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Effects Of Tetracycline On Skeletogenesis In Albino Rat (Rattus Norvegicus) Long Bones

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Abstract
The usage of Tetracycline's, a broad spectrum group of antibiotics in pregnancy has for years been associated with debate about its teratogenic effects on the fetal bones. Present studies show that not all tetracycline's are bone teratogenic. Notably, minocycline and doxycycline augment bone synthesis at low doses. In addition, doxycycline has been shown not to be teratogenic. The study utilized 30 Albino rat dams weighing between 200-250g and were grouped into a control group(n=3) and experimental group (n=27) rats which were further grouped into 3 different trimesters i.e. trimester 1, 2 and 3 groups each having 9 rats. 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 1st day of pregnancy upon which treatment was began for rats in the 1st trimester, whereas for those in the 2nd trimester treatment began on day 8 and those in the 3rd trimester on day 15 of pregnancy all to the 20th day of gestation when animals were sacrificed and fetuses harvested, fixed in 10% formalin for 24hrs, tibia harvested and tissue processing for paraffin wax embedding and later hematoxylin and eosin staining. The study findings showed that at low therapeutic doses, tetracycline maintains bone histoarchitecture as evidenced by comparable distribution of new bone in the experimental groups and controls across trimesters. Conversely, at medium and high dose groups, the amount of new bone was less, notably in a linear fashion with regards to period of exposure. In conclusion, at low therapeutic doses tetracycline preserves bone histoarchitecture in a non-time dependent fashion and at high doses they are bone chelating. From the study, it was also shown that tetracycline does not influence the histomorphological contribution of the reserve cartilage and proliferation zone of the epiphyseal growth. 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.

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I. Introduction

The usage of Tetracycline's, a broad spectrum group of antibiotics in pregnancy has for years been associated with debate about its vague tetratogenic effects on the fetal long bones. Upon its discovery, the prototype drug, tetracycline, was noted to chelate calcium in bone thus contraindicated in pregnancy. This was extended to all other tetracycline drug molecules subsequently discovered (Cheng *et al.,* 2012). In the face of this generalization, present studies have shown that not all tetracycline's chelate calcium in bone. Notably, minocycline and doxycycline have been shown to augment bone synthesis 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 setting of this background that the current study was anchored in which the effects of the prototype drug tetracycline on bone upon in utero exposure was evaluated by varying the period of exposure and dosages.

II. Materials and methods

In carrying out this study, 30 Albino rat dams weighing between 200-250g were used. The 30 dams were grouped into a control group(n=3) and experimental groups (n=27) rats which were further grouped into 3 different trimesters i.e. trimester 1, 2 and 3 groups each having 9 rats. The rats in each trimester 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 1st day of pregnancy upon which treatment was began for rats in the 1st trimester, whereas for those in the 2nd trimester treatment began on day 8 and those in the 3rd trimester treatment began on day 15 of pregnancy. All animals were sacrificed on day 20 of gestation using concentrated CO_2 and fetuses harvested fixed in 10% formalin for 24hrs and thereafter underwent tissue processing for paraffin embedding and later hematoxylin and eosin staining. Representative sections were obtained at a section thickness of 3µm using systematic uniform random sampling technique and viewed under the Labomed light microscope mounted with the IVU 3100 camera at a total magnification of x40. Qualitative description of the different histological layers of the epiphyseal growth plate at the level of the anterior cruciate ligament and the groove of Ranvier was made as well as that of the primary spongiosa.

III. Results

Low Dose Tetracycline Group

The resting cartilage (RC) chondroblasts showed a comparable distribution pattern as compared to control group rats. 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 those in 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. 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. These findings were noted across the trimesters (trimester 1,2 and 3) as shown in figure 1below.

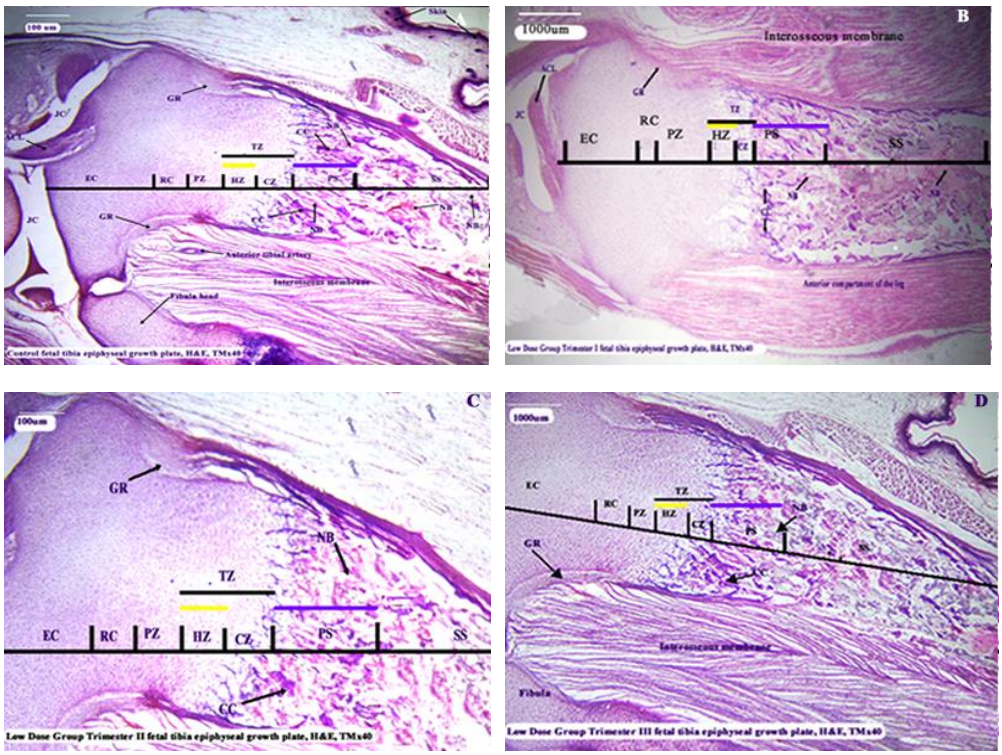

Figure 1: comparative distribution of core cartilage and new bone in the PS in the Low Dose Tetracycline Groups across trimesters (TM_1, B:TM_2, C: TM_3, D) when compared to control group.

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 2, 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 2, 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 tissue 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 2, photomicrograph B).

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 2, 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 2, 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 2, 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 2, photomicrograph B).

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 2, 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 2, 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 2, 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 2, photomicrograph D).

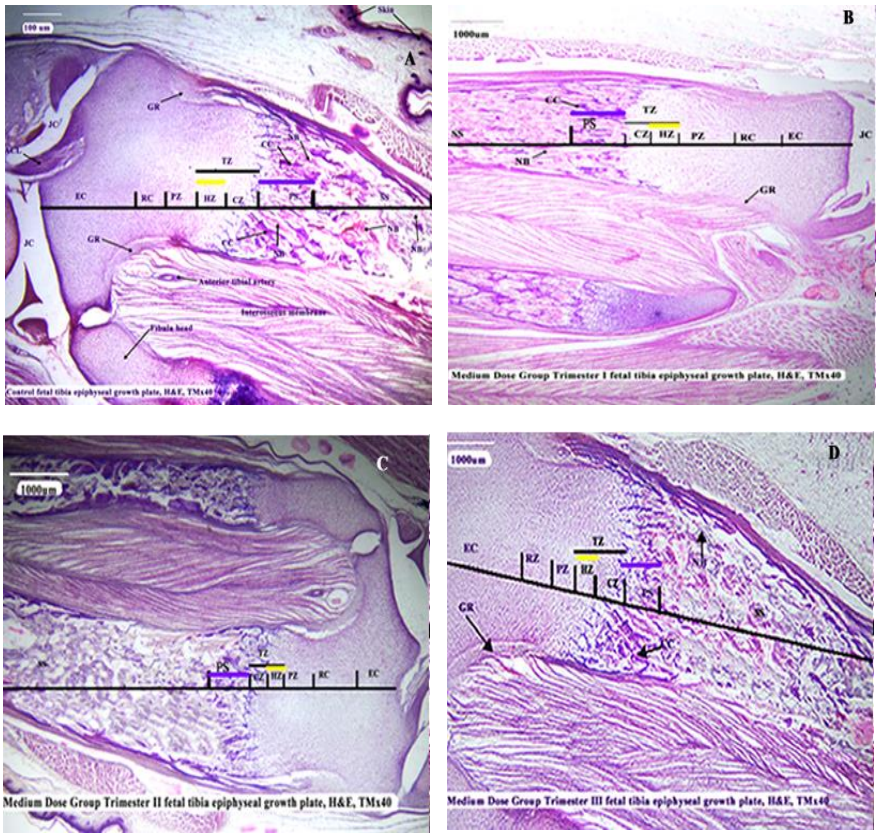

Figure 2: comparative increase in the Hypertrophic zone: Primary Spongiosa proportionate thickness as well as an increase in the distribution of core cartilage with a reduction in new bone in the PS in the Medium Dose Tetracycline Groups across trimesters (TM_1, B:TM_2, C: TM_3, D) as compared to control group.

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 3, 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 3, 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 3, photomicrograph B).

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 3, 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 3, 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 3, 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 3, photomicrograph C).

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 3, 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 3, 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 3, 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 3, photomicrograph D).

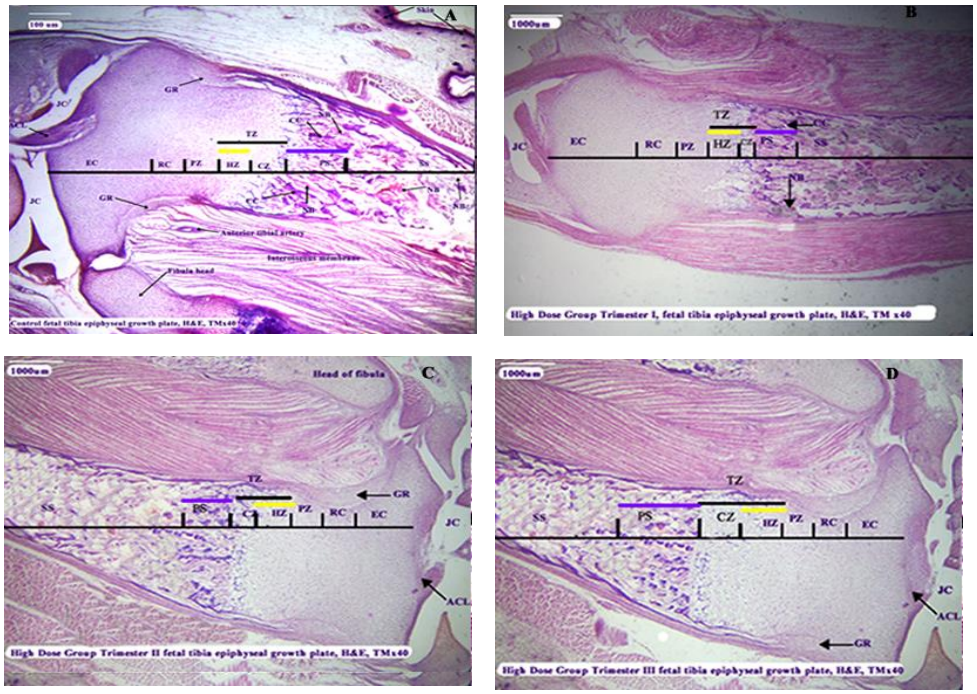

Figure 3: shows a relative increase in the Hypertrophic zone: Primary Spongiosa proportionate thickness as well as an increase in the distribution of core cartilage with a reduction in new bone in the PS in the Medium Dose Tetracycline Groups across trimesters (TM_1, B:TM_2, C: TM_3, D) as compared to control group.

IV. Discussion

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. 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 (Duivenvoorden 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) (Duivenvoorden et al., 2007).

V. 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.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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