%concentration

xv) Xylene

xvi) Isopropyl alcohol

xvii) Van Grisons stain

xviii) Glass ware for preparation of dilutions

xix) Wood blocs

xx) Beakers

xxi) Egg albumin

xxii) Dropper

xxiii) Cedar wood oil

b) The procedure for processing the fetal brain for light and stereology

1. The brains were fixed in the Boiun's (Zenkers' solution) for 24 hours
2. They were dehydrated in an ascending concentration of alcohol (50%, 60%, 70%, 80%, 90%, 95% and 100% (absolute) each for one hour.
3. They were cleared by immersion with cedar wood oil for 12 hours.
4. They were then infiltrated with paraplast© wax for 12 hours at 56^0c
5. The brain tissue was then orientated in the longitudinal axis (frontal to occipital lobe)
6. They were then embedded in paraffin wax on the wooden blocs
7. Excess wax was trimmed-off till the entire length of the brain tissue was exposed
8. Longitudinal sections (5µm) thick were cut from head to tail regions with Leitz sledge rotary microtome
9. The cut sections were floated in water at 37^0C to spread the tissue
10. The sections were stuck onto glass slides using egg albumin, applied as thin film with a micro-dropper.
11. The slides were then dried in an oven at 37^0C for 24 hours
12. Blinding was done by coding all the slides by the research assistant in absence of the researcher
13. They were stained with different stains including: -Hematoxylin and Eosin (H&E), based on the cellular structures that needed to be studied.

3.15: Stereological analysis

3.15.1 Estimation of total brain volume using Archimedes principle

Immediately after the removal of the entire fetal brain from both the control and experimental groups the total brain volumes (Archimedes volume) were determined using the water/fluid displacement methods that employed the Archimedes' principle. Through this method the Archimedes brain volumes were obtained by inserting the whole brain tissue into graduated beakers containing normal saline, and the amount of fluid displacement upward was measured.

The normal saline displaced by the brain represented the actual brain volume as described by a study by (Hughes, 2005). The Archimedes volumes were used as the reference volumes when determining the cavalieri stereological densities and volume densities. This method was compared to the cavalieri methods and the mean and standard error of the mean (± SEM) of the measurements were calculated.

3.15.2 Determination of stereological total brain volume and volume densities using cavalieri and point counting methods

The stereological total brain volume and the estimation of the volume densities of both the cortical and subcortical layers of the brain structurers was determined by using a combination of both the cavalieri and point-counting method (Cruz-Orive, 1999).

The following steps were followed: i) Preparation of brain cavalieri sections (5µm) thick sections) ii) Selection of the spacing for the point probe iii) The point probe was tossed randomly onto each section iv) The points that hit the region of interest was counted using STEPnizer stereology tool v) All sections were processed keeping a tally of counts per section vi) The volume was then calculated.

Twenty sections of 5µm thickness from each longitudinal brain section were selected by systematic uniform random sampling, (Bural *et al.,* 2015). Using the microscope's

stage Vernier, images were viewed at magnification of 10x. The volume was obtained by fully sectioning the brain into a series of cuts which was the product of the sum of the cut areas (starting with the first to the last section). Point counting was done using the STEPnizer software (Bolender & Weibel, 1973).

The digital images of the brain tissue were captured using stereological sampling rules with same magnification and saved in the jpeg (joint photograph expert group) file format at adequate resolution. The picture height was ensured that it matched the height of the computer monitor, both defined in pixels.

All images captured both for the control and experimental groups were organized appropriately and saved in one folder. A calibrated scale bar was added to one image of a batch to define the real dimensions of the structures under investigation, and placed on left hand side. Where stereological estimation required the use of a guard area it was set and were not be changed in the course of the whole experiment to obtain consistent results **(figure 3.11)**

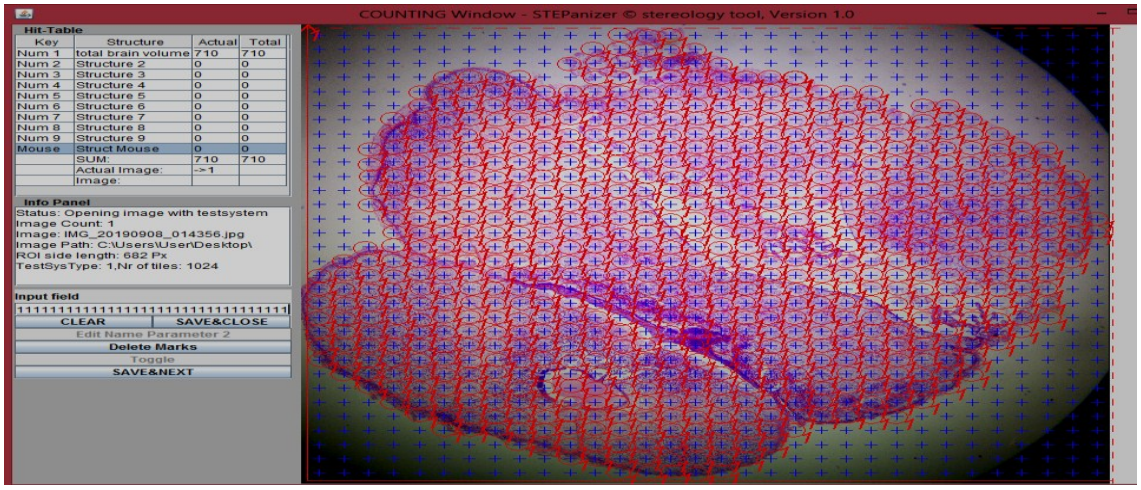


Figure 3.11: showing a cavalieri 5µm (mag X 40) section image of a fetal brain in a superimposed an equidistant point counting grid - (The tissue slide is sampled from a control group whereby, the points that hit the brain tissue are indicated by blue in the red area).

Point counting using the cavalieri principle was employed to estimate the total fetal brain volume using the formula:

$$_{Est}V = \frac{\sum_{i-1}^{m} P.\ a / p.\ t}{M^2}$$

Where: $_{est}V$= was the estimation of the volume of the brain,

$\sum P$= was the sum of the number of points landing within the various

components of the fetal brain profiles,

$^{Mi-1}$. All points from the first to the last

A/p was the area associated with each point,

t was the distance between sections and **M**= was represent the magnification (Welniak–Kaminska *et al*., 2019)

On each sampled section, five fields were selected in a systematic random manner with the aid of mounted grid scale on the Newscast computer screen projected by the stereology new cast microscope. A transparent test system on the grid was then superimposed on the images projected on the computer screen on the cortical and sub-cortical brain structurers and points hitting these areas counted at a final magnification of x10. Then estimates of their volume density, (Vv) of the in the reference space were obtained using the formula:

EstVv = P (part)/P (ref),

Where **P (part)** and **P (ref)** were the number of test points falling in all structure profiles and in the reference space, respectively (Keller *et al*., 2018)

3.15.3: Stereological correction for brain tissue shrinkage

The following method was applied to quantify the percentage brain tissue shrinkage caused by fixation and histological procedures. The volume of removed fresh brain was calculated by Archimedes displacement method. After tissue processing and exhaustively sectioning, the brain volume was estimated with cavalieri method. The brain volume shrinkage was then calculated as follows (Tran *et al., 2015*);

$$\text{Shrinkage} = \frac{\text{Volume before-Volume}}{\text{Volume before}}$$

NB/Volume before is Archimedes volume while volume after cavalieri volume.

3.16 Photography (materials and procedure)

a) Materials

1. Digital camera (32 megapixel)

2. BP Olympus microscope

3. Memory card

4. Histological glass slide

b) Procedure followed in taking photomicrographs

1. Histological slides were mounted on the stage of the microscope
2. The focus was adjusted until the image to be photographed was in focus
3. The field was magnified appropriately
4. Photographs of the regions were taken as they were viewed best under the focus of the microscope
5. Photographs were transferred to the computer by use of a memory card

6. The photographs were uploaded and labelled using the Adobe fireworks programme

3.17 Statistical data management and analysis

The histomorphological data that entailed the qualitative data was collected using photomicrographs at different magnifications using a 32 megapixel digital camera then exported to Adobe fireworks for qualitative analysis.

Data on pregnancy and histostereological outcomes that formed the parametric data was collected using structured checklists and stereological data sheets respectively, stored and coded in excel spreadsheets windows 10, version 2013 and then exported for analysis to SPSS programme windows version 25 for analysis (Chicago Illinois). The data was statistically tested using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison tests. Kruskalwalis test was employed for non-parametric data.

Pearson correlation comparison test was used to compare the results. Results were expressed as mean ± standard error of the mean (SEM) for all values in parametric test, and median values for the non-parametric test. All results whose $P<0.05$ were considered to be statistically significant.

3.18 Ethical consideration

-All procedures for animal handling, feeding, human sacrificing and harvesting of organs were performed as per laid down protocols, with approval from Animal Ethics Committee of Jomo Kenyatta University of Agriculture and Technology (JKUAT) as well as the laid down protocols and regulations by International Animal Research Institute (IARI) of USA as outlined by (Gomez *et al.,* 2010).

-The study went through the legal and administrative requirements as stipulated by JKUAT and the laws of Kenya, (See document attached in the appendices; REF: JKU/2/4/896A**).**

CHAPTER FOUR

RESULTS

4.1 Objective 1; The Maternal and Fetal Pregnancy Outcomes

This is presented as follows:-

4.1.1: Influence of carbamazepine on mean daily maternal weight trends from GD_1 to GD_{20}.

The comparative maternal weight treads in (TM_1), the first three to four days following carbamazepine treatment in all the treatment groups were marked with significant decrease in weight and then a steady increase in weight gain up to GD_{20} (**figure 4.1)**. This phenomenon could be attributed to the carbamazepine acclimatization factor. In (TM_2) there was a marked sudden weight drop in all carbamazepine groups from day 7 for a period of 3 days that were then followed by a steady increase in weight gain up to GD_{20}. However, the control group had a steady weight gain throughout the gestation period ($GD_{1\text{-}20}$), **(figure 4.2)**. Similarly, a marked sudden weight drop in all carbamazepine groups on GD_{15} following introduction of carbamazepine treatment at TM_3. The animals then rapidly regained their weights up to GD_{20}. However, the control groups had a steady weight gain throughout the gestation period ($GD_{1\text{-}20}$), **(figure 4.3).**

When the analysis was done on whether or not the mean maternal weight gain (grams) has a time dependent relationship (**table 4.1)**, it was established that when treatment was instituted in trimester one and two (TM_1 &TM_2), there was significant (P=0.002) reduction in maternal weight gain in all the treatment groups (LCG, MCG, HCG), unlike in TM_3 where there was no significant difference (P=0.21) for the low and medium dose groups compared with the control group.

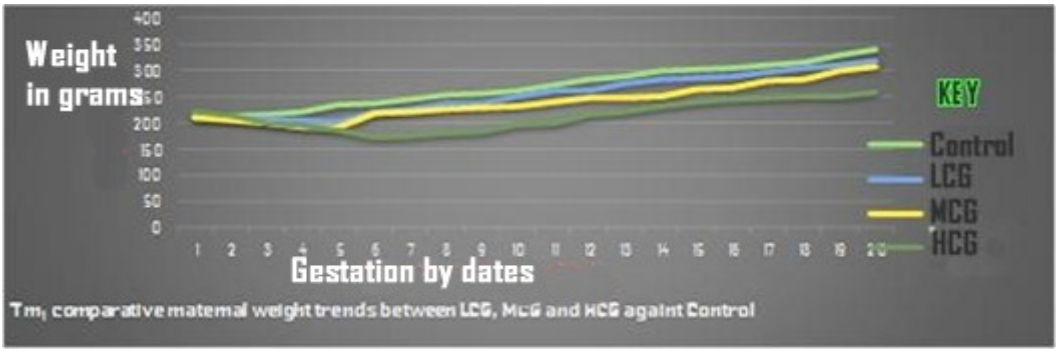


Figure 4.1: Comparative maternal weight gain trends for the carbamazepine treated groups (LCG, MCG, and HCG) against control at TM_1.

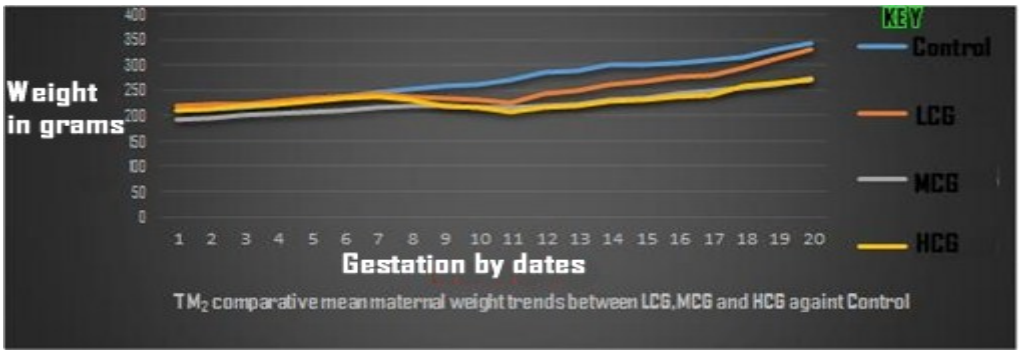


Figure 4.2: Comparative maternal weight gain trends between the carbamazepine treated group (LCG), (MCG), and (HCG) against control at TM_2

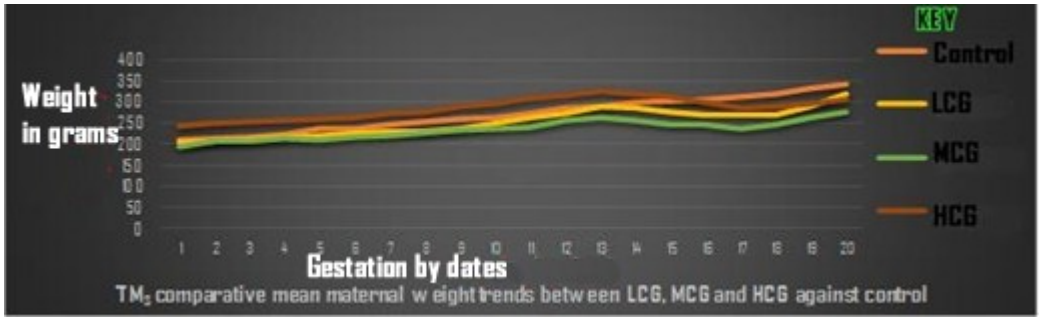


Figure 4.3: Comparative mean maternal weight trends between the carbamazepine treated groups (LCG, MCG, and HCG) against control at TM_3

Table 4.1: The comparative means for maternal weight gain for the LCG, MCG and HCG with the time of exposure (TM_1, TM_2 and TM_3) against the control group

The Study groups	The time of exposure to the CBZ treatment	Mean maternal weight gain (g) ± SEM
Control group	none	130.67±5.78
Low dose carbamazepine group (LCG, 20.7mg/kg)	TM_1	$105.7±5.55^{bc*}$
	TM_2	$112.67±7.42^{bc*}$
	TM_3	116.7±13.860
Medium dose carbamazepine group (MCG, 72.3mg/kg)	TM_1	$88.3±12.1^{bc*}$
	TM_2	$81.00±3.512^{bc*}$
	TM_3	$83.33±5.840^{bc*}$
High dose carbamazepine group (HCG, 124mg/kg)	TM_1	$37.7±7.3^{bc*}$
	TM_2	$60.33±5.93^{bc*}$
	TM_3	$60.67±2.67^{bc*}$

Key: *All value that bear (*) as a superscript indicates that they depict statistical significance differences ($p<0.05$) when compared with the control. Values with (^{b}) &(^{c}) superscripts have a statistical significance difference ($p<0.05$) in the intragroup and intergroup comparisons respectively using one way ANOVA with Turkey post-hoc t-tests*

4.1.2 Influence of carbamazepine on the litter size

The comparative median litter sizes between the carbamazepine treated groups against the control by use of the Kruskal Wallis non-parametric comparison test depicted inverse dose response relationship in that as the dose increased, median litter size reduced significantly (P=0.035) in TM_1, (0.023)in TM_2 and (0.035) in TM_3. The statistical significance difference was observed when high dosages were compared with the control **(table 4.2).**

Table 4.2: Shows the comparison of the median litter size in the low, medium and high carbamazepine groups (LCG, MCG, and HCG) in (TM_1, TM_2 and TM_3) against the control group

The time of exposure to the CBZ treatment	The Study groups	Median
Trimester one (TM_1)	Control	10.83
	LCG	7.67
	MCG	4.50
	HCG	3.09*
Trimester two (TM_2)	C	10.67
	LCG	7.83
	MCG	5.60
	HCG	2.00*
Trimester three (TM_3)	C	10.17
	LCG	8.00
	MCG	5.83
	HCG	2.00*

Key: *All value that bear (*) as a superscript indicates that they depict a statistical significance differences ($p<0.05$) when compared with the control.*

4.1.3. Influence of carbamazepine on placental weights, resorbed endometrial glands and the percentage embryolethality

The mean placental weight that is usually a key indicator of maternal nutritional exchange with the fetus was also observed to have similar inverse dose response relationship with the dose of treatment and the time of exposure. It was found that the lowest placental weight recorded was in high carbamazepine treated group when treated at TM_1 and lowest in the low carbamazepine group at TM_3 (**table 4.3**).

The mean number of the resorbed endometrial gland were also seen to directly vary with the dose of exposure as well as with time of exposure in that with increasing doses of carbamazepine exposure, there was a corresponding significant increase

(P=0.010) in number of endometrial glands resorptions particularly in TM_1 and TM_2 across all the carbamazepine treated groups as compared with the control. At TM_3 the mean number of the resorbed endometrial glands had a significant difference (P=0.001) at TM_1 and TM_2 but only in the high carbamazepine treated group (HCG) when compared with the control (P=0.048).

On the mean percentage embryolethality, it was observed that the comparative percentage mean number of dead fetuses in utero increased with carbamazepine dose and the time of exposure. When carbamazepine was administered at TM_1 the mean percentage embryolethality in HCG was at 1.67±0.882 followed by MCG at 0.67±0.67 and lastly at LCG, it was statistically higher (P=0.001) when compared with the control. When carbamazepine treatment was instituted at TM_2, the embryo-lethality was 1.33±0.667 for the HCG, which was statistically higher (P=0.010) than the control, while MCG and LCG embryo-lethality at TM_2 were not statistically different (P=0.34). When treatment was done at TM_3 the percentage embryo-lethality did not show statistical significance difference (P=0.60) with the control (**table 4.3**).

Table 4.3: The comparative means litter size, placenta weight, resorbed endometrial glands and percentage embryo-lethality in LCG, MCG and HCG with time of exposure (TM1, TM2 and TM3) against the control group (CG).

Study groups	Period of CBZ treatment	Mean placenta weights(g) ± SEM	Mean resorbed endometrial glands ± SEM	Mean embryo lethality (%) ± SEM
Control group	-none-	5.58±0.021	0.67±0.67	0.333±0.333
Low carbamazepine group (LCG, 20.7mg/kg)	TM_1	4. 53±0.049^{bc*}	0.67±0.67	0.33±0.333 ^{bc}
	TM_2	5.0±0.049 ^{bc*}	0.67±0.667	0.33±0.333 ^{bc}
	TM_3	5.40±0.050	0.667±0.333	0.33±0.333 ^{bc}
Medium carbamazepine group (MCG, 72.3mg/kg)	TM_1	4.633±0.034 ^{bc*}	1.33±1.33 ^{bc*}	0.67±0.67 ^{bc*}
	TM_2	5.03±0.034 ^{bc*}	1±0.577 ^{bc*}	0.33±0.333
	TM_3	5.133±0.0612 ^{b*}	0.83±0.333 ^{b*}	0.33±0.333
High carbamazepine group (HCG, 124mg/kg)	TM_1	4.23±0.018*	7±1.53 ^{bc*}	1.67±0.882 *
	TM_2	4.64±0.0200*	1.3±0.000 ^{bc*}	1.33±0.667 ^{bc*}
	TM_3	4.96±0.030 *	0.900±0.6506*	1.00±0.577 ^{bc*}

Key: *All value that bear (*) as a superscript indicates that they depicts a statistical significance differences ($p<0.05$) when compared with the control. Values with (b) &(c) superscripts have a statistical significance difference ($p<0.05$) in the intragroup and intergroup comparisons respectively using one way ANOVA with Turkey post-hoc t-tests*

4.1.4: Influence of carbamazepine on congenital anomalies

A total of fetuses 41 fetuses among 286 fetuses were found with one type or a combination of a number of congenital anomalies. The most common types of anomalies observed were musculoskeletal abnormalities. These abnormalities were found to be concentrated in the high and medium carbamazepine groups (MCG and HCG). This was particularly so when carbamazepine was administered in the first (TM_1, and the second trimesters (TM_2) **(table 4.4).**

Table 4.4: Showing the types of congenital anomalies observed, their numbers and their distribution LCG, MCG & HCG against the control.

Types of congenital abnormalities	Control	Low carbamazepine group (20.7g/kg)	Medium carbamazepine group (72.5g/kg)	High carbamazepine group(124g/kg)	Total number of fetuses
Spina bifida	0	1	2	6	10
Hypospadias	0	0	1	3	4
Cleft lip	0	1	1	2	4
Anencephaly	0	1	2	4	7
Microcephaly	0	1	2	3	6
Meromelia	0	0	1	1	2
Sadactyly	0	0	0	1	1
Oligodactyly	1	0	1	2	3
Microphalmia	0	0	1	1	2
Aphalagia	0	0	1	1	2
Total	1	4	12	24	41

4.1.5 Correlation analysis on maternal pregnancy outcomes

The correlational statistics using Pearson correlation comparison procedure shown that there was a strong significant association ($r>0.5$, $p<0.05$) between the dose and time of exposure across all the three experimental groups when compared with the control in all the maternal pregnancy outcomes including; mean maternal weight gain, mean placenta weight, mean congenital abnormalities and mean percentage embryolethality at TM_1 and TM_2 and TM_3 (**table 4.5)**.

Table 4.5: Intra and inter-group correlational comparisons on the various maternal Pregnancy outcomes for the LDG, MDG and HDG at TM_1, TM_2 and TM_3 against the control.

		Weight gain(gms)	Initial weight (gms)	Terminal weight (gms)	Placenta weight (gms)	Number of resorbed glands	litter size	Dead fetuses	Congenital abnormalities
Weight gain(gms)	r	1							
	p								
Initial weight (gms)	r	-.284	1						
	p	.093							
Terminal weight (gms)	r	.844**	.274	1					
	p	.000	.105						
Placenta weight (gms)	r	.831**	-.116	.768**	1				
	p	.000	.500	.000					
Number of resorbed glands	r	-.569**	.059	-.538**	-.649**	1			
	p	.000	.733	.001	.000				
litter size	r	.793**	-.131	.722**	.892**	-.668**	1		
	p	.000	.447	.000	.000	.000			
Dead fetuses	r	-.517**	.051	-.490**	-.450**	.482**	-.438**	1	
	p	.001	.769	.002	.006	.003	.008		
Congenital abnormalities	r	-.313	.072	-.274	-.399*	.447**	-.470**	.381*	1
	p	.063	.677	.107	.016	.006	.004	.022	

***key**: r is the Pearson's correlation coefficient, P is the p-value, * and ** indicate significance i.e. p<0.05, while (+/-) depict the direction of the relationship*

4.1.6 Influence of carbamazepine on fetal body weight, CRL, head circumference, head length and bi-parietal diameter.

It was observed that the mean fetal weights, the mean crown rump lengths, the head lengths the head circumference and the bi-parietal diameters depicted an inverse dose response relationship while at the same time depicting a direct dose response relationship with the time of exposure. This was particularly observed when the treatment was done in TM_1 and TM_2 across all the carbamazepine treated groups (LCG, MCG and HCG). The treatment at TM_3 did not show statistical significant

difference (P=0.95) across the three experimental groups as well as when compared with the control in all the four fetal parameters (**table 4.6)**.

Table 4.6: The intra and inter group comparative means of the fetal body weight, head circumference, head length, CRL and bi-parietal diameter of LCG, MCG and the HCG in (TM_1, TM_2 and TM_3) against the control (C).

Study groups	Period of CBZ treatment	Mean fetal body weight (mg) ± SEM	Mean Head Length (mm) ± SEM	Mean head circumference (mm) ± SEM	Mean CRL (mm) ± SEM	Mean bi-parietal diameter (mm) ± SEM
Control group	----------	6.73±0.026	1.34`±0.002	3.89±0.010	4.723±0.030	0.7155±0.018
Low carbamazep group (LCG) 20.7mg/kg	(TM1)	6.42±0.007 bc*	1.304±0.00082 bc*	3.22±0.025 bc*	4.123±0.009 bc*	0.659±0.00073 bc*
	(TM2)	6.57±0.011 bc*	1.32±0.0008 bc*	3.453±0.029 bc*	4.5±0.009 bc*	0.686±0.0008 bc*
	(TM3)	6.66±0.0168	0.297±0.0033	3.57±0.037	4.55±0.027	0.692±0.0026
Medium carbamazepine group (MCG) 72.3mg/kg	(TM1)	6.31±0.046 bc*	1.28±0.0008 c*	2.95±0.018 bc*	3.865±0.044 bc*	0.6296±0.0033 bc*
	(TM2)	6.42±0.018 bc*	1.31±0.0004 c*	3.22±0.019 bc*	4.15±0.0029 bc*	0.655±0.0005 bc*
	(TM3)	6.53±0.004	0.216±0.008	3.52±0.015	4.44±0.028	0.69±0.004
High carbamazepine group (HCG) 124mg/kg	(TM1)	5.42±0.02 bc*	1.252±0.001 bc*	2.35±0.013*	3.4±0.023d*	0.579±0.002 bc*
	(TM2)	5.92±0.00 bc*	1.30±0.0069 c*	3.04±0.021	3.85±0.0024*	0.635±0.0011*
	(TM3)	6.21±0.010 b*	0.1200±0.012 b*	3.41±0.021	4.34±0.011	0.68±0.0028

Key: *All value that bear (*) as a superscript indicates that they depicts a statistical significance differences (p<0.05) when compared with the control. Values with (^{b}) &(^{c}) superscripts have a statistical significance difference (p<0.05) in the intragroup and intergroup comparisons respectively using one way ANOVA with Turkey post-hoc t-tests*

4.1.6 Correlation statistics on fetal pregnancy outcomes

The correlational statistics using Pearson correlation comparison procedure shown that there was a strong significant association(r>0.5, p<0.05) between the dose and time of exposure across all the three experimental groups when compared with the

control in all the fetal pregnancy outcomes (fetal weight, litter size, crown-rump length, head circumference, head length and bi-parietal diameter) at TM_1, TM_2 and TM_3 (**table 4.7)**.

Table 4.7: The correlation statistics on the Intra and intergroup comparison on fetal pregnancy outcomes between the carbamazepine treated groups (LDG, MDG and HDG) at TM_1, TM_2 and TM_3 against control.

		Fetal weight	Litter Size	Crown Rump length	Head Circumference	Bi-parietal diameter	Head length
Fetal weight	r	1					
	p						
Litter Size	r	.836**	1				
	p	.000					
Crown Rump length	r	.912**	.856**	1			
	p	.000	.000				
Head Circumference	r	.913**	.872**	.985**	1		
	p	.000	.000	.000			
Bi-parietal diameter	r	.915**	.867**	.994**	.993**	1	
	p	.000	.000	.000	.000		
Head length	r	-.059	-.016	-.204	-.125	-.177	1
	p	.734	.926	.233	.466	.301	

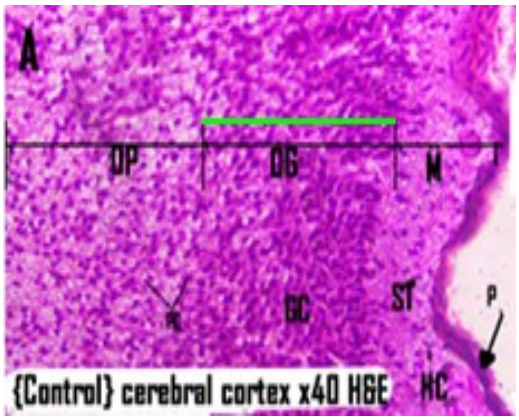
***Key**: r is the Pearson's correlation coefficient, P is the p-value, * and ** indicate significance i.e. p<0.05, while (+/-) depict the direction of the relationship.*

4.2 Objective 2: The Histomorphological Findings On The Developing Fetal Brains

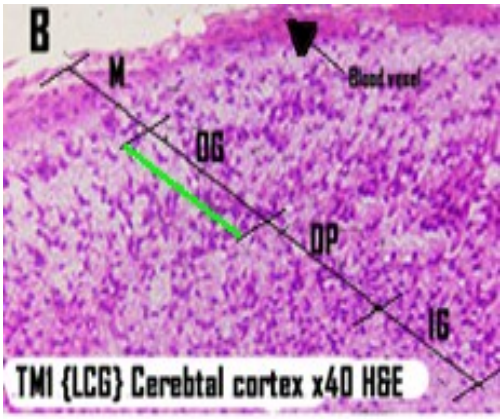
In describing the histomorphological findings on the effects of prenatal exposure to varied doses of carbamazepine, the following parameters were compared in the treatment groups (LCG, MCG and HCG) against the control group; cerebral and cerebellar cortical organization and thickness of the cellular layers, organization of corpus calossal fibres, ventricular size, organization of choroid plexus as well as the epidymal cellular layer.

4.2.1 Influence of carbamazepine on the morphological thicknesses of the cerebral cortical layers

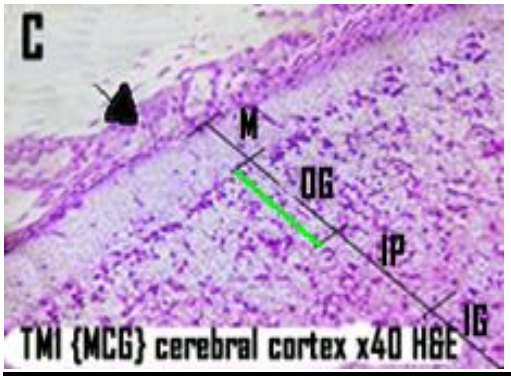
The thicknesses of the six cerebral cortical layers that include (i) Molecular layer (ii) Outer granular layer (iii) Outer pyramidal layer (iv) Inner granular layer (v) Inner pyramidal layer (vi) Multiform layer in that order from outside to the inside were seen to variably differ in their differentiation thickness based on the dose of carbamazepine exposure as wells as with the time of exposure. For instance, the cortical thickness and the accompanying cell distribution and cellular densities per each of the six cortical layers were seen to reduce appreciably among all the carbamazepine treated groups (LCG,MCG, HCG) when treatment was done in trimester one ($\mathbf{TM_1}$), **figure 4.4 and figure 4.5 respectively.** This was also replicated when the treatment was instituted at $\mathbf{TM_2}$ where both the outer and the inner cortical layers reduced with the dose of exposure **(figure 4.6 and figure 4.7 respectively).** When the treatment was done at $\mathbf{TM_3}$, there was no marked significance different in both the outer and the inner cortical layer thicknesses as well as in cellular densities between the carbamazepine treated groups against the control group **(figure 4.8 and figure 4.9 respectively).**



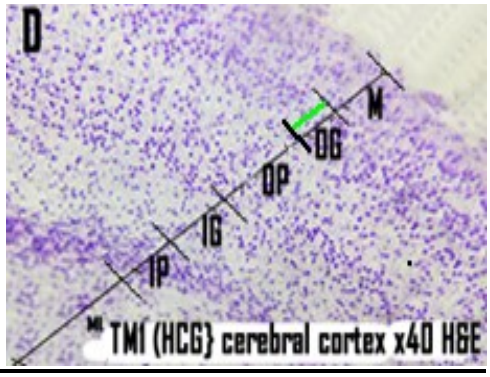
A: <u>Control</u>: Showing the cortical thickness, the cellular densities and cellular distribution in the (a) outer Molecular layer -M, (b) outer granular layer OG and (c) OP-outer pyramidal layers. Note other layers are not visible in this field x40 while the cells are densely packed



B: <u>The LCG</u>: showing the reducing cortical thickness, the cellular densities and cellular distribution in the (a) outer Molecular layer -M, (b) outer granular layer OG and (c) OP-outer pyramidal layers. Note the inner Granular layer (IG) has now become visible in the same field of magnification of x40 and cellular densities are reducing.

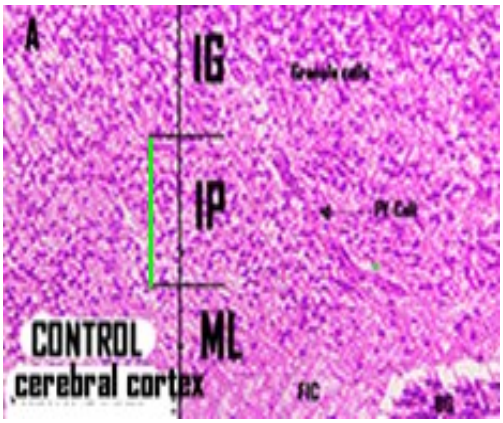


C: MCG: shows further reduction in the various cortical layers thickness of (a) outer Molecular layer -M, (b) outer granular layer OG and (c) OP-outer pyramidal layers. And inner granular layer. Note further reduction in cellular densities in each of the layers.

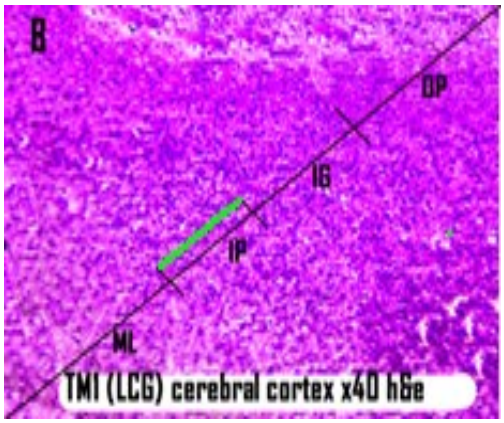


D: HCG: shows highly reduced cerebral cortical thicknesses in all layers that are now all visible; (a) outer Molecular layer -M, (b) outer granular layer OG and (c) OP-outer pyramidal layers. (d) Inner Granular layer (IG), e) inner pyramidal-IP; and (f) multiform layer. (Magnification of x40).

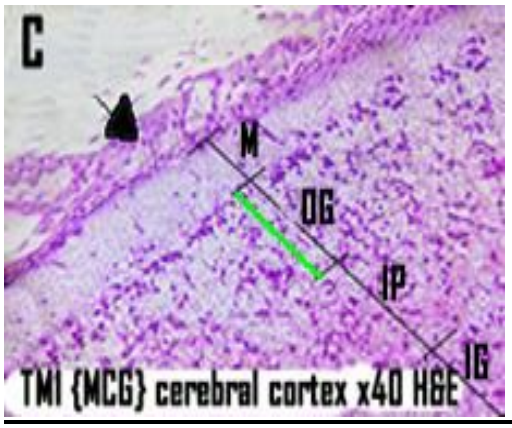
Figure 4.4: The TM_1 comparative thicknesses of the outer cortical layers with their cellular distribution: (a) control (b) LCG, (c) MCG, and (d) the HCG



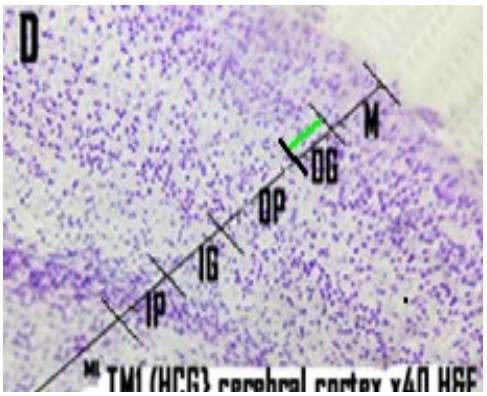
A: Control: Showing the inner layers cortical thickness, the cellular densities and cellular distribution in the (a) inner granular-IG; (b) inner pyramidal-IP; and Multiform layers ML Note other layers are not visible in this field x40 while the cells are densely packed



B: LCG: showing the relative reduction of the Inner cortical histological layers with IP layer in the middle (green line). Note some of the other outer layers are visible (mag x40). The outer cortical layer can as well be observed due to the cortical reduction in thickness and cellular densities

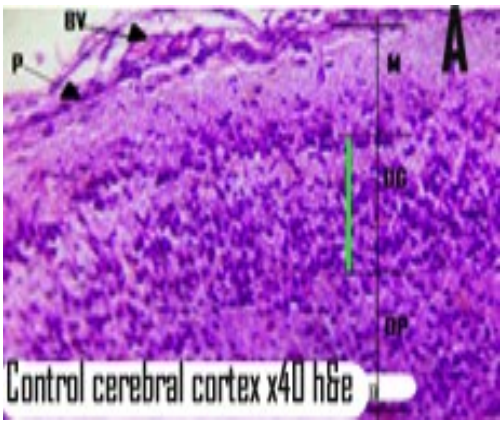


C: MCG: showing further reduction in the inner histological cortical layers and further reduction in cellular densities and sparse distribution of the cell in each inner layers (green line) (mag x40). Four further reduced layers can be observed as well as sparse and disorganized cells

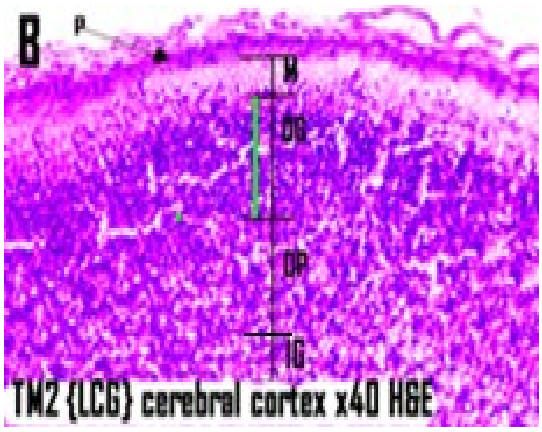


D-HCG: The most reduced inner histological cortical layers at **TM_1, HCG** (mag x40). The IP layer is much more reduced (green line) and two outer cortical layers; OP and OG are visible due to the thinness of layers. The cells are the most disorganized and sparsely distributed

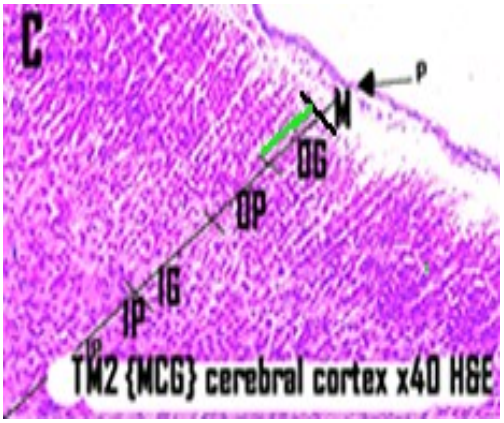
Figure 4.5: The TM_1 comparative thicknesses of the inner cortical layers and their cellular densities/distribution: (a) control (b) LCG, (c) MCG, and (d) HCG.



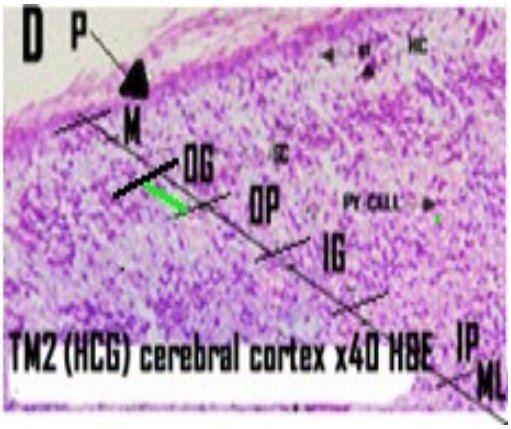
A: Control: A: Showing the cortical thickness, the cellular densities and cellular distribution in the (a) outer Molecular layer -M, (b) outer granular layer OG and (c) OP-outer pyramidal layers. Note other layers are not visible in this field x40 while the cells are densely packed



B: <u>The LCG</u>: showing the reducing outer cortical thickness, the cellular densities and cellular distribution in the (a) outer Molecular layer -M, (b) outer granular layer OG and (c) OP-outer pyramidal layers. Note the inner Granular layer (IG) has now become visible in the same field of magnification of x40 and cellular densities are reducing

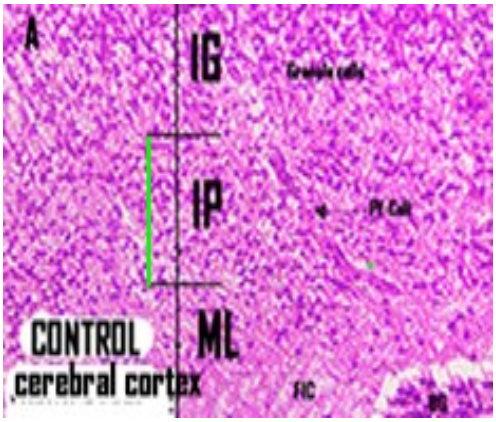


C: <u>MCG</u>: Further reduction in the thickness of outer histological layers at **TM_2, MCG** (magnification x40) depicted by the outer granular layer (green line) with further sparsely distributed cells. IG and IP layers are visible due to reduction of layers

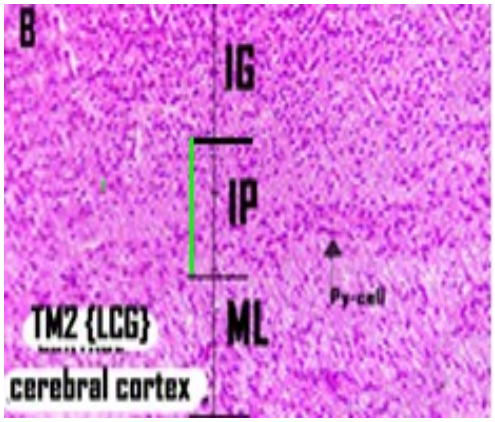


D: <u>HCG</u>: showing the highest reduction in the outer cortical thickness and cellular densities in the outer cortical histological layers at **TM_2, HCG** (mag x40) as depicted by OG layer. In addition, all the 6 cerebral cortical layers are visible and distributed cells.

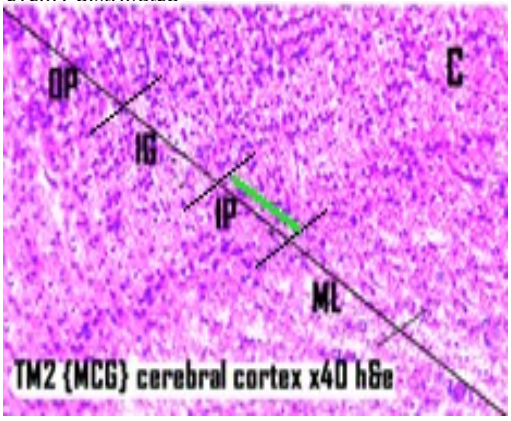
Figure 4.6: The (TM_2) comparative thickness of <u>the outer</u> cortical layers, plus their cellular densities and distribution: (a) control (b) LCG, (c) MCG, and (d) the HCG.



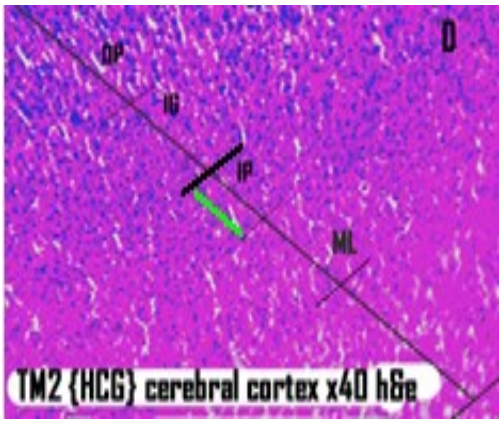
A: Control. Showing the normal inner thickness and cellular distribution in the inner cortical layers of the cerebral cortex included; (1G) Inner granular, (IP) Inner pyramidal and (ML) multiform layer in **Control group** (mag x40). The cells are also densely packed and evenly distributed



B: LCG: showing relatively reduction in thickness and cellular densities in the Inner histological layers of the cortex when at **TM_2, LCG** (mag x40). The cells are also relatively sparse as compared with those of the control

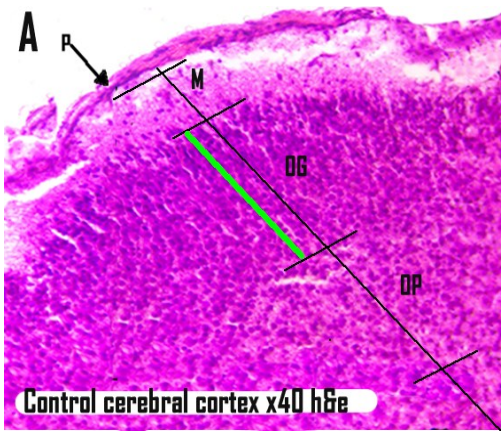


C MCG: showing further reduction and sparse distribution of cells in the inner histological cortical layers at **TM_2,** (mag x40). The cells are becoming fewer and sparsely distributed

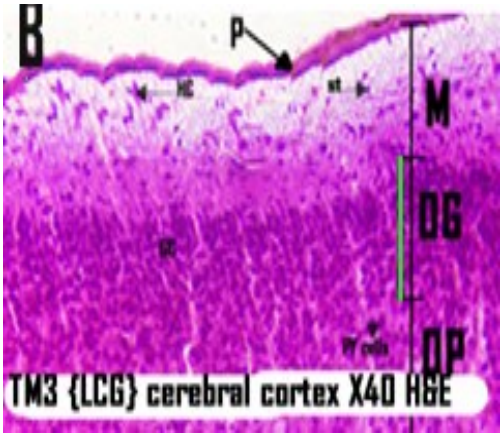


D: HCG: Shows the least reduction and sparse distribution of cells in the inner histological layers of the cerebral cortex at **TM_2,** (mag x40) and sparsely distributed cells

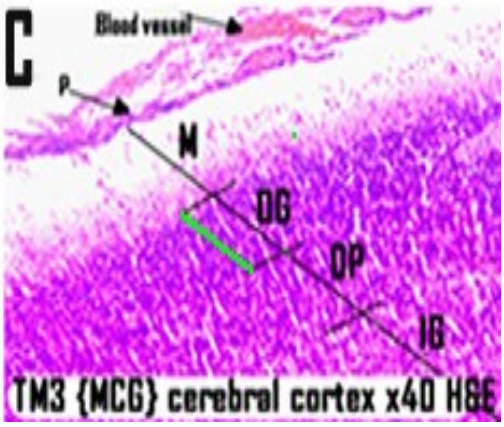
Figure 4.7: The TM_2 comparative thicknesses of the inner cerebral cortical layers with their cells distribution: (a) control (b) LCG, (c) MCG, and (d) the HCG.



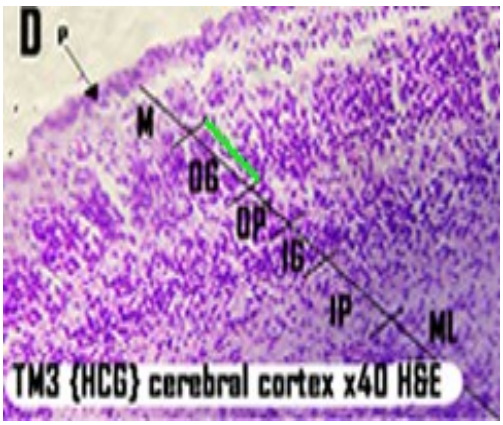
A: Control: Normal thickness of the outer three cerebral cortical layers; M-molecular, OG-outer granular and OP-outer pyramidal layers (mag x40). The densely packed outer cerebral cortical layers for control group.



B: LCG: no much noticeable differences in the outer Inner cortical histological layers as depicted by the IP layer (green line) at **TM_3, LCG** (mag x40).Cells are slightly relatively sparse in the axonal plexus.

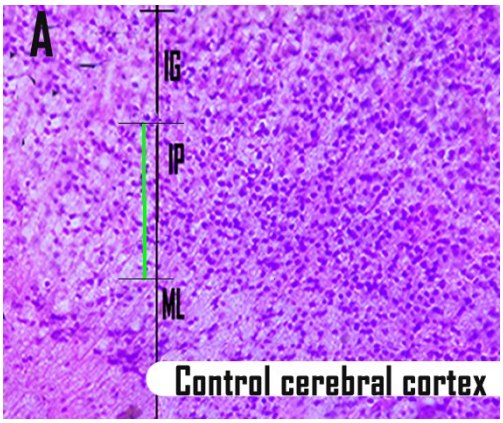


C: MCG: Further slight reduction in the thickness of outer histological layers at **TM_3, in MCG** (magnification x40)

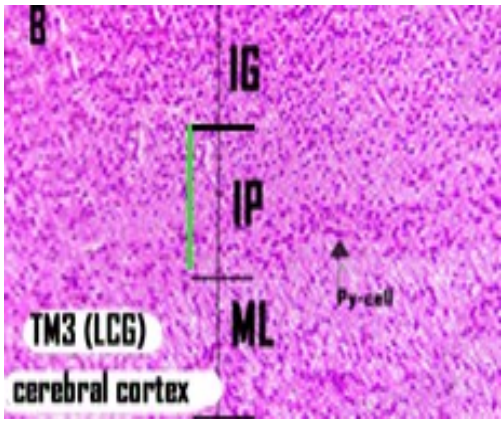


D: HCG: The most reduced outer cortical histological layers at **TM_3, HCG** depicted by OG layer. In addition, all the 6 cerebral cortical layers are visible and reduced with sparsely distributed cells (mag x40).

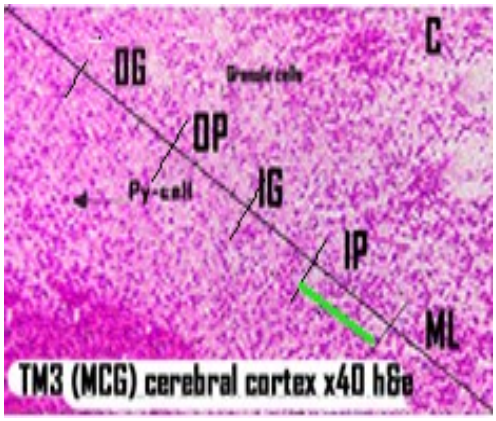
Figure 4.8: The (TM_3) comparative thickness of the outer cerebral cortical layers and their cellular densities/distribution; (a) control (b) LCG, (c) MCG, and (d) the HCG



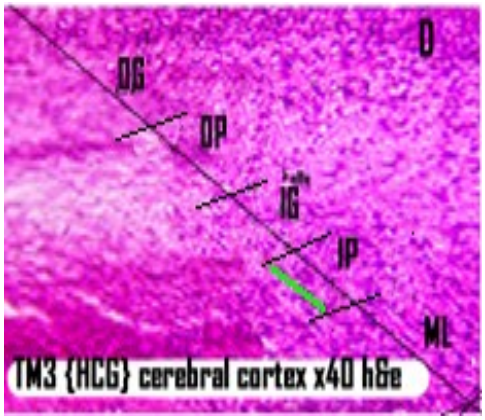
A: Control: showing the normal cortical thickness of inner three histological layers namely; (1G) Inner granular, (IP) Inner pyramidal and (ML) multiform layer (mag x40).



B: LCG: showing no reduction in thickness in the inner cortical histological layers at **TM_3**, (mag x40). The cells are also relatively sparse as compared with those of the control



C: MCG: showing slight reduction on the inner histological cortical layers at **TM_2, MCG** as depicted by the IP layer (green line) (mag x40). OG and OP layers can be observed as a result of thinness of layers as well as sparse and disorganized cells

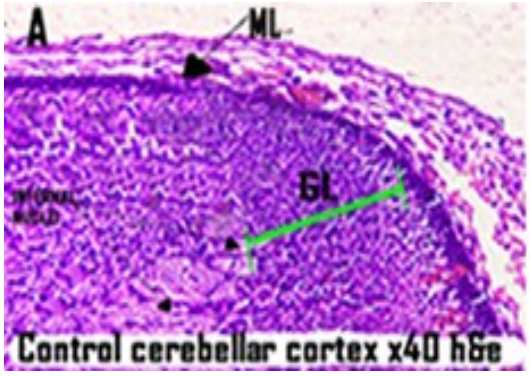


D: HCG: showing marked reduction in thickness of the **i**nner histological cortical layers at **TM_3, HCG** (mag x40). The 6 cortical are visible due to the thinness of layers. The cells are the most disorganized and sparsely distributed

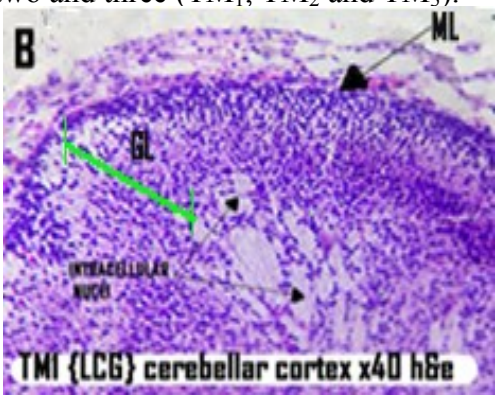
Figure 4.9: The TM_3 comparative thickness in the inner cerebral cortical layers with their cellular densities in (a) the control (b) LCG, (c) MCG, and (d) HCG.

4.2.2: The influence of carbamazepine on the development of the cerebellum

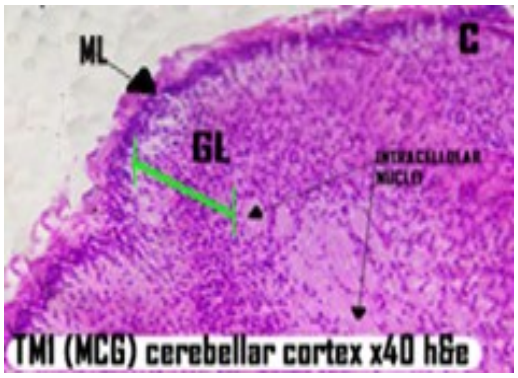
The histological examination of the cerebellum did not show much significant differences in thicknesses of cerebellar cortices but rather it affected the distribution of cells in the cerebellar cortical layers (figure 4.10, 4.11, and 4.12) respectively when the treatments were done in trimester one, two and three (TM_1, TM_2 and TM_3).



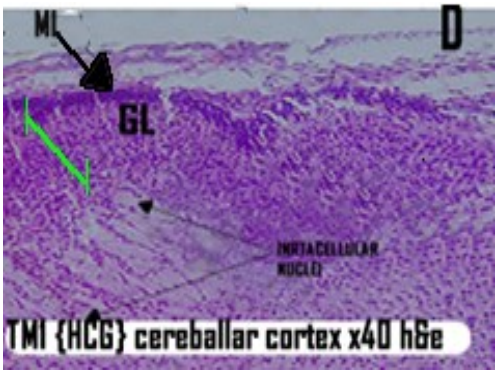
A: control: showing the normal thickness and cellular distribution in the cerebellar cortex i.e. the Molecular layer (M)**; and the** Granule layer (GL**)**. The plexiform layer is not fully developed hence invisible (mag x40)



B: <u>LCG</u>: Showing slight reduction in cerebellar cellular densities in the Molecular layer (M)**;** and the Granule layer (GL**)**. The plexiform layer is not fully developed hence invisible (mag x40)

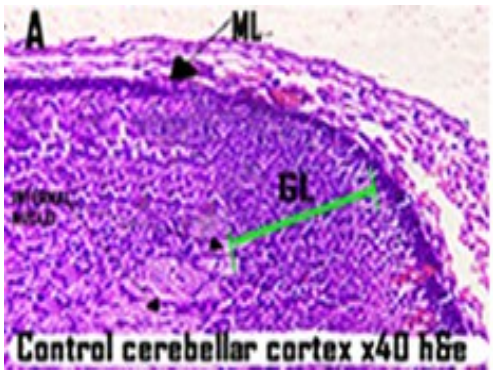


C: <u>MCG:</u> showing further reduction of cellular densities in the Molecular layer (M)**;** and the Granule layer (GL**)**. The plexiform layer is not fully developed hence invisible (mag x40)

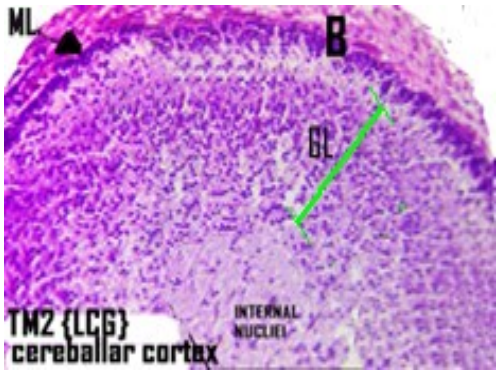


D: <u>HCG:</u> showing a sparse distribution of cells in the Molecular layer (M)**;** and the Granule layer (GL**)**. The plexiform layer is not fully developed hence invisible (mag x40)

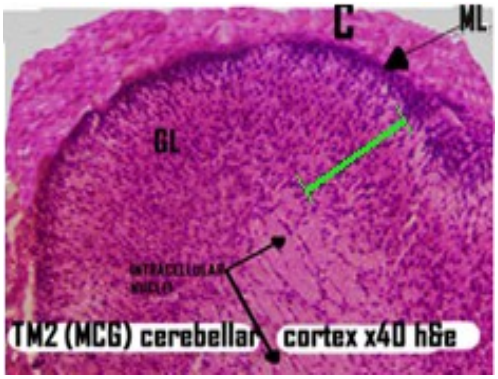
Figure 4.10: Showing the trimester one (TM_1) comparative cells distribution in the cerebellar histological cortices in: (a) the control (b) LCG, (c) MCG, and (d) HCG



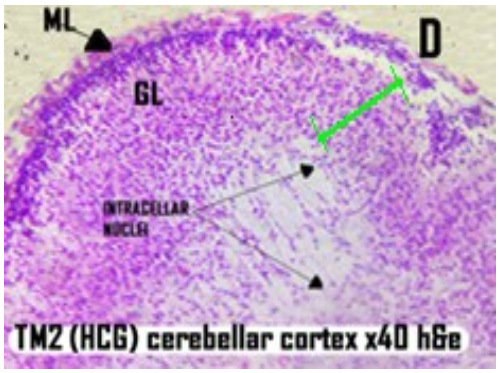
A: Control: showing the normal thickness and distribution of cells i.e. the Molecular layer-(M)**; and the** Granule layer-(GL**)**. The plexiform layer is not fully developed hence invisible *mag X40*



B: <u>**LCG**</u>: showing some slight reduction in cellular densities in the cerebellar histological layers at **TM_2 i.e. the** Molecular layer-(M)**;** Granular layer (GL**)**. The plexiform layer is not fully developed hence invisible (Mag x40)

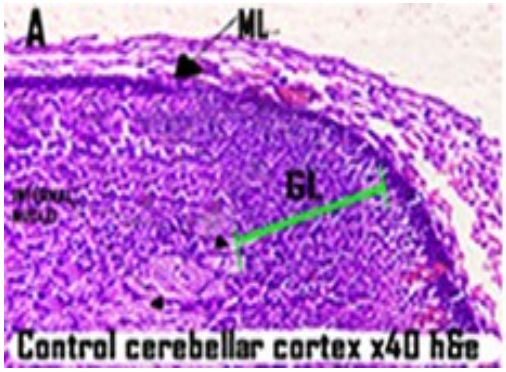


C: MCG: showing reduction in cellular densities in the cerebellar cortical layers at **TM_2 i.e.** molecular layer-(M)**;** Granule layer-(GL**)**. The plexiform layer is not fully developed hence invisible

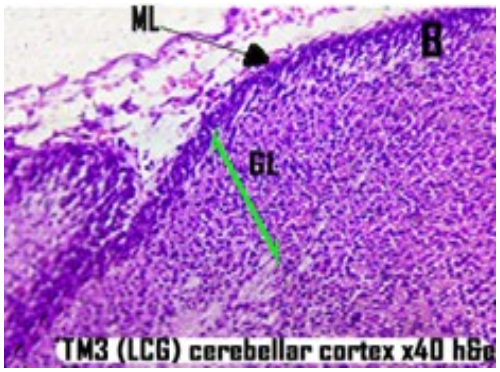


D: HCG: showing the sparsest distribution of cells in the cerebellar cortex at **TM_2 i.e.** molecular layer-(M)**;** Granule layer-(GL**)**. The plexiform layer is not fully developed hence invisible

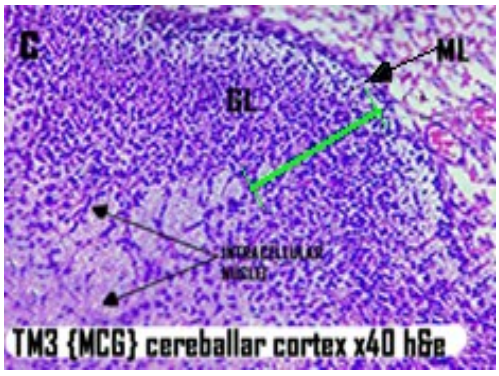
Figure 4.11: The TM2 comparative cellular distribution in the cerebellar cortices in:(a) Control; (b) LCG; (c) MCG; and (d) HC



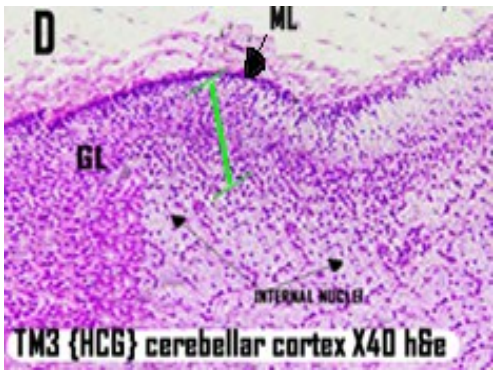
A: control: Normal distribution of cells in the cerebellar cortical layers (mag x40) with normal cellular densities of: (M) Molecular layer**;** (GL**)** Granule layer. The plexiform layer is not fully developed hence invisible



B: LCG: showing no difference in cellular densities and cellular distribution in the cerebellar cortices at TM_3 compared with control (a)



C: MCG: showing no differences in cellular distribution in the cerebellar cortical layers at **TM_3. Mag x40.**



D: HCG: showing so slight reduction in the cerebellar cortical layers in TM3 **(mag x40)**

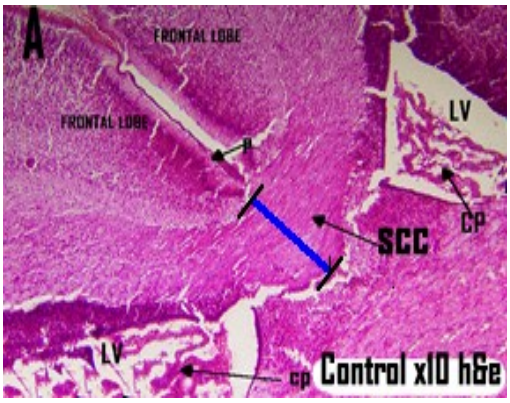
Figure 4.12: TM3 comparative cellular distribution in the histological layers of the cerebellar cortices in (a) the control; (b) LCG; (c) MCG; and (d) HCG

4.2.3 Influence of carbamazepine on the development of the corpus callosum

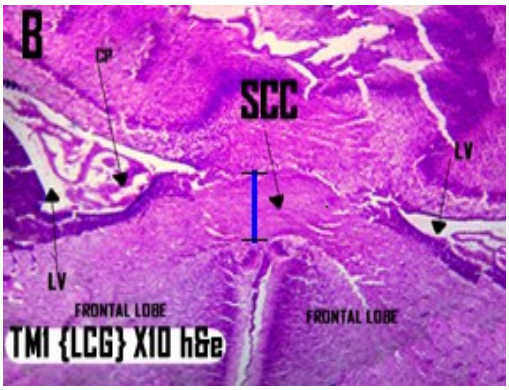
The prenatal exposure to carbamazepine was histologically seen to perturb the differentiation of the fibers of corpus callosum particularly those of the splenium. These perturbations were seen to range from a significant reduction in the corpus callosal thickness and disaggregation of the commissural fibers particularly those of the splenium. This disaggregation and reduction in thickness was seen to be dependent on the dose of exposure rather than on gestation stage when carbamazepine was administered in trimester one(**TM_1**) and two(**TM_2**) the pattern on the effects in the organization (thickness and aggregation of the callosal fibres) were seen to depict similar morphological appearance in both the TM_1 and TM_2 photomicrographs across all the carbamazepine treated groups (LCG,MCG and HCG) when compared with the control group **(**figures 4.13 and figure 4.14 respectively).

When the carbamazepine was administered in the third trimester (**TM_3**), there was no difference in the morphological appearance of the commissural fibers nor the thickness of the corpus callosum at the level of the splenium between the LCG and the MCG as compared with the control group. It is only in the HCG that slight disturbances in the arrangement and thickness of the corpus callosum compared with the control group (f**igure 4.15)**.

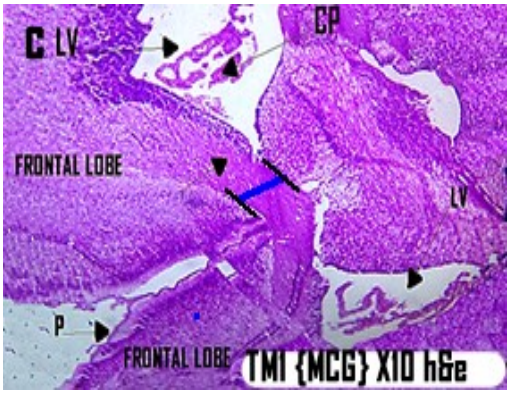
From these observations it was clear that carbamazepine teratogenic effects on the development of corpus callosum was at high dosages (**MCG** and **HCG**). These two critical teratogenic doses of carbamazepine exerted their morphogenetic inhibitory effects to the differentiation of the corpus callosal commissural fibres in the first and in the second trimester (**TM_1 and TM_2**).



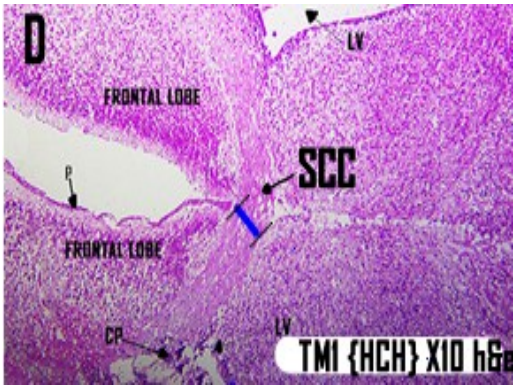
A: control: showing the normal thickness a well-developed fibers of the splenium indicated with a blue line (mag x40)



B: **LCG:** showing reduction and slight disaggregation of the fibers of splenium of the corpus callosum (SCC) at **TM_1 (LCG)** as indicated by a (blue line) (mag x40)

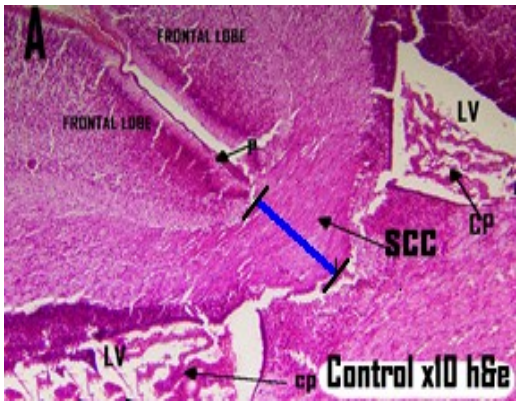


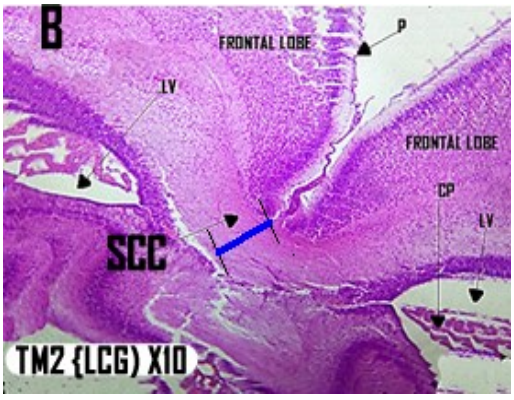
C: MCG: showing further reduction in thickness of the of splenium of the corpus callosum (SCC) at **TM_1 (MCG)** as shown by the (blue line) and reduction in commissural fibers (mag x40)



D: HCG: showing the most significant reduction in thickness of the splenium of the corpus callosum (SCC) at **TM_1** and high disaggregation the fibers (blue line) (mag x40)

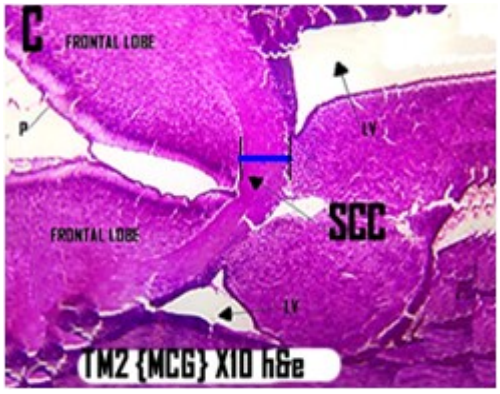
Figure 4.13: The TM1 comparative thicknesses and organization of the commissural fibres of corpus callosum (Splenium) in: (a) the control; (b) LCG; (c) CG; and (d) HCG

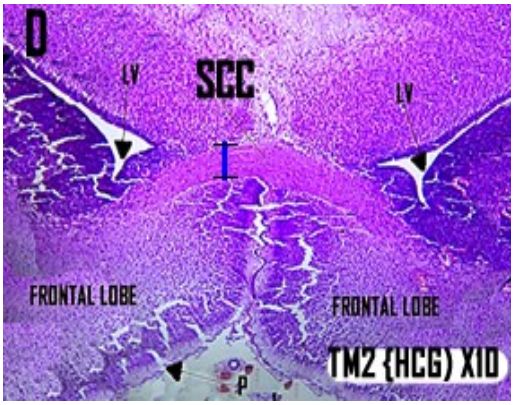




A: control: showing the normal thickness and normal organization of the commissural fibres of splenium of the corpus callosum (SCC) indicated by a blue line, (mag x10)

B: LCG: showing marked disorganization and reduced thickness of the commissural fibres of splenium of the corpus callosum (SCC) at **TM_2** (blue line) - (mag x10)

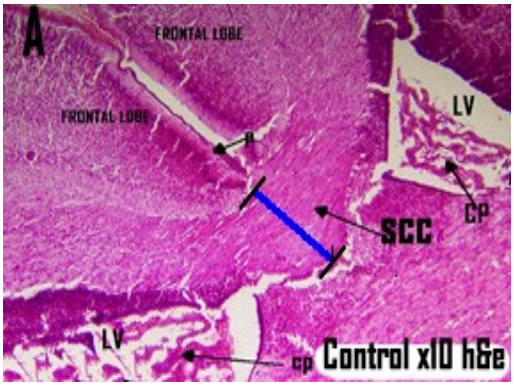




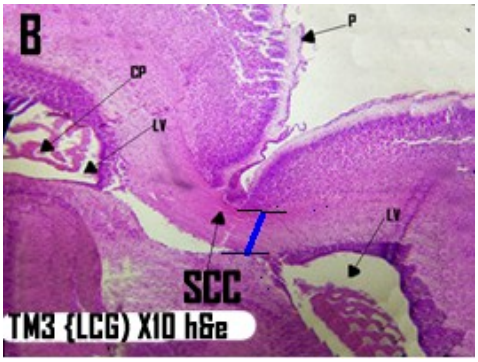
C: MCG: showing further reduction in thickness of the fibres of splenium of the corpus callosum (SCC) at **TM_2** (blue line) (mag x10)

D: HCG: showing the thinnest layer of the fibers of the corpus callosum at the Splenium (SCC) and its disorganization of its fibers at **TM_2** (blue line) with (mag x10)

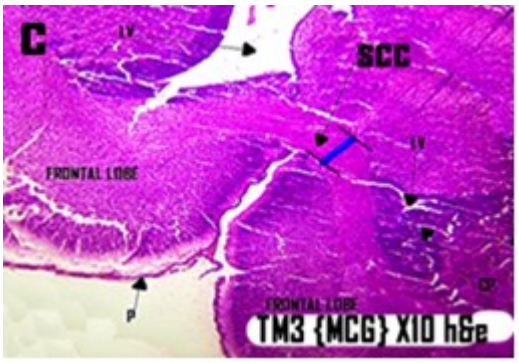
Figure 4.14: The TM_2 comparative thicknesses and organization of the commissural fibres of corpus callosum (Splenium) in: - (a) the control; (b) LCG; (c) MCG; and (d) HCG



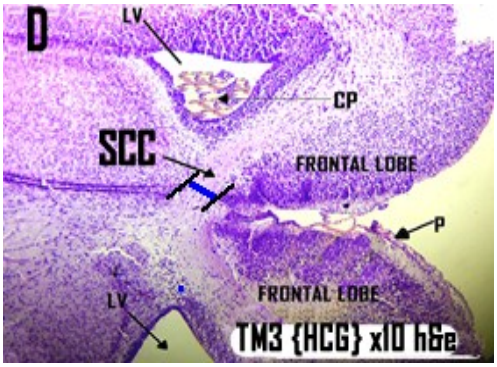
A: Control: The normal thickness and normal organization of the fibres of splenium of the corpus callosum (SCC) of the control fetal brain (blue line, mag x10)



B: <u>LCG:</u> showing reduction in thickness and disorganization of the fibres of corpus callosum (SCC) at **TM_3 (LCG)** as indicated by a (blue line) and when compared with the control (mag x10)



C: <u>MCG</u>: further showing reduction in thickness and in disorganization of the commissural fibers of the splenium of the corpus callosum (SCC) at **TM_3** (mag x10)

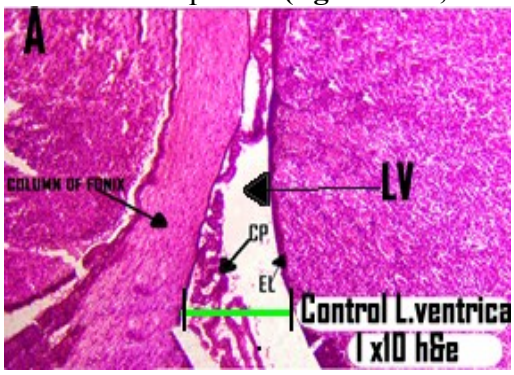


D: HCG: .showing the greatest marked disorganization of the fibres of corpus callosum (SCC) at **TM_3 (HCG)** and reduction in thickness (mag x10)

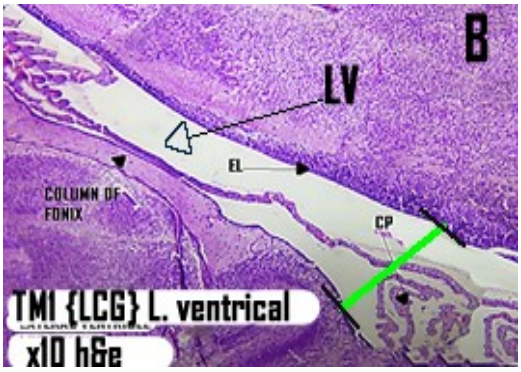
Figure 4.15: The TM_3 comparative thicknesses and organization of the commissural Fibres of corpus callosum (Splenium) in: (a) the control; (b) LCG; (c) MCG; and (d) HCG

4.2.4: Influence of carbamazepine on the development of the ventricular system

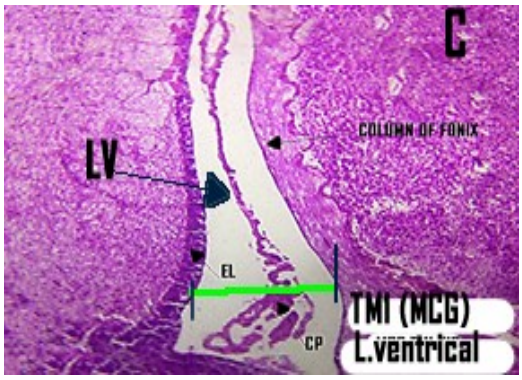
The histological examination of the ventricular system following in-utero exposure to varied doses of carbamazepine at **TM_1, TM_2 and TM_3** revealed that carbamazepine caused ventricular hypertrophy, disaggregation and inhibitory differentiation of the cholloid plexuses inclined more to the dose of exposure than to the time of exposure **(figure 4.16, 4.17 and 4.18 respectively)**



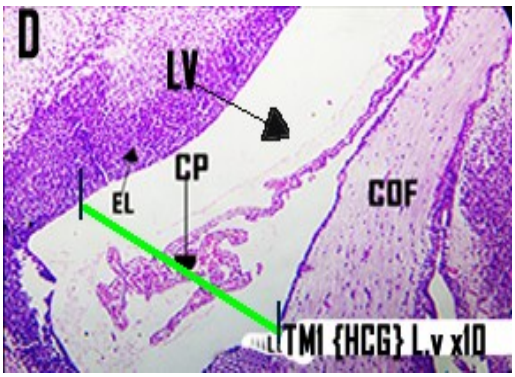
A: **control:** Normal thickness of lateral ventricle (as indicated by green line), well-organized choroid plexus (CP) (mag x10)



B: **LCG**: showing an enlarged lateral ventricle at **TM_1 (LCG)**, shown by green line, with clustered mal-developed and disjointed choroid plexus (CP) mag x10

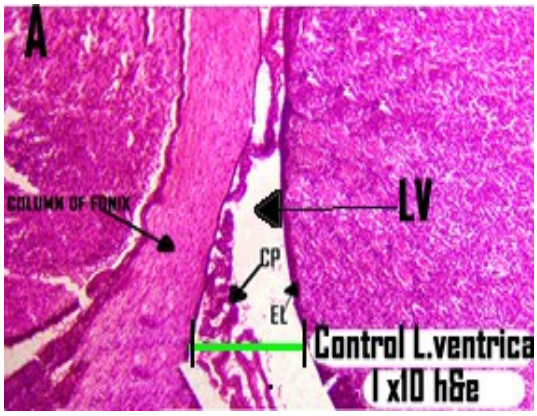


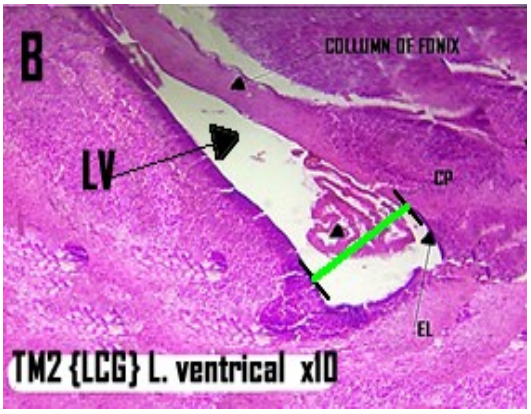
C: **MCG:** showing further hypertrofication of the lateral ventricle as shown by green line at **TM_1 (MCG)** declustred and further mal-developed choroid plexus (CP) (mag x10).



D: **HCG**: showing the most enlarged lateral ventricle as shown by the green line at **TM_1 (HCG)** and the most declustered mal-developed choroid plexus (mag x10).

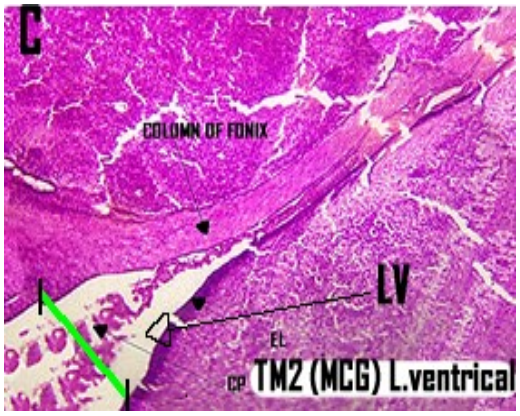
Figure 4.16: The TM_1 comparative thickness and organization of the ventricular system in: - (a) the control; (b) LCG; (c) MCG; and (d) HCG

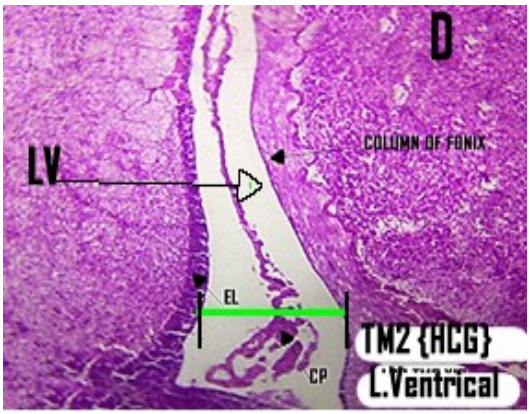




A: **Control:** showing the normal thickness ventricle (as indicated by green line), well- choroid plexus (CP) as well as a ne ependymal cells of the Control fetal brain (m

B: LCG Enlarged lateral ventricle at TM_2 (LCG) showing clustered mal-developed choroid plexus (CP) and disjointed ependymal layer fetal brain MAG x10 H&E)

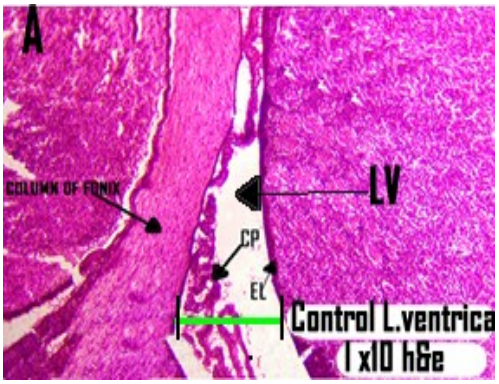




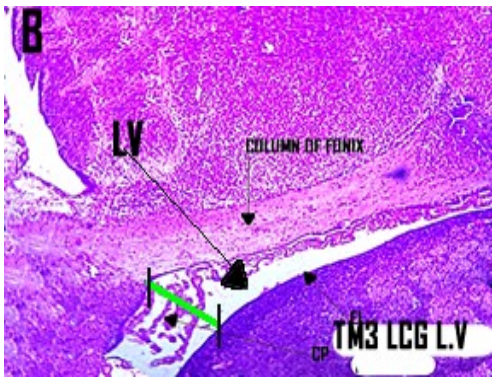
C: **MCG:** showing marked enlargement of the lateral ventricle at TM_2 (MCG) showing clustered further mal-developed choroid plexus (CP) and disjointed ependymal layer (mg x10).

D: **HCG: showing the highest** enlargement of the lateral ventricle at TM_2 (HCG) showing *most* clustered mal-developed choroid plexus (CP) and disjointed ependymal layer (mag x10).

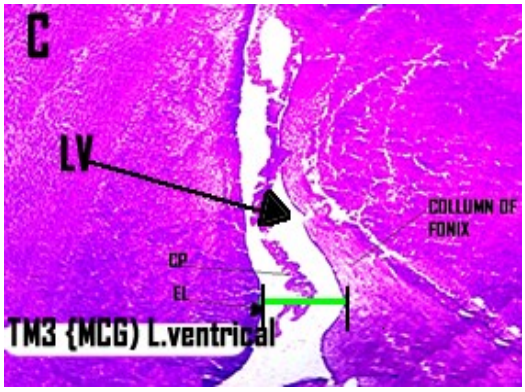
Figure 4.17 : The TM_2 comparative thickness and organization of the ventricular system in: - (a) the control; (b) LCG; (c) MCG; and (d) HCG



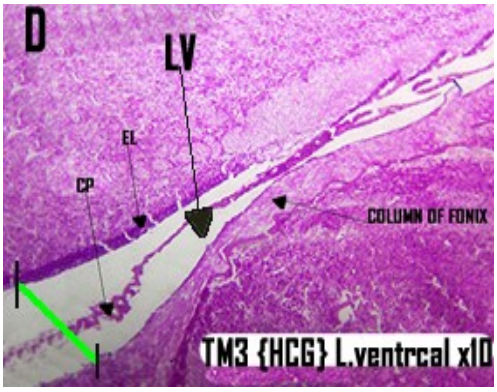
A: **Control:** Normal thickness of lateral ventricle (as indicated by green line), well-organized choroid plexus (CP) as well as a network of ependymal cells of the Control fetal brain (mag x40)



B: LCG: Photomicrograph B: disaggregated choroid plexus in lateral ventricle at TM_3 (LCG) showing clustered mal-developed choroid plexus (CP) and disjointed ependymal layer



C: **MCG:** More disaggregated choroid plexus in the lateral ventricle at TM_3 (MCG) showing clustered further mal-developed choroid plexus (CP) and disjointed ependymal layer.



D: **HCG:** showing ventricular hypertrophy in the lateral ventricle at TM_3 (HCG) showing *most* clustered mal-developed choroid plexus (CP) and disjointed ependymal layer.

Figure 4.18: The TM_3 comparative appearance and organization of the lateral ventricular system in: - (a) the control; (b) LCG; (c) MCG; and (d) HCG

4.3 Objective 3; Stereological Findings on the Fetal Brain

The stereological parameters evaluated and reported in this study included morphometric measurements on total fetal brains weights, brain length and width, total brain volume by use of both water immersion method (WIM) and cavalieri method of point counting, volume densities of both cortical and sub-cortical layers of the fetal brain stractures.

4.3.1 Influence of carbamazepine on the fetal brain size, weight, length and width

The comparative gross appearance of the fetal brain from the carbamazepine treated groups showed that the gross appearances of fetal brains from the experimental groups looked relatively small in size with poorly defined lobes, sulci and gyri when compared with the control group. Similarly, when the intra and the intergroup fetal brain weights comparisons were done for the experimental groups, there was a marked intra-group and inter-group variances in the total gross weights and brain sizes based on the dose of exposure and the time of exposure. For instance, it was observed that when carbamazepine treatment was done at $\mathbf{TM_1}$, the mean total brain weight (in grams) was found to be lowest in HCG group at 0.12±0.012 followed by MCG at 0.216±0.008 and LCG at 0.297±0.0033. When carbamazepine was administered in $\mathbf{TM_2}$, the mean totals of the fetal brain weight in grams was found to be 0.247±0.0071 in the HCG at followed by MCG at 0.300±0.000, then LCG at 0.347±0.0059 as compared with control group (P=0.001). When treatment was done at $\mathbf{TM_3}$, the mean brain weight for the HCG group in millimeters was 0.33±0.0028, followed by MCG at 0.370±0.008 and LCG at 0.394±0.0051. The values were found to be statistically significant (p=0.002) when the comparisons were done within and across groups and when compared with the control group **(table 4.8).**

For the mean brain length and width (in centimeters), a similar scenario was observed. Mean brain length at $\mathbf{TM_1}$ HCG group was at 1.02±0.012 followed by MCG at 1.12±0.008 and LCG at 1.197±0.0033. This was found to be statistically lower as compared with the control group (p=0.001) at 1.295±0.005. At $\mathbf{TM_2}$, brain

length was found to be lowest in HCG at 1.285±0.1433 followed by MCG at 1.2000±0.000, then LCG at 1.247±0.0033. This was not statistically different as compared with control group at 1.295±0.005 (P=0.783).At **TM_3**, the mean brain length was lowest at HCG group at 1.287±0.0018, followed by MCG at 1.31±0.0024 and LCG at 1.304±0.136. The brain width in millimeters were also seen to follow the same treads (**table 4.8**).

Table 4.8: Showing a comparative means fetal brain weight, brain length, and width for LCG, MCG and the HCG treated at TM_1, TM_2 and TM_3 against the control.

Study groups	Period of CBZ treatment	Mean brain weight(g) ± SEM	Mean brain length(mm) ± SEM	Mean brain width(mm) ± SEM
Control group	----------	0.395±0.005	1.295±0.005	1.097±0.003
Low dose carbamazepine group (LCG, 20.7mg/kg)	Trimester one (TM1)	0.297±0.0033 ^{bc}*	1.197±0.0033 ^{b}*	0.997±0.0033 ^{b}*
	Trimester two (TM2)	0.347±0.0059 ^{bc}*	1.247±0.0033 ^{bc}*	1.0711±0.0066 ^{b}*
	Trimester three (TM3)	0.394±0.0051	1.304±0.136	1.09±0.009
Medium dose carbamazepine group (MCG, 72.3mg/kg)	Trimester one (TM1)	0.216±0.008 ^{bc}*	1.12±0.008^{b}*	0.916±0.0079 ^{b}*
	Trimester two (TM2)	0.300±0.000 ^{bc}*	1.2000±0.000^{bc}*	1.023±0.012 ^{b}*
	Trimester three (TM3)	0.370±0.008	1.31±0.0024	1.073±0.009
High dose carbamazepine group(HCG, 124mg/kg)	Trimester one (TM1)	0.12±0.012 ^{b}*	1.02±0.012 ^{bc}*	0.813±0.007 ^{b}*
	Trimester two (TM2)	0.247±0.0071 ^{bc}*	1.285±0.1433	0.948±0.0024 ^{b}*
	Trimester three (TM3)	0.33±0.0028	1.287±0.0018	1.02±0.00696 ^{b}*

Key: *All value that bear (*) as a superscript indicates that they depict statistical significance differences ($p<0.05$) when compared with the control. Values with (^{b}) &(^{c}) superscripts have a statistical significance difference ($p<0.05$) in the intragroup and intergroup comparisons respectively using one way ANOVA with Turkey post-hoc t-tests*

4.3.2 Influence of carbamazepine on the total fetal brain volume and volume densities

The reference and calculated mean total fetal brain volume as determined by use of water displacement method (WIM) and calculated by the calvarieri method was found to depict an inverse dose response relationship in that when the dose of exposure to carbamazepine increased the mean total brain volume had a corresponding decrease in total brain volume and *vice versa,* (**table 4.9**). On the other hand, when the total brain volume was compared with the time of exposure, it depicted a direct response relationship to the time of exposure in the when carbamazepine treatment was administered at different trimesters (**TM_1**, **TM_2 TM_3),** the brain volumes decreased directly with the time of exposure. For instance when the carbamazepine treatment was done at **TM_1** the total brain volume (mm^3) was lowest in the HCG at (0.220±0.001), followed by MCG at (0.231±0.001) and lastly LCG at (0.232±0.002). All the intra and intergroup comparisons were also found to be statistically significant (P=0.001) when compared with the control group (**table 4.9)**

Table 4.9: A Comparative reference, calculated and percentage shrinkage on total mean fetal brain volume using (WIM) and cavalieri method in the LCG, MCG and the HCG treated at TM_1, TM_2 and TM_3 against the control.

Study groups	Period of CBZ treatment	Mean total fetal brain volume (WIM) (mm^3) ± SEM	Mean total fetal brain volume (Cavalieri method) (mm^3) ± SEM	Mean shrinkage ((mm^3) ± SEM	Mean cortical volume density((mm^3) ± SEM	Mean sub-cortical volume density((mm^3) ± SEM
Control group	----------	0.248±0.002	0.244±0.001	0.017±0.001	0.073±0.000	0.171±0.001
Low dose carbamazepine group (LCG, 20.7mg/kg)	TM1	0.233±0.002 ^{b*}	0.232±0.001 ^{b*}	0.015±0.005 ^{bc}	0.070±0.000 ^{b*}	0.162±0.001 ^{bc*}
	TM2	0.239±0.001 ^{b*}	0.235±0.002 ^{b*}	0.016±0.001 ^{bc}	0.071±0.000	0.162±0.001
	TM3	0.247±0.002	0.243±0.001 ^{b*}	0.244±0.005 ^{bc}	0.073±0.000	0.171±0.001
Medium dose carbamazepine group (MCG, 72.3mg/kg)	TM1	0.232±0.001 ^{b*}	0.231±0.001 ^{b*}	0.013±0.001 ^{bc}	0.069±0.000 ^{b*}	0.161±0.001 ^{bc*}
	TM2	0.238±0.001 ^{b*}	0.233±0.002 ^{b*}	0.019±0.006 ^{bc}	0.070±0.001 ^{b*}	0.162±0.001 ^{bc*}
	TM3	0.242±0.000 ^{c*}	0.239±0.001 ^{b*}	0.244±0.006 ^{bc}	0.072±0.000 ^{b*}	0.170±0.001 ^{bc*}
High dose carbamazepine group(HCG, 124mg/kg)	TM1	0.222±0.001 ^{b*}	0.220±0.001 ^{bc*}	0.009±0.003 ^{bc}	0.066±0.002 ^{b*}	0.154±0.001 ^{bc*}
	TM2	0.233±0.002 ^{b*}	0.230±0.002 ^{bc*}	0.244±0.004 ^{bc}	0.069±0.000 ^{b*}	0.154±0.001 ^{bc*}
	TM3	0.242±0.002 ^{c*}	0.236±0.001 ^{bc*}	0.244±0.004 ^{bc}	0.071±0.000 ^{b*}	0.167±0.001 ^{bc*}

Key: *All value that bear (*) as a superscript indicates that they depict statistical significance differences ($p<0.05$) when compared with the control. Values with (^{b}) & (^{b}) superscripts have a statistical significance difference ($p<0.05$) in the intragroup and intergroup comparisons respectively using one way ANOVA with Turkey post-hoc t-tests*

4.4 Objective 4; Findings Of Carbamazepine Teratogenicity On Fetal Brain On Time Of Administration And In Relation To Dosages

The current study has established that teratogenic effects of in-utero administration of carbamazepine had a direct correlation with the dosage and the time of exposure as illustrated when the intra and inter group comparisons were done in the maternal pregnancy outcomes, histomophological results as well as the quantitative results. It was established that when carbamazepine was administered in the first and the second trimester across all the experimental groups i.e. low carbamazepine group

(20.7mg/kg), medium carbamazepine group (72.3mg/kg) as well as the high carbamazepine group (124mg/kg), both the qualitative (i.e. the histomorphological) as well as the quantitave stereological findings were statistically significant ($P<0.05$) as compared with the control. The exposure in the third trimester did not show any significant teratogenic effects ($P>0.05$) except at high dosages.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMEDATIONS

5.1 Influence of carbamazepine on the maternal and fetal pregnancy outcomes

The current findings on the maternal and fetal pregnancy outcomes clearly demonstrates that the maternal and fetal pregnancy outcomes are an important pointer to the kind of perturbations caused in the development of the fetal organs like the brain. This study established that there is a strong correlation ($P>0.5$) between the maternal and fetal pregnancy outcomes with the overall gross, histo-morphological and stereological teratogenic outcomes to the developing fetal brain **(table 4.4).** This corroborates with study recommendations by Aberg *et al.,* (2013), who suggested a holistic approach in conducting teratogenic studies needed to be adopted always as teratogenesis is a factor of a cascade of events that happen *in-utero* and not a stand-alone event.

For instance, in this study, it was established that any significant reduction in the mean maternal weight gain had a subsequent significant reduction ($P<0.05$) in all pregnancy growth parameters including median litter sizes, percentage embryolethality, mean numbers of the resorbed endometrial glands, mean placenta weight, types and numbers of congenital abnormalities, mean fetal weight, mean bi-parietal diameters, mean head lengths and head circumferences, mean crown-rump length, mean brain weight, brain length and width in all the carbamazepine treated groups (LCG,MAG and HCG).These perturbations on these stated parameters were found to directly impact negatively to the fetal brain development including; significant reduction in mean fetal gross brain weights, total brain volumes, brain sizes, reduction in the cortical and subcortical thicknesses, as well as in all the histo-stereological parameters as was also observed in a study (Agarwal *et al*., 2010).

The findings of the maternal and fetal pregnancy out comes were also seen to depict a dose and period of gestation dependent relationship in that when high doses of carbamazepine that included 124mg/kg/bw for HCG and 72.3mg/kg/bw for MCG in the first and second trimesters (TM_1 and TM_2) there was a marked increase in embryolethality, increased rates of spontaneous abortions as well as intra-uterine growth retardations of the fetuses as previously reported (Ayano *et al.*, 2016; Verez-luiz *et al.,* 2015). The current study findings observed the teratogenic effects to the pregnancy outcomes were more in high carbamazepine dosages. A study by Salehnia *et al,* (2013) established that high doses of oxycarbazine had adverse effects on corpus luteum of female rats that plays an important role in reproduction as it progesterone and 20-hydroxy progesterone that maintains in-utero fetal growth and development. The results in this study also concurs with another study that associated high carbamazepine dosages with reduced number of corpus luteum and increased cases of devoured embryo implantations (González-Orozco *et al.,* 2019; Naderifar *et al.,* (2015).

The current study also demonstrated that high carbamazepine dosages administered in trimester one and trimester two leads to increased rate of congenital defects **(table 4.3)** including spina-bifida, anencephaly, cleft lip among others. These anomalies were found to be significantly higher (P=0.001) in the high (HCG) and medium carbamazepine groups (MCG) when compared with the control group (table 4.3). The findings of this study are in tandem with studies by Wlodarczyk *et al.,* (2012) and Hamadi *et al.,* (2017) who demonstrated external congenital malformations related to high dose antiepileptic administration. Low carbamazepine dosage administered in the current study did not result in a wide range of congenital anomalies. Similar results were reported by another study findings by Bank *et al.,* (2017), whose findings depicted that low anticonvulsant doses during pregnancy does not result into significant alteration in the external developmental measures in the morphological parameters of the fetuses as well as congenital malformation.

The mechanism of carbamazepine interference with the overall fetal development *in-utero* is thought to be associated with its anti-proliferative inhibitory effects of on fetal tissues that suppresses the mitotic index and causes persistent block of the boundary between metaphase and anaphase stages of cells, leading to growth retardation manifested by reduction in weight and length of the fetus including mal-development of associated fetal viscera as well as the developing nervous system that includes the brain (El- gaafarawi *et al.,* 2015; Belguis *et al.,* 2007).

5.2 Influence of carbamazepine on the histomophology of the developing fetal brain

The current findings on the teratogenic effects of carbamazepine on the histomorphological layers of both the cortical and subcortical structures of the fetal brain have demonstrated that carbamazepine suppresses the differentiation and development of the neuroblast cells as well as the neuroglial cells of the brain. This was clearly demonstrated by the marked reduction in thicknesses of all the six cerebral cortical layer that includes (i) Molecular layer (ii) Outer granular layer (iii) Outer pyramidal layer (iv) Inner granular layer (v) Inner pyramidal layer (vi) Multiform layer in that order from outside to the inside.

The findings of this study corroborate with findings of other studies by Agarwal, *et al., (*2010) ; Ahmed, (2017) who observed that cortical layers of the developing brain can be suppressed following the suppressive inhibitory effects of anticonvulsants. This study observed that the suppression to the cortical layers thickness was variably seen to differ based on their differentiation based on the dose of carbamazepine exposure as wells as with the time of exposure. For instance, the study established that the cortical thickness was a factor of the accompanying cell distribution and cellular densities in each of the three outer cortical layers that were seen to reduce appreciably among all the carbamazepine treated groups (LCG,MCG, HCG) when treatment was done in trimester 1(TM_1) and trimester two TM_2 **(figure 4.4 and 4.6).** Similarly, this was also replicated in the inner cortical layers that reduced with increase with the dose of exposure **(figure 4.5 and figure 4.7).**

The study also established that when the treatment was done at TM_3, there was no marked significance difference in both the outer and the inner cortical layer thicknesses as well as in cellular densities between the carbamazepine treated groups against the control **(figure 4.8 and figure 4.9).** These findings are in line with another study by Gedzelman & Meador (2012) who also reported reduction in all cortical layers of the brain.

This study found that carbamazepine causes histological alterations to the thickness and cellular distribution and cellular densities in both the cerebral and cerebellar cortices following in-utero exposure to varying doses of carbamazepine were as well found to be dose and time dependent. For instance, it was established that the effects were seen more in the high, medium carbamazepine while the low carbamazepine groups did not show much significant differences with the control particularly when administered in the third trimester (TM_3). These findings are in line with a previous animal study by Gedzelman & Meador, (2012) who reported that early exposure to anticonvulsants at TM_1 and TM_2 in habited mechanisms that result into altered neuronal proliferation and migration, synaptogenesis, and apoptosis, processes that are vital for normal neural development leading to cortical dysplasia.

Another study by Ayano G, (2016), associated effects on developing neuronal structures to the effects leading to the induction of apoptosis, especially in neural tube cells, and production of the free radicals such as epoxide during metabolism a hypothesis supported by a study by Badawy *et al.,* (2019) who reported that gabapentin, an anticonvulsant for seizure management showed a highly significant decrease in brain weight, alteration of the cerebral cortex and hippocampus cellular layers, vacuolated neuropil and massive cell degeneration with cavity formation in the brain tissue.

In the cerebellum, there was marked reduction in cellular densities and disappearance of intermediate zone with basket cells and this could be attributed to two factors including a decrease in proliferation of brain cells and induction of cell death in the cerebellum of fetuses treated with high doses of carbamazepine. Probably, this

resulted in neuronal apoptosis during late gestational period. A study by Abd *et al.,* (2015) reported that cell necrosis detected in different zones of both cerebellar and cerebral cortex causes narrowing and irregularity of the cortices as well as in the lateral ventricle. Similarly, a study by Sah *et al.,* (2013) reported that antiepileptic drugs like carbamazepine were found to hinder the endogenous neuroprotective system in the brain that is crucial for neuronal survival during development. This could have been the case in the present study since the microscopic examination of fetal brain tissue samples from untreated mother apparently showed normal histological structure of different parts of cerebellar cortex with densely backed cells without distinct layers.

This current study also observed that in all the experimental groups including the high (HCG), medium MCG) and low (LCG) carbamazepine dosages administered in the first, second trimesters (TM_1 and TM_2) resulted into significant dilatation of the ventricles as well as reduction in thickness of the fibers of the splenium of corpus callosum, **(figures 4.13-4.15).** These findings were in tandem with those from a previous study on histomophological effects of lamotrigine on fetal brain that revealed that in the treated group, the lateral ventricles were dilated and the plexiform layer of the cerebral cortex was relatively less differentiated (Sah *et al*., 2013). This was attributed to several histopathological alterations including pyknotic and degenerated neurons, fibrin deposition (fibrosis), disorganization of the cerebral cortex, dilated and enlarged blood vessels and dilated ventricles (Werler *et al.,* 2011 ; El-gaafarawi *et al.,* 2015).

5.3 Influence of carbamazepine on the Stereology of the developing fetal brain

This study established that the fetal brains from the carbamazepine treated groups had their gross appearances being relatively small in size with poorly defines lobes, sulci and gyri when compared with the control group. Similarly the calculated mean total fetal brain volume was also smaller and the variances in size and volumes were found to depict an inverse relationship with the dose administered **(table 4.8).** Further, the reduction in total brain volume and the volume densities of both the

cortical and the subcortical layers of both the cerebral and the cerebellar cortices were seen to depict a direct correlation with the time of exposure in that when carbamazepine treatment was administered at trimesters two **TM_2,** and three **TM_3** the effects were not as pronounced as in the first trimester **TM_1**.

The reduction in brain volumes were seen to decrease directly with the time of exposure in that when carbamazepine treatment was done at TM_1 the mean total brain volume (in mls) was lowest in the HCG at (0.222±0.001), followed by MCG at (0.232±0.001) and lastly LCG at (0.233±0.002), **(table 4.8).** These findings were in line with a study by (Berghuis *et al*, 2017) and another one by Bath & Scharfman, (2013).

The findings on the brain length and width were also seen to depict a similar scenario on dose and time response relationship with the doses of exposure and with time of exposure, **(table 4.7).** For instance, when a comparative mean brain length (in centimeters) was done across the three trimesters TM_1, TM_2 and TM_3, it was depicted that at TM_1 the mean fetal brain length was lowest in at HCG group at 1.02±0.012 followed by MCG at 1.12±0.008 and LCG at 1.197±0.0033. This was found to be statistically lower as compared with the control (P=0.003) at 1.295±0.005. At TM_2, brain length (in centimeters) was found to be lowest in HCG at 1.285±0.1433 followed by MCG at 1.2000±0.000, then LCG at 1.247±0.0033. This was not statistically different as compared with control group at 1.295±0.005 (P=0.64). At TM_3, the mean brain length (in centimeters) was lowest at HCG group at 1.287±0.0018, followed by MCG at 1.31±0.0024 and LCG at 1.304±0.136, (table 4.7).

Similarly, when the intra and the intergroup fetal brain weights comparisons were done for the experimental groups, there was marked intra-group and inter-group variances in the total gross weights and brain sizes based on the dose of exposure and the time of exposure. For instance, it was observed that when carbamazepine treatment was done at TM_1, the mean total brain weight (in grams) was found the lowest in at HCG group at 0.12±0.012gms followed by MCG at 0.216±0.008 and

LCG at 0.297±0.0033. When carbamazepine was administered in $TM_{2,}$ the mean totals of the fetal brain weight (in grams) was found to be 0.247±0.0071gms in HCG at followed by MCG at 0.300±0.000, then LCG at 0.347±0.0059 as compared with control (P=0.010).

When treatment was done at $TM_{3,}$ the mean brain weight (in grams) for the HCG group was 0.33±0.0028, followed by MCG at 0.370±0.008 and LCG at 0.394±0.0051. This values were found to be statistically significant (P=0.001), when the comparisons were done within and across groups and when compared with the control. The results of the present study are also in tandem with findings from studies by Erisgin *et al.,* (2019), Afshar *et al*., (2009) and Sah *et al.,* (2013) who reported that cortical alterations and the destructive changes especially of pyramidal cortical layer during fetal brain development (day 14 to day 19) are dose and time dependent.

5.4 Influence of carbamazepine on the dose and time of administration

The current study has established that teratogenic effects of in-utero administration of carbamazepine are time and dose dependent as shown by the histo-morphological and histo-stereological effects, with the most vulnerable period being the first trimester when organogenesis occurs as high carbamazepine dosages are administered at 124kg/kg. Matlow *et al*., (2010) indicated that the overall risk of all major congenital malformations after administration of antiepileptic medicine like phenytoin and valproic acid occurs during the first trimester of gestation period, when administered as monotherapy at high dosages. Other study findings by Armon *et al.,* (2019) indicated that high dosages of oxycarbazine administered during pregnancy are associated with increased risk of congenital malformations.

5.5 Conclusion

In conclusion this study has established that carbamazepine in the doses of **72.3 mg/kg/bw and 124 mg/kg/bw** during pregnancy are teratogenic to the developing fetal brain particularly when administered during the first (**TM_1**) and second trimester (**TM_2**), regardless of the dosage. Its teratogenic effect to the developing

brain when administered in trimester three has no significant outcomes except when administered in high doses. The most vulnerable gestation period for carbamazepine teratogenicity was however established to be the first trimester while the most critical dose was **124 mg/kg/bw.**

5.6 Recommendations

The study recommends that;

1. The of use Carbamazepine during pregnancy should be avoided as it has been shown to be teratogenic to the developing fetal brain particularly in first and second trimesters by seeking appropriate alternatives that are safer to the fetus.
2. Should expectant mothers be on chronic use of carbamazepine and the drug cannot be withdrawn because of associated withdrawal side effects to the mother, the doses should be adjusted to the minimal effective dosages that would confer the maximum maternal benefits and reduce the teratogenic risks to the developing fetal brain.
3. Further studies be carried out in non-human apes that have close phylogenetic relations to humans, to ascertain its teratogenicity in relation to doses.
4. Due to time and dose dependent teratogenic effects of carbamazepine, health care workers including clinicians, nurses, midwives and others, need to be educated on how they will need to be educating women of reproductive age and are on chronic usage of carbamazepine of its teratogenicity during pregnancy, on the need for early planning of their pregnancies for effective introduction of alternative medicines, to enable them avoid use of carbamazepine during pregnancy.

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APPENDICES

Appendix I: 1st Publication

IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS) e-ISSN: 2278-3008, p-ISSN: 2319-7676. Volume 14, Issue 3 Ser. III (May – June 2019), PP 26 www.Iosrjournals.Org DOI: 10.9790/3008-1403032634 www.iosrjournals.org 26 | Page

The Maternal Pregnancy Outcomes Following Prenatal Administration of Varied Doses of Carbamazepine in Albino rats (Rattus *norvegicus*)

Mwangi A. Wairimu$_{1*}$, Kweri J. Kariuki$_1$, Malik A. Nyabola$_1$, Thuo Reuben$_1$, Kafaya G. Kibe$_1$ *{Department of Human*

*Anatomy, School of Medicine (SOMED), College of Health Sciences (COHES) Jomo Kenyatta University of Agriculture and Technology (JKUAT) Kenya} *Corresponding Author Mwangi A. Wairimu*

Abstract: *Their-utero exposure to carbamazepine has been shown to perturb maternal digestion as well as cell and tissue metabolic processes when used during pregnancy. Literature has shown that it perturb the endogenous bioelectrical mechanisms and voltage gradients that guides maternal metabolic pathways of cells and tissues. Further, it also disturbs the fetal growth and development as it interferes with fetal patterning programs, cell division, cell positioning, and cell differentiation as it readily crosses the maternal placental barrier. Though, literature has shown an association between carbamazepine use and adverse pregnancy outcomes when applied in utero, the specific adverse effects on pregnancy outcomes including; maternal weight trends during the gestational period, the numbers of litter sizes, fetal resorptions, congenital malformation, and morphological features of the placenta are yet to be elucidated. Further, whether or not the observed adverse pregnancy outcomes are dose and time dependent is yet to be determined. This study aimed to establish the adverse maternal pregnancy outcomes following administration of varied doses of carbamazepine at different gestational trimesters .In carrying out the study, a total of 30 nulliparous female Albino rats (Rattus norvegicus) weighing between 150 - 250g were randomly assigned into four study groups follows; 3 control, and 27 experimental groups of LCG, MCG and HCG each composed of nine rats each, these 9 rats in each of the experimental group were further subdivided into 3 study rats into TM1, TM2, and TM3 with three rats each. Carbamazepine was administered to the treatment groups though the oral route by use of a gavage needle. The control group received food and water ad-libitum/day while the experimental groups received varied doses of carbamazepine as follows 20.7, 72.5, 124mg/kg/Bw of carbamazepine/day for Low dose, medium and high doses groups respectively as well as water ad-libitum. Daily maternal trends were taken and recorded. At 20th day of gestation, all animals were euthanized and sacrificed by hysterectomy. Litter size, fetal weight, placenta weight, number of resorbed/devoured endometrial glands, number of fetuses with congenital malformations and dead fetuses were examined and recorded accordingly. Data was then entered into the computer and analyzed using Statistical Package for Social scientists version 24 for windows Chicago Illinois. The liner regression statistics, intra and intergroup comparisons were done using one-way analysis of variances (ANOVA) and P-values of less than 0.05 were taken to be significant. The finding of the study showed that there was statistical significant decrease in daily maternal trends (p=0.0001), Litter size (P=0.003), Fetal weight (0.0001) and placenta weight (p=0.002) when experimental groups were compared with the control. Consequently, there was a*

significant increase in the number of devoured/resorbed fetuses (p=0.010), number of fetuses with congenital malformations (0.042) and the dead fetuses (0.004) in the treatment groups when they were compared with the control group. It was therefore established that the effects of carbamazepine on maternal pregnancy outcomes were dose and time dependent .The findings of this study sets a basis for further studies with higher primates as well as advocate for clinical trials that would lead in carbamazepine dose rationalization to enhance maximum maternal benefits when used during pregnancy, while on the other hand, enhance safety of the fetuses. ----------
---------- Date of Submission: 24-06-2019 Date of acceptance: 06-07-2019 ----------
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Appendix II: 2nd Publication

IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS) e-ISSN: 2278-3008, p-ISSN: 2319-7676. Volume 14, Issue 4 Ser. I (Jul – Aug 2019), PP 05-18 www.Iosrjournals.Org DOI: 10.9790/3008-1404010518 www.iosrjournals.org 5 | Page

Effects of In-utero Exposure to Varied Doses of Carbamazepine on Fetal Growth and Development in Albino Rats (*Rattus norvegicus)*

Mwangi A. Wairimu[1*], Kweri J. Kariuki[1], Kanyoni J. Mwangi[1], Thuo Reuben[1]

*[1]{Department of Human Anatomy, School of Medicine (SOMED), College of Health Sciences (COHES) Jomo Kenyatta University of Agriculture and Technology (JKUAT) Kenya} *Corresponding Author: Mwangi A. Wairimu*

Abstract: *Prenatal exposure to carbamazepine has been shown to interfere with the normal morphogenesis and differention of the various fetal organs when applied in management of various maternal conditions like bipolar disorders, trigeminal neuralgia, and seizures associated with epilepsy among others. Such perturbations on normal development of the fetal organs in-utero can help in explaining the cause of some of the structural, behavioral and functional mental disorders observed in adulthood whose cause is yet to be established. Such disorders of embryological origin are currently a major contributor to the disability-adjusted life years (DALY) in adulthood. Though, the existing literature has linked the prenatal exposure to alteration in various growth and development parameters, data on whether these effects on the fetuses are time and dose dependent is yet to be elucidated. The broad objective of this study was therefore to determine the fetal growth and development outcomes of prenatal exposure to varied doses of carbamazepine, as well to establish whether the outcomes are also time dependent. In carrying out the study a total of 30 nulliparous female Albino rats (Rattus norvegicus) weighing between 150 - 250g were assigned four groups by use of Simple random sampling. One group was used as a control and the other groups i.e. Low carbamazepine group (LCG), Medium carbamazepine group (MCG) and high carbamazepine group (HCG) were used as the treatment groups. Treatment groups were further subdivided according to trimesters as trimesters one, two and three (each n=3). Carbamazepine was administered to the treatment groups though the oral route by use of a gavage needle. The control group received food and water ad-libitum/day while the experimental groups received varied doses of carbamazepine as follows 20.7, 72.5, 124mg/kg/Bw of carbamazepine/day for Low dose, medium and high doses groups respectively as well as water ad-libitum. At 20th day of gestation, all animals were euthanized and sacrificed by hysterectomy. The fetal Anthropometric body parameters such as weights, Crown-rump length, and head circumference among others were taken and recorded. The visceral morphometric and morphological growth parameters were also examined and recorded accordingly. Data was then entered in to the computer and analyzed using EPI-Info and Statistical Package for Social scientists version 24 for windows Chicago Illinois. The liner regression statistics and intra-and inter group comparisons were done using one-way analysis of variances and P-values of less than 0.05 were taken to be significant. The finding of the study showed that there was statistical significant decrease*

(p=0.0001) which was less than 0.05 significant level) in the fetal growth and development between the carbamazepine treated groups as compared with the control as illustrated by the decrease in all growth parameters including, fetal weight (p=0.01), crown rump length (P=0.0001), head circumference (0.0001), head length (0.0001), and bi-parietal diameter (p= 0.0001). This was also collaborated in fetal viscera like the brain, liver, lungs, heart, kidney, spleens among others when their gross morphometric analysis was done between the experimental groups versus the control (p=0.0001)which was less than 0.05 significant level). It was also established that the effects of carbamazepine on fetal growth and development were dose and time dependent as high dose carbamazepine group fetuses treated in the first trimester shown the highest effects compared with Low dose carbamazepine treated in the third trimester(P=0.0001). The findings of this study sets a basis for further studies with higher primates like baboons that would lead in carbamazepine dose rationalization and its application during pregnancy for the attainment of maximum maternal benefits and reduction of fetal teratogenic effects while exposed in utero. Clinical trials should also be emphasized to come up with more literature on carbamazepine safety during pregnancy in human beings. ----------
---------- Date of Submission:

29-06-2019 Date of acceptance: 15-07-2019 ----------

Appendix III: Data Capture Sheets

DATA CAPTURE SHEET FOR EXPECTANT ALBINO RATS

ALBINO RAT IDENTITY……

INITIAL WEIGHT……DOSE CALCULATION……

DATE	WEIGHT IN GRAMS	CARBAMAZEPINE DOSE IN 5% DMSO(g\kg)	GENERAL CONDITION OF RAT

DATA CAPTURE SHEET FOR THE ALBINO FETUSES

Albino Rat Identity

(**Mother**)……

Date of Harvesting…….Fixative Used……..

Total No. Of Fetuses…….

Total No. Of Resorptions……

Total Number of Fetuses with Congenital Malformations…….

No. Of Dead Fetuses…….

	F1	F2	F3	F4	FT	F6	F7	F8	F9	F10	F11	F12
GROSS APPEARANCE												
FETAL WT(g)												
FETAL CRL (mm)												
OBVIOUS CONGENITAL ABNORMALITIES												
RESORPTIONS/DEVOURED FETUSES												
PLACENTA WEIIGHT												
HEAD CIRCUMFERENCE(mm)												

HEAD LENGTH (mm)												
BI-PARIETAL DIAMETER(mm)												
BRAIN												
GROSS APPEARANCE												
OBVIOUS CONGENITAL ANOMALIES OF THE BRAIN												
BRAIN WT(g)												
LENGTH (CM)												
WIDTH(CM)												
TOTAL BRAIN VOLUME (mm^3)												
VOLUME DENSITY OF THE CEREBRAL CORTEX (mm^3)												

SUBCORTICAL VOLUME DENSITY (mm^3)												

Appendix IV: Ethical Approval Form

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
P. O. Box 62000-00200 Nairobi, Kenya Tel 0675870225 OR Extn 3209
Institutional Ethics Review Committee

April 19th, 2018 **REF: JKU/2/4/896A**

Ann Wairimu Mwangi
Department of Human Anatomy.

Dear Ms. Mwangi,

RE: HISTOMOPHOMETRIC STUDY ON THE TERATOGENIC EFFECTS OF CARBAMAZEPINE ON THE DEVELOPMENT OF THE FATAL BRAIN IN ALBINO RATS.

The JKUAT Institutional Ethics Review Committee has reviewed your responses to issues raised regarding your application to conduct the above mentioned study with you as the Principal Investigator.

The is to inform you that the IERC has approved your protocol. The approval period is from April 19th 2018 to April 19th 2019 and is subject to compliance with the following requirements:

a) Only approved documents (informed consent, study instruments, study protocol, etc.) will be used.
b) All changes (amendments, deviations, violations, etc.) must be submitted for review and approval by the JKUAT IERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the IERC immediately.
d) Any changes, anticipated or otherwise that may increase the risks to or affect the welfare of study participants and others or affect the integrity of the study must be reported immediately.
e) Should you require an extension of the approval period, kindly submit a request for extension 60 days prior to the expiry of the current approval period and attach supporting documentation.
f) Clearance for export of data or specimens must be obtained from the JKUAT IERC as well as the relevant government agencies for each consignment for export.
g) The IERC requires a copy of the final report for record to reduce chances for duplication of similar studies.

Should you require clarification, kindly contact the JKUAT IERC Secretariat.

Yours Sincerely,

DR. PATRICK MBINDYO
SECRETARY, IERC

J.K.U.A.T
DIRECTOR,
RESEARCH DEPARTMENT (RPE)
P O Box 62000-00200
NAIROBI

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