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Use of in vitro assays to identify antibiotics that are cytotoxic to normal equine chondrocytes and synovial cells

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Summary

Background: Intra-articular (IA) antibiotic usage is prevalent in equine practice. However, recent emergence of antimicrobial resistance prompts re-evaluation of antibiotic selection, particularly when used prophylactically. Furthermore, many commonly used antibiotics exert direct cytotoxicity to equine cells, and appropriate IA doses have not been defined.

Objectives: To screen antibiotics *in vitro* as an initial assessment of cytotoxicity against normal equine joint cells in monolayer culture and explant tissues.

Study design: *In vitro* experimental study.

Methods: Chondrocytes and synovial cells were harvested from 3 horses and plated on 24-well plates (100,000 cells/wells in triplicate) for 48 hours prior to addition of antibiotics. Joint cells were exposed to antibiotics (n=15) at various doses (25 to 0.39mg/ml in complete DMEM media) for 24h and viability was assessed by trypan blue dye exclusion. The half maximal inhibitory concentration (IC50) was determined for each antibiotic. Cartilage explants were obtained from 3 horses, minced and exposed to antibiotics (n=5) for 72h. Live/dead staining was performed, and fluorescence was visualised using Olympus IX83 spinning disk confocal microscope. Percentage of live versus dead cells was quantified.

Results: Antibiotics from different antimicrobial classes expressed dose-dependent but variable cytotoxicity to equine joint cells *in vitro*. Aminoglycosides and doxycycline had the lowest IC50

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Authorship

L. Pezzanite and L. Chow contributed to study design and implementation, data analysis and interpretation, and manuscript preparation. G. Piquini contributed to study implementation. D. Ramirez and G. Griffenhagen contributed to statistical analysis and IC50 calculation. S. Dow and L. Goodrich contributed to study design and data interpretation. All authors contributed to and approved the final manuscript.

Authors' declaration of interests

The authors have no competing interests.

Ethical animal research

This study was approved by the Institutional Animal Care and Use Committee at Colorado State University.

Owner informed consent

Not applicable.

Data accessibility statement

The data that support the findings of this study are available from the corresponding author upon reasonable request

Manufacturers' addresses

(most toxic). Ampicillin sulbactam, imipenem, tobramycin, ceftiofur sodium and amoxicillin had IC50 >25mg/ml for at least one cell line, representing potentially less cytotoxic alternatives.

Main limitations: Further studies are necessary to extrapolate these *in vitro* data results to the *in vivo* joint environment.

Conclusions: Targeted IA antibiotic therapy would involve selection of the safest antibiotics (highest IC50) with efficacy based on bacterial culture/sensitivity. Antimicrobial selection and evidence-based dosing may minimise damage to native articular cartilage and synovial cells and development of antimicrobial resistance when IA antibiotics are used in equine practice.

Keywords

horse; antibiotics; cytotoxicity; joint; septic arthritis

Introduction

Intra-articular antibiotic usage is prevalent in equine clinical practice [1,2]. Intra-articular polysulfated glycosaminoglycans (PSGAGs) have been shown to potentiate infection experimentally [3], and the combined usage of PSGAGs and corticosteroids was identified in one study as a risk factor for increased likelihood of joint infection [4], which has been cited as rationale for inclusion of antibiotics in routine joint injections. However, regardless of the pharmaceutical injected, and despite widespread use of prophylactic antibiotics by equine practitioners in joint injections, a protective effect of antimicrobial use in preventing septic arthritis has not been shown retrospectively in any previous study [2,4–6].

All intra-articular administration of antibiotics is considered “off-label” usage. Although there are anecdotal reports of IA antibiotic doses used by equine practitioners, [7,8], the appropriate dose to maximise efficacy while minimising iatrogenic side effects to the joint have not been defined in dose titration studies. Furthermore, the increasing incidence of antimicrobial resistance across veterinary and human medical practice has prompted re-evaluation of antibiotic usage in many instances [9–17]. Several recent articles have reviewed antimicrobial prophylaxis in equine patients and reported prophylaxis is only indicated when the likelihood of infection incidence exceeds 5% without antibiotics [9]. However, the reported overall incidence of joint infection following injection is much lower (<0.1%) [5–6,18]. Reconsideration of intra-articular antibiotic usage is warranted in light of these findings.

The cytotoxicity of several antibiotics commonly used in equine practice (e.g. amikacin, gentamicin, ceftiofur sodium, tobramycin, enrofloxacin) on joint tissues have been evaluated in separate studies with varying results [19–26]. Multiple studies have demonstrated the chondrotoxic effects of enrofloxacin *in vitro*, confirming the clinical observation of quinolone induced arthropathy in juvenile animals, which was found to be the result of irregular integrin signaling and reduced cartilage metabolism [24–25]. Aminoglycosides have also been demonstrated to exert direct cytotoxicity against various mammalian cells *in vitro* [8, 26–30]. Since amikacin is one of the most commonly used IA antibiotics in equine practice [1,2], our group has recently further investigated amikacin toxicity against normal

equine chondrocytes, synovial cells and adipose and bone marrow derived stem cells *in vitro* [30]. Amikacin induced apoptosis in all four cell lines in a rapid, dose-dependent manner that was independent of pH reduction and not mitigated by the presence of synovial fluid [30]. The half maximal inhibitory concentration (IC50), or concentration at which 50% of the cells were dead following amikacin exposure was less than 1mg/ml for all four cell types [9], which would be easily exceeded in the joint with all reported dosing (125–500mg) [7,8,30].

Transient cytotoxic effects on the joint tissues have also been demonstrated *in vivo* after IA administration. Repeated IA administration of amikacin caused increased values of total protein and nucleated cell counts to within the range of septic arthritis in some cases [21]. Gentamicin sulphate administered IA caused elevation in red and white blood cell counts and higher refractive indices in synovial fluid, with increased oedema, leukocyte infiltration and loss synovial lining cells [22]. Lescun *et al.* further demonstrated that continuous infusion of gentamicin resulted in loss of synovial intimal cells from villi [23]. In contrast, there were no significant differences in synovial fluid cytological parameters or histology of synovium and cartilage following ceftiofur sodium treatment (150mg) of equine joints *in vivo* [19]. These studies, taken together, suggest that if intra-articular antibiotics are used by equine practitioners, transient cytotoxicity may be mitigated by antibiotic selection and dose titration. However, the relative toxicity to joint tissues of antibiotics commonly used by equine practitioners has not been previously established or compared.

The primary objective of this study was to determine relative cytotoxicity of antibiotics commonly used in equine practice as an initial *in vitro* investigation of safety prior to performing further *in vivo* studies. The long-term goal of these efforts is to develop evidence-based strategies in selecting and dosing intra-articular antibiotics. Specifically, the objectives were to determine the half maximal inhibitory concentration (IC50), which reflects the concentration of antibiotic at which 50% of the cells are viable and to compare IC50s between antibiotics as a preliminary screen to determine which antimicrobials may be safest on normal equine joint tissues. We hypothesised that all antibiotics evaluated would be cytotoxic to normal equine chondrocytes and synovial cells in a dose-dependent manner, but that the degree of toxicity would vary between drugs with certain antibiotics being less toxic than others, providing candidates for future IA administration studies.

Materials and Methods

Animals –

Joint tissues were harvested immediately post-mortem from the femoropatellar joints of each of three donor horses (Quarter horses; two geldings and one mare; aged four, five and six years), euthanised for other reasons and without gross evidence of osteoarthritis at time of tissue collection based on previously described methods [31]. Horses had no externally palpable abnormalities associated with the femoropatellar joints and were sound at the trot in the hindlimbs at the time of euthanasia.

Tissue collection –

Using aseptic technique, full-thickness cartilage (approximately 2mm thick) was shaved to the level between calcified and non-calcified layers from the lateral trochlear ridge of the distal aspect of the femur and from the caudal surface of the patella using a #10 scalpel blade as previously described [26]. Synovial tissues were obtained by shaving the synovial lining of the femoropatellar joint using a #10 scalpel blade to remove four sections of synovium from the dorsomedial, dorsolateral, plantaromedial and plantarolateral aspects of the joint. Samples were immediately stored in complete DMEM to incubate at 37°C in 5% carbon dioxide and 95% air for further processing.

Monolayer cell culture –

Cartilage samples were minced immediately following collection using a #10 scalpel blade and digested overnight in complete supplemented Dulbecco's Modified Eagle's Medium (DMEM) (10% FBS, penicillin (100units/ml), streptomycin (100μg/ml), 1M HEPES) with 7.5mg type II collagenase at a concentration of 0.75mg/ml or 7.5mg collagenase per gram cartilage tissue digested to a final volume 10X tissue weight in spinner flask at 37°C with 5% CO_2. Digested cartilage was passed through two cell strainers (70uM and 40uM), centrifuged at 400g for 10 minutes and cells counted. Chondrocytes were plated in complete DMEM media (10% FBS) and expanded to second passage. Cells were frozen in 95% fetal bovine serum, 5% dimethyl sulfoxide at 10×10^6cells/ml and stored in liquid nitrogen until use.

Synovial tissue samples were digested as for chondrocytes for 4 hours at 37° C with 5% CO_2, filtered, centrifuged (400g for 10 minutes), and counted. Synovial cells were plated in complete DMEM media (10% FBS) at 10,000 to15,000 cells per cm^2, expanded in culture to second passage, then frozen at 5×10^6cells/ml in liquid nitrogen until use.

For cell viability experiments, cells were thawed quickly in a 37°C water bath and recovered for at least 48 hours under standard incubation conditions (37˚C with 5% CO_2). Cells from each of the three donor horses were then washed three times in phosphate buffered saline (PBS) to remove residual penicillin/streptomycin antibiotics and plated in triplicate in DMEM media lacking antibiotics on 24 well plates at 100,000 cells/well for 48 hours prior to the addition of antibiotic treatments. Plates were stored in the incubator (37˚C at 5% CO_2) following addition of antibiotics for all experiments.

Cartilage explant cultures –

Cartilage explant tissues from the three donor horses were pooled, minced to approximate 2mm, and aliquoted into 24-well tissue culture plates (approximately 100mg of wet weight per well). Tissues were incubated in complete DMEM media supplemented with 10% fetal bovine serum for 24 hours prior to the addition of antibiotics.

Effect of antibiotic and concentration on joint cell viability in monolayer culture –

The effect of antibiotics on joint cell (chondrocyte, synovial cell) viability from three donors each in triplicate (nine total replicates) was assessed at different concentrations in complete DMEM media (25, 12.5, 6.25, 3.125, 1.56, 0.78 0.39mg/ml versus control) following 24

hours exposure. Antibiotics evaluated included aminoglycosides (amikacin[a], gentamicin[b], neomycin[c] and tobramycin[d]), penicillins (ampicillin sulbactam[e], potassium penicillin[f], amoxicillin[g]), cephalosporins (cefazolin[h], ceftazidime[i], ceftiofur sodium[j]), and miscellaneous drug classes (enrofloxacin[k], vancomycin[l], imipenem[m], doxycycline[n], florfenicol[o]). Antibiotics were chosen to represent multiple drug classes as an initial *in vitro* screen for cytotoxicity to joint cells. As any intra-articular use would be off-label from the recommended route of administration, antibiotics labeled for different routes (i.e. intramuscular, intravenous, oral) may conceivably be injected in solution in the joint based on culture and sensitivity results and were therefore evaluated. The doses assessed were based on previous reports of amikacin administered by equine practitioners at 250mg per joint [7,8], which when administered into a joint with 10ml volume would presumably reach a maximum concentration of 25mg/ml within the joint and titrated to lower concentrations from there. Potassium penicillin was evaluated over the same range of concentrations and converted from units to mg, which did not precisely coincide with mg/ml doses evaluated for other antibiotics (Figures 1,2). Cell viability was assessed in both cell types using trypan blue exclusion staining to determine percentage of live cells.

Effect of antibiotic and concentration on cartilage explant cultures –

Following initial culture in complete DMEM media for 24 hours, explants were washed three times in PBS, and incubated in DMEM media supplemented with 10% fetal bovine serum with multiple concentrations of antibiotics (25, 5mg/ml versus control) for 72 hours at 37℃ 5% CO_2. Given the time-consuming nature of data analysis with explant tissue techniques, all antibiotics evaluated in monolayer culture were not evaluated in explant tissues. Antibiotics evaluated with explant tissues were chosen based on their common use in the authors' clinical practice, including amikacin[a], gentamicin[b], enrofloxacin[k], cefazolin[h] and potassium penicillin[f]. Following culture, explant tissues were transferred to 8-chamber covered glass slides. Live/dead staining was performed with LIVE/DEAD™ Cell Imaging Kit (Thermo Fisher[p]) according to manufacturers' instructions and visualisation of fluorescence staining was done using Olympus IX83 spinning disk confocal microscope. Z-stack images were taken at 10um intervals for each piece of explant in the indicated condition and merged using Olympus cellSens software[q]. Image J was used to count the live/dead pixels of each separated z stack image as previously described [32–34].

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[a]Amikacin sulfate (250mg/ml). Teva Pharmaceuticals INC. North Wales, Pennsylvania, USA.
[b]Gentamicin sulfate. Sigma Life Science. St. Louis, Missouri, USA.
[c]Neomycin trisulfate salt hydrate. Sigma Life Science. St. Louis, Missouri, USA.
[d]Tobramycin sulfate salt. Sigma Life Science. St. Louis, Missouri, USA.
[e]Ampicillin sulbactam salt. Meitheal Pharmaceuticals. Chicago, Illinois, USA.
[f]Penicillin G potassium, USP. Pfizer Inc. New York City, NY USA.
[g]Amoxicillin trihydrate. Sigma Life Science. St. Louis, Missouri, USA.
[h]Cefazolin salt. Sigma Life Science. St. Louis, Missouri, USA.
[i]Ceftazidime salt. Sigma Life Science. St. Louis, Missouri, USA.
[j]Ceftiofur sodium. Zoetis Inc., Kalamazoo, Michigan, USA.
[k]Enrofloxacin (22.7mg/ml). Bayer Healthcare LLC. Shawnee Mission, Kansas, USA.
[l]Vancomycin. Sigma Life Science. St. Louis, Missouri, USA.
[m]Imipenem. Sigma Life Science. St. Louis, Missouri, USA.
[n]Doxycycline. Sigma Life Science. St. Louis, Missouri, USA.
[o]Florfenicol. Sigma Life Science. St. Louis, Missouri, USA.
[p]LIVE/DEAD ™ Cell Imaging Kit. Thermo Fisher. Waltham, Massachusetts, USA.
[q]Olympus cellSens software. Olympus Life Sciences. Tokyo, Japan.

Data Analysis.

For monolayer cultures, the half maximal inhibitory concentration (IC50), or concentration of antibiotic at which 50% of cells were viable, was determined by normalising dose response for each concentration to control, transforming data to normalised dose response vs. log10(concentration) and estimating IC50 by nonlinear regression in GraphPad Prism v8.4.1 (GraphPad Software Inc., La Jolla, CA)[r] by fitting the data to a three parameter sigmoid function (implemented as “log(inhibitor) vs. dose response”. In instances where the IC50 was outside the range of concentrations evaluated, or the data were not distributed in sigmoid fashion following log transformation, the IC50 data were reported as a range of values as the exact value could be determined based on the concentrations assessed.

Normality was assessed via Shapiro-Wilk tests as well as evaluation of diagnostic plots and data was determined to be normally distributed. Antibiotics were compared to amikacin as the antibiotic most commonly used intra-articularly in equine practice to put others in context as alternatives in terms of cytotoxicity. The effect of antibiotic and concentration on cell viability in cartilage explants compared to control was evaluated by two-way ANOVA with Tukey’s adjustment for multiple comparisons. The effect of antibiotic concentration on viability of both chondrocytes and synovial cells was assessed using linear regression, with percent viability as the dependent variable and antibiotic as the independent variable.

Model fit and selection was performed using R functions *lm* and *anova* (base *stats* package) and *lmer* (*lme4* package), and contrasts between antibiotics were performed by comparisons of estimated marginal means (function *emmeans* from the *emmeans* package). Models tested included simple two-way linear models with concentration + antibiotic, log concentration (calculated as (log(concentration +1)) + antibiotic, the base model with interaction, and a mixed model with interaction + donor as a random effect. The addition of the ‘+ 1’ in the log transformation was necessary to account for the control (zero concentration) group while maintaining the same spacing between groups. The model with random effects fit the synoviocyte data better, while the random effect term did not improve the model for chondrocyte data. Therefore, the interaction model with log transformation and without random effects was selected based on AIC, BIC, and analysis of deviance tests. Following model selection, each antibiotic was fit to the model independently for determination of dose dependent effects, and comparisons between antibiotics were limited to amikacin (as baseline) *versus* all other antibiotics evaluated. The *p*-value was adjusted using Bonferroni’s method for multiple comparisons where necessary. Antibiotics were compared to amikacin as the antibiotic most commonly used intra-articularly in equine practice to put others in context as alternatives in terms of cytotoxicity. All analyses were performed using GraphPad Prism8 Software[r] (ANOVA model) and R[S] (linear modeling). Significance was assumed at $P<0.05$.

[r]GraphPad Software Prism8. San Diego, California, USA.
[S]R package version 3.6.0 (2019–04-26). R Foundation. Vienna, Austria.

Results

Antibiotic exposure decreased cell viability in a concentration-dependent manner in cells in monolayer culture that varied between and within antibiotic classes.

Exposure to antibiotics (n=15) resulted in decreased viability of equine chondrocytes and synovial cells in a dose-dependent manner, which varied between and across antimicrobial classes (Figures 1 and 2). Concentration (chondrocytes $p<0.0001$; synovial cells $p<0.0001$) and antibiotic (chondrocytes $p<0.0001$; synovial cells $p<0.0001$) were found to be significant factors to cell viability for both cell lines when antibiotic dose was titrated from 0.39 to 25mg/mL. The antibiotic dose (mg/mL) at which approximately 50% of the cells were alive (i.e. inhibitory concentration 50 or IC50) was determined for each of the two cell types for each antibiotic (Table 1). Specific comparisons in viability of cells (% live) was made between each antibiotic and amikacin, the most common antibiotic used intra-articularly in equine practice, for each concentration evaluated (Table S1).

Antibiotic exposure decreased cell viability in cartilage explants.

Exposure to antibiotics commonly used in equine practice (n=5) resulted in decreased viability of equine cartilage explant tissues (Figure 3) for all antibiotics at all concentrations (5, 25mg/mL) evaluated compared to control tissues ($p<0.0001$ overall). No significant differences were noted between antibiotics or within antibiotics at different concentrations (5 vs. 25mg/mL).

Discussion

These data provide important information to practitioners as an initial *in vitro* assessment and comparison of safety of antibiotics on equine joint tissues. Our overall hypothesis that all antibiotics would exhibit cytotoxicity on equine joint cells in a dose-dependent manner was supported. Furthermore, the degree of toxicity varied with certain antibiotics being less toxic than others. Tissues from normal joints (i.e. those lacking clinical evidence of sepsis and gross evidence of osteoarthritis), were used, which may not reflect the clinical scenario in which joints would commonly be injected with antibiotics clinically. However, this study was necessary to provide a platform from which further studies evaluating antibiotics both *in vivo* and with concurrent inflammation would build.

Limitations of this study warrant further discussion, including the *in vitro* nature of study design, lack of evaluation of the potential effect of concurrent injection of other pharmaceuticals, as well as the condition of the joint injected. The results obtained from this *in vitro* experimental design using a relatively low number of individual equine cultured cell lines investigated (n=3) should be reinforced by further dose titration studies investigating the effects of various antibiotics on the equine joint *in vivo* and in a greater number of animals. In addition, concurrent use of other medications may play an important role in decision making surrounding intra-articular antibiotic usage. The addition of pharmaceuticals such as triamcinolone and hyaluronic acid, which are commonly injected for osteoarthritis, to cartilage explant cultures was previously shown to have a mild protective effect against amikacin toxicity *in vitro* [26]. Additionally, the combination of

antibiotics and corticosteroids has been shown to result in precipitation and aggregate formation, which may reduce activity of either or both drugs, as well as cause irritation of surrounding tissues [35]. Investigation of the effect of PSGAGs in an induced model of septic arthritis using sub-infective doses of *S. aureus* in the middle carpal joint of horses demonstrated that PSGAG administration potentiated infectivity of S. aureus, and that the addition of 125mg amikacin immediately after inoculation significantly decreased infection risk [3–4]. For this reason, the recommendation to include amikacin when PSGAGs are administered IA has been made. However, it is important to note that only a single dose of amikacin was evaluated by Gustafson *et al.* [3,4], indicating that lower doses may potentially be used with equal efficacy to reduce cytotoxicity to the joint. In addition, the use of PSGAGs did not carry a higher risk of infection when evaluated retrospectively, except when combined with corticosteroids [18]. This observed lack of risk may in part be attributed to the low joint infection rate overall (<0.1%), where potentially more cases would need to be analysed to see a statistical difference in joint infection rate between those that received antibiotics and those that did not [18].

Furthermore, the degree of inflammation resulting in synovial effusion may affect antibiotic activity *in vivo* and was not assessed in the current studies. In inflamed joints with increased synovial effusion, concentrations of amikacin administered IA were found to be lower than those of normal joints [7], while antimicrobials administered parenterally reached higher concentrations in inflamed vs. normal joints [36,37]. These findings were attributed to increased joint volume and vascularisation associated with synovial inflammation in diseased joints, which may alter distribution of medication both in and out of joints [36–38]. Finally, it is conceivable that the presence of sepsis, or inflammatory mediators associated with sepsis, may alter antibiotic cytotoxicity profiles as well as drug distribution from the joint due to increased inflammation, but this was beyond the scope of the current study. The ability of bacterial pathogens to form biofilms in septic arthritis, either attached to each other free-floating in synovial fluid or along the synovial lining, may limit efficacy of IA antibiotics, requiring higher doses [39]. The initial *in vitro* studies reported here were necessary to determine IC50 levels in normal joint cells and compare cytotoxicity between antibiotics to move forward with additional work that would account for other variables encountered clinically. Investigation of the effect of different antibiotics in live horses in both normal and effusive or septic states, and with concurrent administration of other medications, to assess collagen degradation, cartilage matrix synthesis, and production of inflammatory cytokines following IA antibiotic injection would lend further credence to the results obtained here.

Aspects of explant culture techniques deserve additional explanation. The collection methods used (i.e. obtaining full-thickness cartilage shavings of approximately 2mm thickness) were performed based on previous reports in equine literature [26]. However, alternative methods of cartilage collection, such as using punch biopsies that maintain the three-dimensional cartilage from the superficial to deep zone, as well *as ex vivo* cartilage cultures that maintain the arrangement of chondrocytes in cartilage elements and in relation to surrounding perichondrium and other joints tissues have been described [40,41]. These different methods of culturing cartilage tissues *ex vivo* may affect viability as well as treatment with different pharmacologic agents, with other described methods being

potentially more representative of the *in vivo* environment. Comparison of explant viability between methods was not performed in this study and warrants further evaluation. In addition, no further reduction in viability was seen in explant cultures exposed to antibiotics at concentrations of 25mg/ml vs. 5mg/ml, which was attributed to viability being maximally reduced at the lower concentration to the extent that could be detected *in vitro* and may further reflect the methods of explant culture employed. Finally, given the significant time required for data analysis in explant tissues compared to monolayer culture, five of the fifteen antibiotics that were evaluated in monolayer culture were selected for evaluation in explant tissues. These antibiotics were selected as they are some of the most commonly used in the authors' equine clinical practice. Given differences pointed out between viability in monolayer culture and explant tissues, and if time allowed, all antibiotics would also ideally be assessed using explant cultures. It was considered necessary by the authors to perform all studies initially *in vitro* to determine IC50 values as an initial screen and comparison of safety. These *in vitro* studies provide the platform from which the necessary further *in vivo* studies will be performed.

Interpretation of discordances between the cytotoxicity observed following antibiotic exposure in monolayer cell culture and explant tissues warrants further discussion. It was considered by the authors necessary to perform studies in equine joint cells in monolayer culture initially to understand whether chondrocytes in particular are inherently more resistant to cell death irrespective of the matrix present in cartilage in situ. However, our results demonstrate that chondrocytes are equally sensitive to the cytotoxic effects of antibiotics both in and out of the cartilage matrix with synovial cells acting as a reference cell population in this instance. Stated differently, the equine cell lines evaluated are not uniquely resistant with the matrix component in play but respond equivalently to antibiotic cytotoxicity. This is revealed by further evaluation of antibiotic toxicity in explant tissues. Our results indicate that the cartilage matrix is modifying the activity of drugs, but not changing the inherent susceptibility of cells to cytotoxicity. Multiple explanations may be offered to describe this effect (i.e. the difference in toxicity of antibiotics to cells in monolayer culture versus in explant tissues *in situ*). Explant tissues may be less susceptible to toxicity as a result of a physical barrier of antibiotics to cell entry due to the three-dimensional nature of tissues or as a result of cells behaving metabolically differently when in matrix versus when digested out of matrix (i.e. cells may become metabolically protected as a result of matrix binding the drug, or differences in solubility or electrical charges). These results demonstrate that reduced cytotoxicity in explant tissues may be overcome with increased doses of antibiotic, but several explanations as described above may account for the discordance observed between explants and *in vitro* cell lines. Further *in vitro* evaluations where components of extracellular matrix are added to monolayer culture conditions may help to determine which component of the explant matrix is responsible for this protective effect.

Investigation of the effects of antibiotics on joint tissue viability and synovial fluid parameters *in vivo* is warranted. Orsini *et al.* originally published that the mean synovial fluid concentration of amikacin remained above MIC for approximately 6 hours following systemic administration when dosed at either 4.4 or 6.6mg/kg body weight [42]. Sedrish *et al.* further demonstrated that the concentration of amikacin in synovial fluid remained above

MIC for >24 hours when 500mg was injected into a normal radiocarpal joint [43]. Finally, Taintor *et al.* established that amikacin injected at 500mg in the radiocarpal joint remained at or above MIC in normal joints for 72 hours, but when the same dose was injected into endotoxin-inflamed joints, the concentration was maintained above MIC for only 48 hours, which was attributed to increased vascularity of the synovial membrane [7]. Further *in vivo* dose titration studies evaluating different concentrations of antibiotics and joint are warranted, with expanded evaluation of safety such as biomarkers of cartilage degradation and inflammation. Characteristics of the individual joint to be injected may affect antibiotic doses, as differences in synovial fluid volume, cartilage thickness and metabolism, and lack of uniform force distribution across cartilage between joints may affect antibiotic distribution and toxicity. Antibiotic penetration into articular cartilage *in vivo* is not known, and therefore the clinical relevance of cytotoxicity seen *in vitro* has not been fully established. Furthermore, current studies have not determined whether pre-existing osteoarthritis, which may commonly be present in clinical scenarios where antibiotics are injected prophylactically at the same time as other medications, affects the dose of antibiotics at which chondrotoxicity is observed. In this *in vitro* study, all samples were obtained from horses without previous known history or gross evidence of osteoarthritis. *In vivo* dose titration of multiple antibiotics in horses with both normal and inflamed osteoarthritic joints including evaluation of synovial fluid parameters and biomarkers of collagen degradation and cartilage matrix synthesis would broaden the scope of this work and lend further credence to the *in vitro* results obtained.

Although intra-articular antibiotics have been used in veterinary medicine for decades [44,45], this route of administration has gained recent attention in specific applications in human surgery as well, most notably total knee and hip arthroplasty [46–48]. Prompted by the increased incidence of multi-drug resistant infections, the goal of many of these reports is similar to that of equine practitioners treating septic arthritis: to achieve high local concentrations while minimising side-effects and cost associated with prolonged systemic administration [46–48]. *In vivo* IA antibiotic usage in humans has been associated with local osteo- and chondrotoxic effects which have imposed limitations on the use of antibiotics locally by orthopaedic surgeons [49]. The chondrotoxocity of three antibiotics commonly used against *Staphylococcus aureus* in human medicine, was recently evaluated in human primary chondrocyte cultures, and found to be non-toxic at relatively low, albeit clinically relevant, doses [50]. This study was limited in the fact that only a single concentration of antibiotic was evaluated (vancomycin 16mg/L, teicoplanin 64mg/L, linezolid 32mg/L). The final concentrations of antibiotics used in this study were determined based on their minimum bactericidal concentration (MBC) to common bacterial isolates in human practice [50]. In comparison to concentrations evaluated in our study which are reflective of those used clinically by equine practitioners, the lack of chondrotoxicity observed in human chondrocyte cultures may be attributed to the much lower concentrations assessed [50]. Taking vancomycin for example, the concentration (16mg/L or 0.016mg/mL) evaluated by Dogan *et al.* which is reportedly used by human physicians in clinical scenarios was well below the IC50 determined in this study for vancomycin on equine chondrocytes (7.306mg/mL). Evaluation of current intra-articular antibiotic use in human surgery provides valuable comparative information from which future *in vivo* studies may expand to determine the

upper limit of doses that may be used to maximise efficacy and minimise local side effects of intra-articular antibiotic usage.

The results of this study demonstrate that antibiotics decrease viability of equine chondrocytes and synovial cells *in vitro* in a dose-dependent manner, which varied between and within antimicrobial classes. Dosing for intra-articular antibiotic administration would ideally be determined by the volume of the joint injected to remain below IC50 (mg/ml) of the injected antibiotic. Selection of antibiotic may be guided by culture and sensitivity results when available in septic arthritis, while in general selecting antibiotics with the highest IC50 possible to minimise iatrogenic damage to joint cells. This study serves as a platform from which further *in vivo* evaluation of antibiotic dose and the effects of antibiotics in the presence of inflammation to guide evidence-based dosing strategies. Consideration of antimicrobial selection and dose by equine practitioners may minimise damage to native joint cells and maximise efficacy when antibiotics are administered intra-articularly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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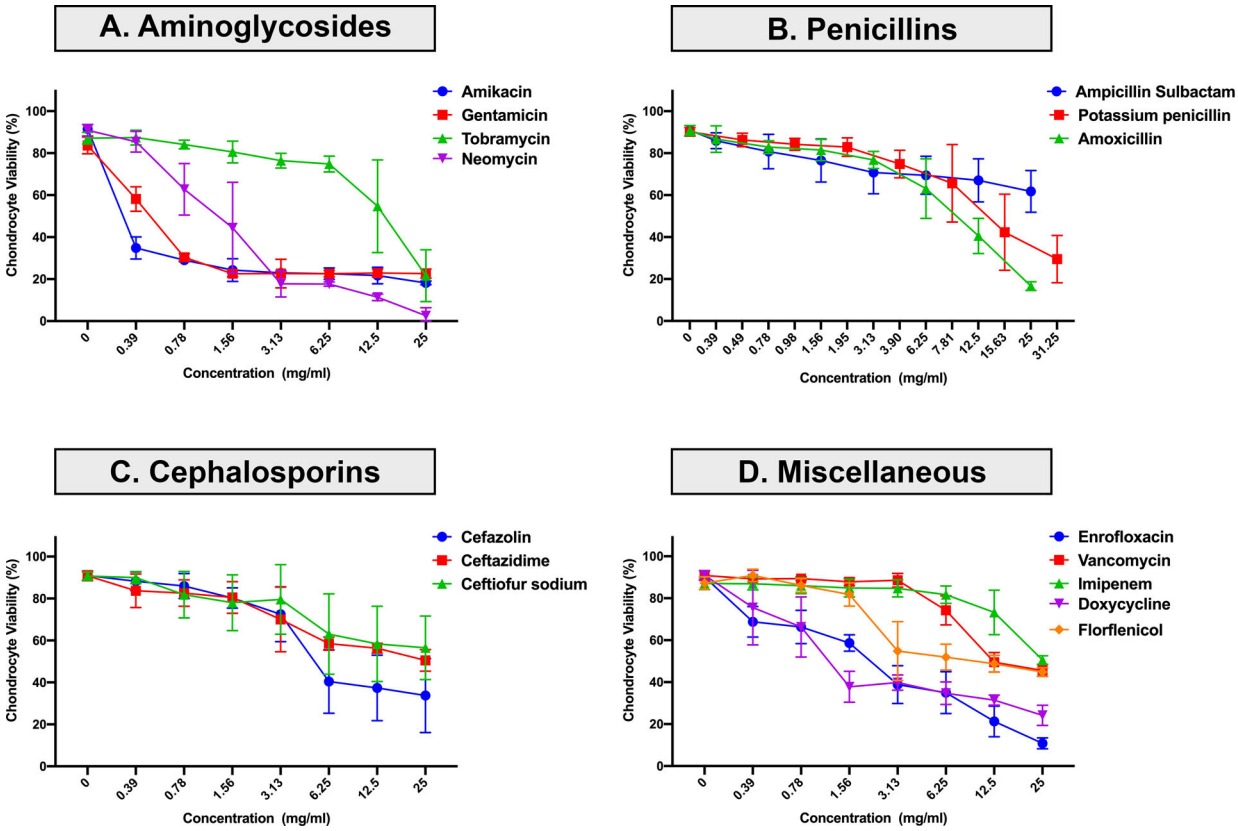

Figure 1:

Effect of antibiotics on chondrocyte cell viability in monolayer culture. Cell viability (mean ± SD) was assessed in chondrocytes from each of 3 donor horses, each in triplicate, using trypan blue exclusion staining to determine percentage of live cells following antibiotic exposure for 24 hours. X-axis represents antibiotic concentration, y-axis represents percentage live cells.

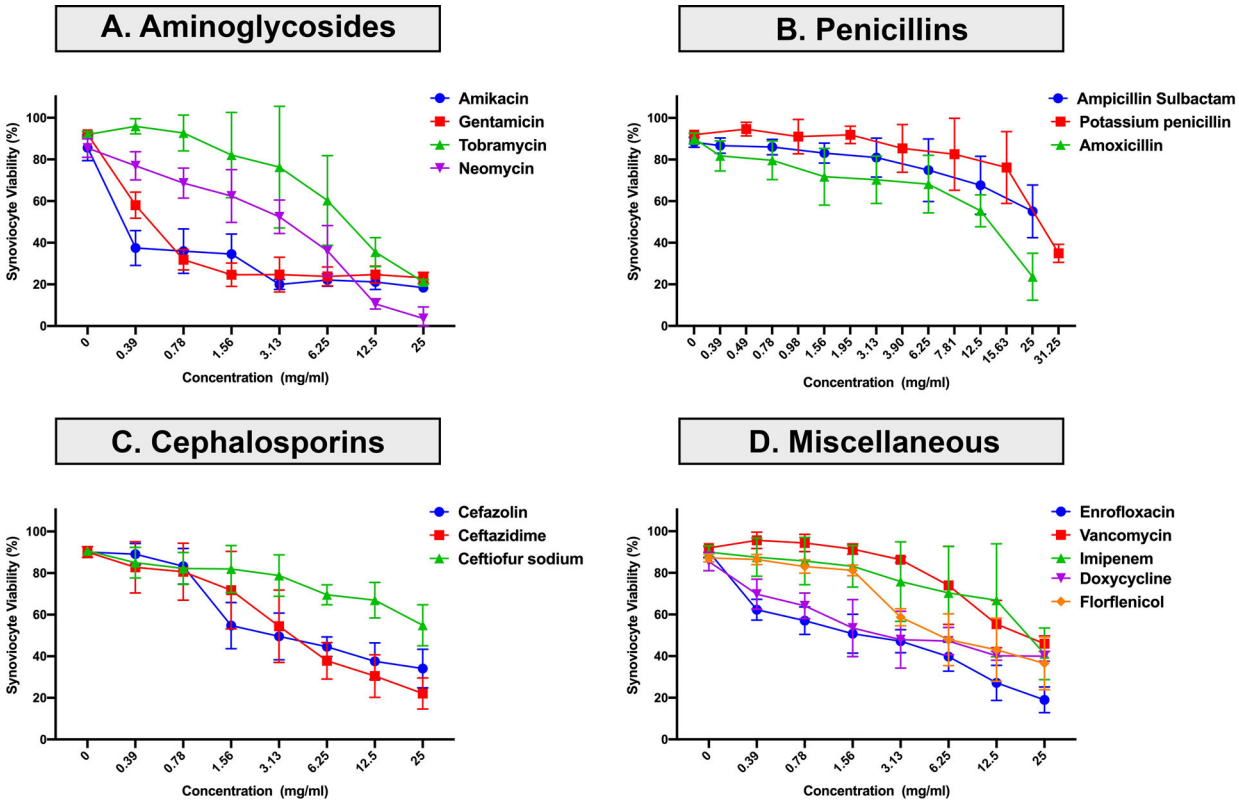

Figure 2:

Effect of antibiotics on synovial cell viability in monolayer culture. Cell viability (mean ± SD) was assessed in synovial cells from each of 3 donor horses, each in triplicate, using trypan blue exclusion staining to determine percentage of live cells following antibiotic exposure for 24 hours. X-axis represents antibiotic concentration, y-axis represents percentage live cells.

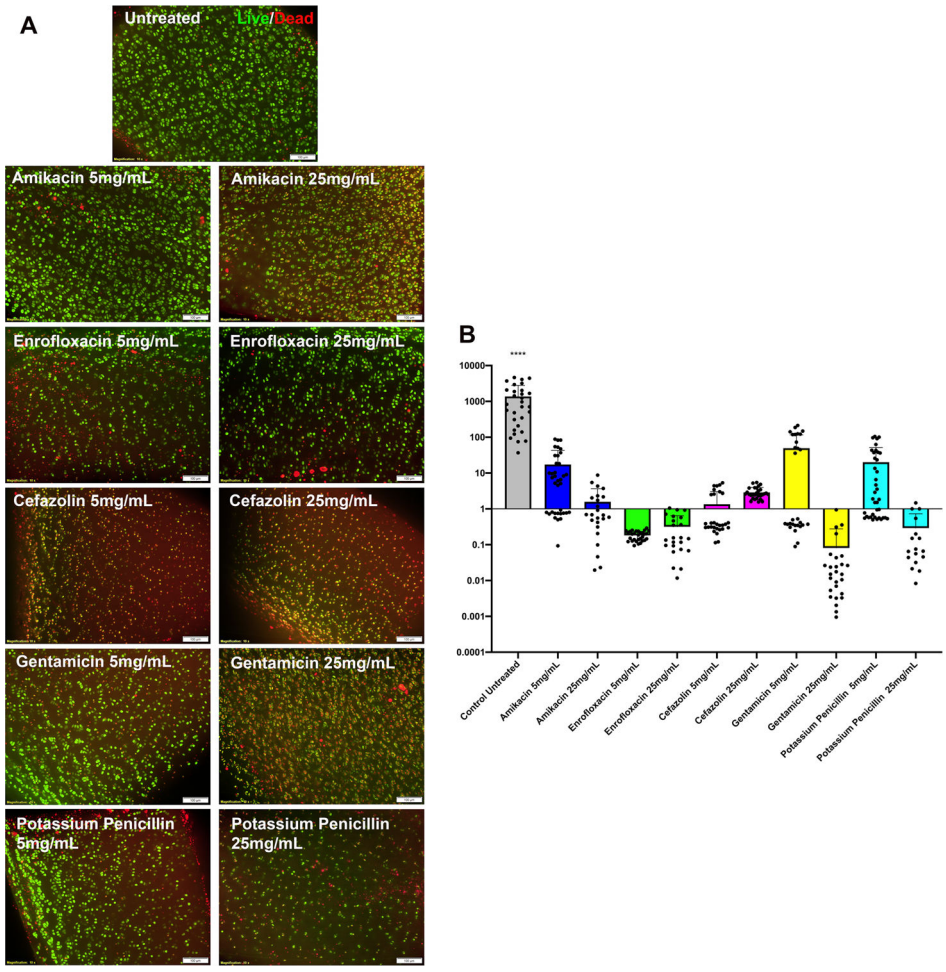

Figure 3:

Viability of equine cartilage explants following incubation with antibiotics. Cartilage explant tissues were cultured in complete DMEM media supplemented with 10% fetal bovine serum or media supplemented with one of five antibiotics at either 25 or 5mg/mL. 3A) Merged z-stack images of equine explants treated with antibiotics for 72 hours. Red staining indicates dead cells, green shows intact, live cells. 3B) Live minus dead pixel counts from n=3 z-stack cartilage explant images after 72 hours of incubation with antibiotics, calculated using Image J. Bar graphs show counts from increasing doses of antibiotics (x-axis).

Table 1:

Determination of half maximal inhibitory concentration (IC50) of chondrocytes and synovial cells in monolayer culture. Cell viability based on permeability was assessed in chondrocytes and synovial cells at multiple concentrations by counting cells that exclude trypan blue to determine percentage of live cells following antibiotic exposure. Antibiotics are listed from top to bottom as least to most toxic. Dose response for each concentration was normalised to control, and the data was transformed to "normalised dose response vs. log10(concentration)" at which point the half maximal inhibitory concentration (IC50) was estimated by nonlinear regression implemented in GraphPad Prism8[r]. In instances where the IC50 fell outside the range of concentrations evaluated, or the data were not distributed in sigmoidal fashion following normalisation and log transformation, the IC50s are reported most accurately as within a range of doses.

Chondrocytes			Synovial Cells		
	Antibiotic	**Concentration mg/mL**		**Antibiotic**	**Concentration mg/mL**
1	Ampicillin sulbactam	>25	1	Ampicillin sulbactam	>25
2	Imipenem	>25	2	Ceftiofur sodium	>25
3	Tobramycin	>25	3	Imipenem	>25
4	Amoxicillin	14.01	4	Amoxicillin	>25
5	Potassium pencillin	11.61	5	Potassium pencillin	15.625<x<31.25
6	Vancomycin	7.306	6	Tobramycin	9.49
7	Enrofloxacin	4.589	7	Vancomycin	7.812
8	Ceftiofur sodium	4.266	8	Neomycin	6.274
9	Cefazolin	3.948	9	Enrofloxacin	3.125<x<6.25
10	Ceftazidime	3.589	10	Ceftazidime	3.359
11	Florfenicol	2.19	11	Florfenicol	2.956
12	Doxycycline	1.031	12	Cefazolin	1.155
13	Neomycin	0.8219	13	Amikacin	0.7993
14	Gentamicin	0.7083	14	Doxycycline	0.7107
15	Amikacin	<0.39	15	Gentamicin	0.5125