Effect of Various Concentrations of Antibiotics on Osteogenic Cell Viability and Activity

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ABSTRACT: Infection is a common complication of open fractures. Systemic antibiotics often cause adverse events before eradication of infected bone occurs. The local delivery of antibiotics and the use of implants that deliver both growth factors and antimicrobials are ways to circumvent systemic toxicity while decreasing infection and to reach extremely high levels required to treat bacterial biofilms. When choosing an antibiotic for a local delivery system, one should consider the effect that the antibiotic has on cell viability and osteogenic activity. To address this concern, osteoblasts were treated with 21 different antibiotics over 8 concentrations from 0 to 5,000 μg/ml. Osteoblast viability and activity will enable clinicians and researchers to choose the optimal antibiotic for treatment of infection and maintenance of healthy host bone. © 2011 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 29:1070–1074, 2011

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The management of severe open fractures with bone loss requires early and aggressive management with thorough debridements, prophylactic antibiotics, and intervention(s) to promote bone growth. Despite these efforts, non-union and infection are still common complications with rates of up to 32% and 23%, respectively, reported in civilian severe lower extremity injuries.1 Similarly, infection is the most common cause of delayed amputation in combat-related open tibial fractures.2 Bacteria produce biofilm that is protective against microbial agents,3 and the antibiotic concentrations needed to eliminate these sessile colonies found in biofilms can be more than 500 times those required to kill planktonic bacteria.4 The high doses of systemic antibiotics that are above the minimum inhibitory concentration required at the fracture site may cause systemic toxicity.5 The local delivery of antibiotics can both avoid adverse systemic effects and achieve the therapeutic concentrations required to eliminate bacteria within the wound milieu.

Antibiotic-impregnated beads, antibiotic-coated cement spacers, and antibiotic-coated implants may reduce infection, but they do little to improve bone regeneration. With an improved understanding and application of growth factors to improve bone regeneration, dual-delivery implants, implants that deliver both a growth factor and antimicrobial, may present a means to simultaneously promote bone growth and prevent infection. Arguably, bioabsorbable carriers that can deliver growth factors to improve bone regeneration6–9 and antibiotics to prevent infection8,10–13 from the same implant may be superior therapeutics in the context of severe open fractures.

Given the increased interest in the development of improving antibiotic delivery, especially via dual-delivery implants, the appropriate selection of antibiotic is warranted and should consider the toxic effects of the antibiotic in addition to the effect on osteogenic activity. It seems intuitive that the number of surviving osteogenic cells impacts bone regeneration, but it is also important to consider the osteogenic activity of the cells. Although the antibiotics may not be overtly toxic to osteoblasts, an alteration of their metabolic profile14 might affect their bone forming potential. Collectively, the determination of cell number and osteogenic activity may provide insight into the effects of antibiotics on bone regeneration. To aid scientists in choosing the most appropriate antibiotic in the development of dual-delivery implants or other antibiotic delivery tools, we determined the effects of antibiotics from several different classes on the cell viability and osteogenic activity of osteoblasts.

MATERIALS AND METHODS

Materials and Reagents
Amikacin sulfate (A2324), cefazolin sodium salt (C5020), cefotaxime sodium sulfate (C7912), ciprofloxacin (17850), colistin methanesulfonate sodium (C1511), doxycycline hydrochloride (D9891), gentamicin sulfate (G1914), levofloxacin (28266), minocycline hydrochloride (M9511), nafcillin sodium salt monohydrate (N3269), penicillin V potassium salt (P4807), rifampin (R3501), tobramycin sulfate salt (T1783), and vancomycin (V8138) were all purchased from Sigma-Aldrich (St. Louis, MO). Azithromycin (NC9229050), cephalixin (NC9229821), daptomycin (NC9634209), imipenem monohydrate (NC9022260), linezolid (NC9838854), meropenem (NC985153), and trimethoprim (NC9822043) were all purchased from Fisher Scientific (Waltham, MA).

Additional supporting information may be found in the online version of this article.

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Cell Culture and Antibiotic Treatments
Human osteoblasts (Promocell, Heidelberg, Germany) were maintained in media consisting of alpha-MEM containing 10% fetal calf serum (FCS), 2 mM L-glutamine, and 0.001% antibiotic–antimycotic (Invitrogen, Carlsbad, CA, 15240-062). For the experiments, cells were seeded at 12,500 cells/cm² in 24-well plates. Twenty-four hours after seeding, cells were treated with osteogenic induction media consisting of alpha-MEM containing 10% FCS, 2 mM L-glutamine, absorbic acid (50 μg/ml), glycerophosphate (5 mM), and dexamethasone (10 nM), and 0.001% antibiotic–antimycotic (Invitrogen, 15240-062). All antibiotics were diluted according to the manufacturer’s recommendations and were used at 0, 10, 100, 200, 500, 1,000, 2,000, and 5,000 μg/ml. Media were changed and fresh antibiotics were added every 3–4 days. Controls consisted of the recommended diluents for the respective antibiotic. Ten and 14 days after the initiation of antibiotic treatments, cell lysates from three wells per time point at each antibiotic dose were collected for cell number and alkaline phosphatase activity (ALP) analyses.

Cell Number
DNA content was measured as an index of cell number similar to that previously described. Cells were washed twice with phosphate buffered saline, and whole cell extracts were obtained with the addition of 200 μl of CelLytic™ M lysis buffer. DNA content was determined using the CyQUANT® assay (Invitrogen, C7026). Thawed cell extracts and a standard curve prepared with DNA diluted in the same lysis buffer were incubated in the fluorescent dye and the cell lysis buffer from the CyQUANT assay kit for 10 min. The fluorescent intensity was determined on a SpectraMax M2 microplate reader with software SoftMax Pro 4.7.1 with excitation at 480 nm and emission at 520 nm, and the results are presented relative to control.

ALP Analyses
ALP was determined as an index of osteogenic activity, similar to that previously described. Ten and 14 days after cell treatment, cells were washed twice with phosphate-buffered saline, and whole cell extracts were obtained with the addition of 200 μl of CelLytic™ M lysis buffer. ALP was determined using a colorimetric alkaline phosphatase assay kit (AnaSpec, 72146, Fremont, CA). Briefly, 50 μl of a sample were mixed with 50 μl of p-nitrophenyl phosphate (p-NPP) substrate solution. Thirty minutes later, the absorbance at 405 nm was read with a SpectraMax M2 plate reader. Results were normalized to protein determined by the Bradford assay (Bio-Rad, Hercules, CA) and are presented as ALP activity per unit protein relative to control.

Statistics
DNA content and ALP per unit protein comparisons within each antibiotic were made using a one-way measure analysis of variance using the Dunnett’s method for multiple test comparisons. Since no significant differences arose at any dose between the 10- and the 14-day samples, they were pooled (n = 5–6 samples at each dose of antibiotic). The data shown are mean ± standard error of the mean relative to control. Significance was set at p < 0.05.

RESULTS
All antibiotics within a family differed either in the dose or the degree to which decrements for cell number and/or ALP were measured (Figs. 1 and 2 and Supplementary Material). With the exception of vancomycin, all antimicrobial agents achieved a >50% decrease in cell number within the range of doses used. Treatment with ≤200 μg/ml of rifampin, minocycline, doxycycline, nafcillin, penicillin, ciprofloxacin, colistin methanesulfonate, and gentamicin reduced both cell number and ALP (Fig. 1). For antibiotics not having an effect on cell number until >200 μg/ml, ALP decreases were observed at doses lower than for cell number (Fig. 1). The antibiotics with the greatest inhibition included rifampin, the tetracyclines, and ciprofloxacin, where >75% decrements in cell number and ALP were measured at 100 μg/ml (Fig. 1). Conversely, amikacin, tobramycin, and vancomycin were the least cytotoxic and did not appreciably affect ALP until very high doses were used (Fig. 1).

Differences among antibiotics with regard to the dose at which cell number and the dose at which ALP was affected tended to depend on the relative toxicity of the antibiotic, that is, antibiotics that were relatively toxic had decreases in cell number and ALP at the same dose, and for those that were less toxic, ALP decrements preceded decreases in cell number. More specifically, for doses ≤100 μg/ml, both cell number and ALP were decreased. Conversely, when decreases in cell number occurred at doses ≥200 μg/ml, decreases in ALP occurred at lower levels than that for cell number, with the exception of vancomycin (Fig. 1 and Supplementary Material).

DISCUSSION
Our primary objective was to determine the effects of a wide variety of antibiotics on cell viability and osteogenic activity. The main justification of the antibiotics we chose was to address antibiotics that are used commonly in clinical practice today delivered either systemically or locally. There is limited basic in vivo and in vitro data to support the current standard of care with regard to antibiotic delivery and bone toxicity. In addition, in the era of multidrug-resistant pathogens, a broader spectrum of antibiotics is required to eradicate or even prevent the development of infection. Even less knowledge exists surrounding these antibiotics’ activity in local delivery and/or toxicity on bone formation. Knowing different characterizations of antibiotics will allow clinicians to better direct therapy at the offending pathogens while minimizing local and systemic toxic effects. It will also allow the fine-tuning of antibiotics based upon delivery site versus systemic toxicity and efficacy on bone/wound healing. Given the increasing interest in high-concentration local antibiotic delivery systems, the varying effects of antibiotics on cell viability, and the paucity of data concerning the effects of antibiotics on osteogenic activity, this study measuring the effects of a wide variety of commonly used antibiotics will serve as a reference to scientists and clinicians for developing and improving local antibiotic delivery systems.

In addition to the high levels that may be achieved with a local delivery system, these levels will be
maintained for long periods. In the current study, we attempted to emulate the long-term effects of antibiotic exposure, and the cells were exposed to the antibiotics for periods of up to 10 and 14 days. For nearly all of the antibiotics tested, we were able to achieve a 50% decrease in osteoblast cell number and/or osteogenic activity. Despite the difference in treatment duration between the current and previous studies, our data are consistent with several other studies in which shorter time periods were used and/or difference cell types were studied.12,14,15,17–23 This similarity both adds validity to our data and suggests that the negative effects imparted by high levels of antibiotics in this study may be a result of changes that occur at early time points.

Although it is obvious that cell toxicity will affect bone regeneration, it may also be important to consider the osteogenic potential of surviving cells as well. Arguably, this is as important of a consideration as cell toxicity, that is, cells that survive but are not osteogenic may do little to aid in bone repair. The observations that changes in metabolic activity with antibiotics14 and decrements in ALP occurring at lower doses than decreases in cell number support this contention. In the current study, when decreases in cell number occurred at doses ≥200 μg/ml, decreases in ALP occurred at lower levels than those for cell number (Fig. 1). Interestingly, this was not seen with antibiotics where cell number decreases were ≥100 μg/ml (Fig. 1). A potential explanation may be that when antibiotics are extremely toxic to cells, subtle differences between metabolic activity and overt cell death are not discernable.

Antibiotics within a family may target a specific genus and species of bacteria similarly. However, our data suggest that differences exist among antibiotics within a class with regard to their effects on cell viability and osteogenic potential. More specifically, all antibiotics within a class differed either in the dose or the degree to which decrements for cell number and/or ALP were measured, which is exemplified by the aminoglycosides (Fig. 2A–C).

In general, our measurements of cell viability and ALP support previous reports on bone regeneration in the presence of antibiotics. We measured a decrease in ALP activity with gentamicin treatment (100 μg/ml), which is the same as reported for osteoblasts by Isefuku et al.,19 and is in agreement with a decrease in ALP activity for C2C12 cells,20 all of which support in vivo observations of bone repair inhibition with local application of gentamicin.24 Similarly, our observation of a decrease in cell viability with ciprofloxacin at 100 μg/ml is consistent with decreases in cell viability reported with doses >80 μg/ml of ciprofloxacin.14,17,22

The in vitro data in our study, and others, with these detrimental antibiotics, support in vivo reports of reductions in bone regeneration, that is, the fluoroquinolones (namely, ciprofloxacin and levofloxacin) were

Figure 1. Effect of treatment with different antibiotics on osteoblast cell number and ALP activity. The mean % decreases in osteoblast cell number (A) and ALP activity (B) are classified as <25%, 26–50, 51–74, and >75% of control after incubation with 0, 10, 100, 200, 500, 1,000, 2,000, and 5,000 μg/ml of each antibiotic for 10 and 14 days (n = 5–6 per dose after data are pooled). Not determined; ALP activity and/or cell number was untestable for some of the antibiotics, presumably because of precipitation and incompatibility with the test assays used. Decreases in osteoblast cell number and ALP activity >25% were significant, p < 0.05, with exceptions indicated by (') where the value at that dose was not different from control.
inhibitive to cell viability and ALP at low doses. The systemic use of fluoroquinolones impairs bone regeneration in vivo.\textsuperscript{25–27} In addition to the dose at which antibiotics are toxic and inhibitory to bone production, it may be important to consider the degree to which these factors are affected. Antibiotics that resulted in >75% decreases at doses $\geq 200$\,\textmu g/ml include the cephalosporins, the macrolide azithromycin, the rifamycin rifampin, the fluoroquinolones, and the tetracyclines (Fig. 1). In contrast, the use of amikacin or tobramycin did not result in a significant change in cell number until $5,000$\,\textmu g/ml (Figs. 1 and 2A–C). Although others have reported negative effects of tobramycin on osteoblast viability and/or proliferation at lower doses,\textsuperscript{28,29} our observed decrease of ALP with $500$\,\textmu g/ml tobramycin is in accord with previous reports of decreases of ALP at $600$\,\textmu g/ml.\textsuperscript{21} The safety of tobramycin is supported by reports that systemic and local deliveries of tobramycin do not impair bone healing.\textsuperscript{30–32} Collectively, the effects of the dose among classes of antibiotics on cell viability and osteogenic activity are different, and our results corroborate in vivo observations of bone repair.

A commonly used antibiotic for local delivery is the glycopeptide vancomycin, which was relatively safe over the wide ranges of doses in this study, similar to that previously reported.\textsuperscript{18} Previous studies showed a potential benefit of antibiotics on osteogenic potential, especially the cephalosporins. For example, the cephalosporin cefuroxime has the potential to increase ALP activity and proliferation,\textsuperscript{23} and a cefazolin-loaded biodegradable polypeptide multilayer nanofilm improved osteoblast viability and proliferation.\textsuperscript{12} Although we did not use the same doses as others for the cephalosporins, and although not significant, we observed a trend for increases in ALP activity for the cephalosporins as well (Fig. 2D–F).

Indeed, future studies are needed to determine how accurately measurements of cell number and ALP in vitro translate into impaired bone healing in vivo. More specifically, bone repair is a complex multifactorial process that also involves angiogenesis, cell migration, and a variety of cell types, including chondrocytes that play an important role in long bone repair. The effects of antibiotics on these crucial steps and on other cell types that play a role in bone healing must be addressed with in vivo experiments before definitive conclusions can be drawn. Nonetheless, these data provide insight into the effects of a wide variety of antibiotics and their potential to affect osteogenesis, giving scientists a means to appropriately select antibiotics for local delivery.

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