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The Effect of Fluoroquinolone Antibiotics on Growing Cartilage in the Lamb Model

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Abstract

Background—The fluoroquinolones are a relatively new class of antimicrobials with an appealing spectrum of activity. Their use in pediatric medicine is limited due to concern over possible growth inhibition, as published reports have documented articular cartilage damage in animal models following their administration. This data, extrapolated to include the epiphyseal cartilage, suggests these agents may reduce growth rates, but limited human data is at the least equivocal, if not strictly contradictory to such claims. Specific investigations into the effects of fluoroquinolones on epiphyseal plate cartilage and growth velocity have not been performed.

Methods—Gatifloxacin and ciprofloxacin were used as representative agents of the fluoroquinolone class. Each drug was administered to experimental lambs over a fourteen day interval, at a dose designed to reflect those used in pediatric medicine. Recumbent versus standing intervals were used to monitor for arthropathy. Upon completion of fluoroquinolone administration, lambs underwent double fluorochrome labeling for determination of growth velocity. Gross and microscopic analysis of articular cartilage was performed to assess for pathologic changes. Age and gender matched lambs served as controls.

Results—Neither gatifloxacin nor ciprofloxacin negatively affected growth velocity of the proximal tibial growth plate as measured by double fluorochrome labeling. Additionally, no difference between experimental and control lambs in regard to recumbent versus standing intervals was noted. Examination of the articular cartilage failed to suggest chondrotoxicity.

Conclusion—Fluoroquinolone antimicrobials do not affect growth velocity in the ovine model when administered along a dosing regimen that closely models that seen in pediatric medicine.

Clinical Relevance—Fluoroquinolones may be acceptable for use in the pediatric population, as concerns over chondrotoxicity and growth inhibition may not be valid. This data suggests that expanded studies in lambs and other species, including humans, with differences in dosing and duration are justified to ultimately demonstrate clinical safety.

Introduction

The growth plate is a cartilaginous structure responsible for longitudinal growth of the developing skeleton. Skeletal growth is precisely regulated by a complex of inputs to chondrocytes during endochondral ossification. These inputs occur at all levels of biological organization including genotype, early embryonic patterning, a number of systemic hormones, a number of paracrine and autocrine signaling molecules, the biomechanical environment, nutrition, and disease.^{3, 4, 11, 15, 18, 33} Interrelationships and integration of multiple control systems are responsible for normal growth velocities. Because normal growth velocity is regulated with precision and influenced by so many different factors, the likelihood that any pharmacological agent will affect growth of the growth plate is high. Although the risks, for example, of corticosteroids and chemotherapeutic agents on immature articular and physal cartilage have been observed and studied, many important drugs have not.^{14, 29}

The fluoroquinolones are a class of antimicrobials with a broad spectrum of activity including resistant gram negative rods, anaerobic bacteria and resistant gram positive cocci. Unfortunately, their use is primarily restricted to the adult population, as these agents are contraindicated for routine use in children. However, given current trends in microbial resistance and enticing features such as enteral bioavailability, these drugs continue to gain interest as potentially effective agents for a variety of pediatric clinical settings, including children with cystic fibrosis, urinary tract infections, pneumonia, otorrhea, and the immunocompromised population, among others. As a result of this mounting interest, approval of various fluoroquinolone agents for use in the pediatric age group continues to be discussed.

These drugs are restricted largely due to a lack of clinical trials in children. The reluctance to perform such studies is based upon decades-old observations of toxicity to the articular cartilage in growing animal models from 1st generation fluoroquinolone exposure. Investigations of later generations of this class of drugs have been deterred by persistent concerns over joint damage.^{9, 16, 23, 30, 32, 36} Compassionate use of these agents in various pediatric populations is increasing, though, owing to both increased need and remarkable efficacy, as well as growing experience showing limited toxicity.^{10, 13} Although children treated with fluoroquinolones have been reported to have reversible arthralgias, the limited clinical studies performed have failed to document significant toxicity in these pediatric populations, and many experts now question both the validity and wisdom of restricted usage.^{7, 12, 13, 17} However, there remains a reluctance to initiate clinical trials, partially due to concerns that articular cartilage effects could portend similar pathology in the growth plate cartilage. Unfortunately these fears cannot be supported or refuted with the limited available data regarding the specific effects of this class of antibiotics on growth and the growth plate.

The purpose of this study was to investigate the effect of both newer and more commonly used fluoroquinolones on the developing growth plate and articular cartilage. Using a recently developed animal model, juvenile lambs were exposed to gatifloxacin and ciprofloxacin, attempting to identify any effects on growth velocity and chondrocyte biology, by examining growth rate, growth kinetics and histological changes of the articular growth plate chondrocytes. Lack of negative side effects would potentially allow for expanded study in animals and consideration for broader clinical trials and expanded usage in children if safety is confirmed. Evidence of an impact on cartilage development in growing lambs might deter further study in pediatrics.

Materials and Methods

After Research Animal Resources Center (RARC) and Institutional Animal Care and Use Committee (IACUC) approval was obtained, four male, mixed-breed lambs were acquired

from local sheep farms as 6–8 week old weanlings and housed in large pens at the University of Wisconsin Veterinary Medicine Teaching Hospital where free ambulation was encouraged. All animals experienced standard animal husbandry.

Vascular Access Ports

To allow vascular access for serial phlebotomy and drug administration, all lambs underwent surgical implantation of two Vascular Access Port (VAP) systems, from Norfolk Vet Products (www.norfolkaccess.com). Ports were placed into the external jugular and lateral saphenous veins, and were sutured to subcutaneous tissue allowing transcutaneous access. Routine care of catheters was performed to prevent embolic occlusion and ensure proper working order, using standardized flushing procedures.

This study was performed using both gatifloxacin and ciprofloxacin as representative agents of the fluoroquinolone class of antibiotics. A critical component to experimental design involved determining an appropriate dosage for each drug in the lamb model to approximate the doses used in human children. For example, gatifloxacin is a relatively new fluoroquinolone with limited pharmacokinetic data, and thus required establishment of a pharmacokinetic profile in lambs. This was not necessary for ciprofloxacin as this is a more commonly prescribed fluoroquinolone with which there is extensive clinical experience. As such, study design for administration of each antibiotic to experimental animals differed, requiring categorical description of each study method, as described below.

Gatifloxacin

Two genetically distinct, age and gender-matched lambs received a one-time intravenous dose of gatifloxacin (40 mg/kg) infused over one hour, with serial plasma samples drawn at specified time intervals after the start of the infusion (15 min, 55 min, 65 min, 75 min, 90 min, 2h, 3h, 6h, 7h, 17h, 24h). High pressure liquid chromatography (HPLC) assay of plasma samples allowed construction of a concentration versus time curve for gatifloxacin, which was used to develop a dosing regimen for the drug. The pharmacokinetic data obtained was used to design a dosing regimen that closely approximates human doses required for achieving therapeutic serum concentrations.

The fluoroquinolone class of antibiotics exhibits concentration-dependent killing of microbes, and clinical efficacy is highly correlated with the systemic concentration of drug (area under the time-concentration curve, AUC). Studies demonstrate that achieving an AUC/MIC (minimum inhibitory concentration) ratio of ≥ 125 during once daily gatifloxacin dosing correlates with clinical efficacy.⁶ The dose of drug required varies with the organism targeted as a function of its specific MIC value. In practice, gatifloxacin is indicated and prescribed primarily for respiratory tract infections, commonly caused in children by *H. influenzae* and *S. pneumoniae*. The gatifloxacin MIC values for these pathogens are approximately 0.03 mcg/mL and 0.5 mcg/mL, respectively. The AUC needed for our specific MIC values of interest was therefore 62.5 mcg-hr/mL. Results of HPLC obtained following the initial dose of 40 mg/kg indicated similar concentration versus time plots for both subjects (Figure 1). Area under the time-concentration curve (AUC) values were calculated to be 99.72 and 95.98 mcg x hr/ml, respectively. As AUC is directly and linearly proportional to the dose of gatifloxacin, obtaining a goal AUC of ≥ 62.5 mcg-hr/mL required administration of 30 mg/kg gatifloxacin, thus achieving an AUC/MIC ratio of >125 for both *S. pneumoniae* and *H. influenzae*.

Four weeks after the initial, single dose pharmacokinetic study, the two lambs who had received 40 mg/kg gatifloxacin infusion for phase I were infused with gatifloxacin (30 mg/kg once daily) for fourteen consecutive days. During the period of drug administration, Position Activity Loggers (PAL) were periodically affixed to forelimb metatarsals to determine recumbency

versus standing intervals over twenty-four hour intervals. In addition, daily weights and serial routine serum testing of hematopoietic, renal, and hepatic function was performed to monitor for signs of systemic toxicity.

Lambs were sacrificed following completion of the fourteen-day antibiotic course. Daily growth velocities were determined for the proximal tibia via double fluorochrome labeling with oxytetracycline (2 mg/kg) and alizarin complexone (15 mg/kg), given 24 hours apart prior to euthanasia (Figure 2).³⁵ Post-necropsy, articular cartilage was analyzed by HPLC to verify penetration of gatifloxacin into the joint space. Gross analysis was performed on articular cartilage to assess for chondrotoxicity (see below).

Ciprofloxacin

Two additional lambs were administered parenteral ciprofloxacin (15 mg/kg twice daily) for fourteen consecutive days. During the period of drug administration, Position Activity Loggers (PAL) were periodically affixed to forelimb metatarsals to determine recumbency versus standing intervals over twenty-four hour periods. In addition, daily weights and serial routine serum testing of hematopoietic, renal, and hepatic function was performed to monitor for signs of systemic toxicity. No abnormal results nor systemic symptoms of toxicity were observed at this dose.

Lambs were sacrificed following completion of the fourteen-day antibiotic course. Daily growth velocities were determined for the proximal tibia via double fluorochrome labeling with oxytetracycline (2 mg/kg) and alizarin complexone (15 mg/kg), given 24 hours apart prior to euthanasia (Figure 2).³⁵ Post-necropsy, articular cartilage was analyzed by HPLC to verify penetration of ciprofloxacin into the joint space. Gross analysis was performed on articular cartilage to assess for chondrotoxicity (see below).

HPLC

The assay for both agents was based upon the gatifloxacin assay reported by Overholser.²⁵ Plasma samples were prepared for analysis by first transferring 50 μ L of sample or plasma standard to a 1.5 ml microcentrifuge tube. A matching volume of gatifloxacin or ciprofloxacin internal standard (3mg/L) was added, followed by 900 μ L of ice-cold 0.1% formic acid in acetonitrile. The microcentrifuge tube was vortexed and centrifuged for 10 minutes. The resulting supernatant was transferred to a 750 μ L auto sample vial from which 5 μ L was injected by a Shimadzu LC-10A auto sampler.

The mobile phase consisted of 4.32 gm sodium dodecylsulfonate (Sigma, St Louis MO), 7.89 gm anhydrous citric acid (Sigma), 4.5 mL of tetrabutylammonium acetate (Aldrich, Milwaukee), 1.5 L deionized water, and 1.5 L HPLC-grade acetonitrile (Fisher). The flow rate was 1 ml/min, and separation relied upon an Alltech Adsorbosphere C18 5 μ m, 0.46 $\times$ 25 cm analytical column. Drug concentration was measured with a McPherson FL-750 spectrofluorometer with a high-sensitivity attachment. Fluorescence detector settings included an excitation wavelength of 295 nm and a 360 nm emission filter. Data acquisition and processing was performed with EZChrom Elite on a Windows XP workstation. Both the intra- and inter-day precision of the assay were greater than 90% throughout the linear range of the assay (0.3–30 mg/L). Area under the time-concentration curve (AUC) was determined by the trapezoidal method.

Positional Activity Logger (PAL)

In order to gain a sense of lamb activity, a PAL (Michael Gorman, Carnegie Victoria Australia) was periodically placed on the outer thigh of each lamb to monitor total average (freeranging) uptime, total daily recumbency, number of intervals, average time per standing and recumbent

periods. These Uptimers have been previously used to determine activity levels in 529 normally developing children.⁸

Articular Cartilage Analysis

Quinolone chondrotoxicity induces gross articular cartilage blistering and fissure formation, eventuating in joint surface erosion with resultant arthropathy, and has been previously described.⁹ Post-necropsy, weight-bearing diarthrodial joint surfaces including the femoral head, distal femur, proximal tibia, distal tibia, humeral head, and glenoid fossa from sacrificed lambs were examined grossly to observe for either the presence or absence of such findings.

In addition, tibial bones were harvested immediately following euthanasia and 1 mm articular cartilage samples (including subchondral bone) were collected from the proximal tibia with either a tabletop bandsaw (Gryphon Corp., Sylmar, California) or low speed, precision saw (Buehler, Isomet 2000, Lake Bluff, IL), under concurrent saline irrigation to maintain hydration and control temperature. Cell viability was determined by staining with the LIVE/DEAD Viability/Cytotoxicity Kit® (L-3224, Molecular Probes, Eugene, Oregon).²² Samples were incubated in 1 ml saline with calcein AM (.5 microliters) and ethidium homodimer (10 microliters) at 37 degrees Celsius for one-half hour prior to viewing. Live cells with an intact membrane and intracellular esterase activity fluoresce bright green as calcein AM is converted to calcein. Dead, dying, or damaged cells allow ethidium homodimer to enter through a porous plasma membrane and bind to nucleic acids producing a bright red fluorescence.

Samples were viewed using the Bio-Rad Radiance 2100 MP Rainbow confocal/multiphoton (W. M. Keck Laboratory, Madison, WI) system using an excitation wavelength of 488 nm (green) or 543 nm (red) and emission filters of 500–530 nm (green) and 605 nm (red). All samples were viewed the same day as collected.

Results

Gatifloxacin

Positional Activity Loggers revealed no increase in time spent in recumbency vs. standing as a result of the gatifloxacin infusion (Figure 3). Determination of growth velocity via double fluorochrome labeling revealed proximal tibial growth velocity to be 237 and 265 $\mu\text{m}/\text{day}$. This is consistent with previously published normative data for proximal tibial growth velocity in lambs at this age.²⁴ Penetration of gatifloxacin into the joint space was verified using HPLC analysis of synovial fluid and articular cartilage (Figure 4).

Gross analysis of diarthrodial, weight-bearing joints was performed. No evidence for articular cartilage blistering, fissuring, or erosion was seen (Figure 5). Microscopic analysis with calcein/ethidium homodimer staining demonstrated no signs of chondrocyte necrosis (Figure 6).

Ciprofloxacin

Two unrelated, age and gender matched lambs received 15 mg/kg intravenous ciprofloxacin infusion twice daily for fourteen days. No untoward systemic effects were observed following this dosing regimen and Positional Activity Loggers revealed no significant difference in time spent in recumbency vs. standing as a result of the ciprofloxacin infusion (Figure 7). Determination of growth velocity via double fluorochrome labeling revealed proximal tibial growth velocity to be 253 and 326 $\mu\text{m}/\text{day}$, respectively. This is consistent with previously published normative data for proximal tibial growth velocity in lambs at this age as well as with the gatifloxacin group.²⁴ Penetration of ciprofloxacin into the joint space was verified using HPLC analysis of synovial fluid and articular cartilage (Figure 8).

Gross analysis of diarthrodial, weight-bearing joints was performed. No evidence for articular cartilage blistering, fissuring, or erosion was seen. Microscopic analysis with calcein/ethidium homodimer staining demonstrated no signs of chondrocyte necrosis (Figure 9).

Discussion

Fluoroquinolones represent a class of antibiotics with broad spectrum antibacterial activity, a generally favorable safety profile; and as a result of current trends in bacterial resistance, increasing importance as potential therapeutic agents. Of this class of drugs, only ciprofloxacin is approved for use in children, and then only for severe urinary tract infections, including pyelonephritis. Restriction of approval is currently justified by concerns about toxicity to the developing skeletal system.

Concerns over the possible chondrotoxic effects of the fluoroquinolone class of antibiotics originated in the late 1970's with two studies which demonstrated deleterious effects on articular cartilage when administered to immature beagle pups.^{16, 32} From these studies it has been inferred that fluoroquinolones could adversely affect the human skeletal system including the growth plate cartilage. Nonetheless, use of these agents in children is sometimes vitally necessary, and is thus becoming increasingly common, particularly among children suffering from cystic fibrosis or cancer.^{13, 26, 27} Moreover, when fluoroquinolones are used in children, effects on the skeletal system have not been observed.^{2, 7, 17, 20, 21, 27, 28}

The goal of these trials was to approximate, as closely as possible with an animal model, the developing growth plate of children. Lambs were chosen due to their similarity to human subjects with respect to skeletal development kinetics, biomechanical loading and activity cycles. In comparison with other animal models, adolescent lambs have relatively slow growth velocities and are relatively older at skeletal maturation reaching adult height at about 42 weeks of age. Using approximate age/body weight scaling, our lambs' age correspond to children about 6–7 years of age. At 10 weeks of age these lambs weigh on average 26 kg, while 6 year-old boys on average weigh about 20 kg.^{19, 34}

The ovine proximal tibial growth plate is elongating at about 200µm/da., a modest growth velocity in comparison with rabbits (500µm/da.) or rats (400µm/da.). Lambs cycle through, on average, thirty-one recumbency versus standing intervals per day with average standing/ambulation intervals of 16.9 minutes, and average recumbency intervals of 29.8 minutes. When converted to total hours per day, lambs stand for about 8.6 hours, and are recumbent for 15.4 hours.²⁴ In a recent large study of 8 year-old children, average activity included 5.4 hours/day in standing/ambulation and 19.6 hours/day in recumbency.⁸ In addition to favorable comparisons to human children of body mass, growth rates, and recumbency/standing intervals, our lambs also go through brief periods of playful romping, running, and jumping, similar to behavior commonly seen in children, with similar stresses and forces being exerted on joint tissue.

The specific effect of fluoroquinolones on growth and the growth plate cartilage is largely unknown. With this study, we aimed to investigate longitudinal bone growth in the setting of exposure to fluoroquinolones, modeling doses currently employed in human therapeutics. Using lambs as a model, we have administered fluoroquinolones at doses approximating those used for pediatric therapeutic indications, and observed no significant effect on growth velocity or articular cartilage. Specifically, proximal tibial growth velocity, as measured by double fluorochrome labeling was consistent with age-matched normative data for the lamb.²⁴ In addition, no subjective or objective evidence of joint arthropathy as a result of chondrotoxicity to articular cartilage was observed, as evidenced by no significant change in recumbency versus

standing intervals prior to and during drug administration nor by gross and microscopic pathologic examination.

This study has significant limitations, most importantly in its small number of enrolled subjects and lack of a negative control group (instead comparing growth velocities in experimental animals to normative age-matched controls). It is possible that plasma levels achieved in our lambs underestimated those seen in children undergoing therapy. **Additionally**, various authors have suggested fluoroquinolone toxicity is a species-specific phenomenon. It is suggested that primates, rats, and canines are extraordinarily susceptible to the chondrotoxic effects of these agents, while others are relatively immune to them.^{1, 5, 31} This prompts the question of whether the lamb was an appropriate species for study, particularly given that no significant diminution of growth velocity or articular chondrotoxicity was observed in the lambs receiving a full, fourteen-day course of antibiotic. **Also, our study only measured chondrocyte activity by evaluating cellular viability and growth velocity. Future studies could specifically focus on chondrocyte biology, including metabolic and synthetic activity, both during and after fluoroquinolone administration. Finally, our study design was not continued over a period of time long enough to detect any delayed or late complications of fluoroquinolone therapy on the articular or epiphyseal cartilage. It is possible that deleterious chondrotoxic effects may not be observed until weeks, months, or even years following administration. A larger study carried out over a longer period of time is warranted in the future.**

Despite these concerns, we have demonstrated in this preliminary study that both gatifloxacin and ciprofloxacin, when administered to juvenile lambs, result in no significant chondrotoxicity as evidenced by articular cartilage physiology and proximal tibial growth velocity. To our knowledge, this is the first study to measure growth velocity following routine administration of fluoroquinolone antibiotics. These findings bolster evidence for continuing use in human therapeutics, and suggest that concerns regarding chondrotoxicity may indeed be ungrounded and at the very least, merit expanded studies. **The authors caution, however, that this is a preliminary study, and should not be viewed as an absolute endorsement for fluoroquinolone administration to pediatric patients.**

In addition to its specific application of studying fluoroquinolone toxicity, our experimental model could be utilized to quantify the effects of any systemic pharmacological agent on growth occurring at the epiphyseal plate. Future animal models employing microtransducer technology will allow in-vivo, real-time growth velocity measurements during administration of any systemic agent thought to exert either deleterious or beneficial effects on skeletal growth. Candidate agents might include corticosteroids, growth hormone, and chemotherapeutic drugs.

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Figure 1.

Concentration versus time curves for two lambs receiving a one-time 40 mg/kg dose of gatifloxacin. Area under the time-concentration curve (AUC) was calculated as $99.72 \text{ mcg} \times \text{hr/mL}$ and $95.98 \text{ mcg} \times \text{hr/mL}$, respectively. AUC values were then used to design fluoroquinolone dosing schedules that replicated those used in pediatric clinical medicine.

Figure 2. Chondro-osseous junction of proximal tibial growth plate following double fluorochrome labeling with oxytetracycline and alizarin complexone. Distance between the two fluorescent lines is measured in microns and represents longitudinal growth in a 24 hour period.

Figure 3. Percent time spent in recumbency on random days both prior to and during gatifloxacin administration ("Day 0" represents the first day of drug infusion). No significant increase in recumbency occurred during gatifloxacin administration.

Figure 4. HPLC analysis of articular cartilage following gatifloxacin administration. The drug demonstrates excellent articular penetration and is almost completely eliminated in 23 hours.

Figure 5.
Gross photograph of distal femur articular surface in lamb receiving 14-day course of gatifloxacin therapy. No blistering, erosions, or fissuring is noted.

Figure 6.

LIVE/DEAD Viability/Cytotoxicity Kit®, utilizing calcein AM/ethidium homodimer to stain for cellular viability in proximal tibial articular cartilage of lamb exposed to gatifloxacin. Calcein AM fluoresces green when it is converted by intracellular esterases to calcein by live cells. Ethidium homodimer enters the nuclei of dead or damaged cells and fluoresces red when bound to nucleic acids. The striking preponderance of calcein (green) on this slide indicates cellular viability throughout the entire thickness of articular cartilage.

Figure 7. Percent time spent in recumbency on random days both prior to and during gatifloxacin administration (“Day 1” represents the second day of drug infusion). No significant increase in recumbency occurred during gatifloxacin administration.

Figure 8. HPLC analysis of articular cartilage following ciprofloxacin administration. The drug demonstrates excellent articular penetration and is partially eliminated after 23 hours.

Figure 9.

LIVE/DEAD Viability/Cytotoxicity Kit®, utilizing calcein AM/ethidium homodimer to stain for cellular viability in proximal tibial articular cartilage of lamb exposed to ciprofloxacin. Calcein AM fluoresces green when it is converted by intracellular esterases to calcein by live cells. Ethidium homodimer enters the nuclei of dead or damaged cells and fluoresces red when bound to nucleic acids. The striking preponderance of calcein (green) on this slide indicates cellular viability throughout the entire thickness of articular cartilage.