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ANTIBIOTICS INHIBIT *IN VITRO* BUT NOT *IN VIVO* CARTILAGE DEGRADATION

By

Tonia L Peters

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ABSTRACT

ANTIBIOTICS INHIBIT *IN VITRO* BUT NOT *IN VIVO* CARTILAGE DEGRADATION

By

Tonia L Peters

Recently, certain antibiotic families utilized in the poultry industry have been found to adversely affect bone formation and cartilage metabolism in dogs, rats, and humans. Therefore, the objectives of this study were to 1) determine if certain antibiotics inhibit *in vitro* cartilage degradation and 2) determine if antibiotics that inhibited *in vitro* cartilage degradation would induce tibial dyschondroplasia (TD) in growing broilers. Ten antibiotics were studied using an avian explant culture system that is designed to completely degrade embryonic tibia over 16 days. Lincomycin, tylosin tartrate, gentamicin, erythromycin, and neomycin sulfate did not inhibit cartilage degradation. Doxycycline (200 μg/ml), oxytetracycline (200 μg/ml), enrofloxacin (200 and 400 μg/ml), ceftiofur (400 μg/ml) and salinomycin (10 μg/ml) significantly decreased proteoglycan and nitric oxide concentrations (markers of cartilage breakdown) in conditioned media. These antibiotics plus chlortetracycline (known not to inhibit *in vitro* cartilage degradation) and thiram (known to induce TD) were then administered to day old broiler cockerels at 25, 100, and 400% of recommended dose levels. At 22 days of age the birds were killed and inspected for TD lesions in both proximal tibia. Incidence of TD did not differ between the antibiotic treatment groups and control birds. These data show that although some antibiotics inhibit *in vitro* cartilage degradation, their administration to growing broilers does not induce TD lesions.

This thesis is dedicated to husband, daughter, mom and dad. Without their love and support, I would not have been able to complete this degree.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CM – Conditioned media

CO_2 – Carbon Dioxide

ECM – Extracellular matrix

FGF – Fibroblast growth factor

IGF – Insulin-like growth factor

LPS - Lipopolysaccharide

MMP – Matrix metalloproteinases

NO – Nitric oxide

NOS – Nitric oxide synthase

PG – Proteoglycan

ppm – Parts per million

QAP – Quinolone arthropathy

TCCS – Tissue culture chick serum

TD – Tibial dyschondroplasia

TGFβ - Transforming growth factor-beta

INTRODUCTION

Tibial dyschondroplasia (TD) is one of the major metabolic skeletal disorders to affect the long bones of fast growing meat producing birds (broilers, turkeys, and ducks). A developmental abnormality of the tibiotarsal bone, TD originates in the growth plate where it disrupts the delicate balance between cartilage synthesis and degradation. Cells within a TD lesion fail to differentiate into mature hypertrophic chondrocytes and remain in a pre-hypertrophic state. Tibial dyschondroplasia affected birds accumulate an avascular, non-mineralized cartilage plug in the metaphyseal region of the tibia. Lameness, decreased feed efficiency, and increased mortality, culls, and condemnations at slaughter can result from TD. Producers see a peak in clinical TD cases at approximately half the market age of the bird, 3 weeks for broilers and 9 weeks for turkeys.

Understanding the etiology of such a debilitating metabolic disorder would benefit the poultry industry. Experimental conditions and compounds that induce TD include: copper deficiency; calcium:phosphorous imbalance, vitamin D deficiency; fusarochromanone, thiram, and antabuse toxicity; excessive dietary levels of cysteine, homocysteine and histidine; and metabolic acidosis. Genetics and environmental factors such as time of year and rearing conditions also induce the disease. Many experimental theories have been presented, but the pathogenicity of TD in commercial industry is still elusive.

Recently, some antibiotics in the tetracycline family have been shown to inhibit cartilage degradation of the embryonic chick tibiae. Other antibiotics implicated

in the disruption of bone metabolism include tobramycin, ceforanide, and quinolones. *In vitro* data on the effects of tetracyclines and other antibiotics, which interfere with bone metabolism, show that further investigation into the concentrations of growth promoting antibiotics used in the poultry industry is needed.

The use of antibiotics as growth promotants began at about the same point in time that TD prevalence increased. Modern broilers have an 18-63% incidence of TD. Although genetic changes were also prevalent over this time period, recent data support the hypothesis of the studies reported herein that certain antibiotics might induce TD lesions *in vivo*. Therefore, the objectives of this research were: 1) To determine if antibiotics commonly used in the poultry industry inhibit growth plate cartilage degradation *in vitro* and 2) To determine if the antibiotics that inhibited degradation *in vitro* would induce TD in the proximal tibial growth plate of broilers *in vivo*.

The following chapters of this thesis describe research projects that address these objectives. Chapter 1 provides background information on normal and TD growth plate cartilage physiology as well as mechanisms of antibiotics used in this research. Chapter 2 is the data, methodology and results of the embryonic chick tibiae project and demonstrates which antibiotics inhibited *in vitro* cartilage degradation. Chapter 3 describes the *in vivo* experiments using antibiotics from the previous chapter to determine if they induce TD lesions in growing broilers. In conclusion (Chapter 4), I discuss the effects of antibiotics on *in vitro* compared to *in vivo* cartilage degradation and their implications to the poultry industry.

Chapter 1

Literature Review

Introduction

Poultry skeletal disorders have been estimated to cause losses of approximately $160 million per year to the United States broiler and turkey industries (Sullivan, 1994). One of the major metabolic skeletal disorders that affects the long bones of fast growing meat producing birds (broiler chickens, turkeys, and ducks) is tibial dyschondroplasia (TD). A developmental abnormality of the tibiotarsal bone, TD originates in the growth plate where it disrupts the delicate balance between cartilage synthesis and degradation. Growth plates of birds affected with TD undergo cell proliferation at a normal rate whereas hypertrophic chondrocytes within these lesions only reach 40% for their normal size (Hargest *et al.*, 1985). Due to inhibited degradation, an avascular cartilage plug accumulates in the metaphyseal region of the tibia, which may caused impairment of long bone growth and clinical TD lesions. Lameness, decreased feed efficiency, and increased mortality, culls, and condemnations at slaughter can result from TD.

Modern broilers have an 18 to 63% incidence of TD (Praul *et al.*, 2000; Roberson, 1999; Elliot and Edwards, 1997). Knowing the etiology of such a debilitating metabolic disorder would benefit the poultry industry. Havenstein *et al.* (1994) reported that when broilers were fed a 1991 commercial diet, birds with 1991 genetics had a 48.6% incidence of TD, whereas birds with 1957 genetics had a 1.2% incidence of TD. However, genetics is not the only factor involved with TD. Other experimental conditions and compounds that induce TD include: copper deficiency;

fusarochromanone, thiram, and antabuse toxicity; excessive dietary levels of cysteine, homocysteine and histidine; calcium and phosphorus imbalance, and metabolic acidosis (Orth and Cook, 1994). Environmental factors such as time of year and rearing conditions also induce TD (Orth and Cook, 1994). Many hypotheses have been presented, but the cause of TD in the commercial industry is still not known.

Recently, some antibiotics in the tetracycline family have been shown to inhibit cartilage degradation in embryonic chick tibiae (Orth *et al.*, 1997). Other antibiotics implicated in the disruption of bone metabolism include tobramycin (Murakami *et al.*, 1996), ceforanide (Smith *et al.*, 1987), and quinolones (Vormann *et al.*, 1997). These and possibly other antibiotics routinely used in the industry may cause TD cases in the field. This hypothesis is circumstantially supported by the parallel increase in standard feeding of growth promoting antibiotics with the rise in commercial TD incidence. Further investigation may reveal these antibiotics to be the common link between many commercial TD incidences.

Avian Growth Plate

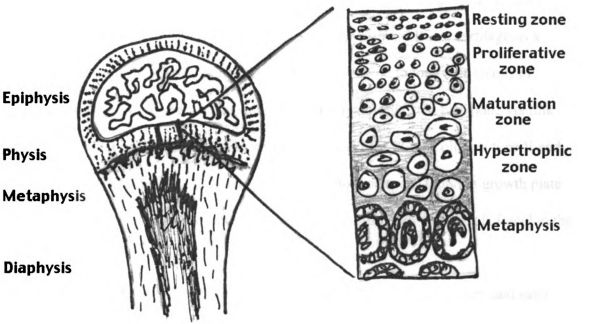
Endochondral ossification is the process by which cartilage is calcified and bone is formed. Growth plates, thin discs of cartilage located between the epiphyseal and metaphyseal regions of long bones, are responsible for the longitudinal growth rate and the ultimate length of bones. The primary center of ossification (growth plate) is responsible for longitudinal growth; the secondary center of ossification is responsible for latitudinal growth and is located in the epiphysis. In the chick tibia, the proximal end

has only a primary center of ossification whereas the distal end has both the primary and secondary center of ossification.

Two main zones exist within the growth plate, the proliferative zone, which is the proximal portion of the plate and the hypertrophic zone (Fig 1). Although chondrocytes, the cells of the growth plate, appear to move through the growth plate, they stay at a spatially fixed location as bone is formed and new chondrocytes are produced. Chondrocytes start out as flat cells in the proliferative zone. These cells are arranged in a column fashion and as they mature, they proliferate and produce extracellular matrix (ECM) proteins including proteoglycans and collagens. The chondrocytes are surrounded by ECM, which gives the growth plate its structural strength as well as its cushioning support. In the hypertrophic zone, the cells enlarge and become round as they differentiate. As they enlarge, hypertrophic chondrocytes synthesize proteinases that degrade the ECM surrounding them (pericellular matrix) (Brighton *et al.*, 1973). Columns are no longer apparent in the growth plate once chondrocytes reach the hypertrophic zone. Although these zones are defined by their metabolic function, the transitions that occur between them are gradual. The rates of synthesis and degradation must be in equilibrium if the growth plate is to properly elongate and ossify.

The avian growth plate is not as highly organized as the mammalian; avian cells are more numerous and are randomly oriented into longer disarrayed columns (Pines and Hurwitz, 1991). Approximately 200 cells per column (Howlett, 1979) are contained in a 4-7 week old chicken, in contrast to the rat growth plate that contains 25 cells (Kember, 1960). In comparison, Leghorn chickens have a more orderly transition than broilers

Figure 1. Diagram representing the location and organization of the primary growth plate (physis).



between the proliferative and hypertrophic zone and a more regular vascularization of hypertrophied cells (Reiland *et al.*, 1978).

Extracellular Matrix

The extracellular matrix undergoes synthesis, reorganization, and eventually degradation. The ECM is composed of 50% water, 25% proteoglycan (PG) complexes, and 25% collagen. The high concentration of water and PG content gives cartilage its gel-like appearance and cushion feature. Aggrecan, the primary PG in cartilage, is a macromolecule consisting of a protein core covalently linked to long side chains of glycosaminoglycans. Aggrecan molecules bind to a hyaluronic acid backbone via link proteins. A collagen network provides structural support for PGs. Collagen type II and smaller amounts of type VI, IX, X and XI are the major collagens found in growth plate cartilage. Orth (1999) published an extensive list of proteins and cell signals found in the growth plate.

Type II collagen is synthesized mainly by proliferative chondrocytes and early hypertrophic chondrocytes; it is the primary collagen in the growth plate that provides tensile strength. Covalently cross-linked to type II collagen is type IX collagen, which is thought to limit the collagen fibril diameter along with collagen type XI (Mendler *et al.*, 1989). Type IX collagen is distributed throughout the matrix (Muller-Glauser *et al.*, 1986) and potentially facilitates the interactions of glycosaminoglycan side chains to type II collagen fibrils (Van Der Rest and Garrone, 1991). A protein unique to the hypertrophic zone is type X collagen, which is 45% of the total collagen for that zone. This collagen is thought to be involved with mineralization or possibly facilitates matrix degradation near the chondro-osseous junction, but the exact function is unknown

(Schmid *et al.*, 1986). Type VI collagen is thought to stabilize the main collagen fibril network to the chondrocytes (Van Der Rest and Garrone, 1991).

The structural organization of the ECM changes throughout the proliferative and hypertrophic zones of the growth plate. As the chondrocytes mature, the matrix is reduced to thin longitudinal and transverse septae due to the enlargement of the chondrocytes in the lacunar spaces. Eggli *et al.* (1985) described the ECM as having three compartments: the pericellular, territorial, and interterritorial. The pericellular matrix compartment immediately surrounds the chondrocyte and is rich in PGs. A collagen fiber network surrounds the chondrocytes to compose the territorial compartment. The interterritorial compartment consists of parallel collagen fibers that connect the chondrocyte columns through the longitudinal matrix septa. The collagen content of the ECM increases as the columns of cells enter the hypertrophic zone. The degradation of the ECM by matrix metalloproteinases (MMP) facilitates growth plate remodeling, vascularization, and maturation into bone.

Proliferative Zone

The proliferative zone is characterized by disk shaped chondrocytes, mitotic cell division, and synthesis of ECM. Proteoglycan content is highest in the proliferative zone. Collagen fibrils are distributed randomly; collagen type II is the main collagen that appears in this zone as well as types IX and XI (Pines and Hurwitz, 1991). Active cell division occurs in the upper proliferative zone, which is the progenitor layer for longitudinal growth; chondrocytes are in a germinal stage having few mitochondria and appear dense due to a large amount of rough endoplasmic reticulum (RER) (Howlett,

1979). Maturing proliferative cells have ample glycogen stores and extensive RER to promote high rates of aerobic glycolysis and protein synthesis. The longitudinal columns of disk-like chondrocytes become disarranged as the cells begin to hypertrophy and become separated by ECM (Howlett, 1979). Golgi apparatus also become more numerous as the chondrocytes enter the hypertrophic zone.

Nutrients, oxygen, and growth factors are supplied to the proliferating chondrocytes by the epiphyseal arteries. Chondrocytes are under direct regulation by hormones such as vitamin D metabolites (Corvol *et al.*, 1977) and growth factors. Basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), transforming growth factor-β (TGFβ), and platelet-derived growth factor (PDGF) have been primarily associated with mitogenic activity in chondrocytes of the proliferative zone (Rosselot *et al.*, 1994).

Hypertrophic Zone

Proliferating chondrocytes eventually differentiate and hypertrophy. As chondrocytes enter the hypertrophic zone, the RER becomes irregularly shaped, the number and size of organelles in the cytoplasm decrease. Hypertrophic chondrocytes are metabolically active even though the area surrounding then becomes anaerobic and lacking in nutrients. The chondrocytes become spherical and increase 5-10 times the size of the cells in the proliferative zone. They synthesize extracellular proteins such as alkaline phosphatase, osteocalcin, osteopontin, and bone sialoprotein. Proteolytic enzymes such as collagenase are expressed which allow the breakdown of collagen facilitating cell expansion (by degrading the matrix) and vascularization. An important

autocrine regulator, TGFβ is a growth factor responsible for chondrocyte differentiation and maturation (Rosen *et al.*, 1986;Rosen *et al.*, 1988). However, terminal differentiation and matrix mineralization appears to be inhibited by TGFβ. It inhibits matrix vesicle phospholipase A_2 activity, an enzyme involved in calcification (Schwartz and Boyan, 1988; Schwartz *et al.*, 1993).

In the degenerative zone of the hypertrophic area, calcification of the matrix and vascularization occur. The aggregated structure of PGs and the irregular distribution of matrix vesicles assist in inhibiting mineralization in other zones of the growth plate. Once in the distal end of the hypertrophic zone the vesicles merge (Poole and Pidoux, 1989) and PGs disaggregate (Anderson, 1989) to initiate calcification. Rapid turnover of broiler chick growth plate cartilage allows for little calcification to occur. However, nutrients cannot penetrate the increasingly calcified matrix and cells closest to the chondro-osseous junction undergo apoptosis (Hatori *et al.*, 1995). Phagocytic cells originating from the metaphyseal blood supply remove apoptotic cells (Howlett, 1980). Capillary sprouts penetrate into the lacunae of apoptotic cells at the last transverse septa of the chondrocyte columns to provide a vascular network. A portion of chondrocytes do not degenerate in avians due to metaphyseal vascular penetration (Howlett, 1979). Fibroblast growth factor is released from terminal hypertrophic chondrocytes and may act as an angiogenic signal for metaphyseal blood vessel vacularization (Twal *et al.*, 1994). Nutrients are supplied through vacularization to matrix vesicles as well as other components of cartilage to support normal function and mineralization (Nie *et al.*, 1995). Therefore, angiogenesis supports cartilage calcification and endochondral bone formation. The majority of matrix vesicles, containing calcium phosphate, are in the

lower hypertrophic zone due to their participation in mineralization. The first mineral crystals appear within matrix vesicles and spread outward between columns by accretion of calcium salts. The cartilage matrix degradation and calcification provides a framework for invading osteoblasts, which deposit bone on the calcified cartilage. This bone is resorbed and replaced by trabecular bone.

Tibial Dyschondroplasia

Tibial dyschondroplasia, a metabolic disorder of the growth plate in rapidly growing meat-type birds, disrupts the formation of endochondral bone by impairing the resorption of cartilage. An avascular, uncalcified cartilaginous plug accumulates in the proximal metaphyseal region of the tibial growth plate. The rapid growth rate of broilers may contribute to a disarrangement of chondrocytes during bone formation, supporting the formation of the plug and therefore predisposes these birds to TD. In severe lesions, the metaphyseal region may weaken resulting in bowing of the long bone and lameness.

The growth plate of TD birds consists of similar components and concentrations as those of a normal bird. However, TD chondrocytes tend to aggregate and have a condensed morphology in comparison to normal chondrocytes (Rath *et al.*, 1997). Dyschondroplastic growth plates also have a decreased level of S and K in the ECM of the upper hypertrophic zone (Hargest *et al.*, 1985). Collagen type II expression (normally a characteristic of proliferative chondrocytes) in the hypertrophic zone of TD growth plates suggests abnormal cell proliferation (Pines *et al.*, 1998). Other researchers have shown that chondrocyte differentiation and hypertrophy are interrupted before maturation is complete (Bashey *et al.*, 1989). Collagenase-gelatinase activity is

significantly decreased in TD cartilage compared to normal cartilage (Rath *et al.*, 1997). Reduced MMP activity may decrease ECM turnover, inhibit vascularization, and lead to the retention of avascular cartilage. Chondrocytes within TD lesions only reach 40% of normal hypertrophic chondrocyte size (Hargest *et al.*, 1985), and undergo premature necrotic changes. These areas within the transitional zone of the growth plate contain large numbers of apoptotic chondrocytes; a decrease in DNA content and an increase in DNA fragmentation indicate cell death in this region (Rath *et al.*, 1997). Maturation of the chondrocytes is therefore inhibited. The inability of phagocytic cells to enter cartilage and remove apoptotic cells may contribute to the accumulation of an avascular plug, leading to the arrest of endochondral bone formation and the pathogenesis of TD. Matrix vesicles, which are responsible for mineral formation in endochondral ossification, are decreased in TD lesions and only vesicles on the perimeter of the lesion mineralize. They primarily lack a functional nucleational core and provide insufficient mineral ions (calcium and phosphorus specifically) to form normal matrix vesicles (Nie *et al.*, 1995).

Levels of proteins associated with bone formation are below normal in the TD growth plate. Type X collagen, calmodulin, alkaline phosphatase, and basic Fibroblast Growth Factor (bFGF) are decreased, suggesting mineralization and vascularization may be impaired (Twal *et al.*, 1996; Bashey *et al.*, 1989). Skeletal tissue collagenase activity can be increased by FGF (Ries and Petrides, 1995). Varghese *et al.* (1995) reported that FGF facilitates angiogenesis. Therefore, a decreased level may not provide a signal for invading capillaries. The exact roles of Type X collagen and alkaline phosphatase are unknown but their presence precedes calcification and proposed roles include stimulation

of angiogenesis (Kwan *et al.*, 1997) and targeting cells for osteoclast resorption (Linsenmeyer *et al.*, 1991). Kwan (1997) also suggested that Type X collagen synthesis was not reduced but a defect in incorporation into the matrix occurred resulting in decreased levels. However, Wardale and Duance (1996) reported that TD cartilage had reduced levels of Type II and Type XI collagen but had increased levels of Type X collagen. They hypothesized that since the chondrocytes were in an arrested state of hypertrophy they would produce the proteins associated with that stage of maturation.

Accumulated cartilage in the metaphysis has increased collagen and non-reducible collagen cross-link concentrations (Orth *et al.*, 1991). Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links assist in the stabilization of the collagen fibril network as well as increase the hydrophobicity of collagen (Eyre *et al.*, 1984). Increased cross-linking makes the collagen more resistant to enzymatic breakdown (Vater *et al.*, 1979). The distal area cartilage of the TD lesion contains over a 10-fold increase in HP concentration (Orth *et al.*, 1991). These abnormalities are likely involved in the increase of time for cartilage resorption from less than 24 hours to weeks (Thorp, 1988). Increasing the time for cartilage resorption will alter the balance of synthesis and degradation of growth plate cartilage, which facilitates impaired bone growth.

The accumulated cartilage can be resorbed and replaced by bone as the growth rate of the bird slows. Rath *et al.* (1997), reported that TD affected cartilage has healthy as well as apoptotic areas, which may allow for the replacement of TD tissue as the bird ages. However, in fast growing birds such as broilers and turkeys, the rate of growth does not slow enough for the cartilage to be resorbed properly. It is during the first half

of growth that the greatest changes in proportional growth occur (Marks, 1979). Therefore producers see a peak in clinical TD cases at approximately half the market age of the bird (broiler 3 weeks, turkey 9 weeks), even though the birds have only achieved 40% or less of their final body weight (Lilburn, 1994). Broiler tibia and femur diaphyseal ash data indicate that the first 7 days of growth are the most critical to overall skeletal development; this data may provide a physiological window in which to manipulate TD incidence and severity (Lilburn *et al.*, 1989).

The etiology of TD is not well known in the poultry industry although many factors and experimental conditions can induce dyschondroplastic lesions including: copper deficiency; fusarachromanone, thiram and antabuse toxicity; cysteine, homocysteine deficiency; metabolic acidosis; calcium and phosphorous imbalance; vitamin D deficiency; genetic selection and environment. One study showed that *in vitro*, lesion chondrocytes have the ability to terminally differentiate and mineralize suggesting that ECM interaction, vascularization or other regulatory factors are contributing to the etiology of TD *in vivo* (Farquharson *et al.*, 1995). Recently, Kestin *et al (*1999) found that Ross (208 and 308 lines) and Shaver broilers had an increased incidence of TD compared to the Cobb 500 broiler line. How these experimental models relate to commercial TD incidences is unclear. They also may induce TD lesions by different mechanisms. Thiram, a thiocarbamate fungicide, was shown to induce TD at nonlethal dietary concentrations (Edwards, 1985; Wu *et al.*, 1993). *In vitro*, chondrocytes exposed to less than 5 μM of thiram by 72 h of culture showed a decreased level of cellular alkaline phosphates, acid phosphatase, and LDH activity (Rath *et al.*, 1995). This effect may be seen since thiram has the ability to create a "leaky" cell

membrane. Thiocarbamates are metabolized to carbon disulfide *in vivo* (World Health Organization, 1979), which may alter cell-membrane proteins (DeCaprio *et al.*, 1992). Thiram is also highly lipophilic which would decrease its clearance rate and allow for retention in adipose tissue and synovial spaces, which may assist thiram's action on developing cartilage. Rath *et al.* (1995) hypothesized that these properties may allow thiram to exert cytotoxic effects and modify cell membranes to induce TD.

Tibial dyschondroplasia is widespread across the United States as well as worldwide in fast growing poultry (Hemsley, 1970; Laursen-Jones, 1970; Riddell *et al.*, 1971; Itakura and Gato, 1973). A common link must exist in order to have such wide spread occurrence. One such common management practice within each species is the addition of growth promotants to diets to improve performance and to create an economic advantage. Antimicrobial agents, which include antibiotics, are the most widely used growth promotants.

Antibiotics and Bone Metabolism

Antibiotics are derived from cultures of microorganisms or produced synthetically. They interfere with the development of bacteria by the following general mechanisms: inhibiting bacterial cell wall synthesis, microbial DNA translation and transcription, or essential metabolite synthesis, and altering membrane permeability. Antibiotic feed additives given at sub-therapeutic levels alter the gut microflora in a way that ultimately enhances nutrient uptake by the animal. This alteration in natural flora concentration suppresses bacteria that cause mild infections, reducing immune stress and freeing energy for growth. Microfloral production of vitamins and other nutrients is

increased and nutrient utilization by the bacteria is reduced. The primary benefit from antimicrobial supplements is an improved feed conversion. Dr. Tomas Jukes discovered in 1949 that chlortetracycline, fed at low levels to chicks or pigs, improved growth and feed efficiency (Swick, 1996). Other benefits include reduced mortality, resistance to disease challenge, and improved pigmentation and litter quality. Depending on grow-out conditions, savings from an improved feed conversion ratio range from 2 to 12 fold on product return (Swick, 1996).

Antibiotics have minimal effects in a new, clean house due to an absence of microbial challenge to the animal. This response depends on animal management, cleaning procedures and downtime between flocks, age of housing facility, and feed quality. Therefore, growth promotant levels of antibiotics are used primarily in houses with less than sanitary conditions.

The poultry industry uses antibiotics worldwide for growth promotion as well as for disease treatment. Broilers and turkeys are exposed to sub-therapeutic levels of growth promoting antibiotics throughout most of the grow-out period. The potential effects of chronic exposure to sub-therapeutic doses are not known on skeletal development. However, a single therapeutic dose of certain antibiotics during a critical postnatal growth period have been documented to cause deleterious effects on bone formation in humans, rats, and dogs (Gale *et al.*, 1981;Papick, 1998;Vormann *et al.*, 1997;Walker, 1992). Growth plate chondrocytes may be affected in the same manner since they are rapidly turning over and are the foundation for bone growth. According to the 1996 Feed Additive Compendium, the following antibiotics are approved as growth promotants for poultry in the United States: Zinc bacitracin, bacitracin methylene

disalicylate, bambermycins, chlortetracycline, lincomycin, oxytetracycline, penicillin, tylosin, tiamulin, and virginiamycin.

Recently, some antibiotics in the tetracycline family have been shown to inhibit cartilage degradation of the embryonic chick tibiae. Other antibiotics implicated in the disruption of bone metabolism include tobramycin, ceforanide, and quinolones. *In vitro* data on the effects of tetracyclines and other antibiotics, which interfere with bone metabolism, show that further investigation into the use of growth promoting antibiotics in the poultry industry is needed.

Tetracyclines

Tetracyclines are broad spectrum antibiotics that have the ability to inhibit protein synthesis as well as chelate cations. Tetracycline derivatives (tetracycline, doxycycline, oxytetracycline, minocycline, and chlortetracycline) were shown to have an anti-metalloproteinase property that is independent of their antimicrobial property (Golub *et al.*, 1984). The minimum requirement for anti-metalloproteinase activity by the tetracyclines is the 4-ring structure and the oxygen molecules located on the B and C rings, at carbons-11 and 12. These sites are responsible for the primary site of cation binding at physiological pH. Whereas, the removal of the dimethylamino group from the carbon-4 position of the A ring results in loss of only the antimicrobial activity.

We know from several sources that the tetracycline family affects the metabolism of bone growth (Golub et al., 1991; Cole et al., 1994; Orth et al., 1997). In humans, tetracyclines are not recommended for children or pregnant women, especially doxycycline, minocycline and tetracycline due to their lipophilic nature (high rate of

diffusion into tissue). The tetracycline family has been found to cross the placental barrier where they bind calcium, interfering with dental and bone growth in the fetus. This adverse effect of the tetracycline family as well as permanent discoloration of teeth in infants and children persists until 8 years of age, but many drug authorities suggest the avoidance of tetracyclines until 12 years of age.

In vitro, tetracyclines have been investigated for their effects on bone formation. At 20-40 μg/ml of doxycycline, MMP activity was reduced, PG loss was prevented, and cell death and deposition of type X collagen was decreased (Cole *et al.*, 1994). Tetracyclines shown to inhibit MMP activity can do so by chelating cations (Golub *et al.*, 1991). By binding Ca^{2+} and more specifically Zn^{2+}, tetracyclines inhibit the activity of collagenase and gelatinase, which require Ca^{2+} as a cofactor and Zn^{2+} at the active site. These MMPs degrade the collagen matrix and allow cartilage to be remodeled and vascularized. *In vitro* data have shown that 20 μg/ml minocycline, 40 μg/ml doxycycline, 80 μg/ml tetracycline, and 80 μg/ml oxytetracycline inhibit cartilage degradation, while chlortetracycline did not inhibit cartilage degradation at any concentration tested (Orth *et al.*, 1997). Tetracyclines easily diffuse into tissue due to their lipophilic nature. Orth *et al.* (1997) correlated the lipophilic nature of the antibiotics to the inhibitory property they possess; the more lipophilic the antibiotic, the more it inhibits cartilage degradation.

Tetracyclines inhibit matrix degradation in several ways: inhibiting MMPS, impairing vascularization, and decreasing protein synthesis. Doxycycline at low levels (5 μg/ml) decreases type X collagen synthesis as well as gelatinase and collagenase activity in hypertrophic chondrocytes (Davies *et al.*, 1996). Degradation of the ECM may release

matrix-bound activating factors of angiogenesis (Brown *et al.*, 1993; Fisher *et al.*, 1994; Hirshman and Dziewiatkowski, 1996). Suomalainen *et al.* (1992), found that doxycycline and minocycline inhibited tumor-induced angiogenesis in rabbit cornea, possibly due to the anticollagenase action of the tetracyclines. Therefore, inhibiting MMPs, which degrade the ECM, may impede penetration of new vessels. Furthermore, tetracyclines inhibit protein synthesis by penetrating intracellular spaces of cells and binding to ribosomes (Chopra, 1985).

Tetracyclines may interfere with the release of cytokines such as interleukin-1 and tumor necrosis factor (McNamara *et al.*, 1997), which are natural stimulants of cartilage degradation. Tetracyclines may also affect skeletal development by decreasing nitric oxide (NO) production through the inhibition of inducible nitric oxide synthase mRNA expression and protein synthesis (Amin *et al.*, 1996). Nitric oxide participates in matrix degradation by up-regulating MMP activity (Murrell *et al.*, 1995); when NO production is reduced, MMPs may not be activated. Doxycycline at 20-50 μg/ml inhibits collagenase and gelatinase activity, PG degradation, and NO production, as well as decrease cell death associated with PG release (Amin *et al.*, 1996; Cole *et al.*, 1994).

Fluoroquinolones

Fluoroquinolones have good tissue penetration ability and target a broad spectrum of gram-negative and gram-positive bacteria. Since they attain high concentrations in the urine, quinolones are used to treat urinary tract infections. Side effects in humans and animals include gastrointestinal disturbances and at high concentrations central nervous system adverse reactions. In humans, tendenitous and tendon ruptures may also occur.

In young, rapidly growing animals, quinolones can induce arthropathy (Gough *et al.*, 1992). Arthropathy is defined as a chondrocyte toxicity causing abnormal conditions within a joint and in severe cases lameness. The severity of symptoms increase with increasing dosages. Quinolones are not recommended for children, adolescents, or pregnant and nursing women due to this chondro-toxic effect.

The mechanism of action for quinolone-induced arthropathy may be related to their magnesium (Mg^{2+}) chelating property. Juvenile rats (3-6 weeks of age) deficient in Mg^{2+} were shown to have identical lesions as those induced by quinolone treatment (Vormann *et al.*, 1997). Vormann *et al* (1997) found reduced Mg^{2+} concentrations in hyaline cartilage of rats receiving a magnesium deficient diet between 3-5 weeks of age compared to the rats fed the same diet at 8-11 weeks of age, suggesting a lower Mg^{2+} turnover in aged rats. By inducing cartilage lesions only at 3-5 weeks postnatally, the Mg^{2+} deficiency correlates to the sensitive time for young rats to develop chondro-toxic effects from quinolone treatment. Ciprofloxacin is a quinolone used in the treatment of bone and joint infections. Further investigation into the use of this antibiotic was needed after animal studies showed chondro-toxic effects. Ciprofloxacin has been shown to decrease chondrocyte proliferation at 0.5 and 50 mg/l, which correspond to therapeutic and toxic serum levels respectively. There was no effect on PG synthesis due to ciprofloxacin. These data suggest that at increasing concentrations, ciprofloxacin affects newly differentiated cells by inhibiting DNA synthesis (Mont *et al.*, 1996).

Enrofloxacin is a more lipophilic quinolone than ciprofloxacin (Papick, 1998) but ciprofloxacin is slightly more active than enrofloxacin. Oral treatment (25 mg/kg Baytril®) of 15-28 week old growing puppies induced abnormal carriage of the carpal

joint and weakness in the hindquarters compared to no signs of joint limitation in puppies 29-34 weeks of age at the same dose level (Baytril® product information sheet, June 1997). According to the drug label, Baytril® is not recommended for small or medium breeds of dogs during their rapid growth period (2-8 months of age). Large and giant breeds have not been studied, but their growth phase can last until 12-18 months of age and therefore limited use of enrofloxacin should be considered.

Cephalosporins

There are four generations of synthetic cephalosporins. Each generation differs in their spectrum of activity against various gram-negative bacteria, in their susceptibility to beta-lactamases, and in their ability to overcome bacterial resistance when other drugs fail. Cephalosporins inhibit bacterial cell wall synthesis by nicking the peptidoglycan net of the cell wall. They readily cross the placental barrier and should not be administered to pregnant women. Rarely are side effects associated with these antibiotics.

Ceforanide, a long acting second-generation cephalosporin, was investigated with respect to its ability to inhibit cartilage degradation of bacterial-induced arthritis. Smith *et al.* (1987) found that glycosaminoglycan loss was inhibited only when the antibiotic was administered before induction of arthritis. When given one day after arthritis induction, decreased collagen loss occurred, but PG loss was only slowed. Proteoglycan tissue concentration was the same as the control by day 21 (Smith *et al.*, 1987). If ceforanide can inhibit collagen loss in articular cartilage, it may also inhibit collagen loss in growth plate cartilage, which would be detrimental to bone development.

Second-generation cephalosporins reach the synovial fluid. However, whether third or fourth-generation compounds reach the synovial fluid is not clear. Ceftiofur, or Naxcel®, is a third-generation cephalosporin. This antibiotic has a longer half-life than most third-generation cephalosporins and is therefore used once daily and administered less frequently. In the poultry industry, turkey poults may be given an injection of Naxcel® at day one to decrease the incidence of poult enteritis. Research regarding bone health has not been studied for this antibiotic. Anemia and thrombocytopenia have been reported in dogs that received 3-5 times the approved daily dose of 2.2 mg/kg (Papick, 1998).

Aminoglycosides

Aminoglycosides include gentamicin, streptomycin, tobramycin, and neomycin. These drugs inhibit protein synthesis and inaccurately translate mRNA at the ribosome. Streptomycin has a site-specific protein inhibition whereas the others in this group act at multiple sites to either inhibit synthesis or mistranslate the message. Side effects include nephrotoxicosis, ototoxicosis, and vestibulotoxicosis.

In vitro, tobramycin was found to be toxic when embryonic chick tibiae where exposed to concentrations higher than 0.5 mg/ml, and was shown to inhibit glucose metabolism and matrix synthesis in bone (Murakami *et al.*, 1996). Additionally, it lowered the pH of the bone microenvironment, which inhibits bone formation. Protein and collagen synthesis were inhibited and did not return to control values even after administration of the antibiotic was discontinued. Murakami *et al.* (1996) showed that

exposure to high concentrations of tobramycin has damaging, long lasting effects on osteoblast function.

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Chapter 2

The effect of antibiotics on *in vitro* cartilage degradation

Introduction

Endochondral ossification, which occurs in the epiphyseal growth plate, is the process by which long bones develop. Chondrocytes undergo a tightly regulated order of proliferation, differentiation and hypertrophy. Subsequent to chondrocyte growth, the extracellular matrix (ECM) is mineralized, vascularized, and finally invaded by osteogenic cells from the bone marrow. This delicate balance of synthesis and degradation (turnover) determines the ultimate health of long bones. Changes in the sequence may result in pathological skeletal disorders.

Tibial dyschondroplasia (TD), which may result in lameness, is characterized by an abnormality of the growth plate in which an avascular, uncalcified cartilage plug accumulates near the metaphysis of the proximal tibiotarsi. The lesion contains more matrix than normal cartilage (Thorp *et al.*, 1991) and has an increased collagen and non-reducible collagen cross-link concentration (Orth *et al.*, 1991). Proteoglycans (PG), a group of matrix proteins, interact with collagen to provide integrity and also regulate matrix cation concentration (Kuettner, 1992). Chondrocytes of TD cartilage have a decreased ability to synthesize matrix PG (Rosselot *et al.*, 1994).

Many factors induce TD, some examples utilized in experimental studies include nutrition imbalances, fungicides, and environmental conditions. The etiology of TD in commercial poultry production remains unknown. Recently, some antibiotics in the tetracycline family have been shown to inhibit cartilage degradation in an embryonic chick tibia explant culture system (Orth *et al.*, 1997). Other antibiotic families implicated

in the disruption of bone metabolism include aminoglycosides (Murakami *et al.*, 1996), cephalosporins (Smith *et al.*, 1987), and quinolones (Vormann *et al.*, 1997). Furthermore, the increase in the standard feeding of growth promoting antibiotics parallels with the rise in commercial TD incidence. Antibiotic families implicated in the disruption of bone formation include tetracyclines, fluoroquinolones, cephalosporins, and aminoglycosides. The poultry industry commonly utilizes antibiotics from these families to treat disease and promote growth. In the present study, we determined whether certain antibiotics inhibited *in vitro* cartilage degradation by quantifying indicators of cartilage catabolism in an embryonic chick tibiae culture system.

Materials and Methods

Dulbecco's modified Eagle's medium (DMEM): nutrient mixture F-12 (Ham) and the Penicillin/streptomycin additive were purchased from Gibco Laboratories (Grand Island, NY). Fibroblast growth factor and human-recombinant insulin-like growth factor were purchased from R&D Systems (Minneapolis, MN). Enrofloxacin (Baytril®) and ceftiofur (Naxcel®) were purchased from the Michigan State University Veterinary Teaching Hospital Pharmacy (East Lansing, MI). Salinomycin was purchased from ICN Biomedical Research Products (Costa Mesa, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Explant Cultures

Chick tibiae were isolated from 12-day-old leghorn embryos. The bone was isolated from the articular cartilage cap and muscle tissue (Cole *et al.*, 1992). At this stage of development, the tibiae contain two cartilaginous ends, a bony sheath, and bone

marrow (Fig 2). The cartilage ends contain a proliferative and hypertrophic zone similar to that of growth plate cartilage (Schmid and Linsenmeyer, 1985).

Each treatment consisted of either two or three wells in a 24-well culture plate (Becton Dickinson, Lincoln Park, NJ). Each of the wells contained three tibiae and 780 μL (260 ul/tibia) of Dulbecco's modified Eagle's medium (Table 1) supplemented with growth factors (fibroblast growth factor 100 ng/ml and insulin-like growth factor-I 100 pg/ml), ascorbate (50 μg/ml) and treated with varying concentrations of antibiotics (Table 2). Tissue culture chick serum (5%) and lipopolysaccharide (10 μg/ml) were added to stimulate cartilage catabolism. Treatments are listed in Table 2. Explants were maintained in a humidified incubator with 7% CO_2 at 37 °C. Medium was replaced every two days for 30 days or until the cartilage ends of the tibiae were degraded. All samples were stored at 4 °C until analyzed for proteoglycan and nitric oxide concentrations; these compounds have been found to be indicators of cartilage degradation in this explant system (Cole *et al.*, 1994; Orth *et al.*, 1999). Each experiment was replicated to validate results.

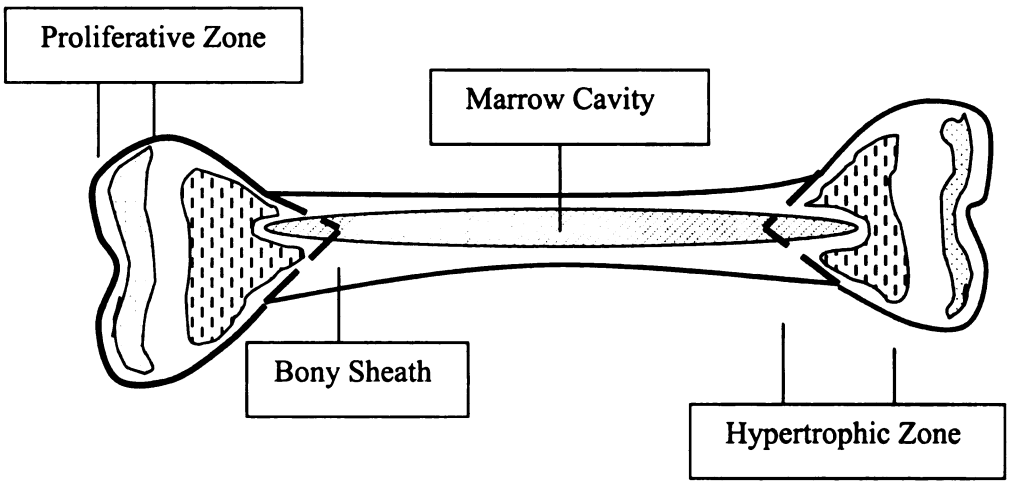


Figure 2. Twelve-day embryonic chick tibia containing bone marrow, a bony sheath, and two cartilage ends. The dotted areas depict the proliferative zone and dashed areas show the hypertrophic zone.

Table 1. Composition of medium used to induce cartilage degradation in the embryonic chick tibia explant culture system.*

Component	Concentration
DMEM:F12 (1:1)	.....
Sodium bicarbonate	44 mM
Lactalbumin hydrolysate	2 μg/ml
Sodium selenite	1 pg/ml
Manganese sulfate	169 ng/ml
Fibroblast growth factor	100 ng/ml
Insulin-like growth factor-I	100 pg/ml
LPS	10 μg/ml
TCCS	5%

* Amino acids were supplemented to enhance chondrocyte viability as outlined in Rosselot *et al.* (1992)
LPS=lipopolysaccharide; TCCS=tissue culture chick serum; DMEM= Dulbecco's modified Eagle's medium

Experiments

The effects on *in vitro* cartilage degradation of lincomycin hydrochloride (Sigma lot # 096H09775), tylosin tartrate (Sigma lot # 085H10166), gentamicin (Sigma lot # 028H2309), neomycin sulfate (Sigma lot # 075H09875), erythromycin (Sigma lot# 026H09865), doxycycline (Sigma lot# 59H0954), oxytetracycline (Sigma lot # 96H0571) enrofloxacin (Baytril® injectable), ceftiofur (Naxcel®), and salinomycin (ICN lot# 0050053) were determined. All antibiotics were tested at three concentrations, ranging from 50 to 400 μg/ml. However, salinomycin had a testing range of 100 ng/ml to 10 μg/ml. A control group (with or without penicillin/streptomycin solution depending on the time of year) was included in each experiment. Treatments are listed in Table 2.

In the months (April-July) that yeast contamination was expected, all treatment groups were supplemented with a penicillin (10 U/ml)/streptomycin (10 μg/ml) mixture. Yeast is a seasonal contaminant ubiquitous in the air, able to penetrate the filtration system of the biological hoods used in these experiments. Preliminary studies showed that 10 U/ml penicillin/ 10 μg/ml streptomycin mixture was an optimal concentration for this tissue culture system and prevented contamination. The addition of penicillin/streptomycin to the antibiotic treatment being tested did not affect PG or NO release from the explants. Therefore, an interaction was not found to occur in this system. These results agree with Murakami (1996), who also reported the addition of an antibiotic solution routinely added to medium such as penicillin/streptomycin does not interact with additional antibiotic treatment.

Table 2. Description of treatments for the tibia explant culture experiments.

Treatment Groups	Concentration Tested
Antibiotic-free Control*	.....
Doxycycline	50, 100, 200 μg/ml
Oxytetracycline	50, 100, 200 μg/ml
Lincomycin HCl	100,200,400 μg/ml
Tylosin tartrate	100, 200, 400 μg/ml
Gentamicin	100, 200, 400 μg/ml
Neomycin sulfate	100, 200, 400 μg/ml
Erythromycin	100, 200, 400 μg/ml
Enrofloxacin	100, 200, 400 μg/ml
Ceftiofur	100, 200, 400 μg/ml
Salinomycin	1.0, 10, μg/ml and 100 ng/ml

* A 10 U/ml Penicillin/ 10 μg/ml streptomycin mixture was used in treatments when yeast contamination was expected (April-July); HCl=hydrochloride

Analyses

Proteoglycan (PG) content of the conditioned media (CM) was determined using the dimethylmethylene blue assay described by Chandrasekhar (1987). Chondroitin sulfate (a proteoglycan) from bovine cartilage was used as the standard. To determine the PG content of the CM, the sulfated glycosaminoglycan content (μg PG/ well) was measured at absorbencies of 540 and 595 nm using a SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA).

Nitric oxide (NO) was measured in the CM using the method outlined by Blanco *et al.* (1995). Quantitative measurements of nitrite, a stable end-product of nitric oxide metabolism, were measured via the Greiss reaction at an absorbency of 540 nm using a SpectraMax 300 plate reader (Molecular Devices, Sunnyvale, CA). A standard curve of sodium nitrite was used and results are expressed as nmol NO/ well.

Statistical analysis

Data were analyzed using the repeated measures option of SAS (1999) PROC MIXED statistical software. The total amount of PG or NO released into the CM between days 6 and 16 of culture was analyzed to determine significance between treatment groups. The first two collection days were not analyzed to allow for equilibration of tibiae to treatments. Treatments were compared using the least squares mean difference procedure. Significance was considered at $p \leq 0.05$ and a trend was considered at $p \leq 0.10$.

Results

Antibiotics shown to inhibit *in vitro* cartilage degradation included doxycycline, oxytetracycline, enrofloxacin, ceftiofur and salinomycin (Table 3). Lincomycin, tylosin tartrate, gentamicin, neomycin sulfate, and erythromycin did not alter the cartilage's catabolic metabolism at any concentration tested (100, 200, or 400 μg/ml). In all treatment and control groups, complete cartilage degradation (visual examination) occurred by day 16 of culture.

Doxycycline at 200 μg/ml inhibited cartilage degradation (Fig 3); PG ($p \leq 0.01$) and NO ($p \leq 0.001$) release were decreased when compared to the control. Another tetracycline, oxytetracycline (200 μg/ml) also showed significant inhibition of degradation. Oxytetracycline inhibited release of PG into the CM ($p \leq 0.008$; Fig 3A) as compared to the control. Nitric oxide release into media was less than the control ($p \leq 0.002$; Fig 3B). The cartilage ends of the tetracycline-treated tibiae remained intact for the entire culture period whereas the control tibiae were degraded by day 14-16 of culture. The bones of the tetracycline treated groups were discolored (brown tinted) by day 8 of culture.

Enrofloxacin inhibited PG release in the CM at 200 ($p \leq 0.003$) and 400 μg/ml ($p \leq 0.004$) compared to the antibiotic-free treatment (Fig. 4A). At the low dose of enrofloxacin, 100 μg/ml, PG release was decreased ($p \leq 0.08$). Nitric oxide concentration in the CM at the highest and intermediate doses of enrofloxacin was lower than in the control ($p \leq 0.007$ and $p \leq 0.006$, respectively; Fig 4B). Once again, the lowest dose only decreased NO concentrations ($p \leq 0.06$). Cartilage ends in all three treatment groups

Table 3. Total release of proteoglycan or nitric oxide into conditioned media between days 6 and 16 of culture. The tables are broken into three groups in order to compare the treatments to the control group within its experiment. Observations without p-values were not different from controls.

Treatment Group (n=2)	Total [PG] μg PG/ well	Total [NO] μM NO/ well
Control	509 ± 62	118 ± 14
200 μg/ml Doxycycline	214 ± 26 ($p \leq 0.01$)	16 ± 3 ($p \leq 0.001$)
200 μg/ml Oxytetracycline	194 ± 21 ($p \leq 0.008$)	21 ± 3 ($p \leq 0.002$)

Treatment Group (n=2)	Total [PG] μg PG/ well	Total [NO] μM NO/ well
Control	711 ± 25	103 ± 5
100 μg/ml Enrofloxacin	480 ± 78 ($p \leq 0.08$)	42 ± 6 ($p \leq 0.06$)
200 μg/ml Enrofloxacin	340 ± 57 ($p \leq 0.003$)	17 ± 3 ($p \leq 0.006$)
400 μg/ml Enrofloxacin	169 ± 20 ($p \leq 0.004$)	22 ± 1 ($p \leq 0.007$)
100 μg/ml Ceftiofur	725 ± 45	141 ± 14
200 μg/ml Ceftiofur	511 ± 38	53 ± 9
400 μg/ml Ceftiofur	224 ± 20 ($p \leq 0.007$)	14 ± 3 ($p \leq 0.005$)

Treatment Group (n=3)	Total [PG] μg PG/ well	Total [NO] μM NO/ well
Control	755 ± 89	143 ± 13
100 ng/ml Salinomycin	319 ± 70	65 ± 17
1 μg/ml Salinomycin	456 ± 72 ($p \leq 0.06$)	70 ± 15
10 μg/ml Salinomycin	244 ± 11 ($p \leq 0.03$)	24 ± 3 ($p \leq 0.03$)

appeared intact after 30 days of culture, indicating inhibited cartilage degradation at all concentrations tested.

Only at 400 μg/ml did ceftiofur inhibit PG release ($p \leq 0.007$; Fig 5A) and NO production ($p \leq 0.005$; Fig 5B) when compared to the control. At the high dose, ceftiofur had intact cartilaginous ends at the end of the culture period (day 30). The cartilage ends of the intermediate dose remained intact through day 22 of culture, and the low dose was completely degraded by day 18 of culture.

The last antibiotic to inhibit cartilage degradation was salinomycin. This antibiotic was lethal to chondrocytes at concentrations $\geq$ 100 μg/ml (data not shown); therefore, lower concentrations were tested. An additional control was added to these experiments because salinomycin had to be dissolved in methanol. The methanol control group was not significantly different from the control for either CM analysis performed. Salinomycin at 10 μg/ml had the same inhibitory effect as the previous antibiotics tested; PG release was lower than the control ($p \leq 0.03$; Fig 6A) and NO production was decreased ($p \leq 0.03$; Fig 6B). At 1 μg/ml salinomycin, a trend for decreased PG release was seen ($p \leq 0.06$), but NO was not reduced. However, at 100 ng/ml, salinomycin appeared to increase the rate of cartilage degradation relative to the control but did not differ significantly in PG release or NO production.

Discussion

These results show that some of the antibacterial agents used in the poultry industry inhibit *in vitro* embryonic chick cartilage degradation. Antibiotics are used in the poultry industry to treat disease, increase disease resistance, reduce mortality,

increase feed efficiency, and promote growth in less than ideal environmental conditions. Although they are used as a management tool to increase the livability and economic value of the animal, certain antibiotics may add to the cull and condemnation rates associated with skeletal disorders.

Tetracyclines are broad-spectrum, rapidly absorbed antibiotics that have recently been found to have an anti-metalloproteinase property that is separate from its antibacterial property. The site for metal binding on the four-ring structure is located in the B and C rings, specifically the oxygen molecules at the 11 and 12-carbon-position (Fig. 7) (Golub *et al.*, 1991; Duarte *et al.*, 1999). The ability of tetracyclines to bind metal ions (Zn^{2+}, Ca^{2+} and Mg^{2+}) may be the primary mechanism by which tetracyclines inhibit cartilage degradation. Matrix metalloproteinases (MMP) are enzymes responsible for the degradation of ECM in many body organs; they require zinc at their active site and calcium as a cofactor. Tetracyclines have been reported to inhibit some of these enzymes including: skin collagenase (Golub *et al.*, 1983), cartilage, synovial, and corneal collagenase (Golub *et al.*, 1991; Greenwald *et al.*, 1992; Burns *et al.*, 1989) neutrophil collagenase (MMP-8) (Suomayor *et al.*, 1992; Smith *et al.*, 1996), gelatinase A (MMP-2) (Lu *et al.*, 1991) and gelatinase B (MMP-9) (Nip *et al.*, 1993). The derivative, concentration, and experimental procedure differed between the aforementioned studies. Smith *et al.* (1999) found that the sensitivity of the MMP to tetracyclines depends on the MMPs structure and whether it can be altered by the binding of tetracyclines to an enzyme-associated Ca^{2+}. Matrix metalloproteinases that have a hemopexin-like domain of MMP-13 or a catalytic domain of MMP-8 show significant inhibition against type II collagen when cultured with concentrations of doxycycline, whereas the MMP-1

structure is resistant to tetracycline reconfiguration (Smith *et al.*, 1999). Smith's results seems logical since in the present explant culture model calcium was present in excess; there would be enough calcium available for MMP activity even if the tetracyclines bound 100%. However, the tetracyclines could have bound zinc and rendered the MMP inactive.

The present study showed that two tetracyclines, oxytetracycline and doxycycline, were able to prevent cartilage degradation of embryonic chick tibiae. This result agrees with previous reports on the tetracycline family (Orth *et al.*, 1997; Cole *et al.*, 1994). Proteoglycan and nitric oxide were decreased in the tissue treated with tetracyclines when compared to the control. Doxycycline was more potent than oxytetracycline, which agrees with earlier studies showing that the more lipophilic the tetracycline, the greater the inhibition of cartilage degradation (Orth *et al.*, 1997).

Nitric oxide is a multifunctional mediator produced by nitric oxide synthases (NOS). Nitric oxide participates in the inflammatory and autoimmune mediated response of tissue degradation. Explant tibiae in the present study were stimulated with the endotoxin, LPS, to initiate cartilage degradation. When treated with tetracyclines, NO production was inhibited compared to the control. Attur *et al.* (1999) reported that doxycycline blocked NO in LPS-stimulated bovine cartilage as well as human osteoarthritis cartilage. Doxycycline and minocycline inhibit expression of inducible NOS (Amin *et al.*, 1996). Nitric oxide is thought to activate MMPs (Golub *et al.*, 1991; Orth *et al.*, 2000) and therefore, if down-regulated, cartilage degradation would decline due to decreased enzyme activity.

Proteoglycan release into media is one way of quantifying cartilage matrix degradation. In the current study, PG release was significantly inhibited by doxycycline and oxytetracycline. In many studies a reduced PG release correlated with inhibition of MMP activity. Cole *et al.* (1994) found that 40 μg/ml of doxycycline prevented PG loss from the matrix, cell death and deposition of type X collagen and increased the hypertrophic region of the cartilage.

Fluoroquinolones, which also chelate cations, have broad spectrum antibacterial properties (they inhibit DNA gyrase, which is responsible for DNA supercoiling) and excellent tissue penetration. Like the tetracyclines, quinolones are ringed structures. In young, rapidly growing animals it is known that these drugs cause arthropathy, a condition characterized by vesicles forming on the articular cartilage surface causing lameness in the affected animal (for a complete review see Gough *et al.*, 1992). Quinolone arthropathy (QAP) may not occur in adults because of the drugs inability affect mature tissue. An experimental trial on racing pigeons showed that 800 ppm enrofloxacin given over a long time period did not induce abnormalities in the adult birds, but increased embryo mortality in eggs of treated birds. Chicks raised from these birds had joint lesions such as arthropathy (Allen, 1998).

In the present study enrofloxacin was tested. It prevented PG loss and NO production in the embryonic chick explant model. Beluche *et al.* (1999) reported that high concentrations of enrofloxacin (>1000 μg/ml) inhibited synthesis and increased degradation of PG in equine articular cartilage explants whereas low doses (2 and 10 μg/ml) did not affect PG metabolism. These high doses of enrofloxacin also were

associated with chondrocyte toxicity. In our work, enrofloxacin did not cause cytotoxic effects (data not shown).

Enrofloxacin is partially metabolized to ciprofloxacin (Allen, 1998). Ciprofloxacin is slightly more active than enrofloxacin, but enrofloxacin is more lipophilic. Mont *et al.* (1996) showed that ciprofloxacin did not inhibit PG synthesis of human chondrocytes, but inhibited cell proliferation at therapeutic serum concentrations. Others have reported that quinolones inhibit glycosaminoglycan synthesis initially and DNA synthesis secondarily (Kato and Onodera, 1988a; Kato and Onodera, 1988b; Kato *et al.*, 1995; Takada *et al.*, 1994; Takayama *et al.*, 1995). One proposed mechanism is the quinolones ability to form chelates with magnesium. Magnesium is needed for cell proliferation and cell-matrix interactions. Juvenile rats have been shown to develop QAP (age $\leq$ 5 wks) whereas adults (age > 8 wks) do not develop lesions (Mayer, 1987; Kato and Onodera, 1988b). Voorman et al (1997) reported that rats less than 4 weeks of age have lower magnesium concentrations and only at this age can cartilage lesions be induced by feeding magnesium-deficient diets. How these drugs affect growth plate chondrocytes is not known

Cephalosporins are widely used in human as well as veterinary medicine. They are broad spectrum, fast acting drugs that inhibit bacterial cell wall synthesis and are known to reach synovial fluid. Ceftiofur is a cephalosporin with a relatively long half-life; it is used to treat most gram-negative urinary tract pathogens as well as systemic infections. To my knowledge, no studies have linked cephalosporins to cartilage abnormalities. However, Smith (1987) showed that a therapeutic dose of ceforanide (15 mg/kg), another member of the cephalosporin family, given before inoculation with

bacteria prevented PG loss in rabbit articular cartilage. In the present study only the high dose of the cephalosporin (400 μg/ml ceftiofur) inhibited PG loss and NO production. Cephalosporins are also a ring structure, but have not been reported to bind cations.

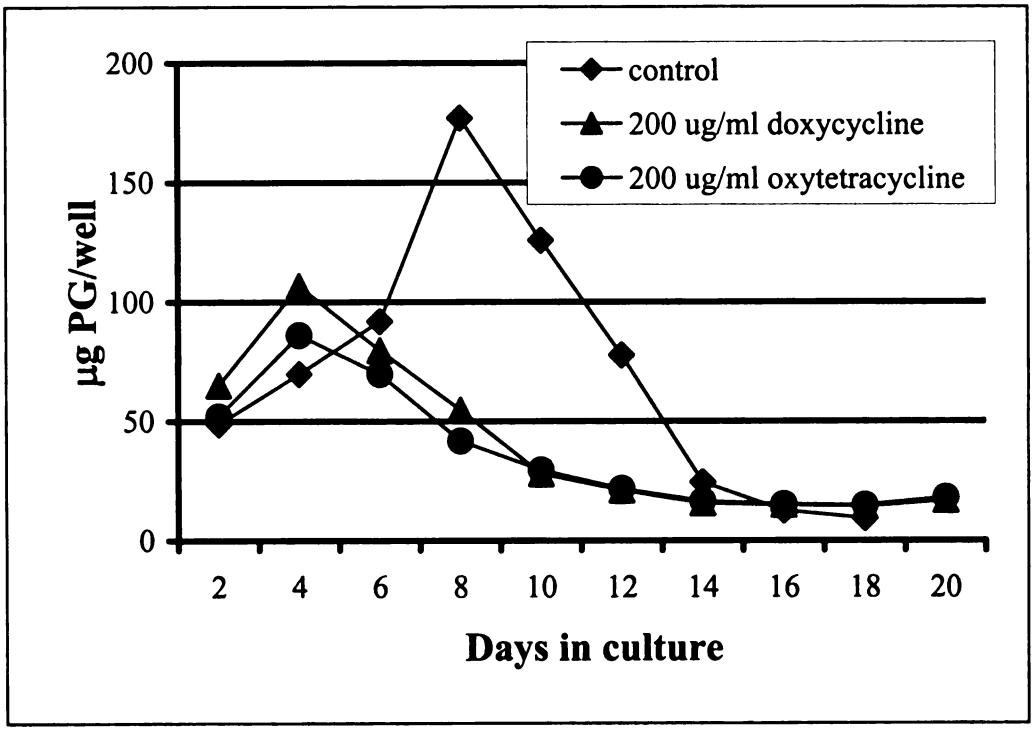
Salinomycin is a polyether antibiotic that has ionophoric properties and is known to transport monovalent cations, especially sodium and potassium, across biological membranes (Dobler, 1981), which may explain why it killed chondrocytes in the present explant model at concentrations over 100 μg/ml. This antibiotic increased the rate of PG release at 100 ng/ml yet inhibited PG loss at 10 μg/ml. Why this occurred in not understood. Possibly cell metabolism was increased due to the excess activity of sodium/potassium channels at the lowest dose, yet at the highest dose it interfered with MMP activity similar to other antibiotics tested. Salinomycin could also interfere with intracellular Ca^{2+} concentrations, leading to changes in chondrocyte metabolism. Ionophore use has been associated with increased leg problems (Leeson and Summers, 1988). However, monensin (another ionophore) fed at 121 ppm to broilers reduced mortality due to leg abnormalities (Chapman *et al.*, 1995).

These data confirm published results concerning tetracyclines, fluoroquinolones, cephalosporins and other categories of antibiotics regarding inhibition of cartilage degradation. For certain diseases such as osteoarthritis, diabetes and periodontal disorders the ability to stop cartilage degradation is utilized for treatment. However, growth plate cartilage develops into bone by tightly regulating cartilage turnover. If this is disrupted, skeletal disorders such as TD could arise. These antibiotics all inhibited PG release and NO production in an embryonic chick explant culture system. However, they may all act by different mechanisms and possibly affect development at critical growth

periods. The ability to chelate cations is a recurring theme that needs further investigation regarding these families of antibacterial agents. Future research should determine if these antibiotics inhibit *in vivo* growth plate cartilage degradation at doses relevant to the poultry industry.

Figure 3. (Next page) Proteoglycan and nitric oxide release into conditioned media from chick tibia explants with two tetracycline treatments (n=2). Panel **A** depicts the proteoglycan release into conditioned media throughout the 30-day culture period. Decreased proteoglycan concentrations were found for oxytetracycline (200 μg/ml) and doxytetracycline (200 μg/ml) between day 6 and 16 of culture compared to the control ($p= 0.008$, $p= 0.01$). Panel **B** depicts the nitric oxide production released into conditioned media throughout the 30-day culture period. Decreased nitric oxide concentrations were found for oxytetracycline (200 μg/ml) and doxycycline (200 μg/ml) between day 6 and 16 of culture compared to the control ($p= 0.002$; $p= 0.001$).

A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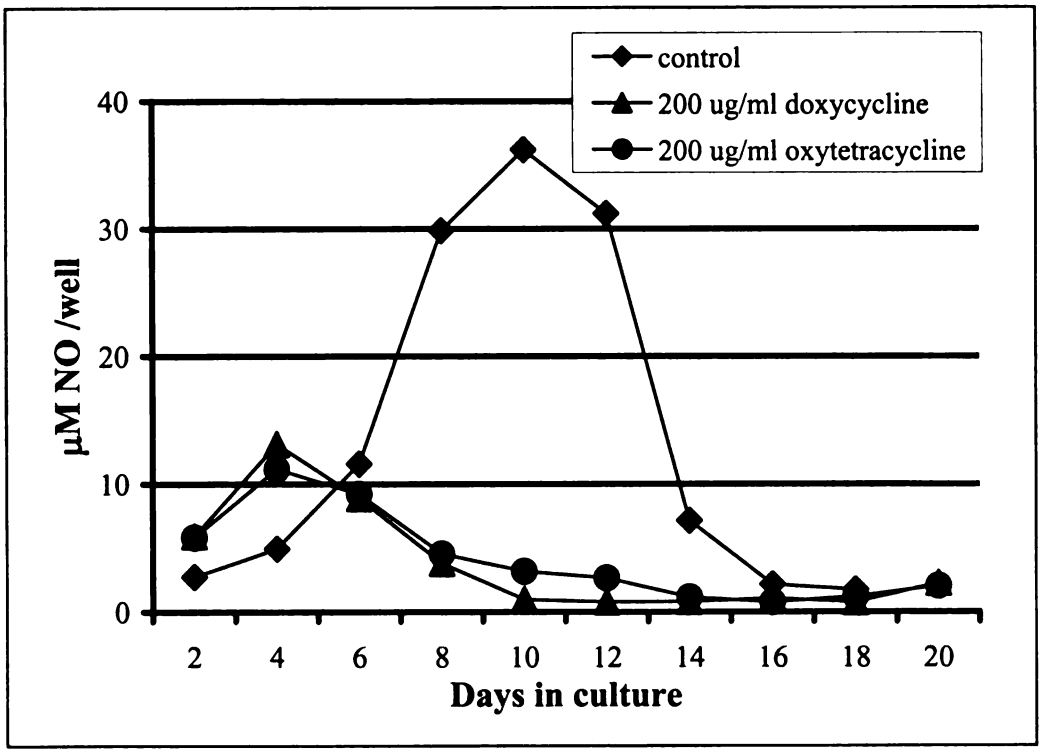
200 ug/ml oxytetracycline
µg PG/well
200
150
100
50
0
2
4
6
8
10
12
14
16
18
20
Days in culture

B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200 ug/ml oxytetracycline
µM NO /well
40
30
20
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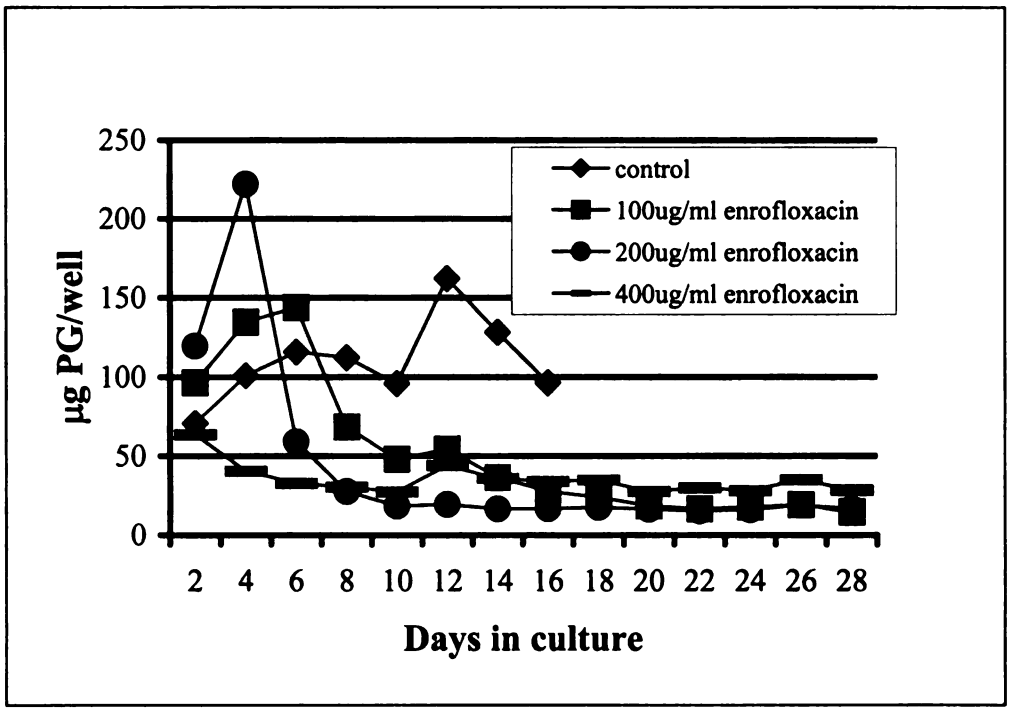
10
12
14
16
18
20
Days in culture

Figure 4. (Next page) Proteoglycan and nitric oxide release into conditioned media from chick tibia explants with enrofloxacin treatments (n=2). Panel **A** depicts the proteoglycan release into conditioned media throughout the 30-day culture period. Decreased proteoglycan release was found for enrofloxacin at the 200 μg/ml ($p= 0.003$) and 400 μg/ml ($p= 0.004$) concentrations between day 6 and 16 of culture compared to the control. A trend for decreased proteoglycan release existed for the 100 μg/ml enrofloxacin treatment ($p= 0.09$). Panel **B** depicts the nitric oxide production released into conditioned media throughout the 30-day culture period. Decreased nitric oxide concentrations were found for enrofloxacin at the 200 μg/ml ($p= 0.007$) and 400 μg/ml ($p= 0.006$) concentrations between day 6 and 16 of culture compared to the control. A trend for decreased nitric oxide production existed for the 100 μg/ml enrofloxacin treatment ($p= 0.06$).

A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200
150
100
50
0
µg PG/well
control
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200ug/ml enrofloxacin
400ug/ml enrofloxacin
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days in culture

B

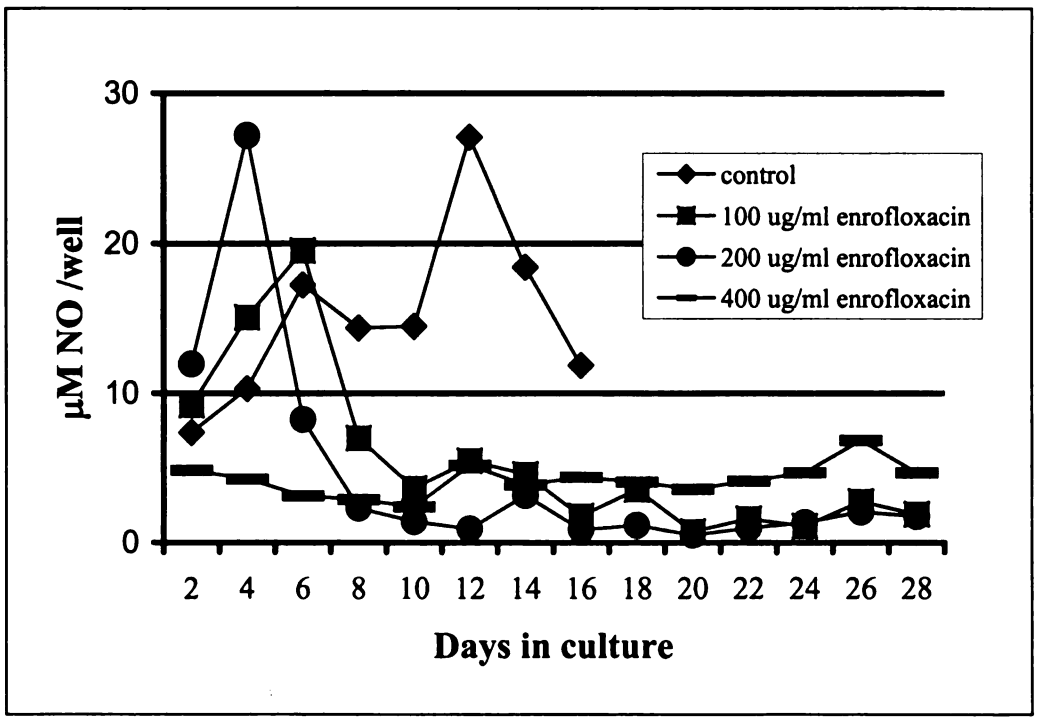
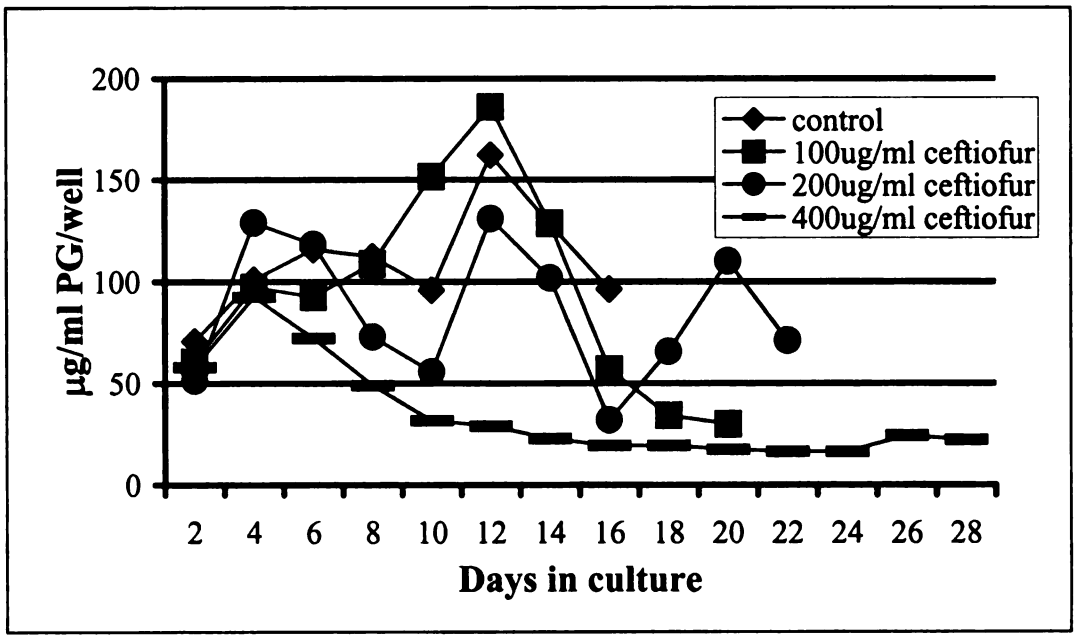

30
20
10
0
µM NO /well
control
100 ug/ml enrofloxacin
200 ug/ml enrofloxacin
400 ug/ml enrofloxacin
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days in culture

Figure 5. (Next page) Proteoglycan and nitric oxide release into conditioned media from chick tibia explants with ceftiofur treatments (n= 2). Panel **A** depicts the proteoglycan release into conditioned media throughout the 30-day culture period. Decreased proteoglycan concentrations were found only for the high dose of ceftiofur (400 μg/ml) between day 6 and 16 of culture compared to the control (p= 0.007). Panel **B** depicts the nitric oxide production released into conditioned media throughout the 30-day culture period. Decreased nitric oxide concentrations were found for the same concentration of ceftiofur between day 6 and 16 of culture compared to the control (p= 0.005).

A


200
150
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50
0
µg/ml PG/well
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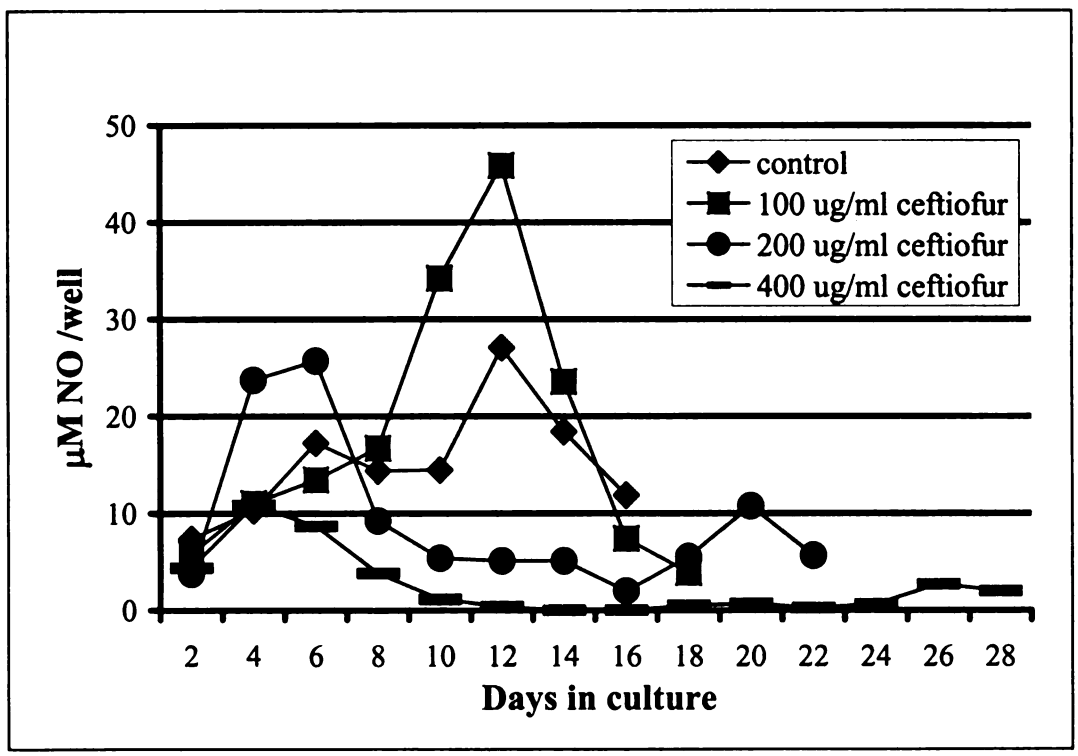
Days in culture
control
100ug/ml ceftiofur
200ug/ml ceftiofur
400ug/ml ceftiofur

B


50
40
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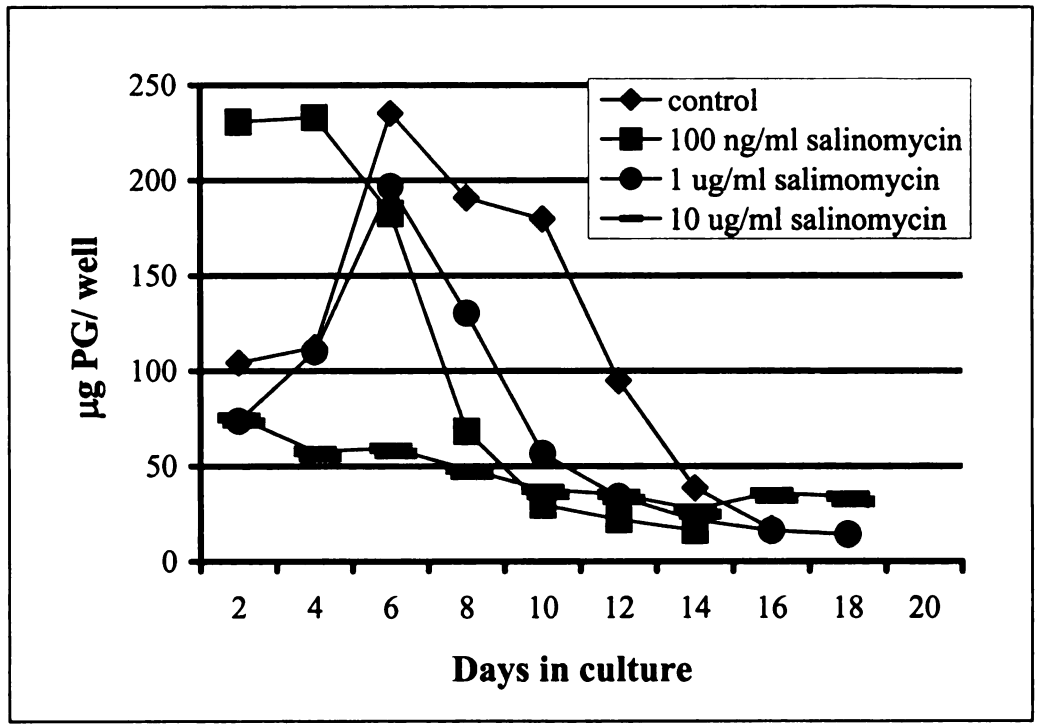
10
0
µM NO /well
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Days in culture
control
100 ug/ml ceftiofur
200 ug/ml ceftiofur
400 ug/ml ceftiofur

Figure 6. (Next page) Proteoglycan and nitric oxide release into conditioned media from chick tibia explants with salinomycin treatments (n=3). Panel **A** depicts the proteoglycan release into conditioned media throughout the 30-day culture period. Decreased proteoglycan concentrations were found for salinomycin at 10 μg/ml between day 6 and 16 of culture compared to the control ($p= 0.03$). A trend for decreased proteoglycan release existed for the 1 μg/ml salinomycin treatment ($p= 0.06$). Panel **B** depicts the nitric oxide production released into conditioned media throughout the 30-day culture period. Decreased nitric oxide concentration was found for 10 μg/ml salinomycin between day 6 and 16 of culture compared to the control ($p= 0.03$).

A


250
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µg PG/ well
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4
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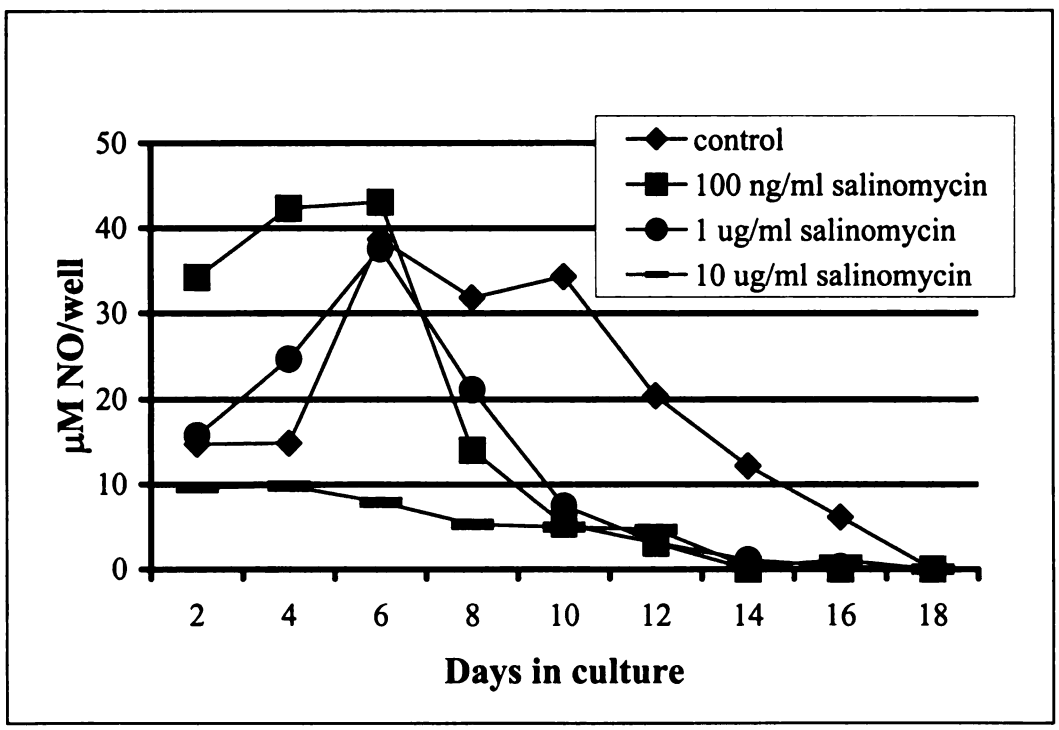
12
14
16
18
20
Days in culture
control
100 ng/ml salinomycin
1 ug/ml salimomycin
10 ug/ml salinomycin

B


50
40
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20
10
0
µM NO/well
2
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6
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10
12
14
16
18
Days in culture
control
100 ng/ml salinomycin
1 ug/ml salinomycin
10 ug/ml salinomycin

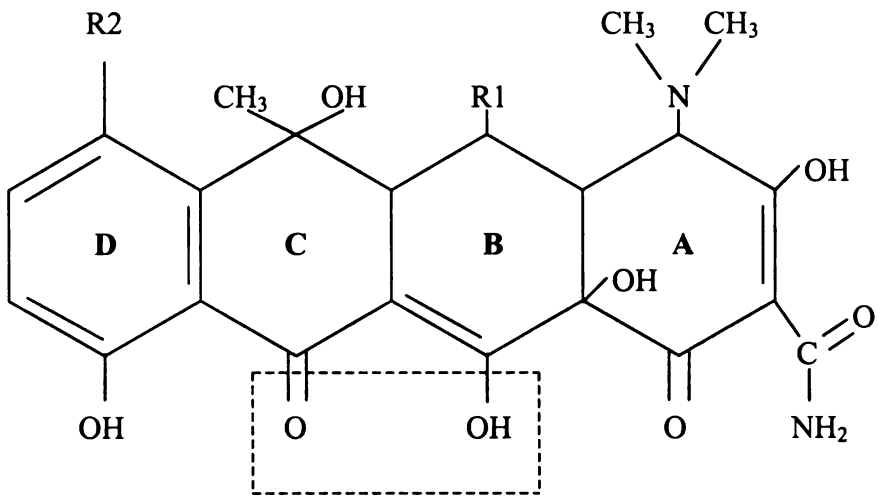

Figure 7. Tetracycline molecule. Dashed box highlights major metal binding site. The four-ring structure and this location are needed for inhibition of collagenase. R1 and R2 are the sites where the tetracycline derivatives differ in molecular structure.

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Chapter 3

The effect of antibiotics on body weight, feed conversion, and tibial dyschondroplasia scores of 3-week-old broiler cockerels

Introduction

Poultry skeletal disorders have been estimated to cause losses of approximately $160 million per year to the United States broiler and turkey industry (Sullivan, 1994). One of the major metabolic skeletal disorders that effect the long bones of fast growing meat producing birds (broiler chickens, turkeys, and ducks) is tibial dyschondroplasia (TD). A developmental abnormality of the tibiotarsal bone, TD originates in the growth plate where it disrupts the normal balance between cartilage synthesis and degradation. Growth plates of birds affected with TD undergo cell proliferation at a normal rate whereas hypertrophic chondrocytes within these lesions only reach 40% for their normal size (Hargest *et al.*, 1985). Due to inhibited degradation, an avascular cartilage plug accumulates in the metaphyseal region of the tibia, causing impairment of long bone growth and clinical TD lesions. Lameness, decreased feed efficiency, and increased mortality, culls, and condemnations at slaughter can be the results of TD.

Modern broilers have an 18 to 63% incidence of TD (Praul *et al.*, 2000;Roberson, 1999; Elliot and Edwards, 1997). Knowing the etiology of such a debilitating metabolic disorder would benefit the poultry industry. Experimental conditions and compounds that induce TD include: copper deficiency; fusarochromanone, thiram, and antabuse toxicity; excessive dietary levels of cysteine, homocysteine and histidine; calcium and phosphorus imbalance, and metabolic acidosis (Orth and Cook, 1994). Genetics and environmental factors such as time of year and rearing conditions also induce TD (Orth

and Cook, 1994). Many hypotheses have been presented, but the cause of TD in the commercial industry is still not known.

Recently, some antibiotics in the tetracycline family have been shown to inhibit cartilage degradation in an embryonic chick tibiae explant culture system (Orth et al., 1997). Other antibiotic families implicated in the disruption of bone metabolism include aminoglycosides (Murakami et al., 1996), cephalosporins (Smith et al., 1987), and quinolones (Vormann et al., 1997). Antibiotics have not been documented to disrupt growth plate cartilage metabolism directly. However, antibiotics routinely used in the industry are derivatives or members of these antibiotic families and may cause TD in the field. We have observed a parallel increase between the use of antibiotics and the rise in commercial TD incidence.

In our laboratory, certain antibiotics were found to inhibit *in vitro* cartilage degradation. Enrofloxacin, ceftiofur, doxycycline, oxytetracycline, and salinomycin significantly inhibited proteoglycan (PG) release and nitric oxide (NO) production from embryonic chick tibia cartilage (Chapter 2). Therefore, the purpose of this research was to determine if these antibiotics induced TD lesions in the proximal tibial growth plate when supplemented to broilers (0-3 weeks of age) at 25, 100 and 400% above the recommended use level.

Materials and Methods

Terramycin® 50 (50 g/lb oxytetracycline HCl; Pfizer Inc., New York, NY) and Aeuromycin® 50 (50 g/lb chlortetracycline HCl; Roche Vitamins, Inc., Parsippany, NJ) were purchased from the Michigan State University feed mill (East Lansing, MI). Naxcel® (50 mg/ml ceftiofur; SmithKline Beecham Corp., Philadelphia, PA) was purchased from the Michigan State University Veterinary Teaching Hospital pharmacy (East Lansing, MI). Bio-Cox® 60 (60 g/ton salinomycin) was generously donated by Roche Vitamins (Gibsonville, NC). Concentrated liquid Baytril® (3.23% enrofloxacin; Bayer, Shawnee Mission, KA) was used in this study. Thiram and doxycycline were purchased from Sigma (St. Louis, MO).

Trials

To determine if certain antibiotics inhibit cartilage degradation and induce tibial dyschondroplasia in growing broilers, day-old cockerels (Ross x Arbor Acres) were purchased from Hoover Hatchery (Rudd, Iowa) and housed at the Michigan State University Poultry Science Research and Teaching Center (East Lansing, MI). All Animal Use and Care regulations were followed. The day-old chicks were randomly wing-banded and placed in an electrically heated wire-floored battery brooder at eight birds per pen with three pens per treatment. A control and three treatment groups per antibiotic were established with 24 birds per group. The chicks were raised on a continuous illumination schedule with both incandescent (in the room) and fluorescent

lighting (in the pen). Feed and water were available *ad libitum* and diets (Table 4) met or exceeded all NRC requirements throughout the 21-day experimental period.

Recommended use levels for poultry of the antibiotics tested were obtained from the 1999 Feed Additive Compendium. Oxytetracycline and chlortetracycline treatment groups were administered antibiotics in their feed at 25%, 100%, or 400% above the recommended use level (except salinomycin, which was administered at 75%, 300% and 1200% above recommended use levels due to a mixing error). Doxycycline was administered at 15, 30, 60 and 200 g/ton in the feed to test its ability to induce TD. Enrofloxacin was administered in the drinking water at 25%, 100%, and 400% above the recommended use level. The three-ceftiofur treatments were injected sub-cutaneously in the neck area of one-day-old chicks (25%, 100% and 400% above the recommended dosage). The control group received no antibiotic treatment. Thiram, a fungicide known to induce experimental TD, was also given at 20 and 40 ppm. The number of available pens was limited; therefore, observations were divided into four separate experiments. Each experiment consisted of a control and two antibiotic treatments (three concentrations per antibiotic and three replicate groups of eight cockerels per concentration). The four experiments, and the antibiotics tested in each, are detailed in Table 5.

Weekly growth rates and feed consumption were tabulated by individual bird weights and pen feed consumption. Tibial dyschondroplasia lesions (degree of physeal thickening) were visually examined at the end of the 3-week trial. Birds were killed by cervical dislocation at 22 days of age. Right and left proximal tibiotarsi were

Table 4. Diet Composition for 0-3 week old broiler cockerels.

Ingredient	% of Diet
Ground yellow corn	53.75
Soybean meal (48%)	36.69
Choice white grease	5.14
Dicalcium phosphate (18.5% P)	1.57
Limestone	1.33
Salt	0.45
DL- methionine	0.20
Vitamin premix[1]	0.25
Trace mineral premix[2]	0.25

[1] Supplied X mg/kg of diet (except where noted): vitamin A* (all-trans-retinol acetate), 5000 IU; vitamin D3, 309 UCU, vitamin E* (alpha-tocopherol acetate), 11 IU; biotin*, 0.3; choline Cl*, 600; ethoxyquin, 125; folic acid, 3; menadione sodium bisulfite, 1.1; nicotinic acid, 44; D-pantothenic acid*, 10; riboflavin*, 4.4; thiamin mononitrate, 2.2; pryridoxine HCl (B_6), 3; vitamin B_{12}, 0.01.

[2] Supplied X mg/kg of diet: cupric sulfate, 10; potassium iodide, 2.1; ferrous sulfate, 60; manganese dioxide*, 120; sodium selenite, 0.1; zinc oxide*, 100.

Vitamins and minerals purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA) except noted by * those were purchased from Sigma (St. Louis, MO).

Table 5. Treatment groups within the 3-wk broiler experiments.

Antibiotic	Recommended Use Level	Treatment Use Level	Method of Administration
Oxytetracycline[a]	7.5 g/ton	9.4, 15, 30 g/ton	Feed
Chlortetracycline[b]	400 g/ton	500, 800, 1600 g/ton	Feed
Doxycycline[bd]	...	15, 30, 60, 200 g/ton	Feed
Salinomycin[a]	60 g/ton	180, 225, 360 g/ton	Feed
Ceftiofur[d]	0.2 mg/chick	0.25, 0.4, 0.8 mg/chick	Injection Sub-cutaneous
Enrofloxacin[c]	25 ppm	31.3, 50, 100 ppm	Water
Thiram[c]	...	20 and 40 ppm	Feed

[a]= treatments administered in Experiment 1; [b]= treatments in Experiment 2; [c]= treatments in Experiment 3; [d]= treatments in Experiment 4 (only the 200 g/ton doxycycline was tested in this experiment, the other 3 treatments were tested in experiment 2)

longitudinally sectioned and growth plate lesions were visually scored. A scale of 1-4 was used with 1 representing no lesion and 4 representing the most severe lesion as previously described by Orth *et al.* (1992). The incidence of TD was determined using the 2 to 4 scores as positive indicators of TD compared to the 1 scored indicating no TD lesion.

Statistical Analysis

Body weight and feed conversion data were analyzed using the general linear model procedure (SAS, 1999). Where appropriate, main effect means were separated by Tukey's studentized range test. The incidence of TD was examined by Chi Square analysis using the PROC FRIC program of SAS, 1999. A score greater than 1 was considered positive for tibial dyschondroplasia. Significance was determined only for the presence of TD.

Results

No difference in the incidence of TD lesions was observed between the control birds and those subjected to any of the antibiotics tested (Table 6). However, both thiram treatments induced TD lesions in 3 wk old broilers when compared to the control group. The 20 ppm treated birds experienced an 92% incidence rate ($p \leq 0.0006$) and the 40 ppm treatment group experienced a 78% incidence rate ($p \leq 0.0004$).

All of the salinomycin treated birds were lighter than the controls ($p \leq 0.001$; Table 6C). Feed conversion (amount of feed consumed to the rate of gain) only differed from the control at the 360 g/ton salinomycin treatment ($p \leq 0.05$). The 31.3 ppm enrofloxacin treated birds were heavier than control birds ($p \leq 0.04$; Table 6C), but there

Table 6. Body weight, feed conversion and tibial dyschondroplasia incidence for 3-week-old male broilers treated with several levels of seven antibiotics or thiram (a fungicide).

A: Experiment 1

Treatment	Body Weight[1] grams	Feed Conversion[1] Ratio	TD incidence[2] %
Control	574 ± 56	1.83 ± 0.04	13
9.4 g/ton oxytetracycline	628 ± 42	1.70 ± 0.24	8
15 g/ton oxytetracycline	602 ± 53	1.37 ± 0.28	0
30 g/ton oxytetracycline	587 ± 60	1.79 ± 0.21	0
180 g/ton salinomycin	434 ± 12**	1.94 ± 0.14	4
225 g/ton salinomycin	387 ± 3**	2.13 ± 0.10	0
360 g/ton salinomycin	232 ± 9**	3.71 ± 0.62*	0

B: Experiment 2

Treatment	Body Weight[1] grams	Feed Conversion[1] Ratio	TD incidence[2] %
Control	599 ± 77	1.83 ± 0.37	13
500 g/ton chlortetracycline	683 ± 21	1.62 ± 0.12	0
800 g/ton chlortetracycline	693 ± 27	1.62 ± 0.31	0
1600 g/ton chlortetracycline	611 ± 55	1.69 ± 0.11	0
15 g/ton doxycycline	689 ± 96	1.68 ± 0.26	4
30 g/ton doxycycline	669 ± 4	1.74 ± 0.18	8
60 g/ton doxycycline	672 ± 32	1.53 ± 0.06	0

C: Experiment 3

Treatment	Body Weight[1] grams	Feed Conversion[1] Ratio	TD incidence[2] %
Control	594 ± 87	1.91 ± 0.44	15
20 ppm thiram	429 ± 13	1.74 ± 0.11	92**
40 ppm thiram	235 ± 42**	1.76 ± 0.18	78**
31.3 ppm enrofloxacin	637 ± 51*	1.81 ± 0.47	4
50 ppm enrofloxacin	619 ± 60	1.91 ± 0.30	17
100 ppm enrofloxacin	676 ± 56	1.73 ± 0.02	12

D: Experiment 4[#]

Treatment	Body Weight[1] grams	Feed Conversion[1] Ratio	TD incidence[2] %
Control	502 ± 53	1.60 ± 0.17	8
200 g/ton doxycycline	547 ± 76	1.57 ± 0.19	0
0.25 mg ceftiofur/ chick	546 ± 68	1.65 ± 0.14	0
0.4 mg ceftiofur/ chick	506 ± 52	1.73 ± 0.16	0
0.8 mg ceftiofur/ chick	534 ± 48	1.67 ± 0.16	0

[1]= Mean of 3 pens; [2]= TD incidence was determine by using a positive indicator of a lesion (scores 2 to 4); [#] = Birds (same strain) were purchased from Townline hatchery (Zeeland, MI)

* significantly different from control group within its experiment at $p \leq 0.05$

** significantly different from control group within its experiment at $p \leq 0.001$

was no difference in feed conversion when compared to control birds. Thiram treated birds also did not differ from controls in feed conversion, but the 40 ppm treated birds were lighter than control birds ($p \leq 0.001$).

Discussion

The antibiotics (oxytetracycline, chlortetracycline, doxycycline, enrofloxacin, ceftiofur and salinomycin) tested in this experiment inhibited *in vitro* cartilage degradation of embryonic chick tibiae (Chapter 2). However, they did not induce TD lesions in 3-week-old male broilers. Tibial dyschondroplasia lesions were evident in thiram treated birds, which agrees with other literature (Orth et al., 1994; Vargas et al., 1983; Wu et al., 1990). Both treatment levels of thiram, 20 and 40 ppm, induced TD lesion when compared to control birds.

The antibiotics may have inhibited cartilage degradation *in vitro* because the antibiotic concentrations were higher than what the growth plate was exposed to in the bird. In the *in vitro* trials, explants are directly exposed to treatments. However, *in vivo* the drugs have to travel through the body to concentrate in growth plate tissue. Absorption factors could prevent the drugs from being active in the growth plate. If Ca^{2+} or Zn^{2+} were in excess, the tetracyclines would bind these molecules and become less available. Blood levels of antibiotics were not tested so possibly the absorption rates of the antibiotics in this study were not as high as those published. Trying to achieve higher concentrations of antibiotics to mimic *in vitro* concentrations would not be pharmacologically relevant.

Another factor that may have influenced the results of this study are the fluorescent lights used in the brooder where the chicks were housed. Edwards (1989,

1990) reported that birds fed adequate calcium and vitamin D_3 had a low incidence of TD when allowed access to ultraviolet light emitted by fluorescent lighting. In 1997, Elliot and Edwards showed that rapidly growing broilers fed diets adequate in both calcium and phosphorus and lacking sunlight or fluorescent lighting had a high incidence of TD, which could not be reduced by supplementing 10 times the recommended dose of cholecalciferol. They also stated that fluorescent lighting and supplementation with 10 μg/kg 1,25-$(OH)_2D_3$ (the active form of vitamin D_3) are equally effective in reducing TD development in broiler chicks. Thiram may act by a different mechanism than the Vitamin D pathway and overcome the effects of fluorescent lighting to induce TD. However, antibiotics that bind cations (tetracyclines and fluoroquinolones are thought to act primary by this mechanism) may not be able to inhibit cartilage degradation in the presence of fluorescent light.

Although skeletal disorders have been associated with tetracyclines, fluoroquinolones, cephalosporins and aminoglycosides, this study showed that doxycycline, oxytetracycline, chlortetracycline, enrofloxacin, ceftiofur, and salinomycin did not induce TD lesions in growing broiler chicks. However, thiram (20 and 40 ppm) significantly induced TD lesions in chicks raised in the same environment and fed the identical basal diet. Orth *et al.* (1999) reported that dithiocarbamates, such as thiram, inhibit *in vitro* cartilage degradation in this explant system. Dithiocarbamates and calcium deficiency both inhibit *in vitro* and *in vivo* cartilage degradation indicating that our explant model may predict whether certain compounds or conditions will induce TD. However, the results of this study show that the antibiotics tested are likely not involved in the etiology of TD in the poultry industry.

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Chapter 4

Summary and Conclusion

Poultry skeletal disorders have been estimated to cause losses of approximately $160 million per year to the United States broiler and turkey industries. One of the major metabolic skeletal disorders that effects the long bones of fast growing meat producing birds (broiler chickens, turkeys, and ducks) is tibial dyschondroplasia (TD). An avascular cartilage plug accumulates in the metaphyseal region of the tibia, causing impairment of long bone growth and clinical TD lesions. Lameness, decreased feed efficiency, and increased mortality, culls, and condemnations at slaughter can result from TD.

If the etiology of this debilitating skeletal disorder could be found, the industry would economically benefit from the solution. Antibiotics are incorporated into the diet of meat-type birds to promote growth and increase feed efficiency. Many of the antibiotics used in the poultry industry for growth promotion and disease treatment have been found to disrupt normal bone formation. Therefore, the objectives of this study were 1) to determine if antibiotics commonly utilized in the poultry industry inhibited *in vitro* cartilage degradation and 2) determine if antibiotics that inhibited *in vitro* cartilage degradation also induced TD in growing broilers.

An embryonic chick tibiae explant culture system was used to answer objective 1. Lincomycin, tylosin tartrate, gentamicin, erythromycin, and neomycin sulfate did not alter the cartilage's catabolic metabolism at any concentration tested. This work determined that doxycycline, oxytetracycline, enrofloxacin, ceftiofur and salinomycin

significantly inhibited proteoglycan and nitric oxide (indicators of cartilage degradation) release into conditioned media. The mechanism by which these antibiotics decrease cartilage degradation is not completely understood. However, binding cations (Ca^{2+}, Zn^{2+}, or Mg^{2+}) or altering cation concentrations is a common theme. If these cations are limited, the normal metabolism of the growth plate is altered and may cause impaired turnover leading to skeletal disorders like TD.

Conversely, when these five antibiotics were administered daily to day-old male broilers, TD lesions were not evident after 3 weeks of treatment. Thiram, a dithiocarbamate, did induce TD lesions when administered in the same environment. Dithiocarbamates have also been shown to inhibit cartilage degradation in the same embryonic chick tibia system that indicated the five antibiotics inhibited cartilage degradation. We believe that our *in vitro* model potentially predicts TD for certain compounds. However, *in vivo* the antibiotics tested were not involved in the etiology of TD.