HISTOQUANTITATIVE TERATOGENIC EFFECTS OF PRENATAL EXPOSURE TO TETRACYCLINE ON FETAL SKELETOGENESIS IN ALBINO RATS (Rattus *norvegicus*)

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JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2020

Histoquantitative teratogenic effects of prenatal exposure to tetracycline on fetal skeletogenesis in Albino rats

(Rattus *norvegicus*)

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A thesis submitted in partial fulfillment for the award of Master of Science in Human Anatomy in the Jomo Kenyatta University of Agriculture and technology

2020

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 wish to dedicate this thesis to my family members (Doris, Malia and Anna-Riita Marja) as well as my mum Auma Nabade, the Kiiveris', Jarkkos', mama Marja-Liisa and the late papa Illka.

ACKNOWLEDGEMENT

I hereby wish to recognize with much appreciation the guidance and support I received from my supervisors; Dr. Joseph Kariuki Kweri, Dr. Reuben Thuo and Dr. George Kibe Kafaya in the research process. The successful completion of this thesis was through their thoughtful guidance. I also wish to acknowledge Mr. Paul Kiarie for his expertise guidance on statistical analysis and interpretation of my research data.

I similarly wish to extend my sincere gratitude to the technical staff in the Department of Human Anatomy; Mr. Chege Bela, Mrs. Pamella Emali, Ms. Grace Gathoni, Mr. Dennis Koech and Mr. William Mwaura for their support and encouragement during histological tissue processing. Finally, I wish to appreciate my classmates in the MSc. Human Anatomy class namely; Ann Mwangi, James Kanyoni, Terry Musa, Rono Walter, Peris Gathoni, Asena Shadrack, Caroline Sigei and Caroline Ndung'u for their help and positive criticism throughout the research process.

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ABBREVIATIONS AND ACRONYMS

ACL	Anterior Cruciate Ligament
ANOVA	Analysis of Variance
Av. TL	Average Tibial Length
BPD	Biparietal Diameter
CC	Cartilaginous Core
CRL	Crown Rump Length
DMSO	Dimethyl Sulphoxide
FDA	Food and Drug Administration of the United States of America
GR	Groove of Ranvier
HDG	High Dose Group
HZ	Hypertrophic Zone
JC	Joint Cavity
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KOH	Potassium Hydroxide
LDTG	Low Dose Tetracycline Group
MDTG	Medium Dose Tetracycline Group
MLBwt	Mean Litter Birth Weight
MMP	Matrix Metalloproteinases
NB	New Bone
OPG	Osteoprotegerin
PS	Primary Spongiosa

PZ	Proliferation Zone
RANK	Receptor Activator of Nuclear factor k B
RANK-L	Receptor Activator of Nuclear factor k B- Ligand
RC	Reserve Cartilage
RZ	Resting Zone
SAJOREC	Sino-Africa Joint Research Centre
SEM	Standard Error of Mean
SS	Secondary Spongiosa
TM_1	Trimester 1
TM_2	Trimester 2
TM_3	Trimester 3
TSA of E	Total Surface Area of Epiphyseal growth plate
TZ	Transformation Zone
WHO	World Health Organization

DEFINITION OF TERMS

Embryogenesis the process by which the embryo forms and develops.

Groove of Ranvier a cuff of chondroblasts surrounding the resting cartilage

Histogenesis differentiation of cells into specialized tissues and organs during growth.

Histoquantitative numerical data of histological parameters.

Morphometry measurement of organs.

Ossification process of bone formation.

Primary spongiosa the immediate histological layer of the metaphysis that is adjacent to the epiphyseal growth plate.

ABSTRACT

The bone teratogenic effects of tetracycline group of medicines following in-utero exposure has been contentious of late in that doxycline and minocycline have been shown to enhance bone formation in low doses. This is contrary to the documented bone calcium chelation effect of tetracycline's. The broad objective of this study therefore was to evaluate the histoquantitative and histomorphological teratogenic effects of tetracycline on fetal skeletogenesis upon exposure to varied doses of tetracycline at different gestational periods in Albino rats (Rattus *novegicus*). In carrying out this study, 30 Albino rat dams weighing between 200-250g were used. The 30 dams were grouped into 3 control group and 27 experimental group rats which were further grouped into 3 different groups each having 9 rats to receive tetracycline over different durations. The 9 rats were further subdivided into 3 different dosing categories as Low (155mg/kg/day), Medium(232mg/kg/day) and High dose (310mg/kg/day) tetracycline groups. Confirmation of pregnancy marked the 1^{st} day of pregnancy, treatment began and animals sacrificed on day 20 of gestation using concentrated CO_2 and fetuses harvested. Gross morphometric measurements were done using a digital Vanier calipers while photomicrographs were analyzed using Image J v1.50i© stereological software after tissue processing and H&E staining. Fetuses for gross qualitative analysis were processed using the Christiene Effting protocol and examined using Leica M205C Stereomicroscope. Data was captured in MS Excel spreadsheets and analyzed using SPSS v25. One-way ANOVA and Pearson Chi square were used in statistical analysis with statistical significance set at $p<0.05$. The study findings showed a statistically significant difference ($p<0.05$) in the ratio of hypertrophic zone to primary spongiosa which was observed more in the medium (232.5mg/kg) and high dose (310mg/kg) tetracycline groups irrespective of duration of exposure. In addition, a statistically significant ($p<0.05$) dose dependent reduction in crown rump length and biparietal diameter was observed in the medium and high dose tetracycline treatment groups with the reduction shown not to be influenced by duration of exposure to tetracycline. Similar findings were also noted for rib anomalies and ossification status ($p<0.05$) whereby rats that received high and medium dose tetracycline had wavy ribs, incomplete ossification and unossification and this as well was not influenced by the duration of exposure to tetracycline. Contrary to these findings, tetracycline was shown not to influence the quantitative histological contribution of the reserve cartilage and proliferation zone to epiphyseal growth plate neither did it influence the tibia length($p>0.05$) regardless of the duration of exposure. From the study findings, it can be concluded that the bone chelation effect of tetracycline is dose dependent, with high tetracycline dose having the highest chelation effects followed by medium tetracycline dose whereas at low doses, chelation effects were not realized. These bone chelation effects were found not to be time dependent. From the study it has been shown that low doses of tetracycline maintain bone synthesis. This unique property can be explored for treatment of demineralization disorders.

CHAPTER ONE

INTRODUCTION

1.1 Background information

The use of tetracycline, a broad spectrum group of antibiotics during pregnancy has for decades been riddled with controversy due to its unclear tetratogenic effects on the developing fetal skeletal structures. The prototype drug, tetracycline, was noted to chelate calcium in bone upon its discovery and at that time it was contraindicated in pregnancy which was also extended to all other tetracycline drug molecules subsequently discovered (Cheng *et al.*, 2012; Larochelle, Maclean & Murray, 1968). Despite this generalization of bone calcium chelation effect of tetracycline, current studies have shown that not all tetracycline's chelate calcium in bone. Notably, minocycline and doxycycline have been shown to enhance osteosynthesis at low doses (Cross *et al.*, 2016) and further to this, doxycycline has been shown not to be teratogenic and has been recommended to be used in pregnancy as well as children below the age of 8 years (Gomes *et al.*, 2017). It is in the context of this background that the current study was anchored in which I sought to evaluate the effects of the prototype drug tetracycline on bone by varying the period of exposure and dosages so as to establish the bone chelation effect of tetracycline with regards to duration of administration and amount of drug given respectively.

Osteoblasts are the key cells that secrete bone matrix that is composed of collagen type I, non-collagenous protein, proteoglycans and inorganic salts like calcium, magnesium and zinc(Dhawan., *et al* 2014). Despite bone extracellular matrix consisting of calcium, zinc and magnesium, calcium and phosphate are the predominant ions found in bone in form hydroxyapatite ($Ca_5(PO_4)_3(OH)$) which combines with collagen and other non-collagenous matrix proteins to offer bone its mechanical properties of strength (Florencio-silva *et al.*, 2015; Gilsanz & Ratib, 2012). Tetracycline's have been shown to bind to these divalent cations and prevent them from contributing to formation of the extracellular bone matrix that plays a crucial role in bone strength which result in poor bone formation and altered epiphysial growth plate histogenesis in the fetus (Dhawan., *et al* 2014).

Contrary to this, some tetracycline's, particularly doxycycline and minocycline have been shown to enhance bone formation at low doses through inhibition of matrix metalloproteinases. Matrix metalloproteinases are a family of zinc-dependent endopeptidases that degrade various proteins in the connective tissue extracellular matrix including that of bone and it has been shown that inhibition of their activity in bone extracellular matrix results in new bone formation (Ong & Taylor, 2002). In this regard, low dose doxycycline and minocycline are widely used in implant orthopedic appliances and in periodontal disease to enhance new bone formation (Patianna & Valente, 2015).

1.2 Problem statement

Currently, there exists a controversy on the teratogenic effects of the different regimens of tetracycline's on the morphogenesis and differentiation of the fetal skeletal structures with some studies showing that tetracycline impairs bone morphogenesis while others showing that some tetracycline's like doxycycline do not (Cross *et al.,* 2016; Penelope & Dawn, 2017). This study sought to look into the bone teratogenic doses as well as the gestational period vulnerability for tetracycline bone teratogenicity.

1.3 Justification of the study

The controversial skeletal teratogenicity of tetracycline's has denied pregnant mothers and children an alternate, cheap, readily available and highly efficacious antibiotic in this era of antimicrobial resistance. This controversy and the benefits lost from the use of tetracycline called for a study on the histoquantitative and histomorphological effects of tetracycline on bone development so as to establish the period and dosage of exposure for teratogenicity to occur. Data generated was useful in illustrating whether or not tetracycline bone chelating effects are dose and time dependent. Such data will be useful in guiding future use of tetracycline in bone demineralization disorders like osteoporosis, bone lytic metastatic tumors and periodontitis.

1.4 Significance of the study

This study informed the scientific community on the dose and time related effects of tetracycline on fetal bone development. These findings can be used in establishing guidelines for use of tetracycline in bone diseases.

1.5 Objectives

1.5.1 Broad objective

To evaluate the histoquantitative and histomorphological teratogenic effects of tetracycline on fetal skeletogenesis (appendicular and axial skeleton) upon exposure to varied doses of tetracycline at different gestational periods in Albino rat (Rattus *novegicus*)

1.5.2 Specific objectives

1. To evaluate the histomorphological teratogenic effects of tetracycline on fetal skeletogenesis in Albino rats.

2. To evaluate the teratogenic histoquantitative effects of tetracycline on fetal skeletogenesis in Albino rats.

3. To determine if the teratogenic (histomorphological and histoquantitative) fetal skeletal changes upon exposure to tetracycline are dose dependent.

4. To determine if the teratogenic (histomorphological and histoquantitative) fetal skeletal changes upon exposure to tetracycline are time dependent.

1.6 Null hypothesis (H_0)

Prenatal exposure to varying doses of tetracycline does not impair skeletomorphogenesis of both axial and appendicular skeleton of the fetus neither are its teratogenic effects dose nor time dependent.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of tetracycline's history

Tetracycline's are group of broad-spectrum antibiotics that are sourced from *Streptomyces* bacteria or chemically modified from already available tetracycline molecules (Agwuh & Macgowan, 2006). Broadly, tetracycline's have been categorized into three groups based on their pharmacokinetic and pharmacodynamics properties. The first group are the early molecules that were developed in the early 1950's and are generally poorly lipophilic and have poor absorption upon oral administration. The second group is more lipophilic and their availability upon oral administration is quite high compared to the first group. The third group includes the newest molecules most of which are still in clinical trials. They have improved pharmacokinetics and pharmacodynamics than the predecessor molecules (Cross et al., 2016).

2.2 Pharmacokinetics of tetracycline

Absorption, distribution, metabolism and excretion

Tetracycline when taken orally, 25-90% of it is absorbed in the stomach, duodenum and the small intestine (Agwuh & Macgowan, 2006). Absorption is greatly interfered with presence of divalent ions like calcium, magnesium and iron to which it actively binds and reduces absorption several nutritional components like carbohydrates, proteins and fat greatly reduce the absorption and bioavailability of tetracycline by up to 50% (Agwuh & Macgowan, 2006).

Serum concentrations of tetracycline upon oral administration rise slowly with a t_{max} of 2-4hrs (Agwuh & Macgowan, 2006) and a volume of distribution of 1.3-1.7L/kg (Agwuh & Macgowan, 2006; Griffin *et al.*, 2010). It is majorly metabolized in the liver and 5% of it is excreted in urine as Δ-epitetracycline and 40% excreted in feces (Agwuh & Macgowan, 2006).

The toxicology of tetracycline's has relied so much on studies done on the prototype molecule which has led to misleading assumptions in-that, despite the drugs having a

common derivative, their pharmacokinetics and toxicological properties are different as confirmed by recent studies (Cross et al., 2016; Penelope & Dawn, 2017).

2.3 Bone embryogenesis

Bone is a type of specialized connective tissue that has been impregnated with minerals majorly calcium. There are several types of cells that are actively involved in bone histogenesis, namely; osteoblasts, osteoclasts, osteocytes and bone lining cells that interact via paracrine, autocrine and endocrine signaling mechanisms making bone to be a very dynamic tissue that entails modeling and remodeling(Florencio-silva *et al.,* 2015). Other than the protective function of bone, the minerals found in bone makes bone to be a very important connective tissue in the body for it stores calcium and phosphate which play a major role in critical homeostatic processes (Florencio-silva *et al.,* 2015).

In the developing embryo and fetus, several phases skeletogenesis take place i.e. progenitor cell migration to future sites of skeleton formation, mesenchymal condensation and lastly chondroblast or osteoblast differentiation (Giffin, Gaitor, & Franz-odendaal, 2019).

2.3.1 Phases of skeleton embryogenesis

a. Cell migration to future sites of skeleton formation

Cells from the somite's migrate ventromedially to surround the notochord marking the beginning of formation of the vertebral column whereas neural crest cells migrate from the hindbrain rhombomeres to the pharyngeal arches where they will contribute to formation of the viscerocraniumn (Giffin *et al.,* 2019).

b. Epithelial mesenchymal transformation

Mesenchymal condensations are the sites for future bone formation and they determine the future bone morphology as well as position. Mesenchymal condensation entails several steps that will eventually result in an appropriately positioned bone that is of correct shape and limits. The processes involved are initiation of mesenchymal condensation, establishment of boundaries, cell multiplication, cytoadherence, growth and cytodifferentiation *(Giffin et al.,*

2019).These mesenchymal condensations appear as collections of previously dispersed mesenchymal cells under the influence of molecular regulators that are involved in bone formation like the Shh, BMP family of genes and Hox (Giffin *et al.,* 2019; Wu, Chen, & Li, 2016).

c. Chondroblast and osteoblast differentiation

After mesenchymal condensation ceases, cytodifferentiation is triggered following expression of Cbfa1 as well as runt-related transcription factor 2 and the condensed mesenchymal cells differentiate into chondroblasts that now begin to secrete bone matrix rich in type II collagen and mucopolysaccharides. Osteogenic cells on the other hand secrete type I collagen and non-collagenous extracellular matrix proteins (Giffin *et al.,* 2019).

2.4 Embryogenesis of bones

Bone is sourced from the mesoderm with the bones of the axial skeleton being sourced from the paraxial mesoderm, limbs from the lateral plate mesoderm and cranial bones from the branchial arches as well as the neural crest cells(Wu *et al.,* 2016). There are two main forms through which bone is formed embryologically; intramembranous or endochondral ossification (Giffin *et al.,* 2019).

2.4.1 Intramembranous ossification

This entails formation of bone by direct transformation of mesenchymal cells into osteoblasts which are the bone forming cells. Through intramembranous ossification, flat bones, some bones of the viscerocranium and cranial suture lines are formed (Berendsen & Olsen, 2015). Mesenchymal to osteoblast transformation is under great influence of epithelia via several growth factors, transcription factors and receptor interactions at different times (Giffin *et al.,* 2019).

2.4.2 Endochondral ossification

This entails an initial cartilaginous model that is later ossified transforming it into bone as exemplified in skeletogenesis of the appendicular skeleton and some parts of the axial skeleton (Berendsen & Olsen, 2015). The cartilaginous model has a cartilaginous shaft which is surrounded by the perichondrium, an epiphysis at the

proximal and distal ends as well as the epiphyseal growth plate juxtaposed between the epiphysis and metaphysis (Berendsen & Olsen, 2015). The epiphyseal growth plate contributes to appositional growth due to its active production of chondroblasts from the groove of Ranvier (Berendsen & Olsen, 2015; Burdan *et al.,* 2009). The chondroblasts from the perichondrium later on differentiate into chondrocytes that secrete type II collagen and components of the extracellular matrix. At the same time, the perichondrium gets invaded by capillaries and at this point it differentiates into the periosteum whereas osteoblasts mature to secrete type I collagen which is the major type of collagen found in bone (Berendsen & Olsen, 2015; Burdan *et al.,* 2009; Giffin *et al.,* 2019). Capillaries from the periosteal collar invade the collagen model and bring in the osteoblast precursors that later on differentiate into mature osteoblasts that orchestrates bone formation (Berendsen & Olsen, 2015).

Chondrocyte hypertrophy begins at the center of the bone shaft and marks the beginning of synthesis of type X collagen and calcification of cartilage to form bone (Giffin *et al.,* 2019). Primary growth plates remain to be sites for longitudinal bone growth as they provide the cartilage source (Berendsen & Olsen, 2015; Burdan *et al.,* 2009). This process of calcification will continue until the whole cartilaginous model is replaced with bone that contains the divalent cations; calcium, zinc and magnesium as well as the anion phosphate which contribute to the mechanical strength of bone.

2.5 Epiphyseal growth plate histogenesis and physiology

Epiphyseal growth plate(growth plate/physis) is a cartilaginous structure located between the metaphysis and epiphysis and it plays a major role in bone lengthening (Burdan *et al.,* 2009; Dirsko & Charles, 2009; Shim, 2015). The epiphyseal growth plate is mesodermal in origin and majorly composed of actively dividing chondrocytes, extracellular matrix and calcium impregnated extracellular matrix (Burdan *et al.,* 2009).

2.5.1 Cartilage differentiation

Cartilage being a form of specialized connective tissue is sourced from mesoderm of the embryonic trilaminar germ disc. Chondrocytes undergo differentiation through

several stages i.e. mesenchymal precursor cells(MPCs), prechondrocytes, early chondroblasts and terminally differentiated chondrocytes (Dirsko & Charles, 2009). Chondrogenesis begins with mesenchymal cell condensation after migration of MPCs to sites of skeleton formation. Upon reaching there, they divide at the core of the condensation giving rise to prechondrocytes that later switch off mesenchymal and condensation markers. The cells then become round with a reduction in cell adhesion and increased endothelial cell proliferation which is under the influence of glycosaminoglycan's and N-Catherin whose synthesis stops at the end of cell condensation (Burdan *et al.,* 2009). Stabilization of mesenchymal-endothelial interactions is made possible by transforming growth factor β(Wu *et al.,* 2016). Chondrogenic cells highly express Sox family genes that oversee synthesis of collagen type II and its isoform IIaI together with collagen IXa1 and XIa2. Sox9 plays an important role in initiation of chondrogenesis. Prechondrocytes express Sox9 which is required for their further differentiation to chondroblasts which as well express Sox9. Chondroblasts Sox9 normally gets turned off when the cells undergo prehypertrophy and hypertrophy stages (Burdan *et al.,* 2009; Dirsko &Charles, 2009). At this stage, chondorblasts are no longer within the cell cycle and have increased in size upon which they undergo programmed cell death type 1 so as to pave way for development of primary ossification centers. Cells within the epiphyseal growth plate undergo a similar transformation to form the secondary ossification center (Burdan *et al.,* 2009). Chondrocytes actively synthesize extracellular matrix as well as matrix vesicles that contain calcium channel molecules (Annexin II, V and VI) in their membranes and the matrix vesicles accumulate calcium within them. Collagens type II and X bind to matrix vesicles which later interact with Annexin V. Collagen type X found in the hypertrophic zone facilitates deposition of calcium in the matrix vesicles (Burdan *et al.,* 2009; Shim, 2015).

2.5.2 Epiphyseal growth plate functional morphology

The epiphyseal growth plate is a multilayered structure in between the epiphysis and metaphysis made up of chondrocytes at different stages of differentiation (Dirsko & Charles, 2009). Four major layers of the epiphyseal growth plate have been defined

i.e. reserve, proliferation, transformation and degeneration zones with the degeneration zone incorporating the primary spongiosa zone which gives rise to the secondary spongiosa zone(Elmer *et al.*, 2011; Shim, 2015). Resting cartilage cells in the reserve zone are small chondrocytes that occur singly or in pairs with lots of lips and cytoplasmic vacuoles. In this zone, there is more of extracellular matrix than cells. Cells undergo mitosis at a slower rate in this zone as well synthesis of collagen type IIB is as well slow. The groove of Ranvier makes a collar around the reserve zone and contributes to the chondrocytes as it has chondrocytes progenitor cells (Burdan *et al.*, 2009; Shim, 2015). The zone of proliferation is the immediate continuation of the reserve zone and is made up of flat chondrocytes organized into columns. Mitotic activity is predominantly seen at the base of the columns. It is a pure germinal layer that shows a hyped synthesis of type II and XI collagen (Burdan *et al*., 2009).

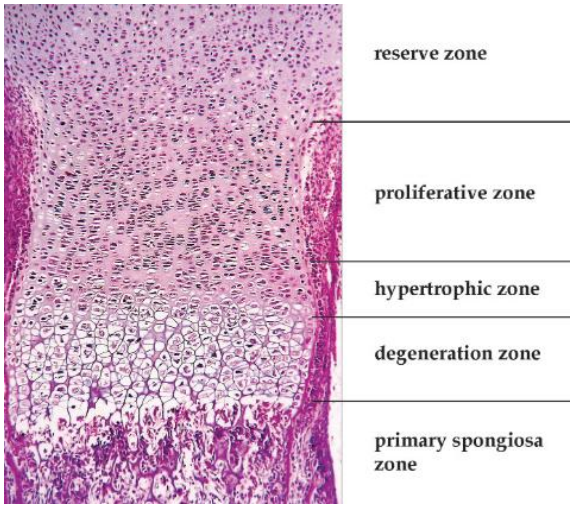

Figure 2.1: Histological zones of an epiphyseal growth plate

(Adapted from Burdan *et al.*, 2009)

Subjacent to the zone of proliferation is the transformation zone that has further been subdivided into the upper and lower hypertrophic zones as well as a zone of degeneration. The most striking feature in this zone is reduced DNA synthesis and no cell division. Instead, there is increased synthesis of extracellular matrix. In this layer, there we have short chain collagen as well as production of alkaline

phosphatase that leads to widening of the growth plate through an increase in phosphate ions that are needed for calcification(Shim, 2015). Chondrocytes in the transformation zone are comparably larger to other chondrocytes and are terminally differentiated and swollen. Chondroncytes closer to the primary spongiosa increasingly show degenerative features and are enclosed in special vesicles formed by extracellular matrix (Dirsko & Charles, 2009). The ultimate phenotypic changes in this zone when the chondroblasts divide into prehypertrophic and hypertrophic chondrocytes in which now the cytoplasmic volume increases nearly ten times and levels of messenger RNA increases for early cartilage matrix genes(Burdan et *al.,* 2009; Elmer *et al.,* 2011; Shim, 2015).

The primary spongiosa resembles the lower level of hypertrophic zone in morphology in addition to presence of osteoprogenitor cells. In this area, mineralization has already began and primary ossified lamella and small blood vessels are present (Dirsko & Charles, 2009). With terminal differentiation of hypertrophied chondrocytes Col1oa1 genes ceases to be expressed in chondorocytes and a new set of genes comes into place. These genes induce mineralization of the extracellular matrix as well as apoptosis of the terminally differentiated chondrocyte which in turn favors invasion by vascular channels and bone marrow stromal cells (Dirsko & Charles, 2009).

2.6 Effects of tetracycline class of drugs on bone

Studies have shown that tetracycline's bone teratogenicity occurs through different mechanisms including chelation of divalent cations like calcium and inhibition of enzymes that regulate bone histosynthesis (Agwuh & Macgowan, 2006; Larochelle *et al.,* 1968).

Calcium and phosphate are the key components of bone matrix that combines with collagen and other non-collagenous matrix proteins to offer bone its mechanical properties of strength (Florencio-silva *et al.,* 2015; Gilsanz & Ratib, 2012). The bone matrix that is secreted by osteoblasts is composed of collagen type I, non-collagenous protein, proteoglycans and inorganic salts like calcium, magnesium and zinc. Tetracycline's interfere with osteoblast function and prevent bone

mineralization by binding to divalent ions like Calcium, Zinc and Magnesium forming complexes that can't be absorbed (Cheng *et al.,* 2012).

Tetracycline's have long been known to interfere with bone morphogenesis, but recent studies are showing that some tetracycline's rather enhance bone formation through inhibition of matrix metalloproteinase that are potent degraders of extracellular matrix (Kalina *et al.,* 2007; Koide *et al.,* 2012; Park, 2011; Wu *et al.,* 2016). Doxycycline, just like other tetracycline drugs binds to divalent ions and in particular chelates Ca^{2+} in bone. Despite this, it has been demonstrated in rats that at low doses (10 and 30mg/kg/bwt) in ovarectimized rats it does improve the amount and quality of cancellous bone being formed (Figueiredo *et al.,* 2019).

Following this discovery tetracycline's were seen as potential therapeutic agents in diseases characterized by excessive matrix metalloproteinase and collagenase activity like periodontitis and osteoporosis(Fowlkes *et al.,* 2015). Besides inhibiting MMP action, doxycycline potentially down regulates enzyme expression at the gene level i.e. inhibits transcription. In addition, the inhibitory effect of doxycycline has been shown not to be related to its antibiotic activity. Further to this, doxycycline has been shown to negatively influence the synthesis of matrix metalloproteinase in human endothelial cells. MMPs are known to breakdown components of extracellular matrix as well as being the key players in connective tissue repair and remodeling. Oral administration of doxycycline has been shown to prevent root and bone resorption in rats making tetracyclines to be effective treatments in dental and orthopaedic implants that exhibit a high degree of dislodgment(Bezerra *et al.,* 2002; Ong & Taylor, 2002). Tetracyclines, majorly doxycycline is a potent inhibitor of osteoclastogenesis and influences the fate of mature osteoclast(Xiong & Brien,2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

Handling, breeding and administration of tetracycline was done at the Safari Animal House in the Department of Biomedical Sciences, while processing of specimens for microscopy was done in the Department of Human Anatomy, School of Medicine, JKUAT and Molecular and Cell Biology laboratory SAJOREC in JKUAT.

3.2 Study design

Laboratory based experimental study design

3.3 Experimental animals

A pure breed of **s**exually mature Albino rats aged 8-9 weeks and weighing 200-250g. Albino rats were chosen because of their low cost of maintenance, a short gestation period of 21 days and a low incidence of spontaneous congenital anomalies.

3.4 Acquisition of the Albino rats

From SAFARI animal house of JKUAT.

3.5 Acclimatization of the animals

Animals were kept in the cages at SAFARI animal house for one week to acclimatize with the experimenter before commencement of the experiments.

3.6 Feeding of the animals

Standard rodent pellets sourced from industrial area Unga Feeds Limited through SAFARI animal house were used. The standard meal contained 75% carbohydrates (3800calories/day), 12.5% proteins and12.5% lipids and water was given *ad-libitum*.

3.7 Breeding of the animals

One male Albino rat was introduced into a cage with three females at 2:30pm (plus/minus 30 minutes). At 09:00am the following morning the males were removed

and put back to their own cages for two hours then returned back to their respective cages.

3.8 Determination of pregnancy

After removing the male from the cage at 9:00 am, pregnancy was determined by doing a vaginal wash and taking vaginal smears for estrus cytological analysis. The animal rat was restrained with a gauze holder and 1ml of saline was introduce into the vaginal cavity using a blunt tipped disposable pipette (vaginal wash) thereafter a vaginal swab was done using a cotton tipped swab moistened with phosphate buffered saline. The cotton tipped swab was rolled onto a microscope glass slide and spray fixed with 95% ethanol, air dried, stained with Giemsa and observed under a light microscope at a TM X400.

Pregnancy was confirmed by observation of large cornified cells, numerous neutrophils and scattered epithelial cells (Ora & Ooistra, 2015).

3.9 Inclusion and exclusion criteria

Inclusion criteria

Healthy Albino rat dams that conceived

All live fetuses

Exclusion Criteria

All Albino rat dams that did not conceive

All Albino rat dams that became sick during experimentation

All dead fetuses

3.10 Sample size determination and grouping for dams

The resource equation method was employed in calculation of sample size because the effect size from previous animal studies was not available, there was no knowledge about expected attrition or death of animals and from the study we were measuring multiple endpoints/outcomes (Charan & Kantharia, 2013).

In this method, the value ‘E’ which is the degree of freedom of analysis of variance (ANOVA) is measured based on a decided sample size. This value (‘E’) should lie between 10 and 20 animals. A value less than 10 necessitates adding more animals which increases the chance of getting significant results. A value more than 20 has been shown to increase the cost of the study without increasing the significance of the results (Charan & Biswas, 2013).

E=Total number of Animals-Total number of groups

Total number of groups=10

Total number of animals=30 (this is the desired total number of animals for the study)

E=30-10

E=20 which falls between 10 and 20, therefore 30 is an adequate and representative sample size for this study.

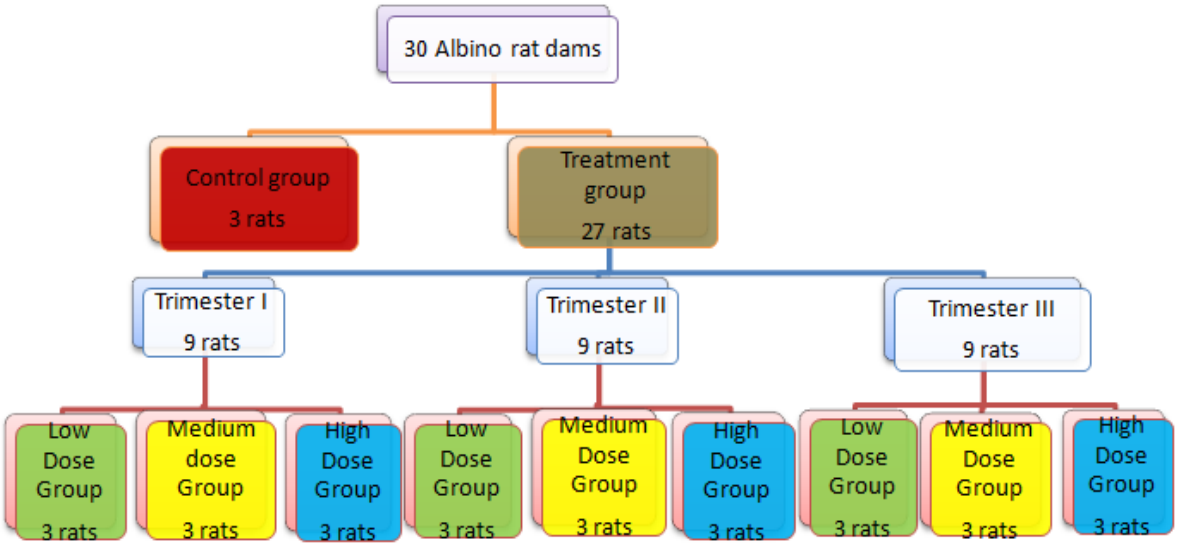

Figure 3.1: Grouping of the dams

3.11 Sample size determination for fetuses and allocation to the different study variables

The average liter size in Albino rats is 8.9±1.8 (Oguejiofor & Ochiogu, 2013). Thus, from the 30 dams, the expected total number of fetuses was 8.9x30=267on average and all were utilized for the study with half the litter size for

histoqualitative/histoquantitative variables and the remaining half for gross morphology/morphometry variables (Effting *et al.*, 2004).

3.12 Schedule for administration of tetracycline doses

The tetracycline doses were administered at 10a.m daily depending on the gestational trimester of the experimental groups as follows, low dose 155mg/kg, medium dose 232.5mg/kg and high dose 310mg/kg beginning pregnancy day 1 for rats in trimester 1, day 8 for 2^{nd} trimester rats and day 15 for 3^{rd} trimester rats.

3.13 Determination of Tetracycline hydrochloride doses

Calculation of the doses:

The lethal dose(LD_{50}) of tetracycline (Hostacycline© by Sanofi Aventis Batch number TC12535270-500mg capsules) in rats has been determined to be 1478.22mg/kg after a single oral administration (Tkachenko *et al.*, 2015). This study utilized this as the reference dose value that will cause death to half of the dams upon single oral administration, thus for the dams to survive through the study, a lower dose than the LD_{50} was adapted. The drug dosages were guided by the Human Equivalent Dose**(HED)** from which the Animal Equivalent Dose**(AED)** was calculated based on the following formula (Aziki, 2014; Sharma & Mcneill, 2009):

AED(mg/kg)=**HED**(mg/kg)×**Conversion factor**$_{km}$

The HED of tetracycline is 25-50mg/kg/day(Fusco & Nichols, 2019) and the conversion factor$_{km}$ from human to rat is 6.2 (Aziki, 2014; Ogasawara *et al.*, 2018; Sharma & Mcneill, 2009; Shin, Seol, & Son, 2010).

To get the low and high dose for the experimental groups, the 25-50mg/kg/day HED was used i.e.

Low dose tetracycline group:

AED(mg/kg)=**HED**(mg/kg)×**Conversion factor**$_{km}$

AED(mg/kg)=25×6.2

=155mg/kg

High dose tetracycline group:

AED(mg/kg)=**HED**(mg/kg)×**Conversion factor**$_{km}$

AED(mg/kg)=50×6.2

=310mg/kg

The medium dose will be taken as the average of HED range i.e.

∑ (25+26+27+…50)/26

=37.5

Thus, the **medium dose tetracycline group** will receive:

AED(mg/kg)=37.5×6.2

=232.5mg/kg

Controls

All controls received 5mls of distilled water through gastric gavage which is the recommended daily fluid intake for rats.

3.14 Determination of the vulnerable period and teratogenic dose for tetracycline on fetuses

The 30 dams that were used in the study were subdivided into 2 broad groups i.e. control group having 3 rats and treatment group having 27 rats. Inorder to determine the vulnerable period for tetracycline teratogenicity, the treatment group rats were further subdivided into 1st trimester(TM_1),2nd trimester(TM_2), rats and 3rd trimester(TM_3) each having 9 rats. The rats in each trimester were further subdivided into Low Dose Tetracycline Group, Medium Dose Tetracycline Group and High Dose Tetracycline Group rats each having 3 rats upon getting pregnant so as to determine the teratogenic dose.

Albino rats have a gestation period of 21 days, thus the 1st trimester was between gestational days 1 to 7, second trimester between gestational days 8-14 and 3rd trimester between gestational days 15-21. The following dosing schedule for tetracycline administration was used:

a. Dams in the 1st trimester (TM_1) received tetracycline from gestation day GD1-GD20
b. Dams in the 2nd trimester (TM_2) received tetracycline doses from gestation day GD8-GD20
c. Dams in the 3rd trimester (TM_3) received tetracycline doses from gestation day GD15-GD20.

3.15 Euthanizing the animals, harvesting and fixation of the fetuses

All animals were sacrificed in a humane manner on day 20 before. Each pregnant dam was placed in a bell jar containing cotton wool and concentrated CO_2 introduced into the bell jar with the lid closed over 3 minutes for the rat to get euthanized. When the rat got euthanized it was opened up along the midline over the anterior abdominal wall and the anterior abdominal wall flaps reflected to expose the gravid uterus upon which the uterine horns were identified, the fetuses probed to check for viability then harvested after making an anti-mesometrial border incision over the uterus. After harvesting the fetuses, they were fixed in situ for 6 hours with BB's fluid fixative which contains 900ml of 95% ethanol, 100ml of 37% formaldehyde and 150ml of de-ionized water-150ml (Chappard , 2009).

3.16 Specimen processing for histoqualitative and histoquantitative analysis

The tibia was chosen after doing a simple random sampling for bones of the appendicular skeleton whereas the ribs and cranium were chosen after simple random sampling of the axial skeleton bones. Ribs and the cranium were selected to represent the different embryological origins for the axial skeleton.

Limbs from fixed fetuses were harvested at the proximal joints and embedded in paraffin wax (Paraplast©) then placed on an electric cold plate for cooling. After 24hours, the tissue blocks were oriented along their long axis on the Medimeas© microtome with the microtome set at 3 micrometer thickness. Slide selection was

done using systematic uniform random sampling technique in which the paraffin embedded tibia block was oriented in the microtome in such a manner that longitudinal sections were obtained. Tissue sections to be stained were picked based on the K^{th} value (skip) of 10 calculated as follows:

Number of sections made from each tibia (N) =100

Number of desired sections from each tibia (n) =10

K^{th} or skip value=N/n

=100/10

=10

Every 10^{th} section was picked beginning from section number 6 upon doing simple random sampling to determine the beginning section. To get a representative sample from both tibia i.e. right and left tibia, each of the picked tissue sections from each tibia (10 sections) were subjected to systematic uniform random sampling as follows:

Number of sections made from each tibia (N) =10

Number of desired sections from each tibia (n) =5

K^{th} or skip value=N/n

=10/5

=2

Every 2^{nd} section was picked beginning from section number 2 upon doing simple random sampling to determine the beginning section. This resulted in 10 sections from each rat i.e. 5 histological sections from the right and left tibia.

The achieved sections were placed in a water bath at 37^0C and fishing done on glass slides then placed in a slide holder for staining as follows.

Deparaffinize and dehydrate sections:

The sections were dipped 3 times for 2 minutes each sequentially in Xylene, 100% ethanol, 95% ethanol, 80% ethanol and for 5 seconds in de-ionized water. Thereafter they were dipped for 2 minutes in Hematoxylin, rinsed with de-ionized water and de-stained with acid ethanol (dip 8 times), rinsed with tap water the de-ionized water. The slides were then stained with Eosin for 2 minutes, rehydrated sequentially with 95% and 100% ethanol (3 dips each) then 3 dips in xylene for 15seconds and cover slips applied using DPX mountant. They were then dried overnight in the hood.

3.17 Histoquantitative methods

Surface area of epiphyseal growth plate

The epiphyseal growth plate surface area was determined using Image J v1.50i©, Java 1.6.0_20© image analysis open source software (Schindelin *et al.,* 2015). Epiphyseal growth plates of proximal tibias were used for the study after tissue processing and staining with Haematoxylin and Eosin.

Upon staining with Haematoxylin and Eosin, photomicrographs were taken using the Labomed light microscope mounted with the IVU 3100 camera at a total magnification of x40 (Objective lens x4, eye piece lens x10). After image capturing, selected photomicrographs were uploaded in Image J v1.50i© stereology analysis software for surface area and area fraction measurements from the Image J main menu bar as follows:

From the main menu bar, the file tab was clicked and from the drop down menu the open tab was clicked so as to import the stored image to the software image analysis window then from the main menu bar the analyze tab was clicked and from the drop down menu in a sequential manner the “select measurements” “select area” “area fraction” “OK” were clicked. From here the scale was set by clicking on analyze from the main menu bar and set (distance in pixels=264.5833, known distance=1µm, pixel aspect ratio=1, unit of length=µm then click OK). To calculate the surface area, the irregular outline tool from the main menu bar was selected and dragged over the outline of the epiphyseal growth plate so as to get the total and zonal surface areas then sequentially click on analyze and area measurements.

3.18 Bone morphometry methods

Tibial bones from the different rat groups were each further processed to remove all soft tissues by placing the forelimbs in 2%KOH for 8 days which led to complete chemical maceration of soft tissues living the bones intact.

The Hercules© digital vernier calipers was used to take tibia lengths upon calibration to 0.00 mark each time a measurement was to be made. The measurements were made from the tibial intercondylar eminence to the medial malleolus.

3.19 Qualitative bone analysis: determination of ossification

The harvested fetuses were sacrificed using concentrated CO_2 and dehydrated in 95% ethanol for 4 days then 100% ethanol for another 4 days. Thereafter they were rinsed in distilled water and eviscerated and placed in 2% KOH solution for 24hours after which they were rinsed and placed in 0.5% KOH solution containing Alizarin red for 24hours. After 24hours the fetuses were drained and placed in 25% glycerol solution for 1 week thereafter in 100%glycerol with a few drops of 0.5phenol solution for preservation (Effting *et al.*, 2004). The thoracic cage was then examined for evidence of skeletal differentiation under the Leica M125 Stereomicroscope mounted with DFC450C camera. All photos were taken at standard optics i.e. objective magnification of x1, eye piece magnification of x10, zoom range of x8, maximum resolution of 432lp/mm, working distance of 61.5mm and at an object field of 28.8mm, saved and processed using the Leica Application Suite (LAS) software.

3.20 Study Variables

Independent variable's: varying doses of tetracycline and gestational periods.

Dependent variable's: birth weight, length of tibia, rib ossification, rib anomalies, crown rump length, biparietal diameter, reserve cartilage percentage of total epiphyseal growth plate surface area, proliferation zone percentage of total epiphyseal growth plate surface area, hypertrophic zone percentage of total epiphyseal growth plate surface area, hypertrophic zone percentage of primary spongiosa surface area.

3.21 Data Management and analysis

Data from the data sheets was entered into a Microsoft Excel Spreadsheet and then transferred to Statistical Package for Social Scientists software version 25.0 for statistical analysis. The statistical tests used were one way Analysis of Variance (ANOVA) with Tukey post hoc test for continuous data to determine variation among and between groups whereas Pearson Chi square was used for categorical variables. A 5% significance level ($p = 0.05$) was assumed with the results being considered to be significant whenever the probability value was $p<0.05$.

3.22 Blinding

Blinding was done during experimentation, data collection and during statistical analysis whereby unique codes were assigned to the study rats and revealed after data analysis and interpretation was done.

3.23 Ethical Considerations

In carrying out the study, ethical approval was sought and granted by the Animal Ethics and Research Committee and handled according to established laboratory animal handling guidelines of JKUAT. The ethical considerations observed were; reduction to the least number of animals that could answer my research objectives as well as provide data that is statistically robust, refinement in which all animals were placed in standard polycarbonated cages for 4 rats, daily changing of beddings or when the beddings got soiled, 12hr day/light cycle, sacrificing of the animals in a humane manner whereby concentrated CO_2 was utilized and sick animals were attended to by the in-house Vet. The that were found to be frail were recalled from the experiments and sacrificed immediately using CO_2 euthanization. Re-use of the animals was considered whereby all the animals utilized in the experiments as well as the body parts not of immediate concern were preserved for future analysis.

CHAPTER FOUR

RESULTS

4.1 Tetracycline teratogenic outcomes on the rib abnormalities when treatment was administered at varied doses in the different window periods against the control.

When tetracycline was administered in Trimester one (TM_1), the observed Rib-anomalies had a direct association to the dose of exposure and there was a statistically significant difference, χ=12.48, p = 0.006, when treatment groups were compared against control rats (Table 4.1). The study findings revealed a dose dependent increase in rib anomalies in which anomalies (Wavy ribs) were seen in 66.7% of MDG rats and nearly 100% of HDG rats whereas Control and LDG rats showed no wavy ribs (Figure 4.1,4.2 and 4.3). Similarly, in TM_2, rib anomalies were associated with dose levels with a statistically significant difference, χ= 12.48, $p \leq 0.006$, being noted when the treatment groups were compared against control rats (Table 4.1). The rib anomalies were seen in 66.7% of Medium dose rats and in nearly 100% of High dose rats whereas in control and Low dose rats there were no rib anomalies (Figures 4.1, 4.2 and 4.3). In TM_3, in-utero exposure to tetracycline at varied doses resulted in rib anomalies as well and there was a statistically significant difference, χ= 16.64, $p<0.001$, when treatment groups were compared against control rats (Table 4.1). The anomalies were seen in almost 100% of Medium dose rats and almost 100% of High dose rats whereas in control and Low dose rats there were no rib anomalies (Figures 4.1, 4.2 and 4.3).

Table 4.1: Tetracycline teratogenic outcomes on the rib abnormalities when treatment was administered at varied doses in the different window periods against the control.

Trimester	Tetracycline treatment group	Rib anomalies		
		None (%)	Wavy (%)	
Trimester 1	Control	3 (100.0)	0	
	Low Dose Tetracycline Group	3 (100.0)	0.0	*
	Medium Dose Tetracycline Group	1(33.3)	2 (66.7)	
	High Dose Tetracycline Group	0 (0.0)	3 (100.0)	
Trimester 2	Control	3 (100.0)	0 (0.0)	
	Low Dose Tetracycline Group	3 (100.0)	0 (0.0)	
	Medium Dose Tetracycline Group	1 (33.3)	2 (66.7)	*
	High Dose Tetracycline Group	0 (0.0)	3 (100.0)	
Trimester 3	Control	3 (100.0)	0 (0.0)	
	Low Dose Tetracycline Group	3 (100.0)	0 (0.0)	*
	Medium Dose Tetracycline Group	0 (0.0)	3 (100.0)	
	High Dose Tetracycline Group	0 (0.0)	3 (100.0)	

*Key: *denotes statistical significance at $p<0.05$*

4.2 Tetracycline teratogenic outcomes on the rib ossification when treatment was administered at varied doses in the different window periods against the control.

The study revealed that in TM_1, ossification was associated with dose levels with as statistically significant difference, χ=17.32, p = 0.008, being noted upon comparison of treatment groups against control rats (Table 4.2). This association was shown to be dose dependent in- that, all the Low Dose Tetracycline Group rats as well as Control rats achieved complete ossification, whereas in Medium Dose Tetracycline Group, 33.3% were unossified and 66.7% were incompletely ossified (Figures 4.1, 4.2 and 4.3). In the High Dose Tetracycline Group rats, 66.7% of the rats were unossified and 33.3% had incomplete ossification (Figures 4.1, 4.2 and 4.3).

Similarly, in TM_2, the study results showed that there was a statistically significant difference, χ= 24.95, p=0.001, when treatment groups were compared against control rats, furthermore; Ossification was associated with dose levels, as all Low Dose Tetracycline Groups and Control rats achieved complete ossification, Medium

Dose Tetracycline Group rats, almost 100% of them showed incomplete ossification whereas the High Dose Tetracycline Group rats showed nearly 100% unossification (Figures 4.1, 4.2 and 4.3 and Table 4.2).

Lastly, in TM_3, the results showed a statistically significant difference, $\chi = 24.95$, $p<0.001$, when treatment groups were compared against control rats for ossification (Table 4.3). Ossification of the ribs was affected in a dose dependent manner whereby, all Low Dose Tetracycline Groups and Control rats achieved complete ossification, Medium Dose Tetracycline Group rats, nearly 100% of them showed incomplete ossification whereas the High Dose Tetracycline Group rats showed almost 100% unossification (Figures 4.1, 4.2 and 4.3).

Table 4.2: Tetracycline teratogenic outcomes on the rib ossification when treatment was administered at varied doses in the different window periods against the control.

Trimester	Tetracycline treatment group	Ossification status			
		Unossified (%)	Incomplete (%)	Complete(%)	
Trimester 1	Control	0 (0.0)	0 (0.0	3 (100.0)	
	Low Dose	0 (0.0)	0 (0.0)	3 (100.0)	
	Medium Dose	1 (33.3)	2 (66.7)	0 (0.0)	*
	High Dose	2 (66.7)	1 (33.3)	0 (0.0)	
Trimester 2	Control	0 (0.0)	0 (0.0)	3 (100.0)	
	Low Dose	0 (0.0)	0 (0.0)	3 (100.0)	*
	Medium Dose	0 (0.0)	3 (100.0)	0 (0.0)	
	High Dose	3 (100.0)	0 (0.0)	0 (0.0)	
Trimester 3	Control	0 (0.0)	0 (0.0)	3 (100.0)	
	Low Dose	0 (0.0)	0 (0.0)	3 (100.0)	*
	Medium Dose	0 (0.0)	3 (100.0)	0 (0.0)	
	High Dose	3 (100.0)	0 (0.0)	0 (0.0)	

*Key: *denotes statistical significance at p<0.05*

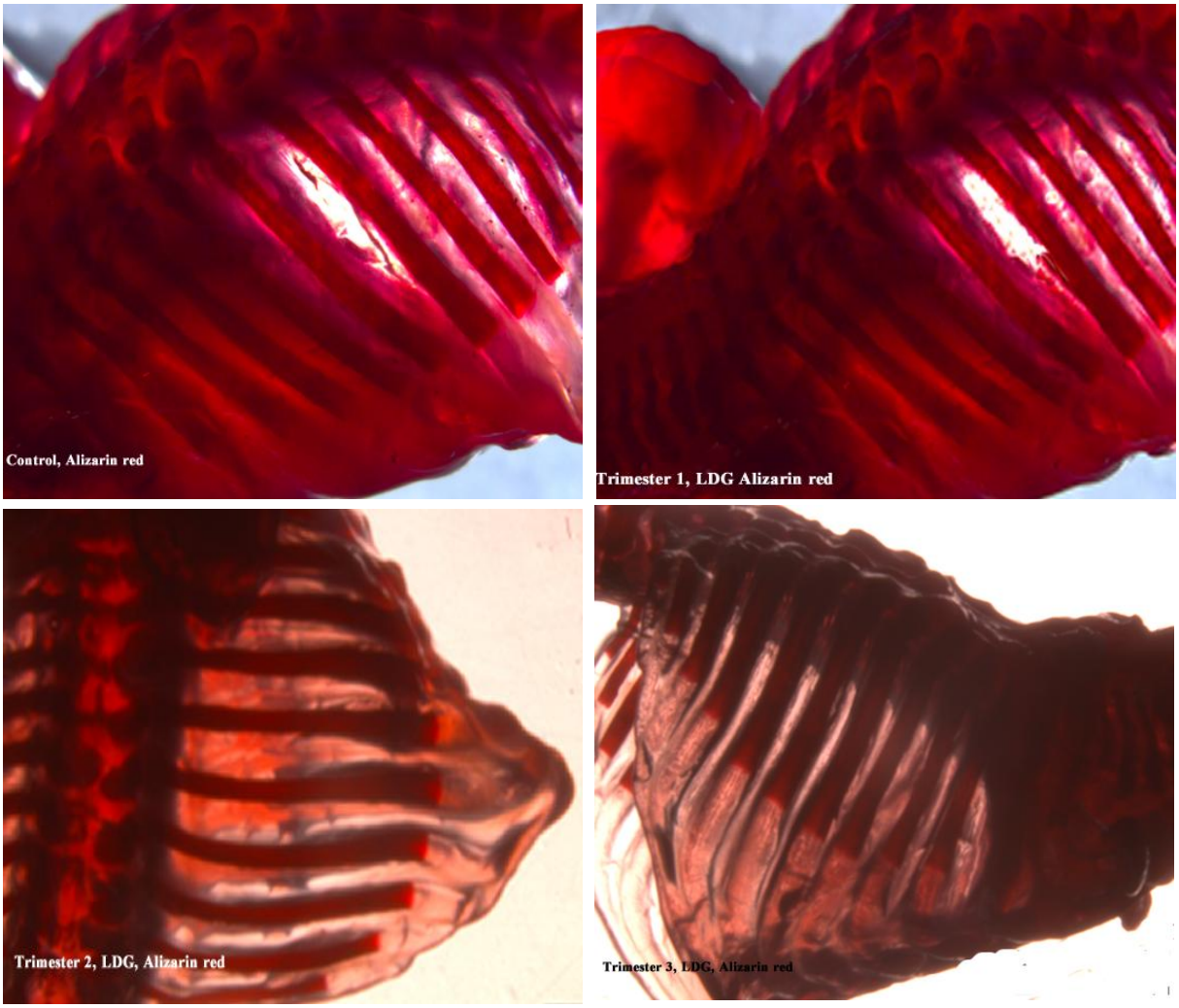

Figure 4.1: shows ossification for Low Dose Tetracycline Groups(155mg/kg/day) compared against control in trimesters 1, 2 and 3 where it is seen that the ossification is similar to control group and there are no wavy ribs. The rib cage was stained

Figure 4.2: shows ossification for Medium Dose Tetracycline Groups(232.5mg/kg/day) compared against control in trimesters 1, 2 and 3 where it is seen that the ossification is reduced compared to control group and the ribs are wavy across

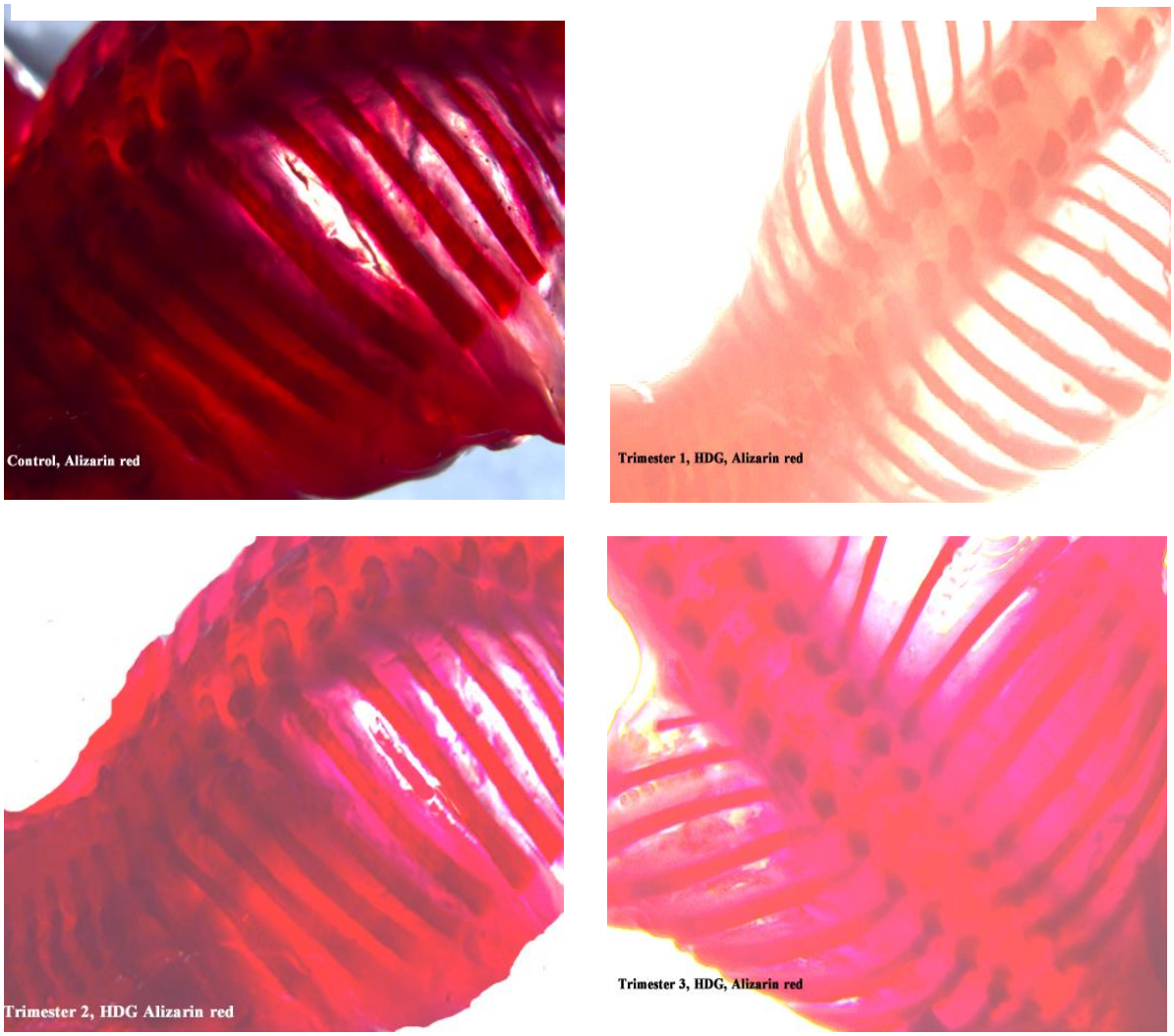

Figure 4.3: shows ossification for Medium Dose Tetracycline Groups(310mg/kg/day) compared against control in trimesters 1, 2 and 3 where it is seen that the ossification is considerably reduced compared to control group and the ribs are wavy across the trimesters. The rib cage was stained using Alizarin red.

4.3 Comparative histological changes of epiphyseal growth plate zones among Low Dose Tetracycline Groups compared to controls in the different trimesters

4.3.1 Low Dose Tetracycline Group Trimester 1

The resting cartilage (RC) have a comparable distribution pattern when the Low Dose Tetracycline Group rats were compared to control in TM_1. The connective tissue in this case is characterized by abundant extracellular matrix in which condroblasts are sparsely distributed and the groove of Ranvier (GR) has made a collar around the resting cartilage. Similarly, in the zone of proliferation, the cells are a little bit bigger with nuclei that are deeply staining with Haematoxylin for cells closer to the groove of Ranvier as compared to the zone of reserve cartilage. The cells are organized into columns and are noted to increase in size as we approach the base of the column (Figure 4.4, photomicrograph B).

Cells in the zone of hypertrophy are the largest and have a pale staining nucleus with some cells lacking nuclei and there is increased spacing between the cells. The primary spongiosa (PS) is characterized by a mixture of terminally differentiated chondrocytes, some cartilage core (CC) with islands of new bone (NB) whose distributions resemble that of control. The Hypertrophic zone percentage surface area of primary spongiosa is relatively similar to that of control as well (Figure 4.4, photomicrograph B).

4.3.2 Low Dose Tetracycline Group Trimester 2

The connective tissue constituents (extracellular matrix and chondroblasts) in the resting cartilage have maintained their general distribution which is analogous to that of the control group (Figure 4.4, photomicrograph C). The chondroblasts have a lightly eosinophilic cytoplasm and not compactly populated. The extracellular matrix is well conserved too and plentiful which accounts for the distribution of the cells not being compactly distributed and the groove of Ranvier (GR) as well surrounds the resting cartilage (Figure 4.4, photomicrograph C).

The zone of proliferation shows larger cells that are intensely staining with Haematoxylin for cells nearer to the groove of Ranvier in comparison to those in the reserve cartilage and the cells are structured into columns with larger ones being closer to the hypertrophic zone (Figure 4.4, photomicrograph C).

Cells in zone of hypertrophy are the largest and have a pale staining nucleus with some cells lacking nuclei with more spacing between the cells. The primary spongiosa is made up of a mixture of terminally differentiated chondroblasts, some cartilage core(CC) and islands of new bone(NB) as well (Figure 4.4, photomicrograph C).

4.3.3 Low Dose Tetracycline Group Trimester 3

The connective tissue components in the resting cartilage have similar distribution to those in control group with the chondroblasts presenting a pale pink cytoplasm and the cells being sparsely distributed (Figure 4.4 photomicrograph D). The extracellular matrix is a lot and well preserved which impacts on the distribution of the cells as being widely distributed and the groove of Ranvier (GR) is seen to encircle the resting cartilage as well.

The zone of proliferation shows intensely basophilic cells closer to the groove of Ranvier in contrast to those in the reserve cartilage with the cells in this zone being organized into columns with larger ones being closer to the hypertrophic zone (resembles an erect cone). Next to the zone of proliferation is the zone of hypertrophy whose cells are the largest, sparsely distributed and has a pale staining nucleus with some cells being anucleate (Figure 4.4, photomicrograph C).

The primary spongiosa is characterized by a mixture of terminally differentiated chondroblasts, some cartilage core (CC) and presence of islands of new bone(NB). The hypertrophic zone percentage surface area of PS is nearly of the same size with that of control with nearly the same density of cartilage core and new bone (Figure 4.4, photomicrograph C). These findings concur with those seen in Low Dose Tetracycline Groups of TM_1, TM_2 and control.

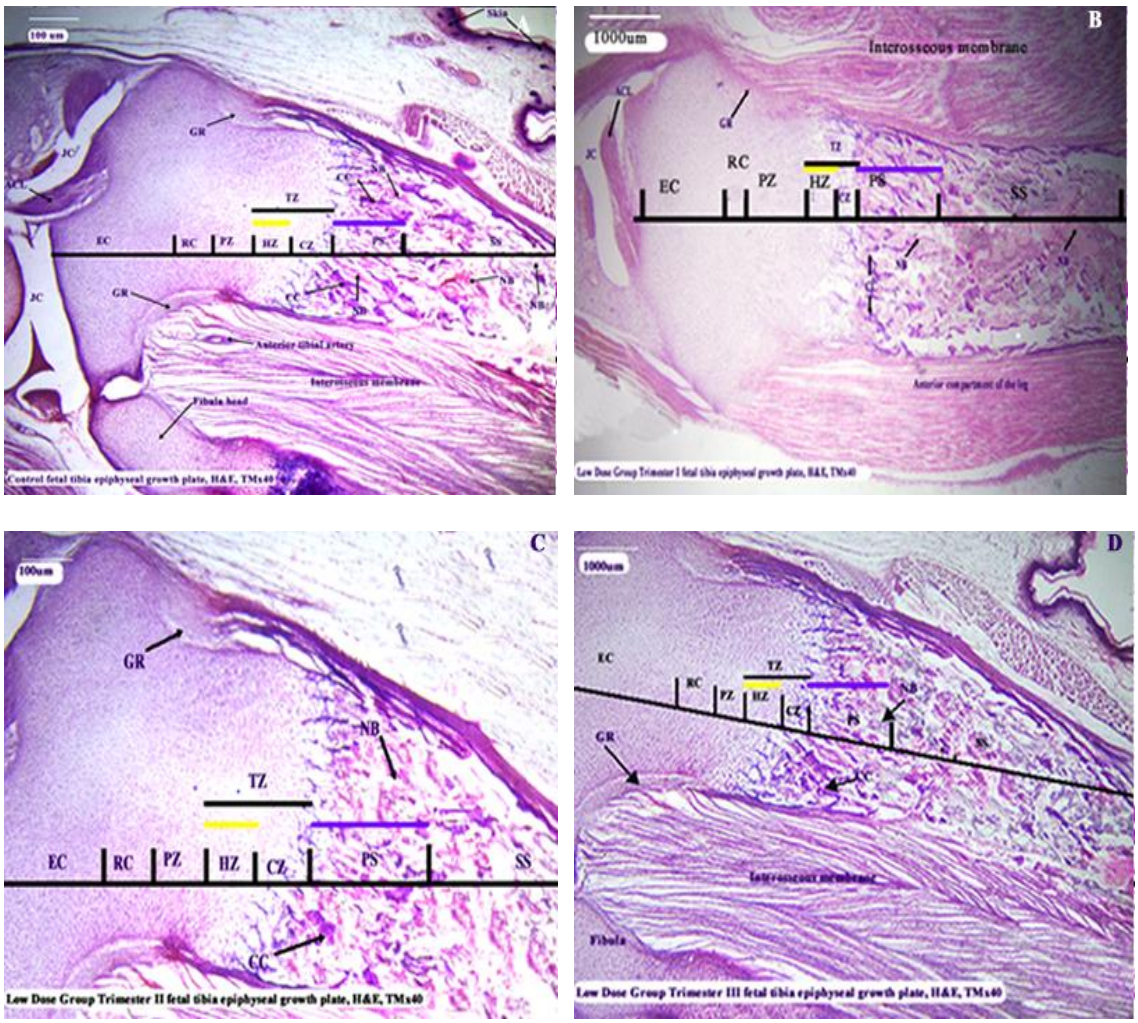

Figure 4.4: shows no comparative difference in the Hypertrophic zone: Primary Spongiosa proportionate thickness as well as the distribution of core cartilage and new bone in the PS in the Low Dose Tetracycline Groups across trimesters (TM_1, B:TM_2, C: TM_3, D) as compared to control group.

Key: EC, epiphyseal cartilage, RC, reserve cartilage, PZ, proliferating zone, HZ/Yellow line, hypertrophic zone, CZ, calcification zone, PS/Blue line, primary spongiosa, SS, secondary spongiosa, GR, groove of Ranvier, TZ, transformation zone, JC, joint cavity, NB, new bone, CC, cartilage core, ACL, anterior cruciate ligament.

4.4 Comparative histological changes of epiphyseal growth plate zones among Medium Dose Tetracycline Groups compared to controls in the different trimesters

4.4.1 Medium Dose Tetracycline Group, Trimester 1

The cells in the resting cartilage have retained their connective tissue structure which is analogous to that of the control group. The chondroblasts in this zone are distributed in an abundance of extracellular matrix and have a pale cytoplasm. The groove of Ranvier(GR) is seen to make a collar around the resting cartilage (Figure 4.5 photomicrograph B).

In the zone of proliferation, the cells are bigger with deeply staining nuclei for cells that are closer to the groove of Ranvier as compared to the zone of reserve cartilage. The cells are organized into columns and are noted to increase in size as we approach the base of the column (Figure 4.5, photomicrograph B).

Cells in the zone of hypertrophy are the largest and have a pale staining nucleus with some cells lacking nuclei. There is abundant connective etissue matrix between the cells as well. The primary spongiosa is characterized by a mixture of terminally differentiated chondroblasts, cartilage core(CC) that is much more as compared to the new bone(NB) as seen in the control group. The hypertrophic zone percentage surface area of PS is greater than that of control as well (Figure 4.5, photomicrograph B).

4.4.2 Medium Dose Tetracycline Group, Trimester 2

The resting cartilage cells have retained their general connective tissue morphology which happens to resemble that of control group. The chondroblasts have a lightly eosinophilic cytoplasm with a scattered distribution courtesy of the abundant extracellular matrix. The groove of Ranvier (GR) in this zone as well encircles the resting cartilage (Figure 4.5, photomicrograph B).

The zone of proliferation shows larger cells whose nuclei stain deeply with Haematoxylin for the cells that are closer to the groove of Ranvier as compared to those in the reserve cartilage. In this zone as well, the cells are structured into

columns with larger ones being closer to the hypertrophic zone (Figure 4.5, photomicrograph B).

A closer look at the zone of hypertrophy the cells are seen to be the largest and have a pale staining nucleus with some cells being anucleate and the cells being surrounded with a lot of extracellular connective tissue matrix (Figure 4.5, photomicrograph B).

The primary spongiosa demonstrates a mixture of terminally differentiated chondroblasts, some cartilage core(CC) which in this case is much more as compared to the new bone(NB) seen in the control. The hypertrophic zone percentage surface area of PS is greater than that of control too (Figure 4.5, photomicrograph B).

4.4.3 Medium Dose Tetracycline Group, Trimester 3

The resting cartilage cells and extracellular matrix show similar morphological characteristics to those of control group with the chondroblasts having a pale pink cytoplasm and scattered within the extracellular matrix which in this case is abundant and thus accounts for the sparse distribution of chondroblasts. The groove of Ranvier (GR) surrounds the resting cartilage (Figure 4.5 photomicrograph D).

The zone of proliferation shows bigger chondroblasts that are intensely basophilic closer to the groove of Ranvier in contrast to those in the reserve cartilage. The cells are arranged into columns with larger ones being closer to the hypertrophic zone just like those in the control group (Figure 4.5 photomicrograph D).

Cells in the zone of hypertrophy are scattered in an abundance of extracellular matrix and show a large pale staining nucleus with some cells lacking a nucleus which is the same case in the control group (Figure 4.5 photomicrograph D).

The primary spongiosa is characterized by a mixture of terminally differentiated chondrocytes, a lot of cartilage core(CC) and presence of islands of new bone(NB). The hypertrophic zone percentage surface area of PS is greater than that of control (Figure 4.5 photomicrograph D).

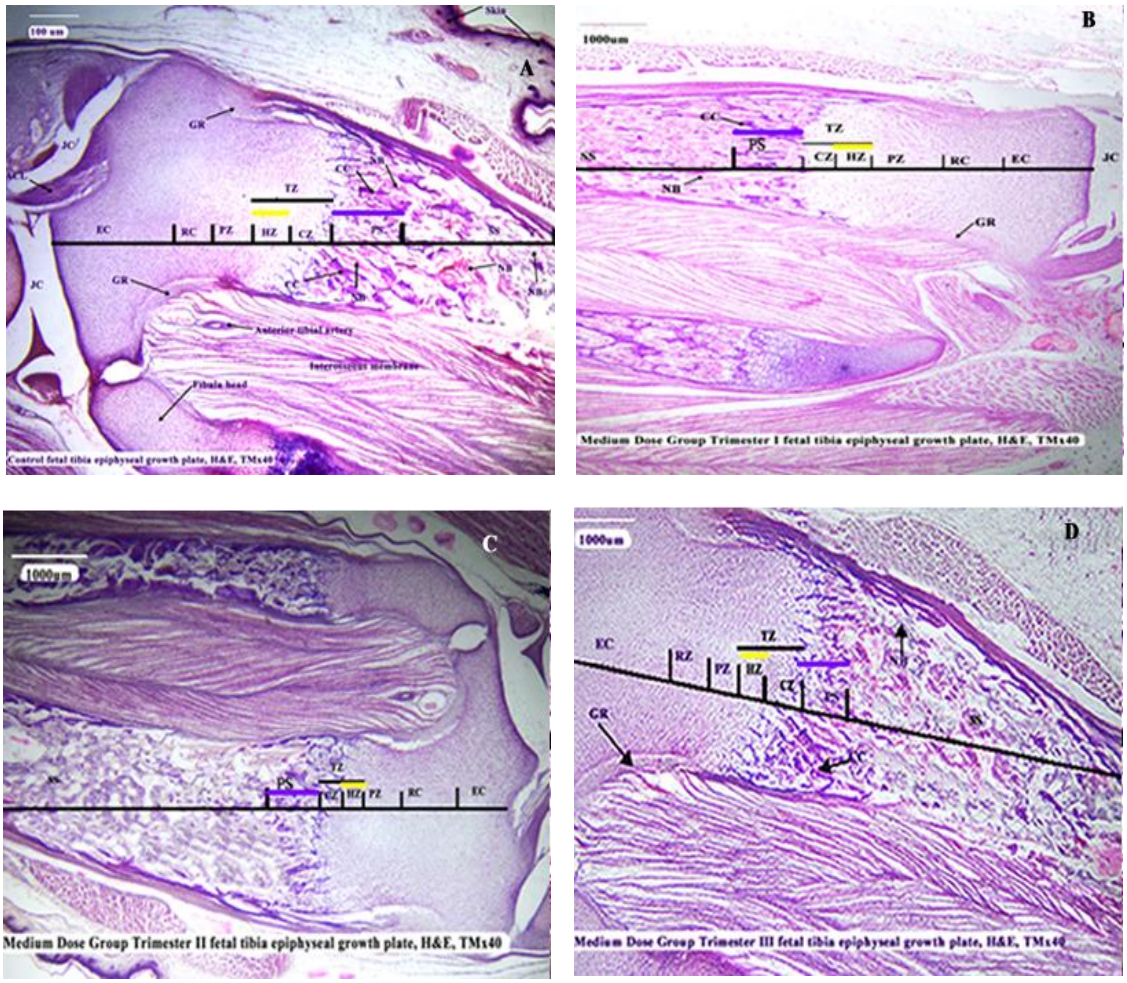

Figure 4.5: shows comparative increase in the Hypertrophic zone: Primary Spongiosa proportionate thickness as well as an increase in the distribution of core cartilage with a reduction in new bone in the PS in the Medium Dose Tetracycline Groups across trimesters (TM_1, B:TM_2, C: TM_3, D) as compared to control group.

Key: EC, epiphyseal cartilage, RC, reserve cartilage, PZ, proliferating zone, HZ/Yellow line, hypertrophic zone, CZ, calcification zone, PS/Blue line, primary spongiosa, SS, secondary spongiosa, GR, groove of Ranvier, TZ, transformation zone, JC, joint cavity, NB, new bone, CC, cartilage core, ACL, anterior cruciate ligament.

4.5 Comparative histological changes of epiphyseal growth plate zones among Medium Dose Tetracycline Groups compared to controls in the different trimesters

4.5.1 High Dose Tetracycline Group, Trimester 1

The tissue architecture of the resting cartilage zone resembles that of control group in that the chondroblasts are sparsely populated, show a lightly staining cytoplasm and there is a lot of extracellular matrix. The groove of Ranvier(GR) is seen to make a collar around the resting cartilage. In the zone of proliferation, the cells are big with nuclei that are deeply staining with Haematoxylin for cells nearer to the groove of Ranvier as compared to the zone of reserve cartilage. The cells are packed into columns with the arrangement presenting a “corn shape” whose base is towards the hypertrophic zone (Figure 4.6 photomicrograph B).

Adjacent to the zone of proliferation is the zone of hypertrophy in which the cells are quite big and have a pale staining nucleus with some cells lacking a nucleus. There is a lot of extracellular matrix as well which accounts for the sparse distribution of the cells in this zone (Figure 4.6 photomicrograph B).

The primary spongiosa, similar to control is characterized by a mixture of terminally differentiated chondroblasts, some cartilage core (CC) and presence of islands of new bone(NB) only that in the experimental groups that received High Dose Tetracycline demonstrate a lot of cartilage core (CC) with diminutive new bone (NB) as compared to control that had a lot of new bone as compared to cartilage core. The hypertrophic zone percentage surface area of primary spongiosa is as well large as compared to that of control (Figure 4.6 photomicrograph B).

4.5.2 High Dose Tetracycline Group, Trimester 2

The connective tissue components in the resting cartilage have maintained their general structure which is corresponding to that of the control group in which the chondroblasts are sparsely distributed in an abundance of extracellular matrix and present a lightly eosinophilic cytoplasm. The groove of Ranvier(GR) surrounds the resting cartilage as well (Figure 4.6 photomicrograph C).

The zone of proliferation shows larger cells that are intensely staining with Haematoxylin for cells closer to the groove of Ranvier in comparison to those in the reserve cartilage. The cells are structured into columns with larger ones being closer to the hypertrophic zone (Figure 4.6 photomicrograph C).

Cells in zone of hypertrophy are the largest and have a pale staining nucleus with some cells lacking nuclei. The spacing between the cells increases as well (Figure 4.6 photomicrograph C).

The primary spongiosa, shows a mixture of terminally differentiated chondroblasts, a lot of cartilage core (CC) and diminutive of islands of new bone(NB) as compared to control that had a lot of new bone as compared diminutive cartilage core. The hypertrophic zone percentage surface area of primary spongiosa is as well large as compared to that of control (Figure 4.6 photomicrograph C).

4.5.3 High Dose Tetracycline Group, Trimester 3

The connective tissue components in the resting cartilage zone demonstrate similar morphological characteristics to those of control group with the chondrocytes having a pale pink cytoplasm and sparsely distributed in a lot of extracellular matrix. The groove of Ranvier(GR) encircles the resting cartilage like in the control group (Figure 4.6 photomicrograph D).

The zone of proliferation shows bigger cells with intensely basophilic nuclei for cells closer to the groove of Ranvier in contrast to those in the reserve cartilage. The cells are organized into columns with larger ones being closer to the hypertrophic zone (Figure 4.6 photomicrograph D).

Cells in the zone of hypertrophy are the largest and have a lightly staining nucleus with some cells being anucleate. There is abundant extracelluar matrix in which the cells of this zone are embedded (Figure 4.6 photomicrograph D).

The primary spongiosa is characterized by a mixture of terminally differentiated chondrocytes, some cartilage core(CC) and presence of islands of new bone(NB). The striking feature is that there is a lot of cartilage core and fewer new bone islands

as compared that observed in the control groups. The hypertrophic zone percentage surface area of primary spongisa is large compared to that of control (Figure 4.6 photomicrograph D).

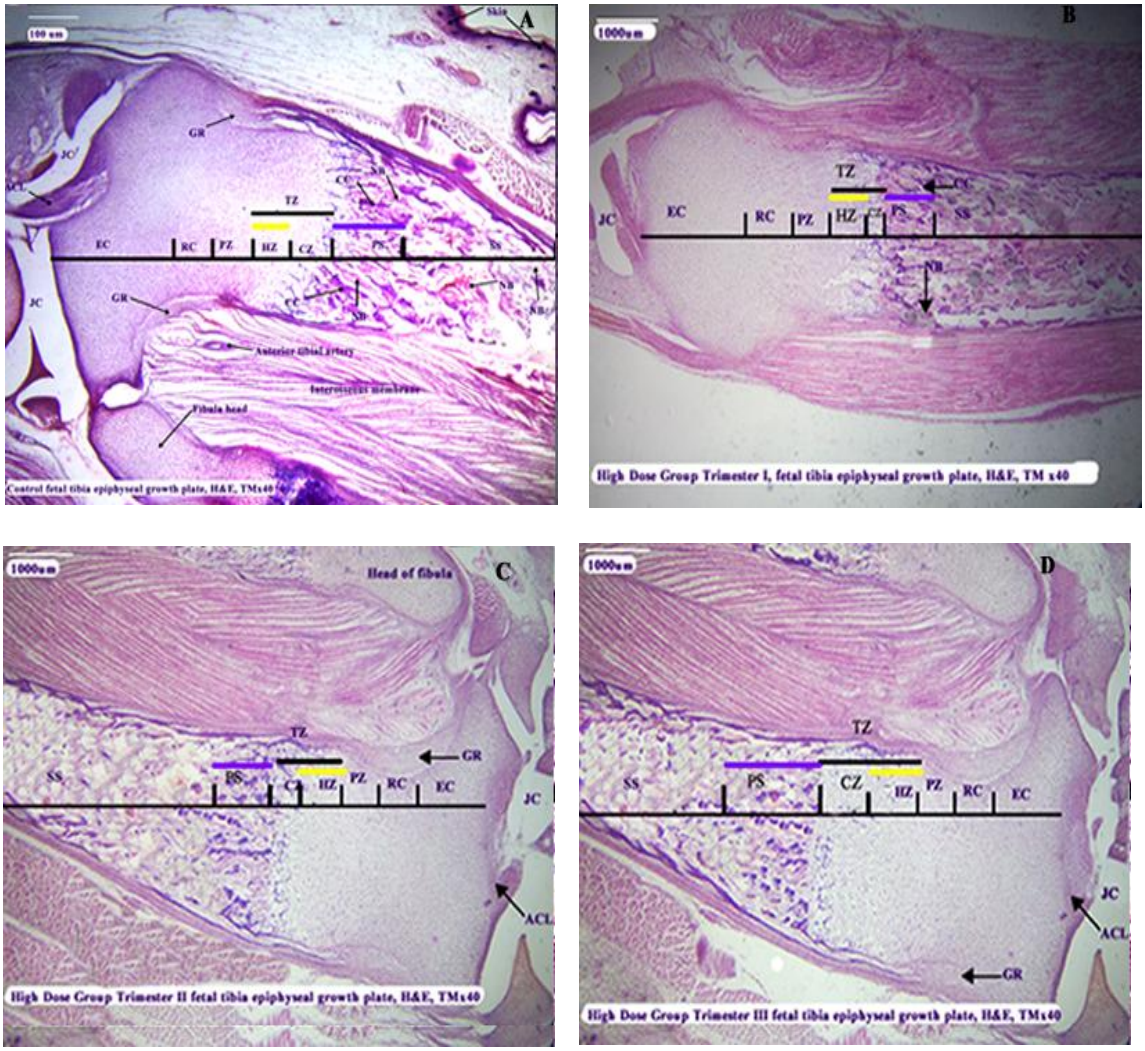

Figure 4.6: shows a relative increase in the Hypertrophic zone: Primary Spongiosa proportionate thickness as well as an increase in the distribution of core cartilage with a reduction in new bone in the PS in the Medium Dose Tetracycline Groups across trimesters (TM_1, B:TM_2, C: TM_3, D) as

Key: EC, epiphyseal cartilage, RC, reserve cartilage, PZ, proliferating zone, HZ/Yellow line, hypertrophic zone, CZ, calcification zone, PS/Blue line, primary spongiosa, SS, secondary spongiosa, GR, groove of Ranvier, TZ, transformation zone, JC, joint cavity, NB, new bone, CC, cartilage core, ACL, anterior cruciate ligament.

4.6 Trimester 1 (TM1) comparative mean Crown Rump Length, Biparietal Diameter, and Mean Litter Birth weight of the tetracycline treated groups against the control.

When tetracycline was administered at trimester 1 (TM_1), the crown rump length (CRL) showed a statistically significant difference (p=0.021) when the treatment groups were compared against control with the statistical significant difference being noted between HDTG and control upon performing the Tukey post hoc t-test (Table 4.3).

The recorded CRL's showed dose dependent linear changes with LDTG (4.2±0.09) registering the greatest length, MDTG (4.0±0.09) and HDTG (3.93±0.33) registering the least as compared to control (4.37±0.09) (Table 4.3). These measurements demonstrate a dose dependent reduction in the CRL.

Similar findings were noted for biparietal diameter (BPD), whereby, the BPD showed a statistically significant difference (p=0.003) when the treatment groups were compared against control with the statistical significant difference established to be between HDTG and LDTG, HDTG and control when the Tukey post hoc t-test was done. The recorded BPD's as well showed dose dependent linear changes with LDTG (0.773±0.015) registering the greatest diameter, MDTG (0.73±0.012) and HDTG (0.70±0.009) as compared to control (0.78±0.012) (Table 4.3). These measurements exhibit a dose dependent reduction in the BPD.

Conversely, the mean litter birth weight showed no statistical significant difference (p=0.969) when the treatment groups were compared against controls despite the variance in the mean litter birth weight between the tetracycline treated groups and the control (Table 4.3).

Table 4.3:Trimester 1 (TM_1) comparative mean Crown Rump Length, Biparietal Diameter and Mean Litter Birth weight of the tetracycline treated groups against the control.

Parameter	Control	Low Dose Tetracycline Group (LDTG-*155mg/kg/ day*)	Medium Dose Tetracycline Group (MDTG-*232.5mg/kg/day*)	High Dose Tetracycline Group (HDTG-310mg/kg/day)
CRL (cm)	4.4±0.09a	4.2±0.09ab	4.0±0.09ab	3.93±0.033b
BPD (mm)	0.78±0.012a	0.773±0.015a	0.73±0.012ab	0.70±0.009b
MLBwt.,(g)	6.4±0.26a	6.5±0.239a	6.52±0.087a	6.6±0.43a

Notes: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA, with Tukey test on post-hoc t-tests. CRL: crown rump length, BPD: biparietal diameter, MLB wt: mean litter birth weight.

4.7 Trimester 2 (TM_2) comparative mean Crown Rump Length, Biparietal Diameter and Mean Litter Birth weight of the tetracycline treated groups against the control.

When tetracycline was administered at trimester 2 (TM_2) the crown rump length (CRL) showed a statistically significant difference (p=0.004) when the treatment groups were compared against control with the statistical significant difference being noted between, HDTG and control, HDTG and LDTG, HDTG and MDTG upon performing the Tukey post hoc t-test (Table 4.4).

The recorded CRL's showed dose dependent linear changes with LDTG recording the greatest length of 4.23±0.09mm, whereas MDTG recorded 4.13±0.09mm and HDTG recorded the least length of 3.77±0.35mm as compared to control that recorded 4.37±0.09mm (Table 4.4). These measurements display a dose dependent reduction in the CRL.

Comparable findings were noted for biparietal diameter (BPD), whereby, the BPD showed a statistically significant difference (p=0.001) when the treatment groups were compared against control with the statistical significant difference being noted between HDTG and Control, HDTG and LDTG, HDTG and MDTG upon performing the Tukey post hoc t-test (Table 4.4). The recorded BPD's too, showed dose dependent linear changes with LDTG registering the greatest mean diameter of

0.763±0.009mm, whereas MDTG recorded a mean diameter of 0.75±0.009mm and the least diameter was recorded in HDTG, 0.69±0.009mm as compared to control which recorded a mean diameter of 0.78±0.012mm (Table 4.4). These measurements exhibit a dose dependent reduction in the BPD as well.

Contrary to these findings, the mean fetal weights (g) between the tetracycline treated groups against the control showed no statistically significant difference (p=0.29) with the weights being, LDTG, 5.65±0.325, MDTG, 6.1±0.36, HDTG, 6.42±0.18 and control 6.4±0.263 (Table 4.4).

Table 4.4: The Trimester 2 (TM_2) comparative mean Crown Rump Length, Biparietal Diameter and Mean Litter Birth weight of the tetracycline treated groups the control.

Parameter	**Control**	**Low Dose Tetracycline Group (LDTG-*155mg/kg/ day*)**	**Medium Dose Tetracycline Group (MDTG-*232.5mg/kg/ day*)**	**High Dose Tetracycline Group (HDTG-*310mg/kg/ day*)**
CRL (cm)	4.37±0.09a	4.23±0.09a	4.13±0.09a	3.77±0.035b
BPD (mm)	0.78±0.012a	0.763±0.009a	0.75±0.009a	0.69±0.009b
MLBwt.(g)	6.4±0.263a	5.65±0.325a	6.1±0.360a	6.42±0.180a

Notes: *The means, followed by the same letter in a row are not statistically different at ($P<0.05$) using one way ANOVA, with Tukey test on post-hoc t-tests. CRL: crown rump length, BPD: biparietal diameter, MLB wt: mean litter birth weight.*

4.8 Trimester 3 (TM_3) comparative mean Crown Rump Length, Biparietal Diameter and Mean Litter Birth weight of the tetracycline treated groups against the control.

When tetracycline was administered at trimester 3 (TM_3) the crown rump length (CRL) showed a statistically significant difference (p=0.032) when the treatment groups were compared against control with the statistical significant difference being noted between HDTG and control, HDTG and LDTG upon performing the Tukey post hoc t-test (Table 4.5).

The recorded CRL's showed dose dependent linear changes with LDTG registering the greatest length of 4.27±0.088mm, whereas MDTG registered 4.2±0.033mm and lastly HDTG registered 4.00±0.057mm which was the least registered mean CRL in

TM_3 tetracycline treated rats as compared to control which registered 4.37±0.09mm (Table 4.5). These measurements demonstrate a dose dependent reduction in the CRL.

Similar findings were noted for biparietal diameter (BPD), in which, the BPD showed a statistically significant difference ($p<0.001$) when the treatment groups were compared against control as well as between the various treatment groups. The statistical significant difference was noted to be between MDTG and Control, MDTG and LDTG, HDTG and LDTG, HDTG and control upon performing the Tukey post hoc t-test with the recorded BPD's as well showing dose dependent linear changes whereby LDTG registered the greatest mean diameter of 0.77±0.009mm followed by MDTG 0.7±0.006mm and lastly HDTG 0.69±0.003 in descending order as compared to control which registered a mean BPD of 0.78±0.012mm (Table 4.5). These measurements reveal a dose dependent reduction in the BPD.

On the other hand, when comparisons were done for the mean fetal weights (g) between the tetracycline treated groups against the control there was no statistically significant difference ($p=0.412$) with the weights being, LDTG, 5.85±0.326g, MDTG, 5.84±0.60g and lastly HDTG, 5.42±0.151g as compared to control which registered a mean fetal birth weight of 6.4±0.263g (Table 4.5).

Table 4.5: The Trimester 3 (TM_3) comparative mean Crown Rump Length, Biparietal Diameter and Litter Birth weight of the tetracycline treated groups against the control.

Parameter	**Control**	**Low Dose Tetracycline Group (LDTG-*155mg/kg/ day*)**	**Medium Dose Tetracycline Group (MDTG-*232.5mg/kg/ day*)**	**High Dose Tetracycline Group (HDTG-*310mg/kg/ day*)**
CRL (cm)	4.4±0.088a	4.27±0.088ab	4.2±0.033ab	4±0.0577b
BPD (mm)	0.783±0.012a	0.77±0.009a	0.7±0.006b	0.69±0.003b
MLBwt.,(g)	6.4±0.263a	5.85±0.326a	5.84±0.600a	5.42±0.151a

Notes: The means, followed by the same letter in a row are not statistically different at ($P<0.05$) using one way ANOVA with Tukey test on post-hoc t-tests. CRL: crown rump length, BPD: biparietal diameter, MLB wt: mean litter birth weight.

4.9 Low Dose Tetracycline Group comparative mean Crown Rump Length, Biparietal Diameter and Litter Birth weight in across trimesters against the control.

When tetracycline was administered at low dose (155mg/kg/day), the CRL showed no statistically significant difference, p=0.485, when experimental groups were compared against controls in the different trimesters (TM_1, TM_2, and TM_3) despite the variation in lengths between the experimental groups and control group (Table 4.6).

Similarly, the BPD showed no statistically significant difference, p=0.679, when experimental groups were compared against controls in the different trimesters (TM_1, TM_2, and TM_3) notwithstanding the variation in lengths between the experimental groups and control group (Table 4.6).

In the same way, mean litter birth weight showed no statistically significant difference, p=0.214, when experimental groups were compared against controls in the different trimesters (TM_1, TM_2, and TM_3) even though there was variation in the measurements between the experimental groups and control group (Table 4.6).

Table 4.6: Low Dose Tetracycline Group comparative mean Crown Rump Length, Biparietal Diameter and Mean Litter Birth weight in across trimesters against control.

Parameter	Control	TM_1	TM_2	TM_3
CRL (cm)	4.4±0.088a	4.2±0.09a	4.23±0.09a	4.27±0.088a
BPD (mm)	0.783±0.012a	0.773±0.015a	0.763±0.009a	0.77±0.009a
MLBwt.,(g)	6.4±0.263a	6.5±0.239a	5.65±0.325a	5.85±0.326a

Notes: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA with Tukey test on post-hoc t-tests. CRL: crown rump length, BPD: biparietal diameter, MLB wt: mean litter birth weight.

4.10 Medium Dose Tetracycline Group comparative mean Crown Rump Length, Biparietal Diameter and Mean Litter Birth weight across trimesters against the control.

When tetracycline was administered at medium dose (232.5mg/kg/day), the CRL showed no statistically significant difference, p=0.084, when experimental groups were compared against controls in the different trimesters (TM_1, TM_2, and TM_3) regardless of the variation in lengths between the experimental groups and control group (Table 4.7).

Conversely, the BPD, showed a statistically significant difference, p=0.02, when the experimental groups were compared against the control group across the trimesters with the statistically significant difference being between TM3 and control group only (Table 4.7).

On the other hand, the mean litter size and mean litter birth weight showed no statistically significant difference, p=0.179 and p=0.620 respectively when experimental groups were compared against controls in the different trimesters (TM_1, TM_2, and TM_3) albeit variation in the measurements between the experimental groups and control group (Table 4.7).

Table 4.7: Medium Dose Tetracycline Group comparative mean Crown Rump Length, Biparietal Diameter and Mean Litter Birth weight across trimesters against the control.

Parameter	Control	TM_1	TM_2	TM_3
CRL (cm)	4.4±0.088a	4.0±0.09a	4.13±0.09a	4.2±0.033a
BPD (mm)	0.783±0.012a	0.73±0.012bc	0.75±0.009ab	0.7±0.006b
MLBwt.,(g)	6.4±0.263a	6.52±0.087a	6.10±0.360a	5.84±0.600a

Notes: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA with Tukey test on post-hoc t-tests. CRL: crown rump length, BPD: biparietal diameter, MLB wt: mean litter birth weight.

4.11 High Dose Tetracycline Group comparative Crown Rump Length, Biparietal Diameter, mean Litter sizes and Mean Litter Birth weight across trimesters against the control.

When tetracycline was administered at high dose (310mg/kg/day), the CRL showed a statistically significant difference, p=0.001, when experimental groups were compared against controls in the different trimesters (TM_1, TM_2, and TM_3) with the statistically significant difference being majorly noted between TM_3 and control only. The registered CRLs were; TM_3 4.00±0.057 which was the highest, followed by TM_1 3.93±0.033 and lastly TM_2 3.77±0.035 as compared to control group which registered a mean CRL of 4.4±0.088 (Table 4.8).

Similarly, the BPD showed a statistically significant difference, $p \leq 0.001$, when experimental groups were compared against controls in the different trimesters (TM_1, TM_2, and TM_3) with the statistically significant difference being majorly noted between TM_3 and control only. The registered BPDs were; TM_1 0.70±0.009 which registered the highest, while TM_2 and TM_3 registered, 0.69±0.009 and 0.69±0.003 respectively (Table 4.8).

Conversely, the mean litter size and mean litter birth weight showed no statistically significant difference, p=0.659 and p=0.069 respectively when experimental groups were compared against controls in the different trimesters (TM_1, TM_2, and TM_3) even though there was variation in the measurements between the experimental groups and control group (Table 4.8).

Table 4.8: High Dose Tetracycline Group comparative mean Crown Rump Length, Biparietal Diameter and Mean Litter Birth weight across trimesters against the control.

Parameter	Control	TM_1	TM_2	TM_3
CRL (cm)	4.4±0.088a	3.93±0.033b	3.77±0.035b	4.00±0.0577b
BPD (mm)	0.783±0.012a	0.70±0.009b	0.69±0.009b	0.69±0.003b
MLBwt.,(g)	6.4±0.263a	6.6±0.43a	6.42±0.180a	5.42±0.151a

Notes: The means, followed by the same letter in a row are not statistically different at (P <0.05) using one way ANOVA with Tukey test on post-hoc t-tests. CRL: crown rump length, BPD: biparietal diameter, MLB wt: mean litter birth weight.

4.12 Gross morphometric measurements of the tibia upon exposure to tetracycline

When the experimental groups were compared to controls upon exposure to tetracycline there was no statistically significant difference in tibial lengths despite a variation in the tibial lengths. The recorded p-values were TM_1, p=0.892, TM_2, p=0891, and TM_3, p=0.521 (Table 4.9).

Table 4.9: Gross morphometric measurements of the tibia upon exposure to tetracycline

Parameter	Control	Low Dose Tetracycline Group(LDTG-155mg/kg/day)	Medium Dose Tetracycline Group(MDTG-232.5mg/kg/day)	High Dose Tetracycline Group(HDTG-310mg/kg/day)
Av. TL in TM_1(cm)	0.403±0.009a	0.397±0.007a	0.400±0.012a	0.393±0.009a
Av, TL in TM_2(cm)	0.403±0.009a	0.397±0.003a	0.400±0.010a	0.403±0.003a
Av. TL in TM_3(cm)	0.403±0.009a	0.410±0.006a	0.397±0.007a	0.397±0.007a

Notes: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA with Tukey test on post-hoc t-tests.

Key: Av. TL: average tibial length

4.13 Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to varied doses of tetracycline throughout the gestational period beginning from day I of Trimester 1 (TM_1)

There was a statistically significant increase (p=0.008) in the hypertrophic zone percentage of total surface area of epiphyseal growth plate when the treatment groups were compared against control rats. This statistically significant increase was found to be between HDTG and LDTG, HDTG and control upon performing the Tukey post-hoc t-test (Table 4.10). Notably, the registered hypertrophic zone

percentages of total surface area of epiphyseal growth plate were found to display an incremental linear relationship with regards to tetracycline dose whereby the HDTG occupied the highest percentage of 28.35%±0.563, MDTG occupied 27.51%±0.160 while LDTG occupied 25.97%±0.268 in comparison to control group which occupied 25.97%±0.53 (Table 4.10).

The hypertrophic zone percentage of primary spongiosa surface area, as well showed a statistically significant increase ($p<0.001$), between the treatment groups and the control. In order to establish which groups were statistically significant, the Tukey post hoc t-test was performed the difference was found to be between MDTG and LDTG, MDTG and control, HDTG and LDTG, HDTG and control with no statistically significant difference between MDTG and HDTG, LDTG and control (Table 4.10).

The recorded hypertrophic zone percentage of primary spongiosa surface areas demonstrated a linear pattern in that HDTG occupied the greatest percentage (HDTG 67.36%±1.24) followed by MDTG 63.37%±0.539 and lastly LDTG 55.33% ±0.689 which occupied the least surface area as compared to control which occupied 55.98%±1.097(Table 4.10).

Conversely, the reserve cartilage percentage of total surface area of the epiphyseal growth plate, showed no statistically significant difference, $p=0.659$ when the treatment groups were compared against control rats with the recorded percentage surface areas being; LDTG 36.78%±1.008 which occupied the largest surface area, followed by HDTG 36.07%±0.674 and finally MDTG which occupied 35.39%±0.849 as compared to control that occupied 36%±0.531 (Table 4.10).

In a similar fashion, the proliferation zone percentage of total surface area of epiphyseal growth plate showed no statistically significant difference, $p=0.785$, between the treatment groups and the control despite showing a variation in percentage surface area of the epiphyseal growth plate. The surface areas obtained were; LDTG 24.89%± 0.690, MDTG 24.38%±0.627, HDTG 25.12%±0.384 as compared to control group 24.42%±0.666 (Table 4.10).

Table 4.10: Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to varied doses of tetracycline throughout the gestational period beginning from Trimester 1

Parameter	Control	Low Dose Tetracycline Group (LDTG-*155mg/kg/ day*)	Medium Dose Tetracycline Group (MDTG-(*232.5mg/kg/ day*)	High Dose Tetracycline Group (HDTG-*310 mg/kg/ day*)
RC % of TSA of E	36.37±0.531a	36.78±1.008a	35.39±0.849a	36.07±0.674a
PZ% of TSA of E	24.42±0.666a	24.89±0.690a	24.38±0.627a	25.12±0.384a
HZ% of TSA of E	25.97±0.530a	25.97±0.268a	27.51±0.160ab	28.35±0.563b
HZ% of PS	55.98±1.097a	55.33±0.689a	63.37±0.539b	67.36±1.24b

Notes: The means, followed by the same letter in a row are not statistically different at (P <0.05) using one way ANOVA, with Tukey test on post-hoc t-tests.

Key: Av.TL, average tibial length, RC% of TSA of E, reserve cartilage percentage of total surface area of epiphyseal growth plate, PZ% of TSA of E, proliferation zone percentage of total surface area of epiphyseal growth plate, HZ% of TSA of E, hypertrophic zone percentage of total surface area of epiphyseal growth plate, HZ% of PS, hypertrophic zone percentage of primary spongiosa

4.14 Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to varied doses of tetracycline from gestational day 8 in Trimester 2 (TM2) to delivery

In trimester 2 rats (TM_2), it is apparent that, when tetracycline was administered the hypertrophic zone percentage of total surface area of epiphyseal growth plate, showed a statistically significant difference, p=0.009, when the treatment groups were compared against control rats with the statistically significant difference being between HDTG and LDTG, HDTG and control upon performing the Tukey post-hoc t-test (Table 4.11).

The registered hypertrophic zone percentages of total surface area of epiphyseal growth plate were found to exhibit an incremental linear relationship with regards to tetracycline dose, in that the HDTG recorded the highest percentage of 28.94%±0.566 while MDTG recorded a percentage of 27.57%±0.079 and lastly

LDTG 25.28%±0.872 as compared to control group which occupied 25.97%±0.53 (Table 4.11).

Similarly, hypertrophic zone percentage of primary spongiosa surface area, also showed a statistically significant difference, $p \leq 0.001$, between the treatment groups and the control. In order to establish which groups were statistically significant, the Tukey post hoc t-test was performed and the difference was found to be between MDTG and LDTG, MDTG and control, HDTG and LDTG, HDTG and control with no statistically significant difference between MDTG and HDTG, LDTG and control (Table 4.11).

The recorded hypertrophic zone percentage of primary spongiosa surface areas demonstrated a linear model in that HDTG occupied the greatest percentage (HDTG 70.62%±1.67) followed by MDTG 65.787%±0.644 and lastly LDTG 55.36%±0.757 which occupied the least surface area as compared to control which occupied 55.98%±1.097 (Table 4.11).

On the contrary, the reserve cartilage percentage of total surface area of the epiphyseal growth plate, showed no statistically significant difference, p=0.646 when the treatment groups were compared against control rats with the recorded percentage surface areas being; LDTG 36.33%±0.45 which occupied the largest surface area, followed by MDTG 35.64%±0.826 and finally HDTG which occupied 35.59%±0.285 as compared to control that occupied 36%±0.531(Table 4.11).

In the same way, the proliferation zone percentage of total surface area of epiphyseal growth plate showed no statistically significant difference, p=0.703, between the treatment groups and the control notwithstanding a variation in percentage surface area of the epiphyseal growth plate. The surface areas obtained were; LDTG 36.33%±0.45, MDTG 35.64%±0.826, HDTG 35.59%±0.285 as compared to control group which occupied 36.37%±0.531 (Table 4.11).

Table 4.11: Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to varied doses of tetracycline from gestational day 8 in Trimester 2 (TM_2) to delivery

Parameter	Control	Low Dose Tetracycline Group (LDTG-*155mg/kg/ day*)	Medium Dose Tetracycline Group (LDTG-*232.5mg/kg/ day*)	High Dose Tetracycline Group (LDTG-*310mg/kg/ day*)
RC % of TSA of E	36.37±0.531a	36.33±0.450a	35.64±0.826a	35.59±0.285a
PZ% of TSA of E	24.42±0.666a	25.13±1.11a	23.88±0.649a	24.52±0.254a
HZ% of TSA of E	25.97±0.530a	25.28±0.872a	27.57±0.079ab	28.94±0.566b
HZ% of PS	55.98±1.097a	55.36±0.757a	65.78±0.644b	70.62±1.67b

Notes: The means, followed by the same letter in a row are not statistically different at (P<0.05) using one way ANOVA with Tukey test on post-hoc t-tests.

Key: Av.TL average tibial length, RC% of TSA of E, reserve cartilage percentage of total surface area of epiphyseal growth plate, PZ% of TSA of E, proliferation zone percentage of total surface area of epiphyseal growth plate, HZ% of TSA of E, hypertrophic zone percentage of total surface area of epiphyseal growth plate, HZ% of PS, hypertrophic zone percentage of primary spongiosa.

4.15 Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to varied doses of tetracycline from gestational day 15 in Trimester 3 (TM_3) to delivery

When tetracycline was administered at trimester 3 (TM_3), the hypertrophic zone percentage of total surface area of epiphyseal growth plate, showed a statistically significant difference, $p \leq 0.0001$, when the treatment groups were compared against control rats with the statistically significant difference being between HDTG and LDTG, HDTG and control, HDTG and MDTG, MDTG and LDTG, MDTG and control when the Tukey post-hoc t-test was performed (Table 4.12).

The registered hypertrophic zone percentages of total surface area of epiphyseal growth plate were found to present an incremental linear relationship with regards to tetracycline dose in that the HDTG recorded the highest percentage of 30.03%±0.062 while MDTG recorded a percentage of 27.86%±0.025 and lastly LDTG

26.12%±0.240 as compared to control group which occupied 25.97%±0.53 (Table 4.12).

In a similar pattern, the hypertrophic zone percentage of primary spongiosa surface area, too showed a statistically significant difference, $p \leq 0.0001$, between the treatment groups and the control. In order to establish the particular groups that showed the statistically significant difference, the Tukey post hoc t-test was performed and the difference was found to be between MDTG and LDTG, MDTG and control, HDTG and LDTG, HDTG and MDTG, HDTG and control with no statistically significant difference between LDTG and control (Table 4.12).

The recorded hypertrophic zone percentage of primary spongiosa surface areas demonstrated a linear pattern in that HDTG occupied the greatest percentage (HDTG 74.28%±0.081) followed by MDTG 68.44%±0.254 and lastly LDTG 57.98%±1.38 which occupied the least surface area as compared to control which occupied 55.98%±1.097 (Table 4.12).

On the other hand, the reserve cartilage percentage of total surface area of the epiphyseal growth plate, showed no statistically significant difference, $p=0.344$ when the treatment groups were compared against control rats with the recorded percentage surface areas being; LDTG 35.96%±0.368 which occupied the largest surface area, followed by MDTG 35.76%±0.308 and finally HDTG which occupied 35.30%±0.317 as compared to control that occupied 36.37%±0.531 (Table 4.12).

In the same way, the proliferation zone percentage of total surface area of epiphyseal growth plate showed no statistically significant difference, $p=0.538$, between the treatment groups and the control despite showing a variation in percentage surface area of the epiphyseal growth plate. The surface areas obtained were; LDTG 23.81%± 0.659, MDTG 24.50%±0.401, HDTG 23.56%±0.246 as compared to control group 24.42%±0.666 (Table 4.12).

Table 4.12: Changes on the Albino rat fetal tibia upon in-utero exposure to varied doses of tetracycline from gestational day 15 in Trimester 3 (TM_3) to delivery

Parameter	Control	Low Dose Tetracycline Group (LDTG- *155mg/kg/ day*)	Medium Dose Tetracycline Group (MDTG- *232.5mg/kg/ day*)	High Dose Tetracycline Group (LDTG- *310 mg/kg/ day*)
RC % of TSA of E	36.37±0.531a	35.96±0.368a	35.76±0.308a	35.30±0.317a
PZ% of TSA of E	24.42±0.666a	23.81±0.659a	24.50±0.401a	23.56±0.246a
HZ% of TSA of E	25.97±0.530a	26.12±0.240a	27.86±0.025b	30.03±0.062c
HZ% of PS	55.98±1.097a	57.98±1.38a	68.44±0.254b	74.28±0.081c

Notes: The means, followed by the same letter in a row are not statistically different at (P <0.05) using one way ANOVA with Tukey test on post-hoc t-tests.

Key: Av.TL,average tibial length, RC% of TSA of E, reserve cartilage percentage of total surface area of epiphyseal growth plate, PZ% of TSA of E, proliferation zone percentage of total surface area of epiphyseal growth plate, HZ% of TSA of E, hypertrophic zone percentage of total surface area of epiphyseal growth plate, HZ% of PS, hypertrophic zone percentage of primary spongiosa.

4.16 Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to Low Dose Tetracycline in Trimester 1, 2 and 3

There was no statistically significant difference ($p>0.05$) when the surface areas for reserve cartilage, zone of proliferation, zone of hypertrophy and the hypertrophic zone percentage surface area of primary spongiosa were compared to those of control across the different trimesters (Table 4.13).

Table 4.13: Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to Low Dose Tetracycline in Trimester 1, 2 and 3

Parameter	Control	TM_1	TM_2	TM_3
RC % of TSA of E	36.37±0.531a	36.78±1.008a	36.33±0.450a	35.96±0.368a
PZ% of TSA of E	24.42±0.666a	24.89±0.690a	25.13±1.11a	23.81±0.659a
HZ% of TSA of E	25.97±0.530a	25.97±0.268a	25.28±0.872a	26.12±0.240a
HZ% of PS	55.98±1.097a	55.33±0.689a	55.36±0.757a	57.98±1.38a

Notes: The means, followed by the same letter in a row are not statistically different at (P <0.05) using one way ANOVA.withTukey test on post-hoc t-tests.

Key: Av.FL,average femur length, RC% of TSA of E, reserve cartilage percentage of total surface area of epiphyseal growth plate, PZ% of TSA of E, proliferation zone percentage of total surface area of epiphyseal growth plate, HZ% of TSA of E, hypertrophic zone percentage of total surface area of epiphyseal growth plate, HZ% of PS, hypertrophic zone percentage of primary spongiosa.

4.17 Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to Medium Dose Tetracycline in Trimester 1, 2 and 3

The reserve cartilage percentage of total surface area of epiphyseal growth plate as well as the proliferation zone percentage of total surface area of epiphyseal growth plate showed no statistically significant difference, p=0.763 and p=0.878 respectively when the Medium Dose Tetracycline Groups were compared to the control group this was despite registering a variation in the measurements (Table 4.14).

On the other hand, the hypertrophic zone percentage of the total surface area of the epiphyseal growth plate (HZ% of TSA of E) as well as the hypertrophic zone percentage of the primary spongiosa (HZ% of PS) showed a statistically significant difference, p=0.006 and $p \leq 0.001$respectivelyl when the Medium Dose Tetracycline Groups were compared to the control group across the trimesters (Table 4.14).

The HZ% of TSA of E demonstrated the statistically significant difference to be between TM_3 and control upon performing the Tukey post hoc t-test despite a difference in the recorded measurements between the experimental groups and the control whereas the HZ% of PS established the statistically significant difference to be between TM_3 and TM_1, TM_3 and control upon performing the Tukey post hoc t-

test despite a difference in the recorded measurements between the experimental groups and the control (Table 4.14).

Table 4.14: Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to Medium Dose Tetracycline in Trimester 1, 2 and 3

Parameter	Control	TM_1	TM_2	TM_3
RC % of TSA of E	36.37±0.531a	35.39±0.849a	35.64±0.826a	35.76±0.308a
PZ% of TSA of E	24.42±0.666a	24.38±0.627a	23.88±0.649a	24.50±0.401a
HZ% of TSA of E	25.97±0.530a	27.51±0.160b	27.57±0.079b	27.86±0.025b
HZ% of PS	55.98±1.097a	63.37±0.539b	65.78±0.644bc	68.44±0.254c

Notes: The means, followed by the same letter in a row are not statistically different at (P <0.05) using one way ANOVA.withTukey test on post-hoc t-tests.

Key: Av.FL,average femur length, RC% of TSA of E, reserve cartilage percentage of total surface area of epiphyseal growth plate, PZ% of TSA of E, proliferation zone percentage of total surface area of epiphyseal growth plate, HZ% of TSA of E, hypertrophic zone percentage of total surface area of epiphyseal growth plate, HZ% of PS, hypertrophic zone percentage of primary spongiosa

4.18 Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to High Dose Tetracycline in Trimester 1, 2 and 3

The reserve cartilage percentage of total surface area of epiphyseal growth plate as well as the proliferation zone percentage of total surface area of epiphyseal growth plate showed no statistically significant difference, p=0.442 and p=0.153 respectively when the High Dose Tetracycline Groups were compared to the control group this was despite registering a variation in the measurements (Table 4.15).

On the other hand, the hypertrophic zone percentage of the total surface area of the epiphyseal growth plate (HZ% of TSA of E) as well as the hypertrophic zone percentage of the primary spongiosa (HZ% of PS) showed a statistically significant difference, p=0.002 and $p \leq 0.001$respectively when the High Dose Tetracycline Groups were compared to the control group across the trimesters (Table 4.15).

The HZ% of TSA of E demonstrated the statistically significant difference to be between TM_3 and control upon performing the Tukey post hoc t-test notwithstanding a difference in the recorded measurements between the experimental groups and the

control whereas the HZ% of PS established the statistically significant difference to be between TM_3 and TM_1, TM_3 and control upon performing the Tukey post hoc t-test despite a difference in the recorded measurements between the experimental groups and the control(Table 4.15) .

Table 4.15: Changes on the Albino rat fetal tibia epiphyseal growth plate upon in-utero exposure to High Dose Tetracycline in Trimester 1, 2 and 3

Parameter	Control	TM_1	TM_2	TM_3
RC % of TSA of E	36.37±0.531a	36.07±0.674a	35.59±0.285a	35.30±0.317a
PZ% of TSA of E	24.42±0.666a	25.12±0.384a	24.52±0.254a	23.56±0.246a
HZ% of TSA of E	25.97±0.530a	28.35±0.563b	28.94±0.566b	30.03±0.062b
HZ% of PS	55.98±1.097a	67.36±1.24b	70.62±1.67bc	74.28±0.081c

Notes: The means, followed by the same letter in a row are not statistically different at ($P<0.05$) using one way ANOVA.withTukey test on post-hoc t-tests.

Key: Av.FL,average femur length, RC% of TSA of E, reserve cartilage percentage of total surface area of epiphyseal growth plate, PZ% of TSA of E, proliferation zone percentage of total surface area of epiphyseal growth plate, HZ% of TSA of E, hypertrophic zone percentage of total surface area of epiphyseal growth plate, HZ% of PS, hypertrophic zone percentage of primary spongiosa

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

Discussion

Tetracycline's have been in use in treatment of bacterial diseases since their discovery in 1950 and were noted to be bone teratogenic which led to their subsequent contraindication in pregnancy. The contraindication was extended to the tetracycline molecules that were discovered henceforth based on studies done on the prototype tetracycline molecule. However, recent literature from experimental studies in animals and epidemiological studies in human beings have shown that doxycycline, a tetracycline, does not impair skeletal morphosynthesis but rather, in low doses, it does enhance skeletal morphosynthesis and it is not skeletal teratogenic (Cross *et al.*, 2016; Larochelle *et al.*, 1968; Safety & Medicine, 2017).

Through these studies, doxycycline was approved for use in pregnancy by the WHO and FDA (Cross *et al.*, 2016; Safety & Medicine, 2017). This study was prompted by these controversies and it sought to find out whether the prototype drug tetracycline was skeletal teratogenic across different dose categories as well as during different gestational periods.

From the study, it is shown that tetracycline does not influence the quantitative histological contribution of the reserve cartilage and proliferation zone to the total surface area of the epiphyseal growth plate neither does it influence the length of tibia.

On the other hand, hypertrophic zone to primary spongiosa ratio was seen to increase linearly with an increase in tetracycline dose which shows that tetracycline dosage is directly proportional to its effect on skeletal tissue. Similar findings were noted for crown rump length and biparietal diameter which showed a linear reduction with an increase in dose.

Rib anomalies and ossification status as well demonstrated direct relationship with tetracycline dosing whereby, rats than received medium and high dose tetracycline were had more wavy ribs, incomplete and unossification implying a dose dependent effect on bone. Contrary to this, the study findings have shown that the effects of tetracycline on the skeleton are not time dependent.

Studies have shown tetracycline's to have a down regulatory effect on Bone Morphogenic Proteins (BMPs), estrogen β and estrogen α that is dose dependent with an inverse relationship(Park, 2011). BMPs, particularly, BMP 2 and 7 have been shown to play a critical role in osteosynthesis through dose dependent induction of osteoblast and pre-osteoblast formation (Park, 2011; Patianna & Valente, 2015).

In one study, doxycylcine was shown to enhance new bone formation in ovarectimized rats treated with doxycycline 10mg/kg/day as compared to ovarectimized rats not treated with doxycycline (p=0.004). At a dose of 30mg/kg/day the p value was recorded at p=0.05 (Figueiredo et al., 2019). In the same study, histologically, rats that received doxycycline at 10mg/kg/day showed well distributed mineralized tissue and of nearly equal distribution as compared to animals that received 30mg/kg/day of doxycycline which showed a poor distribution of mineralized tissue (Figueiredo *et al.,* 2019).

In other studies, tetracycline's have been shown to have an effect on Receptor Activator Nuclear *kappa* Ligand (RANKL), a marker of bone resorption and Osteoprotegerin (OPG), a marker of bone formation (Figueiredo *et al.,* 2019; Koide *et al.,* 2012; Li *et al.,* 2003; Patianna & Valente, 2015). OPG is found in the cell membranes of osteoblasts and bone marrow stromal cells where it binds to RANKL with a very high affinity that is nearly five hundred times that of RANK which is a RANKL receptor. This strong binding of OPG to RANKL prevents the osteoclastic activity of osteoclasts and enhances bone formation (Infante *et al.,* 2019).

Doxycline, has been shown to increase the OPG/RANKL ratio at low doses which in this case favors new bone formation (Figueiredo *et al.,* 2019; Kalina *et al.,* 2007) which confers with the study findings as evidenced with comparable results between control group rats and those of Low Dose Group.

Low doses of tetracycline have been shown to propagate osteoblast proliferation and enhance osteod mineralization (Cheng *et al.,* 2012; Griffin *et al.,* 2010). In a study done on diabetic male DBA/2J mice upon long term exposure to doxycycline which is a Tetracycline, serum Procollagen type 1 N-terminal propeptide (P1NP) which is an indicator of bone development was found to be high in DBA/2J mice treated with doxycline (p=0.04), this implied enhanced osteoblast activity with doxycycline treatment (Fowlkes *et al.,* 2015). Osteoclasts are the major cells that play a role in

bone resorption and have their differentiation and maturation being regulated by osteoblasts which produces the Receptor Activator of Nuclear factor Kappa-B Ligand(RANKL) that is involved in osteoclast formation, function as well as survival whereas Osteoprotegerin (OPG) that inhibits RANKL (Infante *et al.*, 2019; Koide *et al.*, 2012). Osteoclast precursors and mature osteoclasts poses the Receptor Activator of Nuclear Factor Kappa-B (RANK) which is a binding site for RANKL.

In another study, similar results on tetracycline's activity on bone were obtained and it was shown that doxycycline and minocycline inhibit RANKL-induced osteoclast differentiation and maturation at low dosages(Koide *et al.*, 2012).

A study on ovarectimized rats showed an increase in bone trabecular area in rats administered with tetracycline hydrochloride at low doses of 1.2 and 4.8mg/kg/day as compared to ovarectimized rats administered with a placebo which showed a decline in bone trabecular area as well as an increase in bone turnover.

In another study in a bone metastatic cancer mouse model, a combination of zolendronic acid and doxycycline reduced the total tumor burden by 74% as compared to zolendronic alone which reduced the tumor burden by 43% all compared to placebo (Kalina *et al.*, 2007) which implies a bone histosynthetic property in doxycycline. In the same study, doxycycline-zolendronic acid combination was shown to improve bone histomorphometric parameters (osteoid volume, osteoid surface and population of osteoclasts per bone surface area) (Kalina *et al.*, 2007).

The aforementioned studies are in line with this study findings in that the study has demonstrated comparable results between control and low dose group rats i.e. at low doses tetracycline has no bone chelating activity. The bone chelating activity rather, is directly proportional to the amount of dose as evidenced by reduced osseous tissue in the primary spongiosa in the medium and high dose group of rats and this has been shown not to be related to gestation of exposure.

Conclusion

Despite Tetracyclines being known to be bone chelating, at low doses they have been shown to enhance bone formation in a non-time dependent fashion and at high doses

they are bone chelating. From the study, it was shown that tetracycline does not influence the quantitative histological contribution of the reserve cartilage and proliferation zone to the total surface area of the epiphyseal growth plate neither does it influence the length of tibia. In contrast, hypertrophic zone to primary spongiosa ratio was seen to increase linearly with an increase in tetracycline dose which shows that tetracycline dosage is directly proportional to its effect on skeletal tissue. Comparable results were noted for crown rump length and biparietal diameter which showed a linear decline with an increase in dose. Rib anomalies and ossification status as well showed a direct relationship with tetracycline dosing whereby, rats than received medium and high dose tetracycline were had more wavy ribs, incomplete ossification and unossification implying a dose dependent effect on bone. Contrary to this, the study findings have shown that the effects of tetracycline on the skeleton are not time dependent. In conclusion, bone chelation effect of tetracycline on fetal skeletogenesis was found to be tetracycline dose dependent with high tetracycline doses(HDTG) having the highest chelation effects followed by (MDTG) whereas the low dose tetracycline groups (LDTG) had comparable results with control. This bone chelation effect on fetal skeleton was however found not to be time dependent and of importance the Low tetracycline dose groups (LDTG) were shown to enhance maintenance of bone synthesis across the three trimesters compared with high tetracycline doses that were seen to inhibit fetal bone synthesis.

Recommendation

More studies need to be done on the prototype tetracycline at sub-antimicrobial levels to elucidate its bone chelating effects so as to establish the minimum dose levels for chelation and its applicability in long term treatment of Calcium deficiency disorders like osteoporosis.

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APPENDICES

Appendix 1: Data capture sheet

Bone morphometrics and skeletal ossification outcomes													EPIPHYSEAL GROWTH PLATE PARAMETERS														
GROUP	MATERNAL WEIGHT GAIN(g)	LITER SIZE	MEAN LITER Birth Wt.(g)	CRL.(cm)	BPD(cm)	AVERAGE TIBIAL LENGTH(cm)	RIB ANOMALIES	OSSIFICATION	TSA of E	RC TSA	RC % of TSA of E	PZ TSA	PZ% of TSA of E	HZ TSA	HZ% of TSA of E	TSA of PS	HZ% of PS	HZ:PS									
CONTROL A(water only)																											
Rat 1																											
Rat 2																											
Rat 3																											
CONTROL B 5% DMSO																											
Rat 1																											
Rat 2																											
Rat 3																											
EXPERIMENTAL																											
TRIMESTER 1																											
LDG																											
Rat 1																											
Rat 2																											
Rat 3																											
MDG																											
Rat 1																											
Rat 2																											
Rat 3																											
HDG																											
Rat 1																											
Rat 2																											
Rat 3																											
TRIMESTER 2																											
LDG																											
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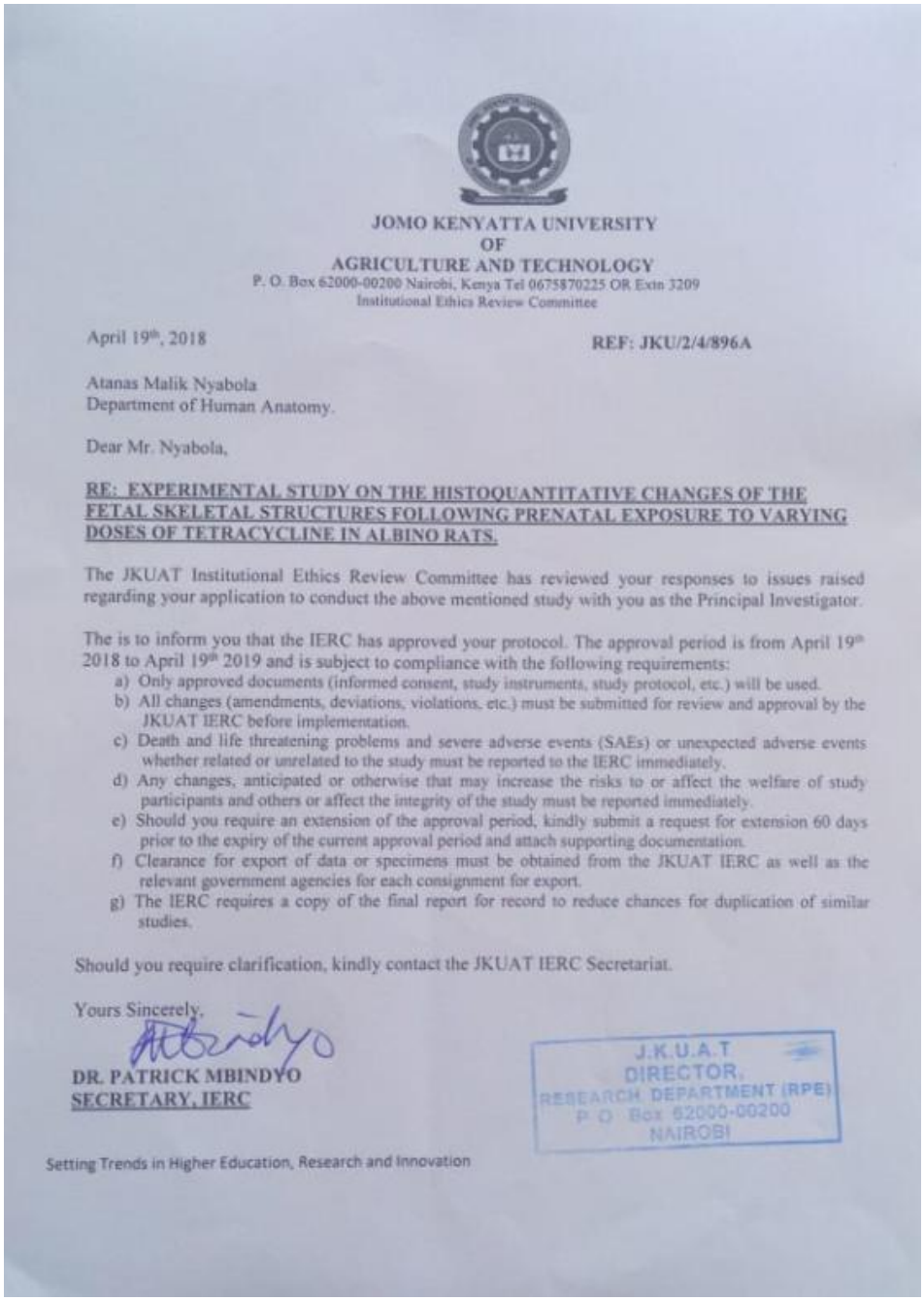
Rat 1																											
Rat 2																											
Rat 3																											
HDG																											
Rat 1																											
Rat 2																											
Rat 3																											
Key																											
Burdan et al Alizarin Red Ossification protocol		Dosing groups		Bone morphometrics																							
1: unossified		LDG: low dose group		BPD: biparietal diameter																							
2: incomplete		MDG: medium dose		CRL: crown rump length																							
3: mishappen		HDG: high dose group																									
4: complete																											

Appendix II: Ethical approval certificate



JOMO KENYATTA UNIVERSITY
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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

Atanas Malik Nyabola
Department of Human Anatomy.

Dear Mr. Nyabola,

RE: EXPERIMENTAL STUDY ON THE HISTOQUANTITATIVE CHANGES OF THE FETAL SKELETAL STRUCTURES FOLLOWING PRENATAL EXPOSURE TO VARYING DOSES OF TETRACYCLINE 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,

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SECRETARY, IERC

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Appendix III: Publication

IOSR Journal of Nursing and Health Science (IOSR-JNHS)
e-ISSN: 2320–1959.p- ISSN: 2320–1940 Volume 8, Issue 4 Ser. VII. (July-Aug .2019), PP 53-58
www.iosrjournals.org

Effect of Prenatal Tetracycline Hydrochloride administration on Skeletal Differentiation in Albino Rat fetuses(Rattus *novegicus*)

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Abstract: ***Objective:*** *To determine the effect of Tetracycline Hydrochloride on Albino rat fetuses skeleton differentiation at different dosages and gestations.* ***Study site:*** *Department of Human Anatomy.* ***Methods:*** *sexually mature 30 Albino rats weighing 150-200g were used upon conceiving. Dams were grouped into 1st, 2nd and 3rd trimester dosing groups each with 3 dams and the drug given via gastric gavaging daily at 9am in 5%DMSO as low dose 155mg/kg, medium dose 231.5mg/kg and high dose 310mg/kg beginning pregnancy day 0 for rats in trimester 1, day 8 for 2nd trimester rats and day 14 for 3rd trimester rats. Animals were sacrificed on day 20 of gestation, fetuses harvested and then killed with concentrated CO_2, dehydrated in 95% ethanol for 4 days then 100% ethanol for 4 days thereafter rinsed in distilled water, eviscerated and placed in 2% KOH solution for 24hours, rinsed then placed in 0.5% KOH solution containing Alizarin red for 24hours. After 24hours, the fetuses were drained and placed in 25% glycerol solution for 1 week then in 100%glycerol with a few drops of 0.5phenol solution. The skeleton was examined for skeletal differentiation and gross anomalies using Leica M205C Stereomicroscope mounted with DFC450C camera.* ***Results:*** *skeletal ossification was associated with dose levels across all trimesters.Trimester one, Ossification χ=17.32, p = 0.008, Trimester two and three χ= 24.95, p=0.001 and χ= 24.95, p=<0.001 respectively.*

Date of Submission: 29-07-2019 Date of Acceptance: 14-08-2019

I. Introduction

Tetracyclines have been shown to have bone chelating effect with resultant poor cortical bone formation and altered epiphysial growth plate histogenesis in the fetus (1–3) which led to their initial categorization as category D medicines, meaning that they were not to be used during pregnancy(1,2,4,5). This categorization was based on studies done on the original molecule which were subsequently adapted for other tetracycline derivatives(5). Currently, there exists a controversy on the teratogenic effects of the different regimens of tetracyclines on the development of the fetal skeletal structures with some studies showing that tetracycline impairs bone morphogenesis and others do not(2,5,6). Recent studies on the other hand have shown that despite this categorization of tetracyclines in class D of the medicines that should not be used in pregnancy, not all tetracyclines affect bone histogenesis (2,5). Tetracyclines regimens are a group of broad spectrum antibiotics that are known to be very efficacious in treatment of atypical microbial infections and their benefits are being lost due to the existing controversy on teratogenicity(2). This study sought to determine the effect of tetracycline hydrochloride on Albino rat fetal skeleton differentiation at varied doses in different gestational periods.

II. Materials and Methods

Two hundred and forty Albino rat fetuses were were harvested on the 20th day of gestation from the 30 pregnant dams for the study. The 30 dams weighed between 150g-200g and were 10-12weeks of age. They were ovulating normally upon acquisition. 2 dams were placed with a fertile male Albino rat for mating purposes and was removed every morning at 0900hrs for 2 hours and a vaginal smear from the dam taken for pregnancy determination. A positive pregnancy was confirmed by observation of large cornified cells, numerous neutrophils on the smear and scattered epithelial cells. The pregnant dams were grouped into 1st, 2nd and 3rd