HISTOSTEREOLOGICAL TERATOGENIC EFFECTS OF PHENYTOIN ON THE FETAL HEART AND VASCULAR TUNICS IN ALBINO RATS (*RATTUS NORVEGICUS*)

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Histostereological Teratogenic Effects of Phenytoin on the Fetal Heart and Vascular Tunics in Albino Rats (*Rattus Norvegicus*)

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DECLARATION

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

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DEDICATION

I dedicate this thesis to my husband Timothy Sigei and my children Miriam Cherop, Emmanuel Kiprotich and Gabriel Kibet for their support and patience throughout the study period.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

AED	Antiepileptic drug
ANOVA	Analysis of variance
BPD	Bi-parietal Diameter
BP	Blood pressure
bwt	Body weight
C	Control
CM	Cavalieri Method
CRL	Crown Rump Length
COHES	College of Health Sciences
g	Grams
GD	Gestational dates
H&E	Hematoxylin and Eosin
HPG	High phenytoin group
IVS	Interventricular Septum
JK**UAT**	Jomo Kenyatta University of Agriculture and Technology
Kg	Kilogram
KM	Kilogram meter

LPG Low phenytoin group

LPG Low phenytoin group

LVL Left Ventricular lumen

LVW Left Ventricular Wall

MAG Magnification

M Meters

mls Milliliters

mm Millimeter

MPG Medium phenytoin group

NIH National Institutes of Health

RVL Right Ventricular Lumen

RVW Right Ventricular Wall

SAFARI Small Animal Facility for Research and Innovation

SEM Standard Error of the Mean

SPSS Statistical Package of Social Sciences

TM_1 Trimester one

TM_2 Trimester two

TM_3 Trimester three

WIM Water Immersion Method

DEFINATION OF TERMS

Dams	Pregnant female rats
Morphometry	The process of measuring the external shape and dimensions of the body structures / organs
Cardiac Perfusion	Passage of fluid through circulatory system
Prenatal Exposure	Introduction of drug or chemical during pregnancy period.

ABSTRACT

Studies have shown that the normal morphogenesis of fetal heart and the vascular tunics is perturbed by in-utero exposure to anticonvulsants such as phenytoin when used during pregnancy. However, the histo-stereological teratogenic effects of phenytoin on the heart and vascular tunics development is not well understood. The broad objective of the current study therefore was to evaluate the histomophorlogical and histo-stereological teratogenic effects of phenytoin on the fetal heart and vascular tunics following administration of varied doses of phenytoin at different gestational periods. This study was carried out in SAFARI animal house of JKUAT and a static-group controlled-experimental study design was adopted. A total of 30 Albino rat dams weighing between 200-250g from a pure colony were used. These 30 dams were randomly assigned into two study groups of 3 control and 27 experimental. The 27 rats in the experimental group were further subdivided into 3 groups of 9 rats each; LPG-31mg/kg, MPG-62mg/kg and HPG-124mg/kg. The 9 rats in each of the three dose groups were further sub-divided into three sub groups with 3 rats each according to the time of exposure; TM_1, TM_2, and TM_3. All rats were fed with standard rodent pellets and water *ad-libitum*, while those in the experimental group also received phenytoin treatment. All rats were humanly sacrificed at GD_{20} then 3 fetuses with the lowest, median and highest weights from each rat selected and their hearts harvested, weighed and processed for histo-morphological and stereological analysis. Data was collected using structured and coded tally sheets, entered and stored in Microsoft excel, then exported to SPSS version 21. One-way Analysis of Variance (ANOVA), followed by Tukey's post hoc tests were done and results expressed as mean ± standard error of the mean (SEM) for all values. All results with $p < .05$ were considered to be statistically significant. The findings on the placental and fetal weights, CRL, BPD and the HC show statistical significant reduction ($p < .05$) among phenytoin treated groups compared with the control. On the histomorphological effects, it was established that the fetal heart left ventricular wall layers were distorted and that of the vascular tunics showed reduction in wall thickness among phenytoin treated groups. On the stereological findings, there was significant reduction $p = .001$ in total heart volume in phenytoin treated groups particularly at TM_1 high dose group (311.40±10.4) when compared to that of the control (378.13±4.57). In conclusion, phenytoin has teratogenic histomorphological and stereological effects on the fetal heart and vascular tunics particularly when administered in TM_1 and TM_2 HPG and its teratogenicity is both dose and time dependent. The study recommends that high dose phenytoin use during pregnancy should be avoided particularly in the first trimester that presents the window of opportunity to its teratogenic effects on the cardiovascular development. Further studies also need to be carried out with higher primates in order to determine phenytoin dose rationalization and its application during pregnancy.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Phenytoin an anticonvulsive medicine under the brand name Dilantin among others has a molar mass of 252.268g/mol, a chemical formula of $C_{15}H_{12}N_2O_2$ and bioavailability of 70-100% oral, and 24.4% for rectal administration is usually used in treatment of convulsive disorders like seizures and epilepsy across all the age groups and even during pregnancy (Patocka *et al*., 2020). Its usage during pregnancy has however been a subject of controversy based on its unclear histostereological effects on the developing fetal heart and the vascular tunics.

On the other hand, the normal morphogenesis of the fetal heart and the vascular tunics has been shown to be perturbed by the in-utero exposure to anticonvulsant medicines in the same class with phenytoin when used during pregnancy in the treat conditions like epileptic seizures, convulsions, neurosurgery among others (Lynch & Abel., 2015). These teratogenic effects of phenytoin have been shown to be due to various mechanism such as its ability to cross the maternal placental barrier because of low molecular weight hence accumulating in the fetal tissues, blocking the potassium ion channels in developing fetal mesenchymal cells of the heart and great vessels causing hypoxia and bradycardia (Azarbayjani *et al*., 2006). In addition, these teratogenic effects have also been linked to the accumulation of metabolites that result from its saturable kinetics in the maternal liver during its metabolism hence these metabolites cross the maternal placental barrier during embryonic period interfering with development of the fetal cardiovascular structures (Danielsson *et al*., 2005).

The arising disruptions on the fetus developing cardiovascular tissues may help to explain some of the rising cardiovascular diseases of unknown cause at adulthood such as cardiac conduction complications like sino-ventricular tarchycardia, congenital heart diseases, blood vessels stenosis, hypertension, heart failure

(Galappatthy *et al*., 2018). Though phenytoin use in pregnancy has been shown to have effects on the developing fetal heart and the great vessels, data on the specific histomorphological and quantitative effects of vessels is yet to be elucidated. Further, whether the effects of phenytoin are dose and time dependent is yet to be established.

The development of the cardiovascular structures in the rat and humans show significant comparative similarities in terms of the origin from the mesoderm beneath the developing foregut, morphological expression and differentiation of cells to ultimate four chambered heart (Kelly *et al*., 2011). Likewise the process of morphogenesis in both involves organized sequence of events involving the primitive heart cells that begins as cardiac crescent that form single tubular structure , looping, differentiation of the wall and Septation (Ratas & Etapas, 2014). However, the timing of these events differ in human and rat for instance straight heart tube formed at day 9 in fetal rat is formed during the 2^{nd} week in human and looping process occur at day 10 of development in fetal rat and 4^{th} week in human embryo (Sagiroglu *et al*., 2012) .

The human and rat embryo originate from the mesodermal beneath the developing foregut, and have common morphological expression and differentiation of cells up to the formation of a four chambered heart (Mei *et al*., 2020). Likewise, the process of morphogenesis in both involves organized sequence of events comprising the primitive heart cells that begins as cardiac crescent that form single tubular structure, looping, differentiation of the wall and septation (Garibay *et al*., 2012). However, the timing of these events differs in human and rat because of their different gestation periods (Wessels & Sedmera, 2020).

Antiepileptic drugs such as phenobarbital, carbamazepine, valproate and phenytoin majorly cause malformations such as spina bifida, cardiac, skeletal and cleft malformations among others seen at birth (Katsiki *et al*., 2014). Though studies have shown teratogenic effects various antiepileptic drugs phenytoin included, there

is lack of data on the histostereological outcome when used prenatally on the heart and vascular tunics.

1.2 Statement of the problem

The Cardiovascular diseases such as hypertension, cardiac dysfunction, coronary heart diseases, myocardial infarction among others are reported to be on the increase globally currently affecting approximately 17.9 million people annually according to WHO (2020) statistics. Maternal use of phenytoin during pregnancy has been shown to partub the normal development of the fetal heart and vascular tunics hence increasing the predisposition risks factors of cardiovascular defects 2-5 times in childhood and as well as increasing the risks of developing cardiovascular diseases (CVS) ten-fold in the adulthood (Lynch & Abel, 2015). There is however, lack of data on the anatomical histoquantitative effects of phenytoin on the heart and vascular tunic when prenatally exposed in different window periods. Therefore, there is need to establish histo-morphological and quantitative effects of the drugs known to affect the heart and vascular tunics prenatally like phenytoin. In addition, the critical period and doses of phenytoin teratogenicity need to be established.

1.3 Justification of the study

To curb the rising cases of cardiovascular diseases like hypertension, myocardial dysfunction among others particularly those of unknown cause, teratogenic studies on medicines known to disrupt cardiovascular system development cannot be over emphasized. This teratogenic histostereological studies on phenytoin would therefore help in tracing back the embryological origin of some of these CVS disorders of unknown cause and subsequently guide the rational application of these medicines during pregnancy. Preventing seizures that could cause more harm to both mother and fetus during pregnancy may outweigh the teratogenic risks of phenytoin. Therefore, there is need to establish specific histomorphological and histoquantitative effects of phenytoin on the differentiation of the fetal heart and vascular tunics in order to generate data that will rationalize use of phenytoin during pregnancy in terms of dosage and the appropriate gestation period to be used. This

will protect the mother from convulsive disorders and find solutions to increasing number of children born with heart abnormalities of unknown cause alongside re-solving some postnatal cardiovascular problems such as coronary artery disease, hypertension among others that are of unknown cause.

1.4 Significance of the study

The findings of this study will help in forming a scientific platform where scientists can carry out further studies on phenytoin use in treatment of convulsive disorders during pregnancy. The study findings will also form a basis for further studies with non-human primates to improve management of maternal CVS conditions.

1.5 Research Question, Objectives and Hypothesis

1.5.1 Research Question

What are the teratogenic pregnancy outcome, histomorphological and histostereological effects of phenytoin on the fetal heart and vascular tunics in Albino rats.

1.5.2 Broad Objective

To evaluate the teratogenic pregnancy outcome, histomorphological and histostereological effects of phenytoin on the fetal heart and vascular tunics in Albino rats.

1.5.3 Specific Objectives

1. To evaluate the maternal pregnancy outcomes following prenatal exposure to phenytoin in albino rats.
2. To evaluate the histomorphological changes that occur on the fetal heart and vascular tunics following exposure to phenytoin in albino rats
3. To evaluate the histo-stereological changes that occur on the fetal heart and vascular tunics following exposure to phenytoin in albino rats

4. To establish whether the teratogenic effects of phenytoin on the developing fetal heart and vascular tunics are time and dose dependent

1.5.4 Hypothesis (H_o)

There is no relationship between prenatal phenytoin exposure and pregnancy outcome, histomorphological and histostereological changes in the fetal heart and vascular tunics

1.6 The study model assumptions

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

1.7 Study limitations

Some of the anticipated study limitations included, failure of some dams becoming pregnant at the same time following the introduction of the males in the cages, death of the animals along the experimental process following mishaps in drug administration while administering phenytoin using the gastric gavage needle.

1.8 Study delimitations

To overcome these challenges the following delimitation measures were applied:

(i). The albino rats (dams) that did not conceive the first day of the experiment were separated from those that conceive the first day, put in a separate cage then given a second attempt with a male rat that was reintroduced overnight again until prove of pregnancy was established. Then their treatment was done separately as they would have different gestational days with the ones that got pregnant the first day.

(ii). For the rats that got sick or died in the course of the experiment, their study groups were noted as per dosage and the time of exposure. Postmortems were conducted to establish the cause of death then repeat experiments were done after the main experiment was completed.

(iii) A pilot study was done to test the study protocol and to minimize causes of errors as much as possible.

CHAPTER TWO

LITERATURE REVIEW

2.1 Brief introduction of phenytoin and cardiovascular anomalies.

Phenytoin is an anticonvulsive and antiarrhythmic agent commonly known as Dilantin is used in treatment of convulsive disorders like seizures and epilepsy (Patocka *et al*., 2020). Phenytoin administration to epileptic pregnant mothers alone or in combination with other anticonvulsants increase the risk of delivering a child with congenital defects by 2-3 times (Lander *et al*., 2008). Accumulation of phenytoin metabolites cause fetal hypoxia, vascular disruption and necrosis of existing and developing structures hence affecting fetal cardiovascular system histo-morphogenesis and cell cytodifferentiation (Webster *et al*., 1997)

Dysmorphogenesis of the fetal heart is key predictor to postnatal cardiovascular anomalies called congenital heart diseases which are the most common birth defects in man that result in spectrum of heart problems like coronary artery disease, congestive heart problems and pulmonary hypertension among others (Ksoo *et al*., 2017). These defects have been attributed to genetic and environmental factors. However some of the medications such as phenytoin have been shown to affect heart development hence may have a higher risk of having a child with congenital heart disease (Shah, 2010). Congenital heart defects are common in infants born to women with epilepsy taking antiepileptic drugs and the most implicated drug was phenytoin (Waltman, 2003).

2.2 Prenatal development of the heart

The heart's morphological expression appears as cardiac crescent at day 9 of gestation in the ventral side beneath the developing foregut following very dynamic processes from a single tubular structure to the ultimate four chambered heart (Mei *et al*., 2020). The first organ to function in human during embryonic development is

the heart which starts to form at the beginning of third week gestation (Kakkar *et al*., 2013).

Straight heart tube formed at day 9 is transformed to C-shaped looped heart that has 5 primordia after 15 hours consequently S-shaped heart loop is formed at day 10 of development (Sagiroglu *et al*., 2012). Septation of the heart into chambers begin at day 11 of development (Marcela et al., 2012). The aortic sac project into the pericardial cavity as it divides into two ducts and the aortic valves at day 12 and day 13 marks the end of looping and aortic sac division (Ratas & Etapas, 2014). From day 14-16 the heart takes more mature features and day 16-21 very few morphological changes occur within the heart and the great vessels (Garibay *et al*., 2012). Heart begins by pumping blood around day 21 or 22 and fully formed at eight weeks gestation (Kakkar *et al*., 2013)

2.3 Cytodifferentiation of the heart

The control of cardiac cells, gene expression protein coding and cardiac morphogenesis is through genetic network of five transcription factors ; NKX2.5, GATA, MEF 2, Tbx and Hand (Kelly *et al*., 2011). Endocardial cytodifferentiation is biphasic and its mesenchyme transformed by day 11 (Garibay *et al*., 2012). Myogenic cytodifferentiation of the precursor cells of the heart muscle begin before anterior intestinal portal of the primitive foregut forms in the embryo from the lateral plate mesoderm (Ratas & Etapas, 2014).

From the straight heart tube formed at day 10 of the heart development there is appearance of desmin and myosin and these population of cells from the mesoderm settle along the midline of the developing embryo and later fuse to form the primitive heart tube (Mei *et al*., 2020). Connective tissues play a role in mechanical functions of the heart and it is formed by tissue interactions aided by a glycoprotein called tenascin in embryonic extracellular matrices (Sagiroglu *et al*., 2012)

2.4 Heart and Great vessels Gross and Microscopic Anatomy

The heart is an important organ that pump blood to different body structures, cone in shape with a size of a fist located in the middle of the chest between the lungs (Kelly *et al*., 2011). It has a fibrous covering called pericardium and the wall is made up of cardiac muscles (Galfiova *et al*., 2018). The heart consists of four chambers with muscular walls and lies in the pericardial cavity. Left and right chambers do not communicate directly as in the other mammals. Reverse of blood flow is prevented by valves with three leaflets (tricuspid) or two leaflets (bicuspid) which attach to muscular pillars called papillary muscles. Arteries include Pulmonary artery from the right ventricle and Aorta from the left ventricle; these two-carry blood from the heart. Veins include Pulmonary veins which enter the left atria, Superior and Inferior Vena cava which enter the right atria (Ratas & Etapas, 2014)

The heart wall has three main layers; endocardium, myocardium and epicardium. Endocardial cells are found in the endocardium with connective tissue that is loose binding them to the muscle underlying it (Galfiova *et al*., 2018). Endocardial folding forms the valves but contain connective tissue that is fibrous in nature containing minimal cartilage cells at the valve cusps bases. The cardiac muscle bound by connective tissue supporting the neurovascular bundle constitute tissue that is thin and a layer of pericardium called mesothelium (Batulevi *et al*., 2004).

The walls of the most arteries have three distinct layers; Intima, media and adventitia (externa). Loose connective tissue is what constitutes the adventitia. The structures of veins resemble that of arteries but their walls are thinner, softer and have minimal elasticity. Smooth muscles of the media are replaced by connective tissue in small veins and by cardiac muscle in pulmonary veins. Intima has folding that form pairs of semilunar valves (Galfiova *et al*., 2018).

2.6 Chemistry of phenytoin

Phenytoin also called Dilantin is an anti-seizure medication and it is useful when preventing partial and tonic clonic seizures. Phenytoin has a molar mass of 252.268

g/ml with a formula C_{15} H_{12} N_2 O_2 (Patocka *et al*., 2020). Pharmacodynamics: phenytoin exerts its anticonvulsant effects by binding to specific site on voltage dependent sodium channel by suppressing neuronal firing through inhibition of sodium flux through these voltage dependent channels (Nelson *et al*., 2015). Phenytoin protects the sodium pump in the brain and the heart by stabilizing membranes hence minimizing maximal convulsive activity (Bittigau *et al.*, 2002).

Phenytoin is bound to protein 87-93% and it is widely distributed throughout the body. Albumin is the plasma protein almost exclusively bound by phenytoin in individuals whose concentration of plasma albumin is normal (Wu & Lim, 2013). Half-life by route of exposure: when therapeutic doses of phenytoin are administered orally, its half-life is varied and dose dependent ranging from 8-60 hours but the average is 20-30 hours. Adults given high doses, the range is from 23-230 hours (Perucca, 2005).

Phenytoin metabolism takes place exclusively in the liver through parahydroxilation to inactive metabolites 5-(4-hydroxyphenyl)-5 phenyl-hydantoin by cytochrome P 450 enzyme and the p- hydroxylated phenytoin is in turn conjugated to its glucuronide (Guengerich, 2020). Phenytoin metabolism is nonlinear at therapeutic doses and linear at toxic doses (zero order kinetics). 3, 4-dihydroxyphenyl-phenylhydantoin, catechol and 3-o-methylated catechol are metabolites when phenytoin is oxidized. This metabolites cause toxicity which is increased in children due to rapid metabolism (Appleton & Gill, 2003). Elimination of phenytoin: hydroxylated metabolite 23-70% of phenytoin is excreted through urine free or conjugated 5%, 5% through feces and small amounts through milk (Wu & Lim, 2013).

2.7 Mechanism of action of phenytoin on the heart and great vessels teratogenesis.

Studies have shown that birth defects following in utero exposure to phenytoin are caused by embryonic bradycardia which leads to severe hypoxia and alteration in embryonic blood flow and blood pressure inducing cardiovascular defects

(Danielsson *et al*., 2005). In addition, generation of reactive oxygen species within the embryo during reoxygenation possibly result in free radicle damage (Azarbayjani *et al.,* 2006) that leads to vascular disruption and necrosis of existing and developing structures (Nulman *et al*., 1997).

Studies done in mouse embryo, phenytoin caused a decrease in heart rate based on concentration; at 100 micrograms(2-7%) effect increased at 200 micrograms(20%), temporary cardiac arrest at 500 micrograms (Katsiki *et al*., 2014). Another study done histologically showed dilated blood vessels within 2 hours, vascular disruption and hemorrhage at 8 hours, vascular disruption and mesenchymal necrosis depicted after 24-48 hours of administration as well decreasing heart rate and blood pressure of the mother decreased by around 15% consequently resulting in decreased partial pressure of oxygen and increased partial pressure of carbon dioxide as well as low fetal heart rate (Guldiken *et al*., 2015)

Cytochrome P450 enzyme eliminates phenytoin through para-hydroxylation. In overdose this metabolic pathway saturates leading to accumulation of free phenytoin which is possible in therapeutic doses when albumin levels are low, hepatic dysfunction, hereditary dysfunction and inhibition of phenytoin metabolism by other drugs (Patocka *et al*., 2020). In addition, teratogenic effects of phenytoin have been linked to its action in blocking of potassium ion channels both in the maternal tissues as well as in developing fetal mesenchymal cells of the heart and great vessels causing hypoxia and bradycardia (Danielsson *et al*., 2005).Phenytoin administration to epileptic pregnant mothers alone or in combination with other anticonvulsants, the risk of delivering a child with congenital defects is increased by 2-3 times (Lynch & Abel, 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study location/ area

All experiments including breeding, handling, weighing, and phenytoin administration and measurements of fetal parameters was done at the Small Animal Facility for Research and Innovation (SAFARI) in the school of biomedical sciences of Jomo Kenyatta University of Agriculture and Technology (JKUAT).

3.2 Study design

A static-group controlled-experimental study design was adopted.

3.3 Study sample/ subject

A total of 30 nulliparous albino rat dams of the species *Rattus norvegicus* derived from a pure colony were used as the animal experimental model in this study. These rats were sourced from SAFARI animal in the school of biomedical sciences of 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 ranging between 1-16 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 breed colony (iv) Low cost of maintaining the animals , (v) 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 like 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 post-natal dates 45-48 in males defined by

vaginal opening (females) or Balan preputial separation (males). Reproductive senescence in female rats occurs between 15 and 20 months of age (Pallav & Sengupta, 2013).

Their gestation period is estimated at from 21 to 23 days during which the fetuses are viable. 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 feeling the abdomen, noticing weight gain or mammary (breast) development or 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. In the current study, 15 male albino rats of the same breed as females were used.

3.4 Sampling method

The 30 dams were calculated using the resource equation method (E=TA-TG), (Charan & Biswas, 2013) since the standard deviation from previous studies was not available as well as the effect size. The Resource equation states that E=Total number of Animals-Total number of groups.

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; Arifin *et al*., 2017).

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

3.4.1 Sample size calculation for the fetuses used in histo morphology and stereology

The average litter size for both the control and experimental animals in this study was approximately six (6) fetuses per rat. To ensure objectivity of the finding and to eliminate observer or process bias all the fetuses per each dam were weighed and organized in an ordinal sequence starting with one with the lowest fetal weight to the highest. 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 hearts and associated great vessels (i.e. ascending aorta, pulmonary trunk and the veins draining into the heart) were harvested for stereology and for histomorphology. The rest of the fetuses were preserved in zenkers solution for future repository should a problem arise during processing.

3.4.2 Grouping of dams

The 30 dams used in the study were randomly assigned to either three rats as the control and 27 in the experimental category. To determine whether phenytoin 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 phenytoin group (LPG); 9 rats for the medium phenytoin group (MPG); - and 9 dams for the high Phenytoin group (HPG). To determine whether the Phenytoin teratogenicity is time dependent, the 9 rats in each of the three study categories of the low, medium and high phenytoin 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 three (3) rats for trimester three (TM_3)-Figure 3.1.

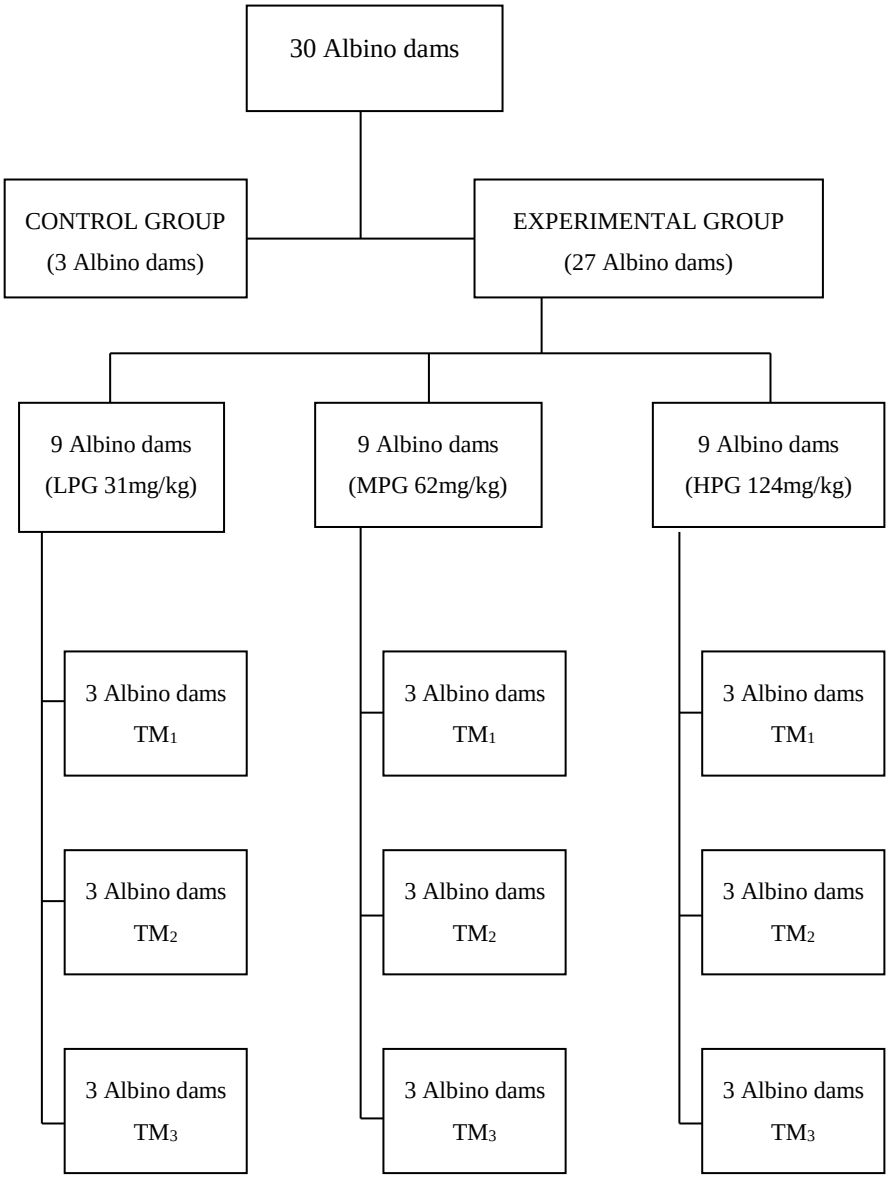

Figure 3.1: Shows how the 30 dams were grouped into the control, the Low phenytoin group (LPG), medium phenytoin group (MPG), and the High phenytoin group (HPG).

3.5 Selection criteria

3.5.1 Inclusion criteria

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

3.5.2 Exclusion criteria

- All animals that later shown signs of sickness in the course of the experiment
- All fetuses in which mother had an underlying disease state

3.6 The feeding of the rats

All rats were fed on standard rodent pellets obtained from Unga feed Limited situated in Thika town, plus water *ad libitum*. Feeding was done every morning at 0800 hours; within their spacious polycarbonate plastic cages as outlined by Curfs *et al*., (2011), 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: -

3.6.1 The Control sub group

Received standard diet as determined by the academy of nutrition and dietetics containing by weight (g/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

3.6.2 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 the received phenytoin

treatment based on their doses of low, medium and High (LPG, MPG, HPG) as well as according to the trimester of exposure (TM_1, TM_2 and TM_3) as follows:

3.6.2.1 The low dose phenytoin group (LPG)

All rats in this group received a standard diet, water *ad-libitum* and a constant daily dose of phenytoin (31.0mgs/kg/bwt)] administered as a single bolus through gastric gavage gauge 16 at 0900hrs. The 3 rats in trimester one (TM_1) received phenytoin treatment from gestation+ day one (GD_1) to gestation day 20 (GD_{20}); those in trimester two (TM_2) received phenytoin treatment starting gestational day 7 (GD_7) all trough gestation day 20 GD_{20}), while those in trimester three (TM_3) received phenytoin treatment from gestational day 14 GD_{14}) all through to- gestational day 20 (GD_{20}) that is the last day of gestation.

3.6.2.2 The medium phenytoin group (MPG)

All rats in this group received a standard diet, water *ad-libitum* and a constant daily dose of phenytoin (62.0mgs/kg/bwt)] administered as a single bolus through gastric gavage gauge 16 at 0900hrs. The 3 rats in trimester one (TM_1) received phenytoin treatment from day one (GD_1) to gestation day 20 (GD_{20}); those in trimester two (TM_2) received phenytoin treatment starting gestational day 7 (GD_7) all trough gestation day 20 GD_{20}), while those in trimester three (TM_3) received phenytoin treatment from gestational day 14 (GD_{14}) all through to- gestational day 20 (GD_{20}) that is the last day of gestation.

3.6.2.3 The high dose phenytoin group (HPG)

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

3.7 The handling of the rats during experimentation

The rats were handled by the investigator only for the purpose of obtaining daily weights between 0800 and 0900 hours, then feeding the with pellets was always done at 0930 hours. All procedures were performed according to the guidelines for care of laboratory animals by the National Institute of Animal Research- (NIAR-USA) as outlined by (Retnam *et al*., 2016); and National Research Council, report of 2011.

3.8 Breeding of the rats

One sexually mature (above fifty days postnatally) male albino rat from a pure colony that were bred at the same time with the female dams was introduced into a standard cage with two female rats at 1430 hours (+/- 30 minutes) and at 0900 hours (+/- 30 minutes) the following morning, the males were removed and returned to their separate cages. The selection of the males applied the following selection criteria.

3.8.1 Inclusion criteria

All male rats whose weights were between 200-300 grams and did not show any signs of sickness of a scar

3.8.2 Exclusion criteria

All males that showed signs of sickness

3.9 Pregnancy determination:

Materials used in determination of pregnancy included:

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) Papanicolaou stain or Giemsa stain

3.9.1 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.

3.9.2 The Procedure that was followed in the determination of pregnancy

1. The animal was restrained with a gauze holder against the body
2. 1ml 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 slide
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 Papanicolaou stain and giemsa stain
9. Observations of the slide followed, and was done under the BP Olympus microscope

3.9.3 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 cornified epithelial cells and neutrophils on the smear was used to determine estrous changes, which was denoted as the first day of gestation (GD_1) (Telendo *et al*., 2019; Hamid & Zakaria, 2013).

3.10 Determination of the phenytoin doses, reconstitution and administration.

A simple guide for conversion of animal dosages from human dosages by Nair & Jacob, (2016) was applied 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) was 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 m^2. Therefore, the Km factor for human is calculated by dividing 60 by 1.62, which is 37]. The Km factor values of various animal species are 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 K ratio Eq.

The Km ratio values are already provided and are obtained by dividing human Km factor by animal Km factor or vice versa.

3.10.1 Determination of phenytoin doses:

The maximum phenytoin dose in humans is 1200mg/day, medium dose is 600mg/day and minimum dose is 300mg/kg

Determination of high phenytoin dose

Highest dose phenytoin 20mg/kg

AED = HED X Km factor

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

Determination of Medium Phenytoin dose

Medium dose phenytoin-10mg/kg/day

AED = HED X Km factor

Therefore 10mg/kg x 6.2 =62mg/kg

Determination of Low Phenytoin Dose

Low dose phenytoin -5mg/kg/day

AED = HED X Km factor

Therefore 5mg/kg x 6.2 =31 mg/kg

Therefore, different phenytoin groups received the doses as follows:

(i) The Medium phenytoin dose group (MPG) - 62mg/kg/ bwt

(ii) The low phenytoin dose group (LPG) - 31mg/kg/ bwt

(iii) The High phenytoin dose group (HPG) - 124mg/kg/ bwt

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Grouping of animals to determine critical period of phenytoin teratogenesis.

In reference to Figure 3.1 on animal groupings, the administration of phenytoin in each of the groups was done as follows:

In each of the groups (LPG, MPG, HPG), the 27 dams were randomly sub divided in three sub-groups the Trimester one (TM_1) = 9dams, Trimester two (TM_2) = 9dams and trimester three (TM_3) =9 dams

NB> the gestation period of a rat is 21 days, therefore trimester I is between gestational day GD_1 to GD_7, while TM_2 is between GD_7-GD_{14} and TM_3 GD_{14-20}.

3.10.2 Reconstitution of phenytoin

Materials; phenytoin, Gavages' needle gauge 18, 5ml syringes, 20 ml beaker for dilution, Syringes, Distilled water (500mls) and a table cloth

The 50mg phenytoin tablets were being reconstituted using distilled water. All the volumes were administered in a standard volume of 3 mls (the standard allowable daily oral volume of a rat per day). The administration of phenytoin was done between 0930 hours and 1030 hours daily

The experimental animals received rodent pellets and phenytoin doses were administered as follows;

All TM_1 animals: - (LPG, MPG, HPG) categories received phenytoin from GD_1-GD_{20}

All TM_2 animals: - (LPG, MPG, HPG) categories received phenytoin doses from GD_7-GD_{20}

All TM_3 animals: - (LPG, MPG, HPG) categories received phenytoin doses from GD_{14}-GD_{20}

3.10.3 The Procedure followed in administration of various doses of phenytoin through gastric gavage

1. The animal was held carefully from the neck region using the left hand
2. The animal was then 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 was gently inserted into the mouth of the animal turning it gently to pass the esophageal constrictions and the cardiac sphincter
5. The phenytoin dose was then dropped in the stomach of the animal
6. The gavage needle was then gently removed

3.11 Sacrificing the animals and harvesting of fetuses

In all cases, the pregnant rats were humanely sacrificed by use of concentrated carbon dioxide on gestational day 20 between 0900 hours and 1100 hours, to prevent the mothers from devouring any damaged offspring (Rai & Kaushik, 2018).

After 5 minutes of carbon dioxide exposure, the anterior abdominal wall of the mother was opened 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 indicated by their movement following a gentle pressure was recorded (total litter size). Also, the number of the "devoured endometrial glands", characterized by yellowish nodules found along the mesometrial margin of the uterine horns that marks any original implantation site will be counted and recorded. Thus, the metrial glands unoccupied by living or recently dead fetuses will represent the number of prior resorptions.

The uterine horns were excised along the anti-mesometrial border to reveal the fetuses, embryonic membranes and placentas. The fetuses were gently removed in totality from the uterus using 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 and weighed and the general fetal morphology examined and recorded immediately.

Fetal crown rump length was determined by measuring the head to tail length in centimeters. External examination was done before and after fixation in formaldehyde solution. For each litter in each rat, three hearts from three fetuses with the low, median and high weights were resected for both light histological and stereological analysis. One of the three hearts was processed for transversely and the other longitudinally for light microscopy and stereology, the remaining was kept for use in case of any error during processing.

3.11.1 Determination of vulnerable periods of phenytoin teratogenesis on the fetal heart

To determine the vulnerable periods of phenytoin teratogenesis, phenytoin was administered daily throughout the gestation period starting on day 1 (GD_1) for the trimester I (TM_1) groups, day 7 (GD_7) for the TM_2 and day 14 (GD_{14}) for TM_3 subgroup.

3.11.2 Inclusion and exclusion criteria

Inclusion criteria-All fetuses born to healthy mothers and alive at the point of sacrificing

Exclusion criteria-All dead fetuses and those from diseased mothers

3.11.3 Grouping of fetuses for light and histostereological analysis

Upon sacrificing the mothers, 3 fetuses with low, medium and high weights were objectively chosen from every mother making a total of 90 fetuses. These fetuses were assigned to different groups depending on the maternal group during experiments that is the control (9 fetuses) or the experimental (81 fetuses). The 81 fetuses in the experimental category were further divided into three broad study groups of 27 fetuses each assigned: - low (LPG), Medium (MPG) and High Phenytoin group (HPG) each of the broad subgroups of the LPG, MPG and HPG were further subdivided into first (TM_1), second (TM_2) and third (TM_3) comprising of 9 rats each.

3.11.4 Humane sacrificing of the fetuses and harvesting of the fetal hearts

Materials; Concentrated carbon dioxide, cotton gauze or cotton wool, bell or dissector jar, mounting board, mounting pins, pair of scissors, a pair of forceps (toothed), scalpel blade, scalpel blade handle, fixatives- 10% formaldehyde solution, hypodermic needle gauge 20, gloves (surgical), magnifying glass, ruler, electronic weighing machine and specimen collection bottles.

3.11.5 The Procedure for anaesthetizing and perfusing the fetuses

1. Concentrated Carbon dioxide was opened
2. It was then introduced into a heavy bell jar with a tight-fitting lid
3. The fetuses were then be put into the bell jar for 3-5 minutes to euthanize
4. They were then removed from the bell jar and mounted onto the board using mounting pins with dorsal side on the board
5. The fetus was mounted on the board using mounting pins (dorsal side facing the board)
6. Using a pair of scissors and forceps the ventral medial side-up to the thoracic cavity between the symphysis pubis up to the root of the neck was opened.
7. The heart was identified
8. Intracardiac perfusion was performed with formalin 10% and 0.9% saline
9. The lungs were carefully retracted to avoid damaging the heart that lies in between.
10. The entire heart was excised
11. The heart was immersed in the preferred fixative (formaldehyde solution) for 24 hours.

3.12 Processing for light microscopy

Materials for staining; Specimen bottles, Zenkers solution (1 litre), distilled water, DPX mountant, glass slides and cover slips, hematoxylin and eosin, glass staining square jars, paraffin wax, microtome knives, rotary microtome (American optical CO), heater and water bath container, slide holders, distilled water, formaldehyde 10% concentration, xylene, alcohol, glass ware for preparing dilutions, wood blocs, beakers, egg albumin, dropper, cedar wood oil, toluidine solution

3.12.1 Procedure that was used for processing the fetal heart specimens for light microscopy

1. The heart was fixed in formaldehyde 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. The hearts were cleared with xylene.
4. Then infiltrated with paraplast wax for 12 hours at 56^0c
5. The heart tissue was then orientated in the longitudinal axis (apex to the base)
6. Embedded in paraffin wax on the wooden blocks
7. Excess wax was trimmed-off till the entire length of the heart tissue is exposed
8. 5µm thick longitudinal sections was cut from the apex to the base regions with Leitz sledge rotary microtome
9. The cut sections were then floated in water at 37^0 to spread the tissue
10. The sections were stacked onto glass slides, applied as thin film with a micro-dropper.
11. The slides were then dried in an oven at 37^0 for 24 hours
12. They were stained with Hematoxylin and eosin (H&E).

NB> 50 slides in each subgroup were selected for light microscopy processing using systematic random sampling, 6 slides from each group were selected for observation (1 from each 10 was selected using simple random sampling)

3.13 Stereological analysis

3.13.1 Estimation of the total heart volume using Archimedes method

The hearts from control and experimental groups were immersed in 10% formalin for fixation and perfusion in situ and, after removal, the Archimedes' principle was used to obtain an independent heart volume. The Archimedes volume was estimated by inserting the whole heart tissue into graduated beakers containing normal saline, and the displacement was measured. The normal saline displaced by the heart represented the actual heart volume (Hughes, 2005) These Archimedes volumes were used as the reference volumes. This method was compared to the other

methods and the mean and standard deviation (± SD) of the measurements were calculated.

3.13.2 Preparation of heart tissues for stereology

The fetal heart for stereological analysis was quickly removed, placed in cold saline solution and trimmed of adipose tissue, weighed, and immersed in formaldehyde solution for 24 hours at room temperature (23^0c) to allow for proper fixation. The samples of the fetal heart tissue were processed using graded alcohol, xylene and paraffin blocks was used for embedding. Each heart was exhaustively sectioned into 5µm thick sections both transversely and longitudinally from one end of the paraffin block using a rotary microtome (Leica RM 2135, Germany)

3.13.3 Staining method

Heart and vascular tunic sections was stained with hematoxylin and eosin (Ahmed, 2016)

3.13.4 Determination of total heart volume using cavalieri principal

The volume estimation was done by applying the Cavalieri method of point counting (Altunkaynak et al., 2009). The total volume of the heart was determined by combining the Cavalieri method of segmentation with point-counting on evenly spaced organ slices. The following steps were followed as employed: (i) Preparation of heart Cavalieri 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 StepaNizer stereology tool (v) All counts per slice was done (vi) The shape factor was finally estimated and the calculation of the volume and the CE (Coefficient of Error)

50 sections of 5um, sampled from each transverse heart section were taken by systematic uniform random sampling (Andrew *et al.,* 2004). The volume was obtained by fully sectioning the heart into a series of cuts which was the product of the sum of the cut areas (starting with the first to the last section), fraction 1 / x of

AT). The sum of points that hit the structure will estimate Ai (the area of the structure 'i'). Point counting was done using the Stepanizer software. The digital images of the heart tissue were captured using stereological sampling rules with same magnification and saved in the jpeg (Joint Photograph Expert Group) file format at adequate resolution. 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.

Point counting using the Cavaliers principle was applied to estimate the total volume of the fetal heart using the formula:

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

Where: $_{est}\mathbf{V}$= was the estimation of the volume of the heart,

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

Components of the fetal heart profiles,

a/p= was the area associated with each point,

t = was the distance between sections and

M= was represent the magnification

$\mathbf{m_{i-1}}$= all points in the heart sections from the first to the last

On each sampled heart slide was moved on the microscope's stage in X and Y and Z directions 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 heart structurers and points hitting these areas counted at a final magnification of x4.

3.13.5 Correction of the heart Tissue Shrinkage

The following method was applied to quantify shrinkage caused by fixation and histological procedures. The volume of removed fresh heart was calculated by Archimedes displacement method. After tissue processing and exhaustively sectioning, the heart volume was estimated with Cavalieri Method. The heart volume shrinkage was then calculated as follows (West, MJ. 2013)

$$\text{Shrinkage} = \frac{(\text{Volume before}) - (\text{Volume after})}{(\text{Volume before})}$$

Volume before were the heart volume determined through water immersion method, while volume after were the volumes determined through the cavalieri method. After estimating the shrinkage, the final volume of the heart was corrected.

3.14 Photography (materials and procedures)

3.14.1 Materials

1. Camera

2. Flash disc

3.14.2 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 using a flash disc
6. The photographs were uploaded and labelled using the Adobe fireworks software.

3.15 Statistical analysis

Data was analyzed using statistical package for social sciences (SPSS) for Windows Version 21 Chicago Illinois, and statistically tested using one-way analysis of variance (ANOVA) followed by Turkey post hoc test. Group means with a significance F-value ($p < .05$) were considered as significant

CHAPTER FOUR

RESULTS

4.1 Introduction

The results of phenytoin teratogenicity on the rat following its prenatal exposure of varying doses (LPG, MPG and HPG) at different gestational periods (TM_1, TM_2 and TM_3) are presented according to the study objectives as follows:

- Effects on the fetal and maternal pregnancy outcome,
- Histomorphological changes on developing fetal heart and vascular tunics,
- Histostereological changes on developing fetal heart and vascular tunics
- Effects of phenytoin on the fetal heart and vascular tunic in terms of dose and time

4.2. Effects of phenytoin on the fetal and maternal pregnancy outcomes

This included the mean litter sizes, mean placental weights, resorbed endometrial glands, and percentage embrolethality and maternal weight treads throughout the entire gestation period

4.2.1 The mean litter size, placental weights, resorbed endometrial glands and the percentage embryolethality.

Litter size

The intragroup and the intergroup comparative mean litter sizes between the phenytoin treated groups against the control depicted an inverse dose response relationship. In HPG, there was reduction in the mean litter size particularly when treatment was done in TM_1 (4.00±0.577) and TM_2 (7.00±0.577) and this was found to have statisticant significant difference ($p < .05$) compared with that of the control (13.00±0.57). Among the treatment groups, LPG litter size was observed to be higher across the three trimesters (Table 4.1).

Placental weights

It was found that the lowest placental weight recorded was in the high dose phenytoin group when treated at TM_1 (4.23±0.018) when compared to that of the control (5.98±0.021). However, there was no statisticant significant difference between the placental weights of the LPG at TM_3 (5.40±0.050) when compared to that of the control and high in the low phenytoin group when treated at TM_3 (5.40±0.050) and this was replicated in TM_2 and TM_3 as well (Table 4.1).

Resorbed endometrial glands

The mean resorbed endometrial glands were observed to vary directly with the dose of phenytoin exposure as well as with the time of exposure in that with increasing doses of phenytoin exposure, there was a corresponding significant increase ($p < .05$) in number of resorbed endometrial glands particularly in TM_1 and TM_2 across all the phenytoin treated groups as compared with the control. At TM_3 the mean number of the resorbed endometrial glands only had a significant difference ($p < .05$) in the High dose phenytoin group (HPG) when compared with the control (Table 4.1).

Embryolethality

On the percentage of embryolethality, it was observed that the comparative mean number of dead fetuses in utero increased with phenytoin dose and the time of exposure. When phenytoin was administered at TM_1 the mean embryo-lethality in HPG was high at 1.67±0.882 followed by MPG at 0.67±0.67 ($p < .05$) when compared with the control. When phenytoin treatment was administered at TM_2, the percentage embryo-lethality was 1.33±0.667 for the HPG, which was statistically significantly higher ($p < .05$) than that of the control while MPG and LPG embryo lethality at TM_2 were not statistically different ($p > .05$). When treatment was done at TM_3 the percentage embryo-lethality did not showed statisticant difference ($p > .05$) with the control (Table 4.1).

Table 4.1: The comparative means of litter sizes, placenta weights, resorbed endometrial glands and percentage embryo-lethality in LPG, MPG and HPG with time of exposure (TM_1, TM_2 and TM_3) against the control.

Study groups	Period of phenytoin treatment	Mean litter sizes ± SEM	Mean placenta weights ± SEM	Mean resorbed endometrial glands ± SEM	Mean embryo lethality ± SEM
Control group	-none-	13.00±0.57[a]	5.98±0.021[a]	0.69±0.67[a]	0.339±0.353[a]
Low dose phenytoin group (LPG, 31mg/kg)	TM_1	10.00±1.15[a]*	4. 63±0.009[c]*	0.63±0.67[a]	0.31±0.333[a]
	TM_2	11.13±0.887[a]*	5.0±0.049[b]*	0.67±0.667[a]	0.33±0.333[a]
	TM_3	12.060±0.567[a]	5.40±0.050[ac]	0.68±0.333[a]	0.33±0.333[a]
Medium dose Phenytoin group (MPG, 64mg/kg/)	TM_1	7.00± 0.577[b]*	4.633±0.034[b]*	1.33±1.33[a]*	0.67±0.67[a]*
	TM_2	10.00±0.577[b]*	5.03±0.034[b]*	1±0.577[a]*	0.33±0.333[a]
	TM_3	11.00±0.577[a]	5.133±0.0612[c]	0.667±0.333[a]	0.33±0.333[a]
High dose phenytoin group (HPG, 124g/kg)	TM_1	4.00±0.577[c]*	4.23±0.018[b]*	7±1.53[b]*	1.67±0.882[a]*
	TM_2	7.00±0.577[b]*	4.64±0.0200[c]*	3±0.000[b]*	1.33±0.667[a]*
	TM_3	8.67±0.333[b]*	4.96±0.030[d]*	0.700±0.6506[a]*	1.00±0.577[a]*

Key: *The mean values in a column that have the same letter in the same column are not statistically significant different at ($P<0.05$) using one-way ANOVA with Tukey test on post-hoc t-tests. Any value that has an asterix star (*) indicates that they depicted both intra and inter group statistical significance differences ($p < .05$)*

4.2.2 The influence of phenytoin on the mean daily maternal weight trends

It was observed that the maternal daily weight gains during TM_1 were significantly lower in the treatment groups ($p < .05$) compared with the control as per the line graph (Figure 4.1). It was notable that the mean maternal weight gain in all the experimental groups LPG, MPG, and the HPG when treated at TM_1 depicted a direct dose response relationship (Figure 4.1).

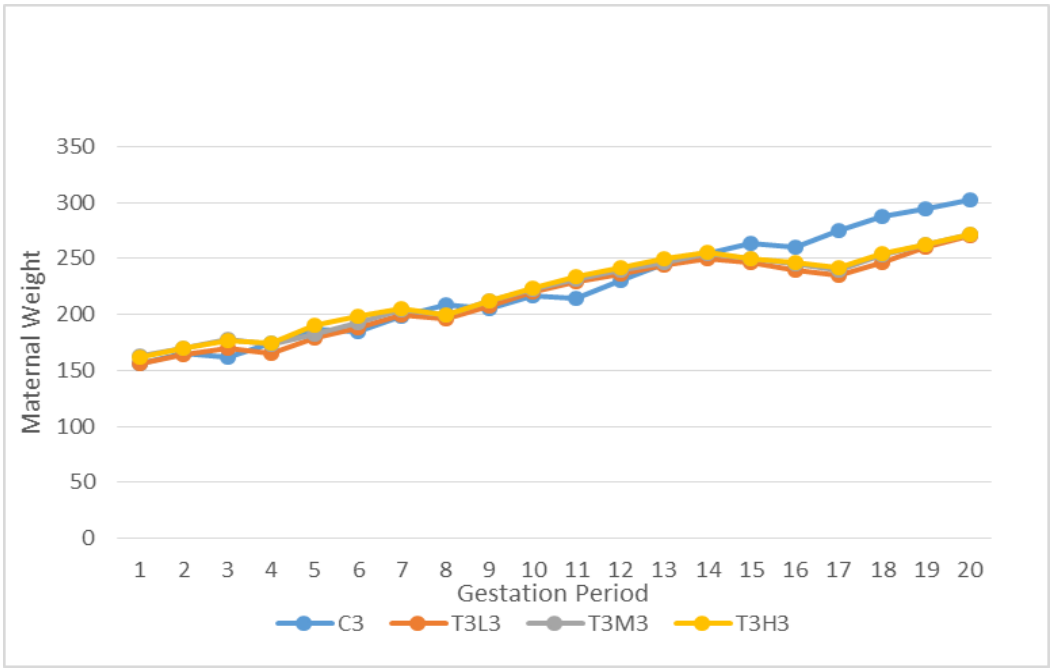


Figure 4.1: Shows the TM_1 mean maternal weight gain trends from GD_1 to GD_{20} in the LPG, MPG and HPG against the control.

Influence of phenytoin on the mean daily maternal weight trends in TM_2

It was observed that the mean maternal daily weight gains during TM_2 reduce in the treatment groups (LPG, MPG and HPG) when compared to those of the control. However, during TM_1 there was no observed differences in the mean maternal weight gain among the treatment groups and the control (Figure 4.2).

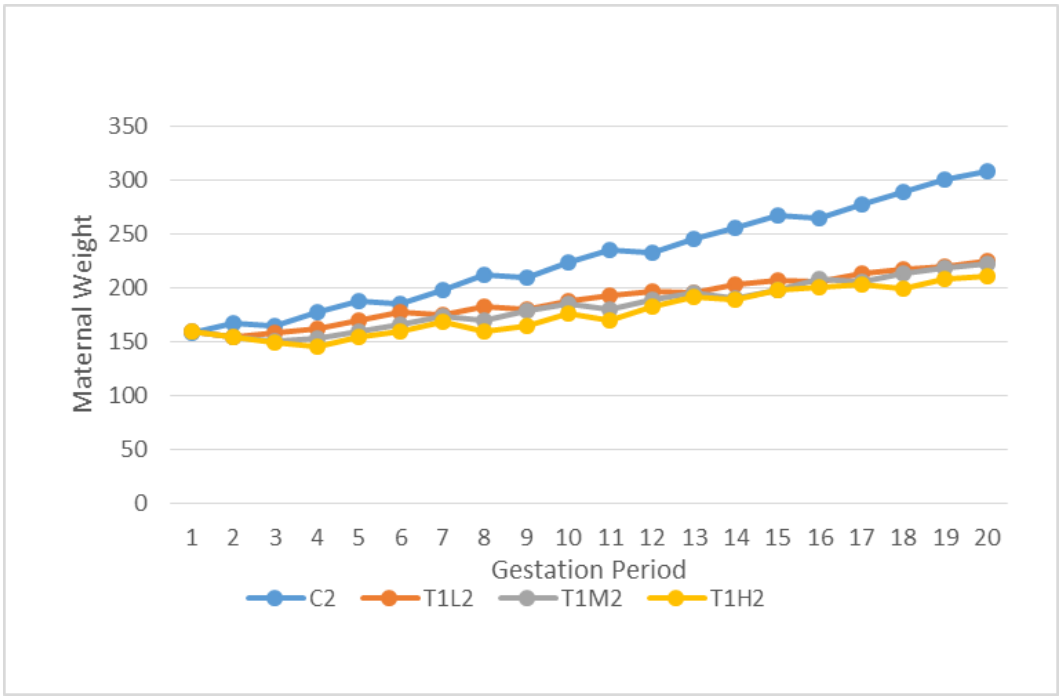


Figure 4.2: Shows the TM_2 mean maternal weight gain trends from GD_1 to GD_{20} in the LPG, MPG and HPG against the control.

Influence of phenytoin on the mean daily maternal weight trends in TM_3

It was observed that the maternal daily weight gains during TM_3 reduce in the treatment groups (LPG, MPG and HPG) when compared to those of the control. However, during TM_1 and TM_2 there was no observed differences in the mean maternal weight gain among the treatment groups and the control (Figure 4.3).

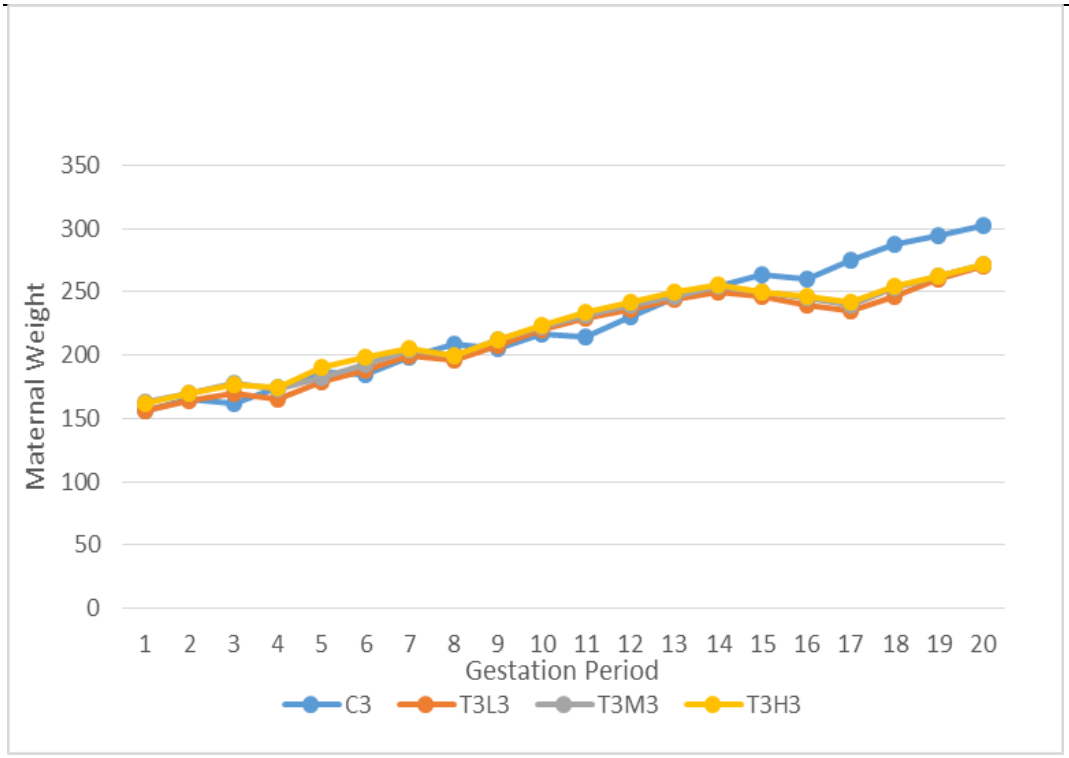


Figure 4.3: Shows the TM_3 mean maternal weight gain trends from GD_1 to GD_{20} in the LPG, MPG and HPG against the control.

4.2.3 The effects of prenatal exposure to phenytoin on the mean fetal body weights, hearts weights, crown rump length, head circumference, and bi-parietal diameter.

It was observed that the mean fetal weights, the mean crown ramp 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 phenytoin treated groups (LPG, MPG and HPG) when compared to that of the control in that all the fetal parameters reduced when high doses of phenytoin were administered at TM_1 and TM_2.

4.2.4 The effects of prenatal exposure to phenytoin on the mean fetal heart weights.

The highest recorded mean fetal heart weights were that of the control group (0.263±0.003) and the lowest among HPG (124mg/kg) at TM_1 (0.157±0.0033g) and TM_2 (0.157±0.003g). The mean of the fetal heart weights depended on dose of phenytoin and the time of exposure with statisticant significant difference of p= 0.001 between the experimental group of MPG, HPG at TM_1, TM_2 and TM_3 as well as LPG at TM_1 and TM_2 from that of the control. MPG-TM_1 (0.157±0.0033g), MPG-TM_2 (0.187±0.003g), MPG-TM_3 (0.22±0.003g); HPG-TM_1 (0.157±0.003g), HPG-TM_2 (0.157±0.003g), HPG-TM_3 (0.187±0.003g), Control (0.263±0.003); LPG-TM_1 (0.177±0.0033g), LPG-TM_2 (0.203±0.003g). However, there was no significance difference of mean fetal heart weights LPG during TM_3 of phenytoin administration and that of the control.

Table 4.2: The intra and inter group comparative means of the fetal body weights, hearts weights, head circumference, crown rump length and bi-parietal diameter of LPG, MPG and the HPG in (TM_1, TM_2 and TM_3) against the control.

Study groups	Period of phenytoin treatment	Mean fetal body weight ± SEM	Mean fetal heart weights ± SEM	Mean head circumference ± SEM	Mean CRL ± SEM	Mean bi-parietal diameter ± SEM
Control group	----------	6.73±0.026a	0.263± 0.003a	3.89±0.010a	4.723±0.030^{a}	0.7155±0.018^{a}
Low dose phenytoin group (LPG, 31g/kg)	TM_1	6.42±0.007^{b}*	0.177± 0.003^{b}*	3.22±0.025^{b}*	4.123±0.009^{b}	0.659±0.073^{b}*
	TM_2	6.57±0.011^{c}*	0.203± 0.003^{a}	3.453±0.029*	4.5±0.009^{b}*	0.686±0.008^{a}*
	TM_3	6.66±0.0168a	0.204±0.003^{a}	3.57±0.037^{b}	4.55±0.027^{b}	0.692±0.006^{b}
Medium dose phenytoin group (MPG, 62gm/kg)	TM_1	6.31±0.046 ^{b}*	0.157±0.003^{b}*	2.95±0.018^{c}*	3.865±0.044^{c}*	0.6296±0.033^{b}*
	TM_2	6.42±0.018^{ab}*	0.187±0.003^{b}*	3.22±0.019^{c}*	4.15±0.0029^{c}*	0.655±0.005^{b}*
	TM_3	6.53±0.004^{c}	0.220±0.003^{a}	3.52±0.015^{b}	4.44±0.028^{b}	0.69±0.004^{d}
High dose phenytoin group (HPG, 124gm/kg)	TM_1	5.42±0.02 ^{b}*	0.157±0.001^{b}*	2.35±0.013^{d}*	3.4±0.023^{d}*	0.579±0.002^{b}*
	(TM_2	5.92±0.00^{b}*	0.157±0.009^{b}*	3.04±0.021^{d}*	3.85±0.0024^{d}*	0.635±0.011^{a}*
	(TM_3)	6.21±0.010^{d}*	0.158±0.001^{b}*	3.41±0.021^{b}*	4.34±0.011^{b}	0.68±0.028^{d}

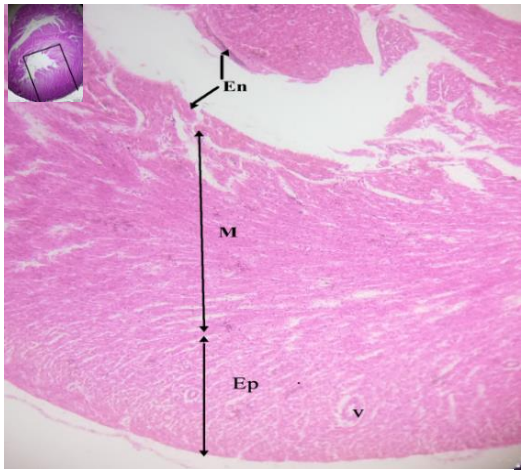
*The means, followed by the same letter in a column are not statistically different at ($p < .05$) using one-way ANOVA with Tukey test on post-hoc t-tests. * indicates significance ($p < .05$)*

4.3 The histomorphological findings on the structures of fetal heart and vascular tunics.

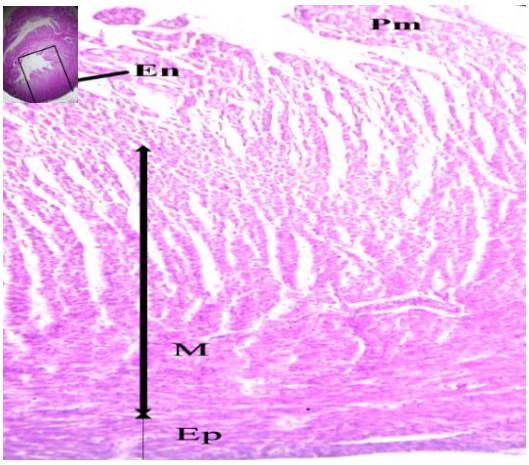
In decribing the histomorphological findings on the effects of prenatal exposure to varied doses of phenytoin on the heart and vascular tunics, the following paramentres were compared between the treatment groups (LPG, MPG and HPG) againt the control; comparative effects on sizes of the heart chambers, the thickness of the heart wall layers, organization of the myocardial muscle fibers, comparative ventricular wall lumens and interventricular septum volumes and comparative thickness of the vascular tunics. (MagX4 and MagX10).

4.3.1 The effects of phenytoin on the ventricular wall layers

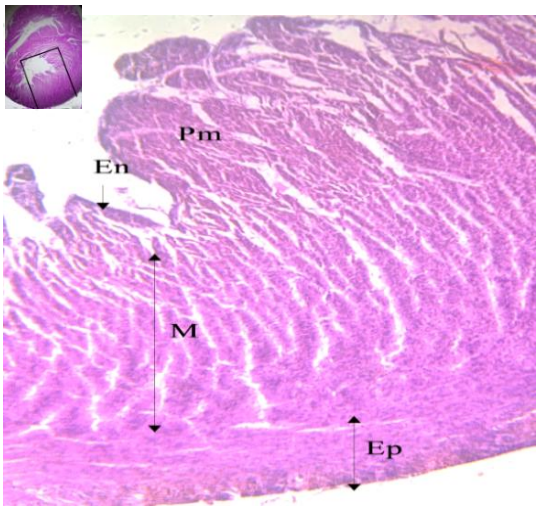
The development and the cyto-differentiation of the various ventricular wall layers that included myocardium, endocardium and the epicardium were shown to be negatively influenced (suppressed) by the in-utero exposure to varied doses of phenytoin.



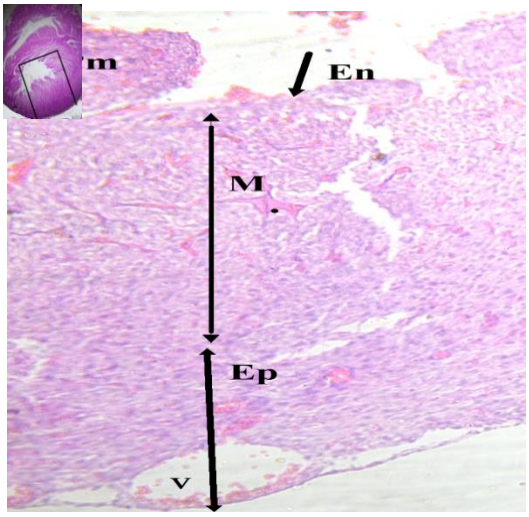
Photomicrograph a: shows well outlined epicardium, densely organized myocardium and well outlined endothelium



Photomicrograph b: shows myocardial disorganisation well outlined epicardium, and endothelium however the myocardial fibers are

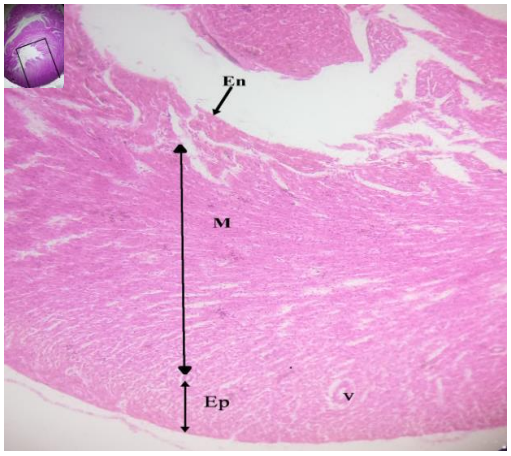


Photomicrograph c: shows disorganised myocardium and poorly outlined endothelium

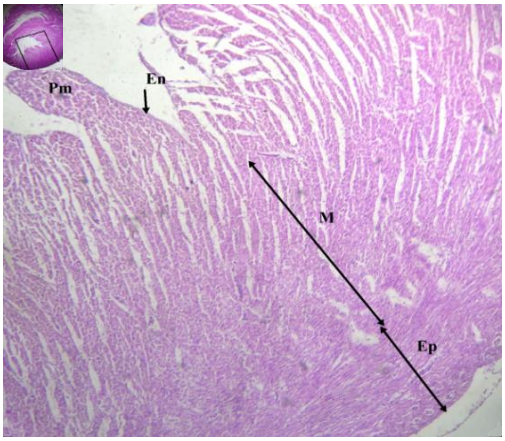


Photomicrograph d: shows poor organization of the myocardium, poorly outlined endothelium

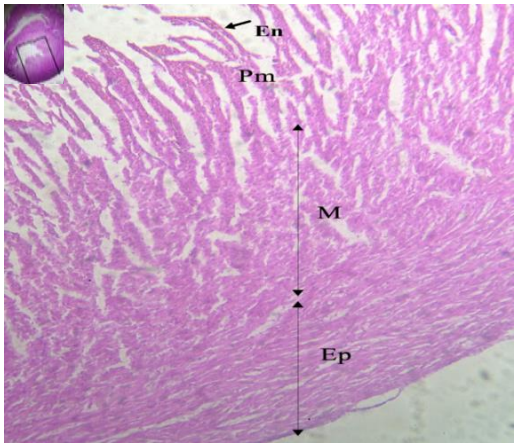
Figure 4.4: The TM_1 comparative left ventricular wall layers; myocardium (M), epicardium (Ep) and endocardium (En) of: (a) control, (b) the LPG, (c) MPG, (d) HPG. Hematoxylin and Eosin (H&E) Mag X10.



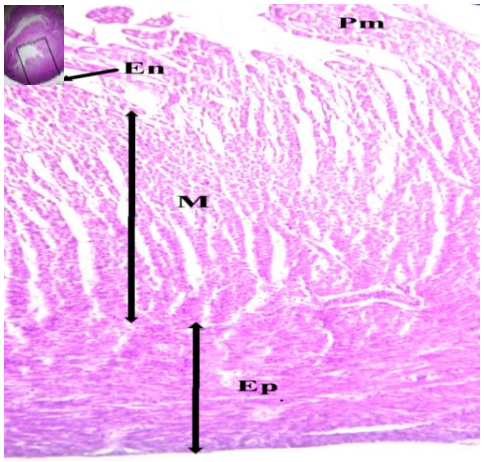
Photomicrograph a: shows well outlined epicardium, myocardium and endothelium



Photomicrograph b: shows well outlined epicardium, myocardium and endothelium however the myocardial fibers are disaggregated

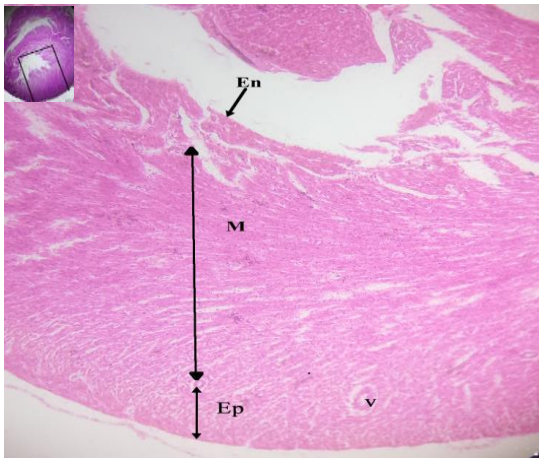


Photomicrograph c: shows disorganization of the myocardial thicknesss, poorly outlined endothelium

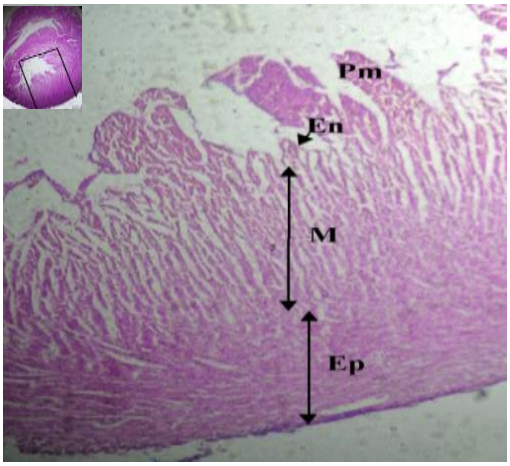


Photomicrograph d: shows disorganized myocardium, poorly outlined endothelium and the epicardium

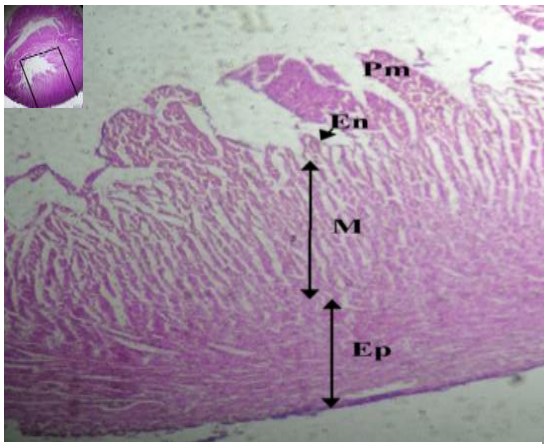
Figure 4.5: The TM_2 comparative left ventricular wall layers; myocardium (M), epicardium (Ep) and endocardium (En) of: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X10.



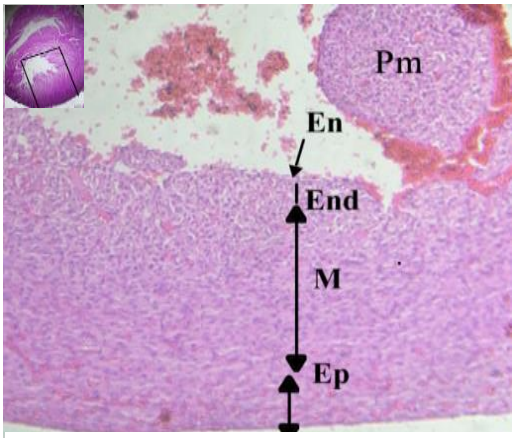
Photomicrograph a: shows well outlined epicardium, densely packed myocardial muscles and well outlined endothelium and vessel



Photomicrograph b: shows disorganization myocardium, poorly outlined epicardium and endothelium



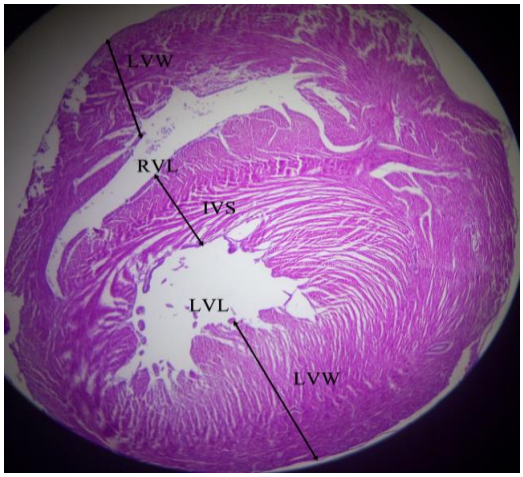
Photomicrograph c: shows poorly outlined endothelium and disassociation of the myocardium



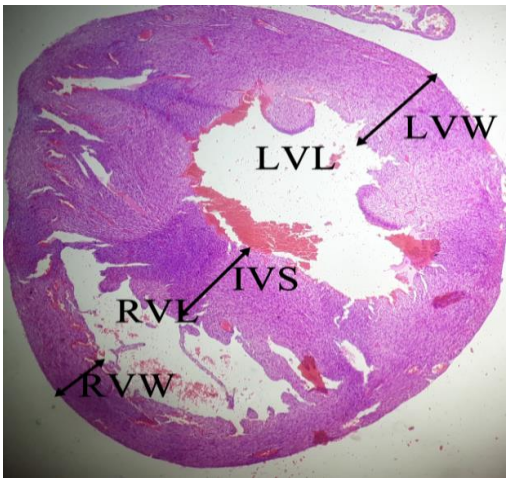
Photomicrograph d: shows disorganization of the myocardium as well as poorly outlined endothelium

Figure 4.6: The TM_3 comparative left ventricular wall layers; myocardium (M), epicardium (Ep) and endocardium (En) of: (a) control, (b) the LPG, (c) MPG, (d) HPG. Hematoxylin and Eosin (H&E) Mag X10.

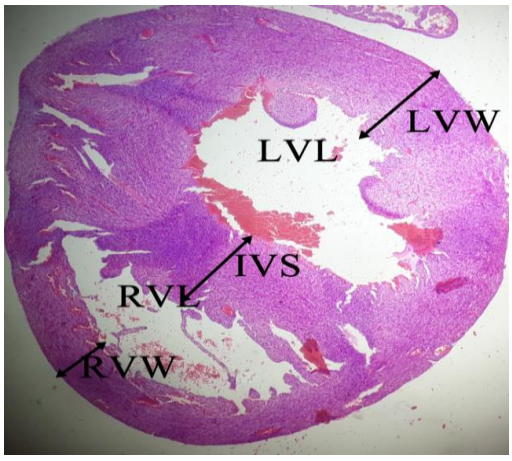
4.3.2 The effects of phenytoin on the left and right ventricular wall, lumen and interventricular septum.



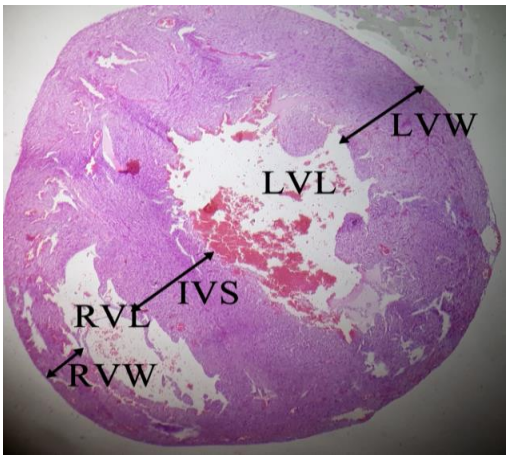
Photomicrograph a: shows well outlined the left and right ventricular wall and lumen with interventricular septum with clearly visible purkinje fibers in the left ventricles



Photomicrograph b: shows decreased left and right ventricular wall thickness, there is no reduction in the IVS and visible purkinje fibers

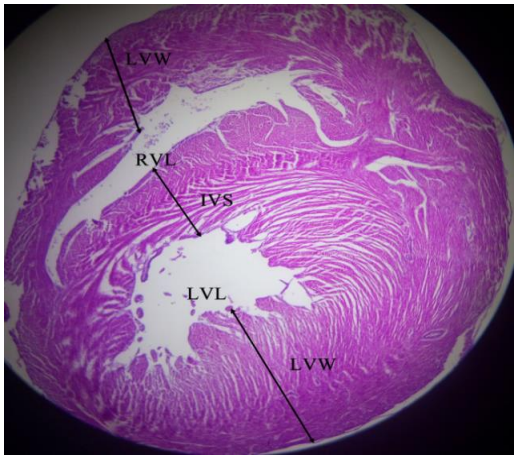


Photomicrograph c: shows decreased left and right ventricular wall thickness, no changes in the IVS thickness and purkinje fibers

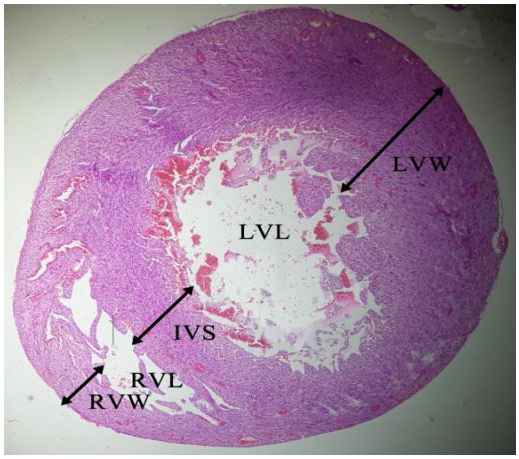


Photomicrograph d: shows decreased left and right ventricular wall thickness, no changes in the IVS thickness and visible purkinje fibers

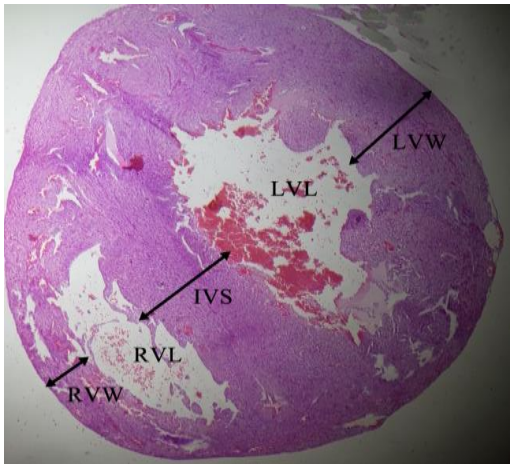
Figure 4.7: The TM$_1$ comparative left and right ventricular wall thickness (LVW, RVW), ventricular lumen (LVL, RVL) and interventricular septum (IVS) in: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X4



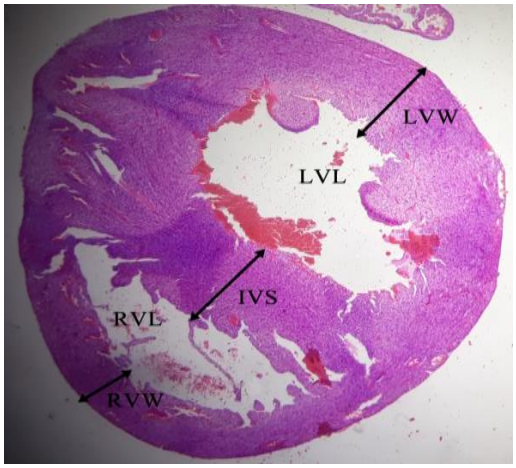
Photomicrograph a: shows well outlined the left and right ventricular wall and lumen with interventricular septum with clearly visible purkinje fibers around the LVL



Photomicrograph b: shows decreased right ventricular wall thickness, poorly outlined interventricular septum thickness and purkinje fibers

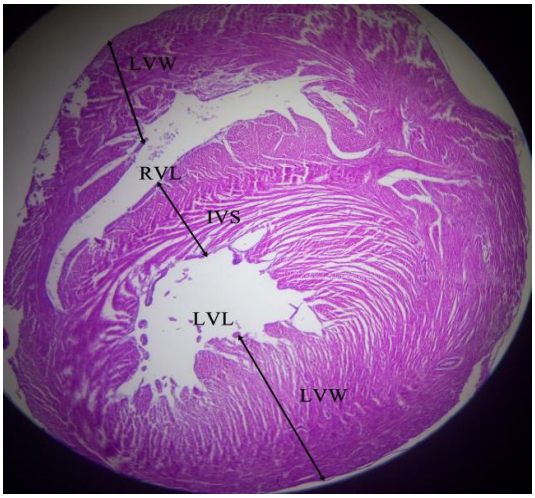


Photomicrograph c: shows decreased left and right ventricular wall thickness, poorly outlined interventricular septum thickness and purkinje fibers

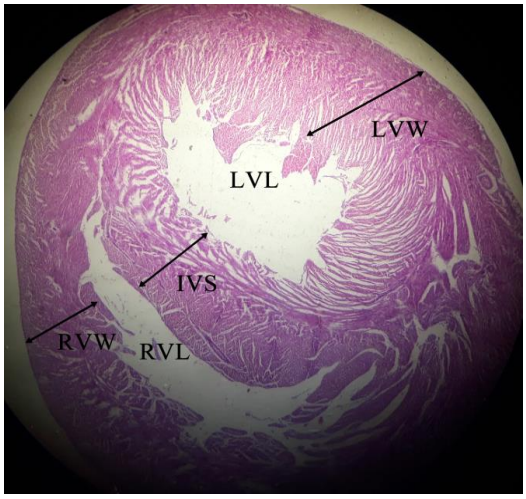


Photomicrograph d: shows decreased left and right ventricular wall thickness, reduced thickness of the interventricular septum thickness and few visible purkinje fibers

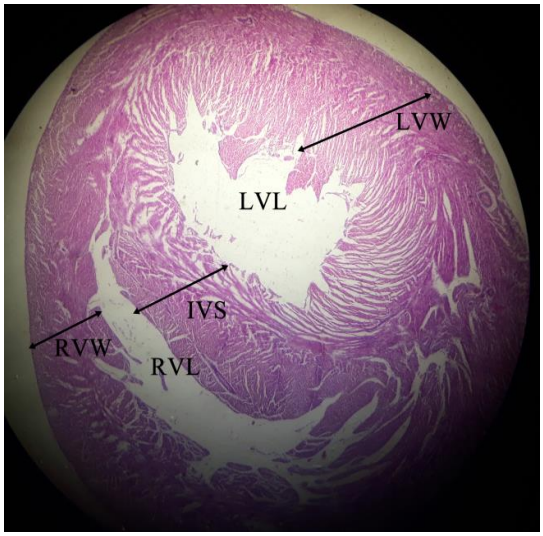
Figure 4.8: The TM2comparative left and right ventricular wall thickness (LVW, RVW), ventricular lumen (LVL, RVL) and interventricular septum (IVS) in: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X4



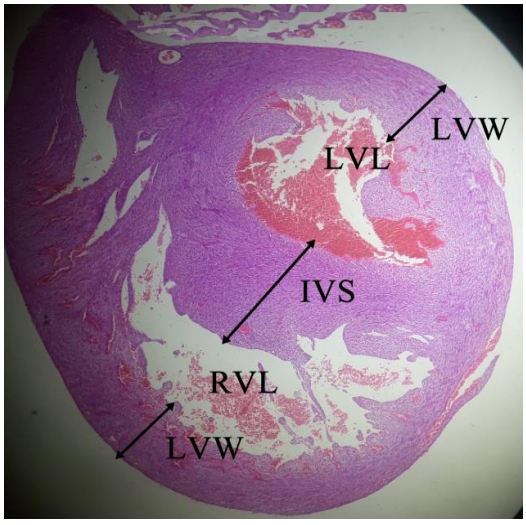
Photomicrograph a: shows well outlined left and right ventricular wall and lumen with interventricular septum with clearly visible purkinje fibers around the lumens



Photomicrograph b: shows slightly decreased left and right ventricular wall thickness, well outlined interventricular septum and purkinje fibers



Photomicrograph c: the left and right ventricular wall did not reduce in thickness, well outlined interventricular septum and purkinje fibers

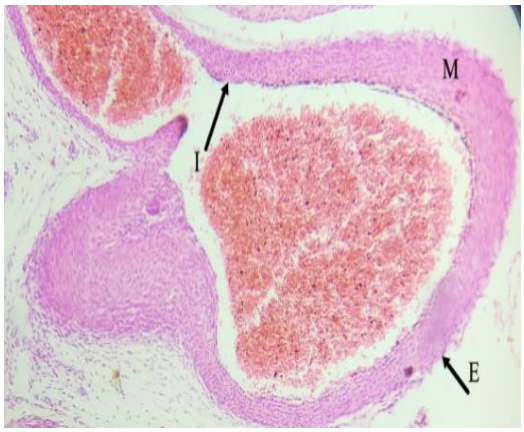


Photomicrograph d: shows decreased left and right ventricular wall thickness, poorly outlined interventricular septum thickness and purkinje fibers

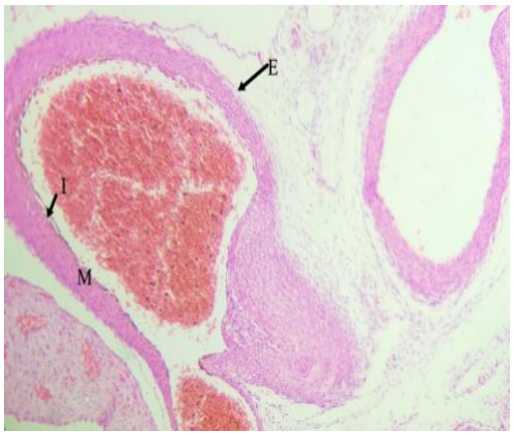
Figure 4.9: The TM_3 comparative left and right ventricular wall thickness (LVW, RVW), ventricular lumen (LVL, RVL) and interventricular septum (IVS) in: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X4

4.4.3 The effects of phenytoin on the fetal heart vascular tunics and the surrounding connective tissues

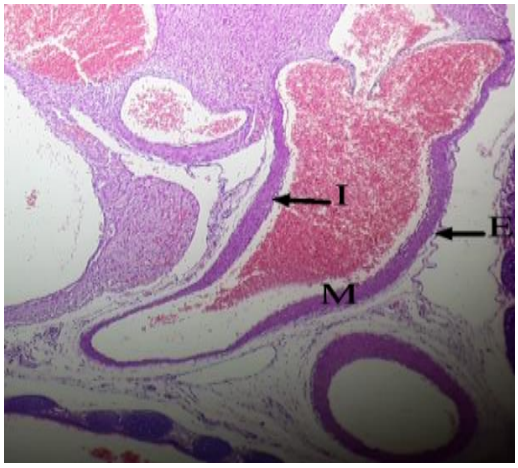
The finding of the study shows that when varied doses of phenytoin was administered in TM_1 (GD_{1-20}), there was relative reduction in the fetal heart vascular tunic wall and the surrounding connective tissues among the treatment groups compared to that of the control. There was marked reduction of the vascular tunic wall and the surrounding connective tissues among the high phenytoin groups followed by medium phenytoin groups and least among the low phenytoin groups



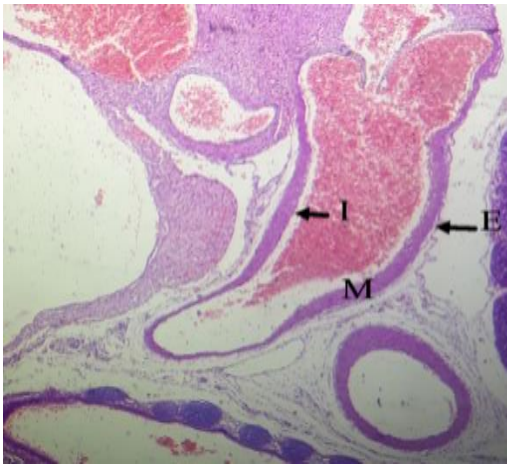
Photomicrograph a: shows the vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the control



Photomicrograph b: shows slightly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the LPG

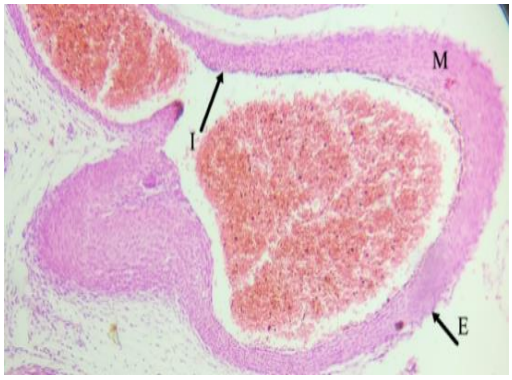


Photomicrograph c: shows markedly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the MPG

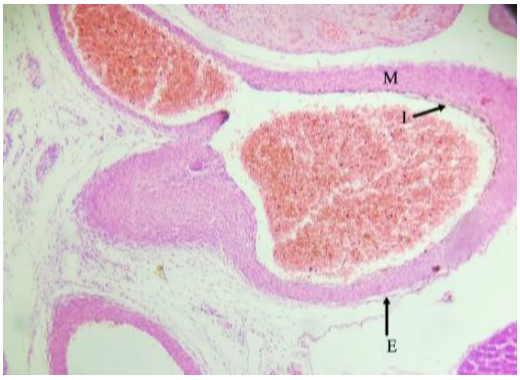


Photomicrograph d: shows markedly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the HPG

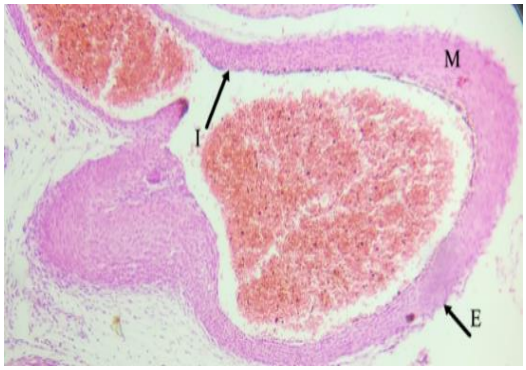
Figure 4.10: The TM_1 comparative vascular tunic wall thickness and the surrounding connective tissues in: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X10



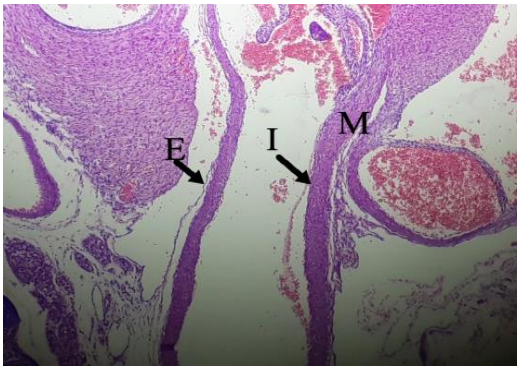
Photomicrograph a: shows the vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the control



Photomicrograph b: shows slightly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the LPG

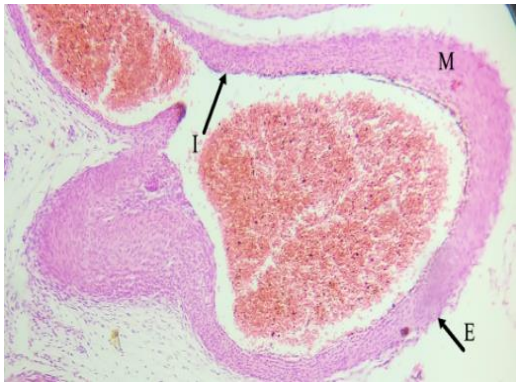


Photomicrograph c: shows markedly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the MPG

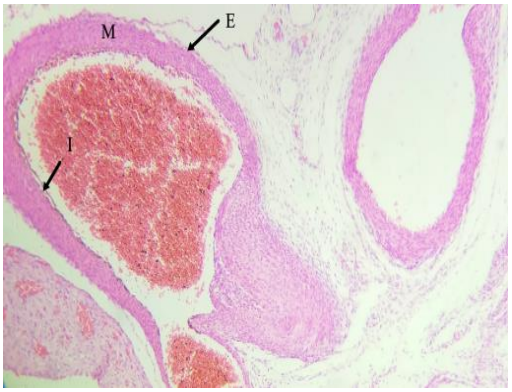


Photomicrograph d: shows markedly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the HPG

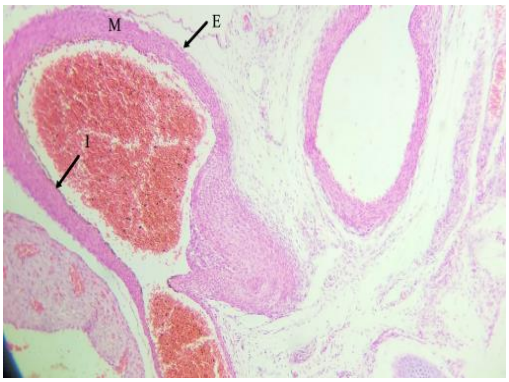
Figure 4.11: The TM_2 comparative vascular tunic wall thickness and the surrounding connective tissues in: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X10



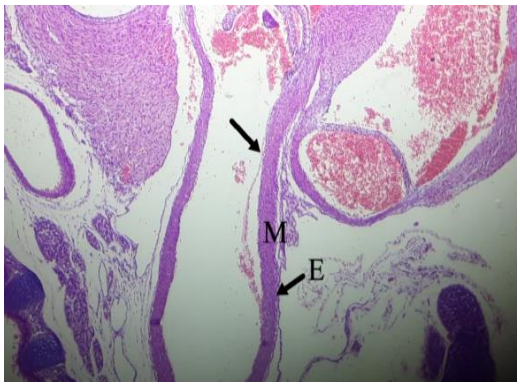
Photomicrograph a: shows the vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the control



Photomicrograph b: shows slightly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the LPG



Photomicrograph c: shows markedly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissue of the MPG



Photomicrograph d: shows markedly reduced vascular wall thickness constituting intima, media and externa and the surrounding connective tissues of the HPG

Figure 4.12: The TM_3 comparative vascular tunic wall thickness and the surrounding connective tissues in: (a) control, (b) the LPG, (c) MPG, (d) HPG Hematoxylin and Eosin (H&E) Mag X10

4.4 The histostereological phenytoin teratogenic outcomes on the fetal heart.

Comparative histostereological teratogenic outcomes on the fetal heart and the vascular tunics are presented in terms of the observed changes on the mean total heart volumes, mean total wall volume, mean total lumen volume, mean total septal volume and vascular tunic wall volume. All these parameters were compared along the varying phenytoin doses (Low-31mg/kg, Medium-62mg/kg, High-124mg/kg) against the period of exposure in TM_1, TM_2 and TM_3 as follows.

4.4.1 The TM_1, TM_2 and TM_3 histostereological findings on the total fetal heart volumes (mm^3) in the LPG, MPG and HPG against the control.

The mean total fetal heart volume decreased with increasing phenytoin dose when administered in TM_1. HPG had the lowest mean total fetal heart volume of 311.40 mm^3 followed by MPG (338.99 mm^3) which was significantly different with that of the control (378.13 mm^3) P=0.001. Similarly, LPG mean fetal total heart volume was lower (353.88 mm^3) from that of the control. The total heart volume among the control group was significantly higher than that of the phenytoin treatment groups in both water immersion and cavalieri methods with P=0.001. However, post hoc test results showed there was no significance difference between LPG and MPG but there was significance difference between MPG and HPG.

When phenytoin was administered in TM_2, it was observed that the mean total fetal heart volume decreased with increasing phenytoin doses. HPG had the lowest mean total fetal heart volume of 317.10mm^3 followed by MPG (345.59 mm^3) which was significantly different from that of the control (378.13 mm^3) p=0. 001.The total heart volume among the control group was significantly higher than that of the phenytoin treatment groups in both water immersion and cavalieri methods p=0.001, the difference in the control and LPG was not significant in cavalieri method. However, post hoc test results showed there was no significance difference between LPG and MPG as well as between MPG and HPG.

From the study findings it was observed that when phenytoin doses were administered in TM_3, the mean total fetal heart volume decreased remarkedly among the high and medium phenytoin dose groups (339.82mm^3, 370.51 mm^3) respectively and this difference was significant when compared to that of the control (378.13) p= 0.001. The mean total heart volume among the LPG was 371.37 mm^3 which was lower than that of the control group, however the difference was not significant. Also post hoc test results showed there was no significance difference between MPG and HPG (Table 4.3).

Table 4.3: The TM_1, TM_2 and TM_3 comparative means of the total fetal heart volumes (mm^3) in LPG, MPG and HPG against the control.

		Control	LPG (31mg/kg)	MPG (62mg/kg)	HPG (124mg/kg)	F-value	P-value
TM_1	WIM	399.53±4.80^a	376.84±4.27^b	351.91±4.79^c	335.33±4.32^c	38.307	<0.001*
	CM	378.13±4.57^a	353.88±3.75^{ab}	338.99±4.36^b	311.40±10.4^c	19.253	<0.001*
TM_2	WIM	399.53±4.80^a	390.90±4.54^a	370.51±4.60^b	357.70±3.52^b	18.771	<0.001*
	CM	378.13±4.57^a	371.37±13.09^a	349.91±4.49^{bc}	339.82±4.14^c	7.388	<0.001*
TM_3	WIM	399.53±4.80^a	390.90±4.54^a	370.51±4.60^b	357.70±3.52^b	18.771	<0.001*
	CM	378.13±4.57^a	371.37±13.09^a	349.91±4.49^{bc}	339.82±4.14^c	7.388	<0.001*

*Notes: The means, followed by the same letter in a row are not statistically different at ($p < .05$) using one-way ANOVA. with Tukey test on post-hoc t-tests. * indicates significance ($p < .05$).*

4.4.2 The TM_1, TM_2 and TM_3 histostereological findings on the mean total fetal heart wall and septal volume densities in the LPG, MPG and HPG against the control.

When phenytoin was administered in TM_1, the mean total heart wall volume, decreased with increasing phenytoin doses with the lowest total wall volume being 155.70mm^3 among the high phenytoin dose group, followed by the median phenytoin group that was found to be 169.49 mm^3 while that of the low phenytoin group was 176.94 mm^3, the volume of the control group was highest (189.07 mm^3). There was significance difference between the mean total volume of the wall, lumen and the septum of the high phenytoin group and that of the control p= 0.001. Similarly, the mean total septal volume and the mean total lumen volume were reducing as per the dosage of phenytoin.

According to the study outcomes it was observed that when phenytoin was administered at TM_2 the mean total heart wall volume was found to be lowest in high followed by median phenytoin group (158.55mm^3, 172.79mm^3 respectively) which was significantly different from that of the control (189.07mm^3) p = 0.001, low phenytoin group fetal heart wall volume was also reduced (178.84mm^3) compared to that of the control, however the difference was not significant. Similarly, the mean total septal volume was reducing as per the dosage of phenytoin.

From the study findings it was observed that when phenytoin was administered at TM_3 the mean total heart wall volume was lowest in high followed by median phenytoin group 169.91, 174.96 respectively which was significantly different from that of the control (189.07) p= 0.001, low phenytoin group fetal heart wall volume was also reduced (188.68.) compared to that of the control, however the difference was not significant. Similarly, the mean total septal volume was reducing as per the dosage of phenytoin

Table 4.4: The TM_1, TM_2 and TM_3 comparative means of the total fetal heart wall and septal volume densities (mm^3) in LPG, MPG and HPG against the control.

Parameter	Control	LPG (31mg/kg)	MPG (62mg/kg)	HPG (124mg/kg)	F-Statistic	P-value
Fetal Heart Wall Volume	189.07±2.29 [a]	176.94±1.87 [ab]	169.49±2.18 [bc]	155.70±5.20 [d]	19.25	<0.001*
Fetal Heart Septum Volume	64.28±0.78 [a]	60.16±0.64 [ab]	57.63.87±0.74 [bc]	52.94±1.77 [d]	15.25	<0.001*
Fetal Heart Wall Volume	189.07±1.60 [a]	178.84±2.24 [ab]	172.79±2.80 [bc]	158.55±6.50 [c]	10.76	<0.001*
Fetal Heart Septum Volume	64.28±0.78 [a]	53.65±0.67 [ab]	51.84±0.84 [bc]	47.57±1.95 [c]	10.76	<0.001*
Fetal Heart Wall Volume	189.07±1.60 [a]	188.68±6.55 [ab]	174.96±2.24 [bc]	169.91±2.07 [c]	7.39	<0.001*
Fetal Heart Septum Volume	64.28±0.78 [a]	57.21±1.96 [ab]	52.49±0.67 [bc]	50.97±0.62 [c]	7.39	<0.001*

*Notes: The means, followed by the same letter in a row are not statistically different at ($p < .05$) using one-way ANOVA. with Tukey test on post-hoc t-tests. * indicates*

4.4.3 The histostereological findings on the mean total fetal heart vascular tunic volume (mm^3) in LPG, MPG and HPG against the control at TM_1, TM_2 and TM_3.

From the study findings it was observed that the mean total vascular tunic volume reduced with increasing dose of phenytoin particularly when administered during TM_1 depicted by high phenytoin group volume of 15.57mm^3 and 15.86mm^3 at TM_1 and TM_2 respectively which was significantly different from that of the control (18.91mm^3) p=0.001. Similarly, the mean total vascular tunic volume of the median and low phenytoin group was different from that of the control, however it was not significant when phenytoin was administered in TM_1, TM_2 and TM_3 (Table 4.5).

Table 4.5: The TM_1, TM_2, TM_3 comparative means of the total fetal heart vascular tunic volumes (mm^3) LPG, MPG and HPG against the control.

Parameter	Control	LPG (31mg/kg)	MPG (62mg/kg)	HPG (124mg/kg)	F-Statistic	P-value
TM_1 mean vascular tunic volume	18.91±0.23 [a]	17.69±0.19 [a]	17.07±0.21 [a]	15.57±0.52 [b]	14.74	<0.001
TM_2 mean vascular tunic volume	18.91±0.23 [a]	17.88±0.22 [a]	17.22±0.31 [a]	15.86±0.65 [b]	7.91	<0.001
TM_3 mean vascular tunic volume	18.91±0.23 [a]	17.07±0.65 [a]	17.54±0.25 [a]	17.54±0.25 [a]	5.42	<0.02

Notes: The means, followed by the same letter in a row are not statistically different at ($p < .05$) using one-way ANOVA.

CHAPTER FIVE

DISCUSSION

5.1 Maternal pregnancy outcomes

The findings of this study established that maternal pregnancy outcome that include the litter size, resorbed endometrial glands, placental weights among others reduced with the increasing dose of phenytoin and at the same time it was marked when administered during the first trimester. The mean litter size significantly decreased with increasing dose of phenytoin among the treatment groups particularly when given during TM_1 4±0.577compared to the control (13±0.577) $p < .05$ (Table 4.1) Resorbed endometrial glands mean was found to be significantly high in high dose group when phenytoin was administered during trimester one and two TM_1, TM_2 (5±0.333g, 3±0.577g) compared to that of the control (0.33±0.333g) (Table 4.1) These finding are in tandem with the study that was done by (Danielsson *et al*., 2005) and the group on phenytoin teratogenic effects due to hypoxia and vascular disruption in that nutrients that include oxygen are important and lay a key role in the normal physiology of pregnancy and also proper development of embryonic structures and therefore hypoxia interfere with the normal physiology of pregnancy hence resulting in undesirable pregnancy outcome .

The Mean Placental weights were found to decrease with increase in phenytoin dose with significant difference between the treated groups (TM_1 high dose group 0.27±0.003g) and that of the control (0.43±0.009g $p < .05$ (Table 4.1) while the mean devoured endometrial glands were found to be high when phenytoin was administered during TM_3 at higher doses HPG-124mg/kg (3.67±0.333g) compared to the control (0.33±0.333g) $p < .05$ (Table 4.1) These findings could be linked to the study that was done by Watkinson that demonstrated phenytoin possibility to interfere maternal cardiovascular functions causing uterine ischemia hence reducing placental oxygen delivery interfering with the pregnancy state (Gelder *et al*., 2010). In addition, these results are in tandem with a study that was done by Cart when phenytoin was compared to class III antiarrhythmic drugs that both block the sodium

channels causing low heart rate and cardiac arrest (Danielsson & Skold, 2001). In TM_3, embryologically the cardiovascular system of the developing embryo has started to function and therefore phenytoin use during this period of gestation may cause bradycardia hence reduction of blood supply to embryonic tissues leading to growth retardation. Furthermore, it may cause cardiac arrest which explains the increased number of devoured endometrial glands (Guldiken *et al*., 2015).

5.2 Fetal outcome

The findings of this study on fetal pregnancy outcome parameters such as fetal weights, crown rump length head circumference among others was decreased with increasing dose of phenytoin marked when administered during trimester I of gestation. There was statistical significance difference between mean fetal weights across the gestational period in MPG and HPG from that of the control. These concur with findings in a study that was done by (Zhu & Zhou, 1989) demonstrating effects of supplementing folic acid to patients using phenytoin prenatally and the results was significantly different between the group that used folic acid supplements in terms of fetal weight and length (higher) from the group that did not use folic acid supplements (low) in terms of fetal weight and length. When the absorption of nutrient such as folic acid is reduced during pregnancy which has been attributed to prenatal phenytoin use, it has an impact to the pregnancy outcome because it is one of the essential requirements for proper development of the embryonic structures.

This study also established that the growth parameters such as head circumference and bi-parietal diameter of the fetuses obtained from the control group had a statistical difference (higher) than the experimental groups (LPG, MPG and HPG) in trimester I, trimester II and trimester III (Table 4.2). These findings are supported by findings of another study that was conducted by (Waltman, 2003) on fetal effects of phenytoin that showed a variety of manifestations such as fetal hyadantoin syndrome, facial clefting, heart malformations, limb deformities and still births explained by phenytoin alteration of vitamin D metabolism through inhibition of

calcium absorption during pregnancy which inturn lead to low level of calcium in the fetus. From the clinical studies, calcium is important in growth of the the fetus particularly the growth of bones and by the fact that this study found reduced crown rump length, head circumfrence and bi-parietal diameter of the experimental group indicates slow growth of bones due to low calcium levels. The reduction in fetal parameters could also be attributed to hypoxia in the embryonic tissue due to low heart rate of the embryo and this is even increased by hemodynamic alterations of the mother such as arryhythmia, hypotention which reduce blood flow through the placenta hence interfering with the developing tissues of the fetus according to Kakkar *et al*., (2013). Nutrients ,oxygen included are very essential in growth and development of various tissues and organs of the embryo, therefore embryonic bradycardia reduce nutrient supply to the tissues hence poor development manifested clinically as low fetal weight with or without malformations. The same effects are also attributed to the hemodynamic alterations of the mother that result in reduced placental blood flow hence reduced nutrients to the embryo by the fact that the placenta is the main site through which nutrients diffuse to the developing embryo.

Antiepileptic drugs(AED's) phenytoin being one of them were earlier postulated to disturb embryonic and fetal developing structures by interfering with moternal folate metabolism which leads to folate deficiency in the mother (Galappatthy *et al*., 2018). Maternal folate deficiency is one of the predisposing factors to fetal malformations particularly the neurotube defects and these supports this study findings of the different (reduced) fetal crown rump length, head circumfrence, head length and bi-parietal diameter of the experimental group from the control group.

5.3 Discussion on histomorphology

The histomorphological findings of this study was poor organization of the ventricular wall layers particularly the myocardium of the left ventricle. At the same time the right and the left ventricular wall thickness reduced. In addition, the study findings showed vascular disruption when phenytoin was administered in high doses as shown in fig 4.4 photomicrograph d. This study findings concurred with another

study done by Webster *et al*., (1997) that found phenytoin to cause fetal hypoxia, vascular disruption and necrosis of existing and developing structures of the fetus.

The findings of this study also showed reduced thickness of the left and right ventricular wall with increasing dose of phenytoin as shown in figure 4.7 photomicrograph b, c and d when administered at trimester I of gestation. This findings could be attributed to another study findings that was done by (Katsiki *et al*., 2014) that showed vascular disruption and necrosis of existing and developing embryonic structures due to generation of reactive oxygen species within the embryo during reoxygenation possibly resulting in free radicle damage. (Appleton & Gill, 2003) also conducted a study of phenytoin and found that embryonic bradycardia which leads to severe hypoxia and alteration in embryonic blood flow and blood pressure induce cardiovascular defects which would possibly present in various histomorphological changes of the heart as described in this study.

5.4 Discussion on stereology

This study examined the quantitative effects of prenatal exposure to different phenytoin dosages on fetal heart while administered at different gestational periods. Results showed that fetuses of the experimental group had reduced total heart volume, wall and septal volume densities among phenytoin treatment groups, particularly when administered in the first and second trimester (TM_1 and TM_2 using high phenytoin dose. In this study, the reduction in fetal heart volumes (total heart volume, wall and septal volume densities) observed in phenytoin treatment groups can be attributed to the fetal heart alterations during its development (Andermann, 1992). This study is in agreement with a previous study by Gelder *et al*., (2010) on effects of antiepileptic drugs; lamotrigine, carbamazepine, phenobarbitone, valproic acid among others on fetal growth and development when exposed in-utero and especially during organogenesis.

This study found reduction in total heart volume that could be attributed to disruption of its development like; alteration of embryonic blood flow and blood pressure (Danielsson & Skold, 2001), fetal hypoxia, vascular disruption and necrosis of existing and developing structures that may be attributed to generation of reactive

oxygen species within the embryo during reoxygenation possibly resulting in free radicle damage (Webster *et al*., 1997). Study done by Bittigau *et al*., (2002) demonstrated effects of antiepileptic drugs including phenytoin through apoptotic neurodegeneration on the fetal structures. Patocka *et al*., (2020) findings showed cerebella atrophy following phenytoin use. These findings help explain the reduction in the total heart volume and vascular tunic volume as well the volume densities of the fetal heart wall and the septum. On the other hand , postnatal study done by Ksoo *et al*., (2017) on various antiepileptic drugs contrast the findings of this study by demonstrating increase in blood vessel wall thickness resulting in arthrosclerosis that manifests with impairment of the cardiovascular system functions.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

In conclusion, the study found out that phenytoin is teratogenic to the developing heart and vascular tunic when used during trimester one and two (TM_1 and TM_2) of pregnancy. There were no significant teratogenic outcomes of phenytoin on the heart and the vascular tunics when used in trimester III except the high dose. The first trimester was found to be the most vulnerable period for phenytoin teratogenicity while the most critical dose was 124mg/kg.

6.2 RECOMMENDATION

1. The study recommends that the use of phenytoin should be avoided prenatally because of its teratogenicity to the fetal heart and vascular tunics particularly during trimester one and two (TM_1 and TM_2)
2. Health education on phenytoin teratogenicity should be done frequently to medical practitioners so that they can sensitize mothers who are using this medication for early planning of pregnancy in order obtained desired outcome possible by using an alternative medication or adjusting the dosages as well as appropriate time of exposure.
3. Further studies also need to be carried out with higher primates in order to determine phenytoin dose rationalization and its application during pregnancy.

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APPENDICES

APPENDIX I - DATA CAPTURE SHEETS

DATA CAPTURE SHEET FOR EXPECTANT ALBINO RATS

ALBINO RAT

IDENTITY……

INITIAL WEIGHT……DOSE CALCULATION……

DATE	**WEIGHT IN GRAMS**	**PHENYTOIN DOSE (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 CROWN RUMP LENGTH(CM)												
OBVIOUS CONGENITAL ABNORMALITIES OF THE FETUS												
RESORPTIONS/DEVOURED FETUSES												
PLACENTA WEIIGHT												

HEAD CIRCUMFERENCE												
HEAD LENGTH												
BI-PARIETAL DIAMETER												
HEART												
GROSS APPEARANCE												
OBVIOUS CONGENITAL ANOMALIES OF THE HEART												
HEART WT(g)												
TOTAL HEART VOLUME(WIM)												

APPENDIX II: ETHICAL CLEARANCE 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**

Caroline Sigei
Department of Human Anatomy.

Dear Ms. Sigei,

RE: HISTOMORPHOMETRIC STUDY ON THE EFFECTS OF PRENATAL PHENYTOIN EXPOSURE IN THE DEVELOPMENT OF THE FETAL HEART AND GREAT VASCULATURE 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,
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APPENDIX III: FRONT PAGE OF THE PUPLICATIION

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Effects of Prenatally Exposed Phenytoin Varying Doses on Fetal Growth of Albino Rats

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Abstract: *Phenytoin is an antiepileptic drug with no sedative effects widely used in treatment of convulsive disorders. However, its use in pregnancy has been associated with various fetal malformations and impairment of fetal growth.Many factors have been attributed to these effects including phenytoin capacity to cause hypoxia in embryonic tissue, interference with maternal folate metabolism, maternal hemodynamic alterations, its inhibition on potassium channels among others,these consequently affect various developing fetus structures and organs that be associated with currently common postnatally problems of unknown cause such as growth retardation, congenital rickets ,congenital heart diseases, hypertension among others. This study aims toinvestigate the effects of prenatally used varying doses of phenytoin(low, medium, high)during different gestational periods (trimester1, 2, 3) on albino rat fetal growth. Pregnant albino rats were divided into four groups; group 1(control) and group 2,3,4 (experimental).The control group receive normal standard diet while the Experimental(group 2,3,4 which are low, medium and high phenytoin group respectively. These three groups of 9 albino rats each received daily phenytoin drug. Group 2-low phenytoin group received 30 mg/kg of phenytoin, group 3-medium phenytoingroup received 60mg/kg of phenytoin and group 4 -high phenytoin group received 120mg/kg of phenytoin. Animals in each group 2,3,4 were randomly assigned to trimesters of 3 animals each(trimester 1,2 and 3).Trimester 1 animals received phenytoin from day 1-20 of gestation, trimester 2 animals received phenytoin from day 7-20 of gestation and trimester 3 animals received phenytoin from day 14-*