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Research Article

The Effects of Intrauterine Tetracycline Exposure on the Proliferating Chondrocytes of the Growth Plate of Mice Long Bones

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Abstract

The proliferating chondrocyte of the growth plate (GP) contributes to the longitudinal growth of long bones. Tetracycline is a broad spectrum over-the-counter antibiotic, which reduces fetal bone growth. This study is meant to investigate the precise effect of intrauterine 12 mg/kg body weight (mbw) of tetracycline on the number, size and arrangement of proliferating chondrocytes on mice longitudinal bone growth. 12 mbw of tetracycline dissolved in normal saline was orally administered to 5 pregnant mice daily between the 11th and 15th day of gestation. Appropriate volume of normal saline was administered to 5 control pregnant mice. Fetuses were harvested on the 20th day of gestation by caesarian section. The humeri and femora were fixed in 10% formal saline, and histological slides were made using Toluidine blue as the stain. Dimensions and cellular population of the proliferating zones were noted using an ocular micrometer. Evidences of growth retardation were not noted in this study. The experimental humeral length was significantly shorter than the control. The proliferating zone cellular density in the distal GP of the experimental humerus was significantly higher than the control. There was a disruption of the columnar arrangement of the proliferating chondrocytes in the experimental humeral GP. Tetracycline (12mbw) does not have any significant effect on mice somatic growth. The disruption of the columnar arrangement of the proliferating chondrocytes in the humeri proximal GP contributed to the limb shortening effect. The sparing of the femur is in keeping with the differences in the timing of limb development.

Keywords; Tetracycline, Growth plate, Proliferating chondrocyte, Long bones, Teratology

INTRODUCTION

Tetracycline is an antibiotic that is available over the counter illegally in most developing countries of the world. It is therefore readily available for use by both pregnant and non-pregnant women. Investigation of its teratologic effects is therefore worthwhile. This is particularly important because of the tendencies of pregnant women to use over the counter drugs. Diav-Citrin and Gideon Karen, (2000) found that about 45% of pregnant women in the United States of America and 10% of British pregnant women use one drug or the other during the period of pregnancy following proper prescription. They opined that a higher percentage of pregnant women will be exposed to unprescribed drug. The data from developing and under-developed nations of the world is expected to be more alarming.

The determination of the teratologic effect of various substances such as radiations, drugs and environmental contaminants is a continuous research focus (Mc-Cormack, 1983). The intensity of research focus on these substances is premised on the fact that the malformations secondary to such teratogen are usually preventable. The embryonic or organogenesis period of pregnancy remains the most critical period due to the high degree of sensitivity of the developing embryo to various substances. During this period the commitment of cell lines is irreversible and their subsequent differentiation is rapid. This period has been found to correlate with between days 8 and 14 post-conception in rats (Skosyreva, 1989, Diav-Citrin and Karen, 2000).

Chondrocyte proliferation and differentiation are closely controlled processes of endochondral ossification. A reduction in the length of long bones is attributed to the attenuation of heparan sulphate which is known to regulate bone formation (Jochmann *et al.,* 2014). Filamin proteins have been implicated in short stature secondary to their effects on the proliferating chondrocytes which results in reduced production of chondrocytes and bone length (Hu *et al.,* 2014). Intracellular cell stress in the resting and proliferating chondrocyte resulted in reduction of bone length without an alteration of the histoarchitecture of the growth plate (Gualeni *et al.,* 2013). Chondrocyte proliferation and differentiation is stimulated by interleukin-10 via the bone morphogenetic protein pathway (Jung *et al.,* 2013), while high concentration of melatonin is deleterious to the processes (Zhong *et al.,* 2013). The commencement and rate of hypertrophic chondrocyte differentiation is accelerated by fibroblast growth factor (FGF) thereby resulting in the reduction in the proliferating portion of the growth plate chondrocytes (Shi *et al.,* 2012). FGF and bone morphogenetic protein (BMP) signaling effects on the regulation of the chondrocyte proliferation and hypertrophic

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differentiation are antagonistic to one another (Minina *et al.*, 2002).

High doses of tetracycline results in the degeneration of proliferating and hypertrophic chondrocytes of the growth plate of juvenile rats with impairment of the calcification processes (Levy *et. al.*, 1980). A long-term silicon deprivation resulted in a reduction in the thickness of the growth plate and an apparent increase in chondrocyte density in the tibia of rats (Jugdaohsingh *et. al.*, 2008). Reduction in the height of the growth plate, particularly the proliferating zone, disorganization of the columnar arrangement of the growth plate chondrocytes and disruption of the shapes of chondrocytes following iodoacetate-induced osteoarthritis were largely reversed by oral doxycycline administration in rats (Cylwik *et al.*, 2004). Though chondrocyte proliferation deceleration and initiation of hypertrophic differentiation are age related in rats, both processes were prevented by bone morphogenetic protein-5 (Bailon-Plaza *et. al.*, 1999). Direct electrical stimulation of the epiphyseal plate resulted in an increase in the height of the growth plate as well as its turn-over rate (Genbun, 1991). A positive linear relationship independent of the location of the growth plate and the age of the animal exists between the rate of longitudinal bone growth and the volume of the hypertrophic chondrocyte of pigs (Breur *et. al.*, 1991).

The teratogenic effects of tetracycline have been widely reported (Balcar-Boron *et.al.*, 1980; Skosyreva 1989; Diav-Citrin and Karen, 2000; Scheinfield and Davis 2004). The effect of tetracycline during embryonic development is most pronounced on gastrulation (Gabrovska and Ruser, 1978). Its embryotoxic properties are evidenced by higher incidence of intrauterine death as well as congenital abnormalities in rat (Skosyreva, 1989). Diastrophic dwarfism has been attributed to the teratogenic effects of tetracycline (Balcar–Boron *et.al.*, 1980). Fetal exposure to tetracycline within the embryonic period has been associated with bone length reduction defects (Zhongjun *et.al.*, 2000). The use of tetracycline after the 20th week of gestation in human results in bone and dental staining in a third of cases (Scheinfield and Davis, 2004). The yellowish bone staining effect of intrauterine and early postnatal exposure to tetracycline is the most widely reported teratogenic effect of the drug. It is for this reason that it is presently contraindicated in pregnancy. However, the limb shortening effect of tetracycline, though reported, has not been fully investigated. For example, the exact mechanism by which it causes reduction in the length of developing bone is yet to be fully elucidated. An investigation into the relationship between such exposure and state of proliferating chondrocytes in the epiphyseal plates of the long bones is the focus of this study.

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MATERIALS AND METHODS

Animals

Ten female mice, 12 weeks old and five adult male mice were obtained from the central animal house, University of Ibadan. The female mice weighed between 19.0 and 25.6 g at the start of the experiment. The animals were fed with growers pellet and drinking water was available ad libitum. The growers pellet was obtained from Bendel Feeds (a reputable animal feed manufacturer in Nigeria). The feed and water trough were cleaned and refilled every day. The animals were allowed to acclimatize for a period of 14 days.

The animals were identified with ear notches. One group of 5 female mice was designated the experimental while the other group of 5 was designated the control group. The male mice were housed in a separate cage throughout the acclimatization period. The animals were subsequently mated and the presence of spermatozoa in the vaginal aspirate was used to confirm mating and the day taken as day 0 of intrauterine life.

Drug administration

A brand of tetracycline known as Tetravin capsule B.P 250 mg manufactured by Maxheal Pharmaceuticals India, NAFDAC Reg. No: 04-4075 was obtained. A dosage of 12 mg /kg body weight (which is similar to the dosage human beings are exposed to) was administered to each female animal in the experimental group. The drug was dissolved in normal saline and administered with an oral cannula mounted on insulin syringe. The mice in the control group received an equal volume of normal saline via the same route. The drug administration was between 10 and 14 day post conception which corresponds with period of skeletal system development in the mice

Fetal harvest

Animals were anaesthetized with the use of chloroform on day 20 and the fetuses were harvested by caesarian section. A total of 24 fetuses were harvested from the experimental group, while the control group produced 25 fetuses. The weight and litter size of the fetuses were recorded. The humeri and femora (left and right) were dissected from their muscular attachments and separated from the adjacent joints. They were fixed separately in 10% formal saline.

Histology and staining

The bones were dehydrated in graded alcohols, cleared in xylene and embedded in paraffin. Sections, 10 µm thick were obtained using a Leica 2450 rotary microtome. Sections were passed through xylene and decreasing concentrations of alcohol before being exposed to water. Sections were immersed in a container of Toluidine blue for 10 seconds. It was subsequently rinsed in distilled water and then allowed to drain before being transferred to the hot plate to dry. Slides were dipped in xylene and mounted in DPX and covered with cover slip.

Microscopic study

The lengths of the humeri and femora were measured on the microscope with X4 objective lens using a micrometer. The diameter and height of the proliferative zone of the growth plate were measured at the same magnification. With the use of X10 objective lens the number of cells in the proliferating zone was counted. The morphology of the cells and their orientation were noted. Statistical analysis was performed using paired student's t-test where appropriate at $p<0.05$. Data were presented as mean values ± standard deviations.

RESULTS

Table 1 shows that the weights gained by and the litter sizes of the experimental and control animals were similar. No area of fetal resorption was found in the uterine horns of the

experimental or control animals. The length of the fetal humeri was significantly shorter in the experimental group while in the femur the difference between the control and the experimental groups were not significant.

Table 2 shows the heights of the proliferating zone of both the proximal and distal growth plate of the humeri and femur. The difference between the experimental and control groups was not statistically significant. The number of proliferating cells in proximal and distal growth plates of the humerus and femur were not significantly different.

Table 3 shows the cellular density of proliferating zone of the proximal and distal growth plates of the humerus and femur. The mean value of distal growth plate of the humerus shows a significant increase in the cellular density of the experimental group. However, the difference in the other growth plates of the humerus and the femur were not statistically significant.

Plate 1 shows the proximal growth plate of the control and experimental humerus. The columnar arrangement of the chondrocytes in the proliferating zone was intact in the control group while it was disrupted in the experimental group.

Table 1; Weight gained, litter size and bone length.

	Control	Experimental
	Mean (SD)	**Mean (SD)**
Weight gained (g)	11.56 (1.59)	13.26 (1.91)
Litter size	5.00 (1.00)	4.80 (0.84)
Humeral length (mm)	3.39 (0.13)	3.05 (0.16)*
Femoral length (mm)	2.67 (0.24)	2.90 (0.23)

***Statistically significant difference at p<0.05**

Table 2; Proliferating zone height and chondrocyte population.

	Proliferating zone height (mm)		**Proliferating chondrocyte population**	
	Control Mean (SD)	Experimental Mean (SD)	Control Mean (SD)	Experimental Mean (SD)
Humeral Proximal	**0.18 (0.07)**	**0.12 (0.08)**	**324 (39.12)**	**212.5 (104.3)**
Humeral Distal	**0.09 (0.04)**	**0.08 (0.05)**	**128.2 (30.0)**	**102 (84.41)**
Femoral Proximal	**0.09 (0.04)**	**0.06 (0.19)**	**154.5 (73.41)**	**129.1 (73.0)**
Femoral Distal	**0.15 (0.22)**	**0.11 (0.06)**	**213.7 (64.65)**	**209.5 (81.25)**

Table 3; Cellular density of the proliferating zones in the different growth plates (/mm^2).

	Control	**Experimental**
	Mean (SD)	Mean (SD)
Humeral Proximal	1.27 (72.09)	1.38 (1.02)
Humeral Distal	27.15 (14.53)	94.13 (66.48)*
Femoral Proximal	77.21 (31.73)	62.05 (39.13)
Femoral Distal	44.52 (43.22)	66.03 (33.92)

***Statistically significant difference at p<0.05**

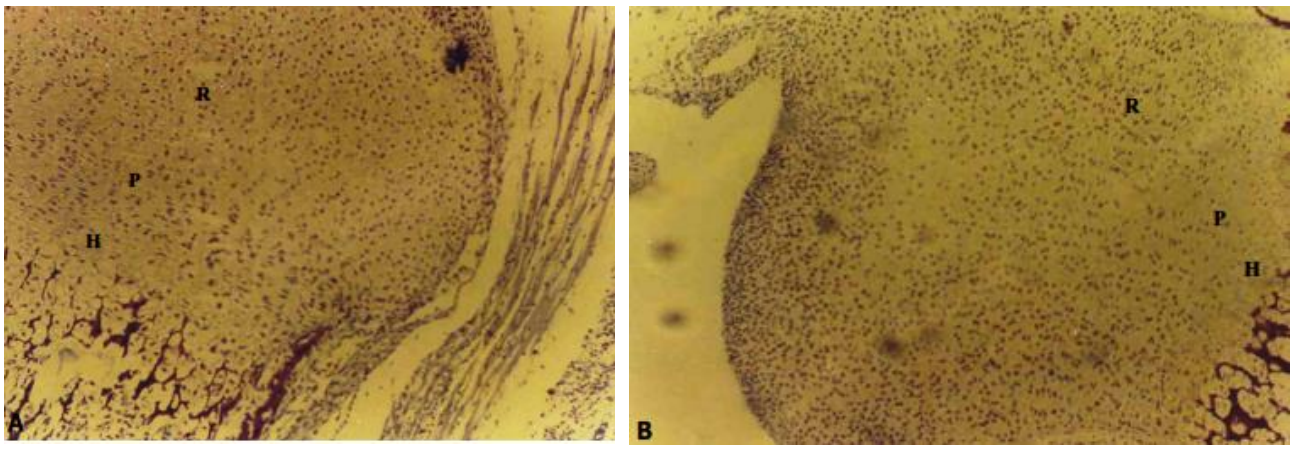

Plate 1;
Photomicrographs of the proximal humeral growth plate of control (A) and Experimental (B) mice. Normal arrangement of the resting (R), proliferating (P) and hypertrophic (H) chondrocytes with columnar arrangement of the proliferating chondrocyte was noted in A. B showed a disruption of the columnar arrangement of the proliferating chondrocytes. Stain; Toluidine Blue. Mag. X40

DISCUSSION

Exposure to tetracycline during intrauterine life at a dose of 12 mg/kg body weight, which corresponds with the usual dosage in humans, did not affect the litter size and weight of the fetuses. This suggests that at this dosage tetracycline does not have a significant somatic growth retarding effect in mice. Despite the limb shortening effect of tetracycline, fetal weight remains unaffected. Also, tetracycline does not have any direct effect on the fertility of the animals at the experimental dosage. This was corroborated by the fact that there is no significant difference between the litter sizes. In addition, the fact that there was no evidence of fetal loss suggests that at the experimental dose, tetracycline is not arbortifacient in mice. However, since the possibility of fetal death, resorption and general intrauterine growth retardation at higher doses of tetracycline was reported by Skosyreva *et.al.* (1989), the intensity of the teratologic effect of tetracycline may be dose dependent. This is a significant issue for developing countries where drug control is minimal.

The control humeri were significantly longer than the experimental humeri, confirming the earlier report of Chelebek *et.al*, (1997). They showed that tetracycline adversely affected the various factors that are responsible for longitudinal bone growth of the humerus. This study has shown that the normal columnar arrangement of the proliferating chondrocytes was markedly disrupted in the experimental group. Such disruption might explain the limb shortening effect of intrauterine tetracycline on the long bones of mice. However, the difference in the length of the femur was not significant. The proximo-distal nature of limb growth during intrauterine life might explain this observed difference in the response of the humerus and the femur. This finding further confirms the critical role of time of exposure in the effect of teratogens. In addition, a significant increase in the cellular density of the distal humeral growth plate was noted, confirming the summative role of the proximal and distal growth plates on the length of the mice long bone.

While the difference in the cellular density of the distal growth plate was significant, there was no significant difference in the cellular population of the distal growth plate between the control and experimental mice. This showed that, while intrauterine tetracycline exposure may not be cytotoxic, it has effects on the extracellular matrix of the distal growth plate resulting in its reduction to the extent that its cellular density was increased in the experimental mice. The non-cytotoxic effect of intrauterine tetracycline was confirmed by the non-significant differences the cellular population of the proliferating zones of the control and the experimental mice.

While this study reaffirmed the limb shortening and proximo-distal pattern of limb development of intrauterine tetracycline, the height and cellular population of the proliferating zone did not appear to contribute significantly to such effect. Though there was an increase in the cellular density of the distal growth plate of the humerus, an increase in the population of chondrocyte in the proliferating zone was not noted in this study.

The effects of intrauterine tetracycline on the extracellular materials of the growth plate as well as the other zones of the growth plate might shed more light on its limb shortening effect.

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