DISSERTATION

USE OF IMMUNE ACTIVATED CELLULAR THERAPYAND RISKS WITH ANTIBIOTIC ADMINISTRATION IN TREATMENT OF SEPTIC ARTHRITIS

Submitted by

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ABSTRACT

USE OF IMMUNE ACTIVATED CELLULAR THERAPYAND RISKS OF ANTIBIOTIC ADMINISTRATION IN TREATMENT OF SEPTIC ARTHRITIS

Septic arthritis causes significant morbidity and mortality in veterinary and human clinical practice and is increasingly complicated by multidrug-resistant infections. Antibiotics have been injected intra-articularly by equine veterinarians for decades, both therapeutically to achieve high local drug concentrations to treat septic arthritis or prophylactically when other drugs are administered intra-synovially for osteoarthritis. This route of administration has also more recently gained attention in human orthopedic clinical practice, particularly as an alternative to systemic antibiotic administration to treat infections following prosthetic arthroplasty. However, the intra-synovial route of administration is considered 'off-label' usage and appropriate doses have not been defined, with relatively minimal attention paid previously to potential local cytotoxicity. In this work, the cytotoxicity of fifteen antibiotics commonly used in equine practice on equine chondrocytes, synovial cells and cartilage explants were evaluated over a range of concentrations, and the half maximal inhibitory concentration (IC50) was determined for each antibiotic. Antibiotics from different drug classes expressed dose-dependent but variable cytotoxicity to equine joint cells *in vitro*, with aminoglycosides and doxycycline having the lowest IC50 indicating the greatest cytotoxicity. Ampicillin sulbactam, imipenem, tobramycin, ceftiofur sodium, and amoxicillin had IC50 >25 mg/mL for at least one cell line, representing potentially less cytotoxic alternatives for intra-synovial use.

Amikacin sulfate was investigated further due to its frequency of use of equine practice and apparent relative cytotoxicity. The effects of amikacin concentration, duration of exposure, pH and presence of synovial fluid to mitigate amikacin cytotoxicity on equine chondrocytes, synoviocytes, adipose and bone-marrow derived MSC were investigated further. At clinically relevant doses, amikacin induced rapid, pronounced cell death in all four equine cell lines investigated in a dose-dependent, pH-independent manner, which occurred primarily by apoptosis. Amikacin cytotoxicity was investigated further using an *in vivo* equine non-inflammatory joint model, where the effects of amikacin injected at three doses (500mg, 125mg, 31.25mg) on immune and cartilage responses were investigated in tibiotarsal joints. Mean amikacin concentrations in synovial fluid reached concentrations over 100 times MIC and remained ≥ MIC (4 μg/mL) for the most common equine joint pathogens at all time points evaluated to 24 hours for all three amikacin doses investigated. Inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1β) increased significantly in SF in the highest amikacin dose group, despite the fact that increases in SF cell counts were not observed. Similarly, biomarkers of cartilage type II collagen cleavage (C2C, C12C) were increased in SF following amikacin injection. Mechanistically, we further demonstrated using *in vitro* studies that chondrocytes and synoviocytes killed by exposure to amikacin underwent apoptotic cell death and were phagocytosed by macrophages in a non-inflammatory process resembling efferocytosis. Neutrophils and T-cells were susceptible to amikacin cytotoxicity at clinically relevant doses, which may result in blunting of cellular inflammatory responses in SF and account for the lack of increase in total nucleated cell counts following amikacin injection. In summary, decisions on whether to inject cytotoxic antibiotics such as aminoglycosides intra-

articularly and what doses to use should take into account the potential harm that antibiotics may cause and consider lower doses than those previously reported in equine practice.

Mesenchymal stromal cells (MSC) possess antimicrobial and immunomodulatory properties and are particularly attractive as adjunctive therapies to antibiotics in treatment of septic synovitis as they are not subject to development of antibiotic resistance. One mechanism by which MSCs pay a role in the inflammatory response to infection is through expression of Toll-like (TLR) and nucleotide-binding oligomerization domain (NOD) like receptors (NLRs). Activation of TLR and NLR receptors of equine MSC was further evaluated here to determine if direct inhibition of planktonic and biofilm bacterial growth and quantitative expression of immunomodulatory cytokines and antimicrobial peptides could be enhanced through this approach. Of the agonists evaluated, TLR-3 polyinosinic:polycytidylic acid (pIC) was most effective to enhance antibacterial and cytokine responses, with reduced viable planktonic colony counts, suppressed biofilm formation, enhanced neutrophil bacterial phagocytosis, increased immunomodulatory cytokine MCP-1 secretion, and enhanced antimicrobial peptide cathelicidin/LL-37 production, which was apparent when serum concentration in media was reduced. Serum source in MSC cell culture was further investigated to maximize antibacterial activity, with cells cultured in fetal bovine serum (FBS) compared to autologous or allogeneic equine serum sources exhibiting greater spontaneous bactericidal activity, antimicrobial peptide cathelicidin/LL-37 and immunomodulatory cytokine (IL-4, IL-5, IL-17, RANTES, GM-CSF, FGF-2, Eotaxin) secretion, and faster population doubling times. These results indicate that MSC culture in FBS generates more functional cells based on a number of parameters, and that the theoretical risks of FBS use in MSC culture should be weighed against the loss of MSC function likely to be incurred from culture in equine serum. Finally, intra-articular administration of

TLR-3 pIC activated equine MSC was investigated to treat infection in an *in vivo* equine model of multidrug resistant *S. aureus* septic arthritis. TLR-MSC treated horses had lower inflammation/pain scores, quantitative bacterial counts in synovial fluid and synovium, synovial fluid total nucleated cell counts, neutrophil percentage, total protein and pro-inflammatory cytokine levels (IL-6, IL-18), as well as peripheral blood neutrophil counts. These findings demonstrate that intra-articular TLR-3 MSC therapy represents a promising adjunctive strategy to mitigate inflammation and infection in multidrug resistant septic arthritis.

The work described here will advance understanding of intra-synovial antibiotic administration, including the overall pros and cons of the approach, and introduces evidence for use of TLR-activated mesenchymal stromal cell therapy as an adjunctive treatment for septic synovitis. Further investigation of MSC dose, route of administration, and mechanism of administration to facilitate longer term bactericidal activity is indicated.

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CHAPTER 1: Introduction and Literature Review of Intra-Synovial Antibiotic Administration in Horses: Justifications, Risks, and Reconsideration of Use[i]

1.1 Summary

Antibiotics have been injected intra-articularly by equine veterinarians for decades, either prophylactically when other drugs are administered for osteoarthritis or therapeutically to treat septic arthritis. This route of administration has also more recently gained attention in human orthopedic clinical practice, particularly as an alternative to systemic antibiotic administration to treat infections following prosthetic arthroplasty. While the rationale for injecting antibiotics intra-articularly has been largely focused on achieving high local drug concentrations, there has been relatively minimal focus on pharmacokinetic parameters of antibiotics administered by this route, or to the potential for local toxicity. The increasing incidence of antibiotic resistance in veterinary and human clinical practice prompts reconsideration of off-label antibiotic usage and evaluation of evidence-based dosing strategies. The purpose of this review is to therefore summarize the current literature describing intra-articular antibiotic usage, including specific studies where pharmacokinetics, potential safety and toxicity have been evaluated. This review will advance practitioners' understanding of the use of intra-articularly administered antibiotics, including the overall pros and cons of the approach.

1.2 Historical rationale for intra-articular antibiotic usage

Intra-articular (IA) antibiotics have been used by equine practitioners for decades when injecting

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[i] A version of this manuscript has been submitted to the *Equine Veterinary Journal*: Intra-articular administration of antibiotics in horses: rationale, outcomes, and risks: Pezzanite L, Hendrickson D, Dow S, Stoneback J, Krause D, Chow L, and Goodrich L.

joints in osteoarthritis or to treat septic arthritis. More recently, IA antibiotic usage has gained attention in human orthopedic clinical practice with the goal of achieving high local drug concentrations in septic arthritis, particularly those associated with orthopedic implants such as after arthroplasty. In horses, the inability to deliver antibiotics systemically for longer durations without risk of severe side effects (e.g. gastrointestinal, renal toxicity) has been the impetus to emphasize local versus systemic administration to treat joint infections. Furthermore, the development of biofilms or biofloats in synovial structures may require higher antibiotic doses to penetrate and therefore effectively treat infection. Finally, antibiotics may be administered intra-articularly based on sensitivity results that would be prohibitively expensive in horses at systemic doses. More rapid resolution of infection through a combination of antibiotic routes of administration (e.g. systemic, regional limb perfusion, and intra-articularly) may also improve owner compliance and convenience in treatment, potentially decreasing cost and complications and improving prognosis with faster resolution of clinical signs compared to long-term administration of oral medications.

However, as the intra-articular route of administration is 'off-label', appropriate doses have not been defined. Furthermore, the increasing incidence of multidrug resistance in both veterinary and human clinical practice has prompted reevaluation of antibiotic protocols in many instances. Relatively little attention has been directed to potential disadvantages of intra-articular approaches including deleterious local tissue effects of high antibiotic concentrations, potential contribution to development of multidrug antibiotic resistance given lack of published dosing strategies, and expense to the owner when used prophylactically given a lack of demonstrated effect in preventing infection. This review will discuss published reports of intra-articular antibiotic administration in horses and humans, including pros and cons of the approach, and

conclude with a summary of supplemental therapies to treat joint infection. A Pubmed search was performed for articles using the search terms 'horse,' 'equine,' 'intra-articular,' 'intrasynovial,' 'antibiotics,' and 'antimicrobials,' with no date limit placed on search results. Additional articles were also pulled from Google Scholar as well as a general internet search using the same keywords to broaden the search and capture non-indexed manuscripts. The objectives of this review are to identify problems with, questions surrounding, and knowledge gaps associated with intra-articular antibiotic use to help equine clinicians make informed decisions in their practice.

1.3 Review of intra-articular antibiotic administration in horses

Intra-articular antibiotic administration in equine practice may be considered either prophylactic when injecting joints with other medications in osteoarthritis or therapeutic in the treatment of septic arthritis. In one recent survey, 78.6% of equine veterinarians reported using IA antibiotics prophylactically in at least some situations, most commonly when injecting corticosteroids or polysulfated glycosaminoglycans, and likely driven by the perceived emotional, financial, and clinical consequences to the horse and client and potential professional consequences to veterinarians following development of an infection following injection.[1] However, the incidence of septic arthritis following joint injection across four large retrospective studies of over 300,000 joints, is reportedly quite low (<0.1%).[2-5] Smith *et al.* most recently reported in 2019, that even in ambulatory practice, where presumedly conditions are less clean than in veterinary hospitals, the rate of joint infection following injection was not higher than that reported in other studies (0.04%).[2-5] Similarly, recent retrospective evaluation of the synovial infection rate following injection by the Surgery, Sports Medicine and Ambulatory

services at Colorado State University supported these findings with an overall infection rate of 0.1% (4 out of 3866 joints) over a five-year period, 2014-2018 (manuscript in review).[6] The four cases resulting in infection had been injected with triamcinolone acetonide, hyaluronic acid and amikacin sulfate (n=2 cases), platelet rich plasma (n=1) and autologous conditioned serum (n=1). Concurrent antibiotic administration was performed in 42.1% of cases overall, and in 2/4 joints that resulted in iatrogenic septic arthritis.[6] Synovial injections were performed in the hospital in 60.2% and in the field in 39.8% of cases.[6] Concurrent administration of antibiotics with injections or environment in which synovial structures were injected were not identified in any of these studies or the co-authors' retrospective evaluation of their own case population as a factor that statistically reduced the risk of development of joint infection, with the exception of antibiotics reducing risk of infection with polysulphated glycosaminoglycans specifically in Smith *et al*.[2-5] However, it is acknowledged that these studies may have been underpowered statistically to detect a difference between cases where antibiotics were used or not, and the effects of environment and weather on risk of development of infection have not been fully explored and are furthermore difficult to model experimentally.

Regardless of clinicians' rationale for injection, doses for IA injection remain undefined with relative efficacy and safety undetermined. Antibiotics such as aminoglycosides have been shown to reduce viability and gene expression of mammalian cells *in vitro*, and for this reason the recommendation has been made to not include antibiotics when injecting stromal/stem cells in osteoarthritis.[7-10] The current use of intra-articular antibiotics by many equine veterinarians raises several questions. First, does good rationale for prophylactic use of antibiotics in routine joint injections exist given the reported low incidence of infection following injection regardless of antibiotic use? Several recent papers on antibiotic administration in equine practice have

described prophylaxis as indicated only when the incidence of infection is greater than 5% when antibiotics are not given, and equine practitioners are well below that with less than 0.1% infection rate following joint injections.[2-4] Furthermore, as IA administration is 'off label', there have not been studies until recently investigating or supporting the doses currently used or comparing relative toxicity of different classes of antibiotics. Here, the co-authors review and summarize the literature describing investigations of the effects of antibiotics using both *in vitro* equine joint tissues (**Table 1.1**) and *in vivo* equine studies (**Table 1.2**).

In vitro studies using equine tissues **(Table 1.1) -** Multiple studies have demonstrated the *in vitro* cytotoxicity of several antibiotics commonly used in equine practice to joint tissues.[11-16] Reduced cartilage metabolism and dysregulated integrin signaling were described in enrofloxacin-treated chondrocytes and cartilage explants, which were more severe in neonatal tissues, providing rationale for the clinical observation of fluoroquinolone-induced arthropathy in young animals.[11,12] Articular cartilage explants treated with amikacin sulfate had increased proportion of empty lacunae based on histomorphometric quantification, indicating toxicity to chondrocytes within cartilage matrix, which was mitigated by the presence of triamcinolone in culture.[13] Co-culture with amikacin (25 mg/mL) and enrofloxacin (1 mg/mL) induced 90% cell death of chondrocytes in monolayer culture.[14] Enrofloxacin further induced production of PGE_2 at all concentrations assessed (0.1, 1 and 10mg/mL), which was reduced by pre-treatment of chondrocytes with a nutraceutical supplement containing avocado/soybean unsaponifiables, glucosamine and chondroitin sulfate.[14]

Using *in vitro* cytotoxicity assays, the co-authors recently expanded on this body of work to investigate the relative effects of several classes of antibiotics on joint cells, including chondrocytes, synovial membrane cells, mesenchymal stromal cells and leukocytes. Amikacin

was investigated initially due to its common use in equine clinical practice and used as the benchmark for cytotoxicity, based on previously reported cellular toxicity.[7-10] Amikacin induced dose-dependent cell death in all four cell lines, which occurred primarily by apoptosis and was not the result of reduced pH of cell culture media by the addition of amikacin.[15] Fifteen antibiotics were then screened and compared for cytotoxicity to chondrocytes and synoviocytes over a range of concentrations *in vitro*. The half maximal inhibitory/cytotoxic concentrations (IC50) were determined to rank relative cytotoxicity of antibiotics. Aminoglycosides and doxycycline had the lowest IC50 (most toxic). Ampicillin sulbactam, imipenem, tobramycin, ceftiofur sodium, and amoxicillin had IC50 >25 mg/mL for at least one cell line, representing potentially less cytotoxic alternatives. Further studies are necessary to extrapolate these data to the *in vivo* joint environment, but these findings suggest that antimicrobial selection and dosing approaches based on achieving drug concentrations between MIC and IC50 may allow equine practitioners to minimize damage to cartilage and synovial tissues if antibiotics are injected by intra-articular route.[16]

Table 1.1: Summary of *in vitro* studies investigating the effects of antibiotics on equine joint tissues.

Investigator	Antibiotic	Dose	Cell type(s)	Outcome Parameters	Main Findings
Davenport *et al.* 2000	Enrofloxacin	1-500 µg/mL	Chondrocytes	Glycosaminoglycan synthesis, degradation	Enrofloxacin had detrimental effect on cartilage metabolism of neonates.
			Cartilage explants	Glycosaminoglycan content	No effect on mRNA, GAG degradation or GAG content.
			(adults, neonates)	mRNA (aggrecan, type-II collagen, biglycan, decorin, link protein, MMP 1,3,13, tissue inhibitor of metalloproteinase 1)	
Egerbacher *et al.* 2000	Enrofloxacin	10-150 µg/mL	Chondrocytes	Cell viability	Cells cultured in quinolone-supplemented media had reduced ability to adhere.
	Ciprofloxacin			Morphology via electron microscopy	Cell shape, actin and vimentin cytoskeleton changed concentration dependently.
				Attachment to collagen II coated slides	Quinolones induced irregular integrin signaling and subsequent cellular changes.
Bolt *et al.* 2007	Amikacin sulfate	25 mg/mL	Cartilage explants	Glycosaminoglycan content	Amikacin treatment increased empty lacunae in non-LPS challenged explants.
				Histomorphometric quantification lacunae	Co-culture with triamcinolone attenuated toxic effects of amikacin.
					GAG content did not differ with treatment or LPS challenge.
Mochal-King *et al.* 2019	Amikacin sulfate	25, 2.5, 0.25 mg/mL	Chondrocytes	Cell viability (MTT assay)	Amikacin induced 90% cell death at 25 mg/mL.
	Enrofloxacin	10, 1, 0.1 mg/mL		PGE2 production	Enrofloxacin induced 98% cell death at 10 mg/mL, 90% cell death at 1.0 mg/mL.
					Enrofloxacin and LPS induced PGE2 production at all concentrations.
					Induction of PGE2 by enrofloxacin was reduced by pre-treatment with soybean/ glucosamine/chondroitin combination
Pezzanite *et al.* 2020a	Amikacin sulfate	0.0003-25mg/mL	Synoviocytes	Cell viability	Amikacin is cytotoxic to equine joint cells (rapid, dose-dependent, pH independent).
			Chondrocytes	Cell proliferation	Amikacin induced cell death occurs primarily by apoptosis.
			Synovial explants	Cell metabolism	Amikacin induced cell death was not mitigated by presence of synovial fluid.
			Cartilage explants	Mechanism of cellular death	
Pezzanite *et al.* 2020b	Amikacin sulfate	0.39-25mg/mL	Synoviocytes	Cell viability	Antibiotics from different antimicrobial classes expressed dose-dependent but variable cytotoxicity to equine joint cells in vitro.
	Amoxicillin		Chondrocytes	Inhibitory concentration 50 (IC50)	
	Ampicillin sulbactam		Cartilage explants		Aminoglycosides and doxycycline had lowest IC50 (most toxic).
	Cefazolin				Ampicillin sulbactam, imipenem, tobramycin, ceftiofur sodium, amoxicillin had higher IC50 > 25mg/mL for at least one cell line, representing potentially less cytotoxic alternatives.
	Ceftazidime				
	Ceftiofur sodium				
	Doxycycline				
	Enrofloxacin				
	Florfenicol				
	Gentamicin				
	Imipenem				
	Neomycin				
	Potassium penicillin				
	Tobramycin				
	Vancomycin				

In vivo and pharmacokinetic studies in horses **(Table 1.2) –** Transient cytotoxicity of intra-articularly injected antibiotics, which differed between drug classes, has been previously noted in several equine studies.[17-20] Aminoglycosides (amikacin, gentamicin, tobramycin) are the most commonly used IA antibiotics in equine practice,[1,5] and have been the most thoroughly investigated.[17-22] Perhaps the most commonly cited rationale for use of IA antibiotics is in conjunction with polysulfated glycosaminoglycans (PSGAGs),[1] which have been reported to potentiate the infectivity of *Staphylococcus aureus* in horses with experimentally induced septic arthritis.[23] Intra-articular injection of amikacin (125 mg) immediately after inoculating the joint with *S. aureus* was subsequently shown to significantly reduce potentiation of infection by PSGAGs.[24] However, all studies investigating aminoglycosides have reported some degree of joint toxicity in horses.[17-22] Gentamicin sulfate injected IA caused loss of synovial lining cells, increased synovial oedema and leukocyte infiltration and higher refractive indices and increased

numbers of red and white blood cells.[18] Continuous infusion of gentamicin also resulted in loss of synovial intimal cells from villi lining the joint.[19] Repeated injection of amikacin, as would be typical in treatment of septic arthritis, induced elevations of both nucleated cell counts and total protein to within the range considered typical for septic arthritis.[17] Finally, both tobramycin and cefovecin sodium induced mild chemical synovitis evidenced by increased nucleated cell counts and total protein in synovial fluid.[21,25] The long-term effects of antibiotic induced synovitis have not been evaluated at this time to the authors' knowledge (i.e. it is unknown to what extent IA antibiotic usage may contribute to long-term progression of osteoarthritis within the injected joint).

Pharmacokinetic distribution of antibiotics following IA injection has been explored for aminoglycosides, ceftiofur sodium, and cefovecin sodium.[20-22,25-28] When the IA route of administration was compared to regional limb perfusion, gentamicin (one gram injected by either route) reached higher peak concentrations in synovial fluid following IA injection, but did not differ in serum or bone.[27] Amikacin concentrations were lower and distribution of amikacin (500mg) from radiocarpal joints occurred more rapidly (48 versus 72 hours) from endotoxin-inflamed versus normal joints.[28] The effect of buffering of the pH of gentamicin was evaluated to potentially decrease antibiotic-induced synovitis and buffering was not found to affect mean peak synovial fluid concentrations in synovial fluid.[26] Tobramycin (240 mg in the middle carpal joint) and ceftiofur sodium (150 mg in the radiocarpal joint) maintained concentrations above MIC for most common equine pathogens (4 µg/mL) for 48 and 24 hours, respectively.[20,21] Cefovecin sodium (240 mg in the radiocarpal joint) remained above 1 µg/mL in synovial fluid for over 24 hours.[25]

The co-authors recently further investigated and compared the effects of IA amikacin injected at a range of doses on synovial fluid drug concentrations and biomarkers of cartilage injury and inflammation using horse models *in vivo*.[22] Healthy horses (n=3/group) were administered amikacin (500 mg, 125 mg or 31.25 mg) or lactated ringers solution in the tarsocrural joint, and synovial fluid samples were obtained at multiple time points (0,1,2,4,8, and 24 hours) and assessed for drug concentrations and biomarkers of inflammation and cartilage degradation. Amikacin concentrations remained greater than or equal to the minimum inhibitory concentration (4 μg/mL) for most common equine pathogens for at least 24 hours following injection of all doses of amikacin assessed. Intra-articular administration of amikacin induced dose-dependent increases in cartilage degradation products (C2C, C12C) and biomarkers of inflammation (IL1-β, TNFα). White blood cells, especially neutrophils, were found to be more sensitive to amikacin-induced apoptosis than synoviocytes or chondrocytes, evidenced by lower determined IC50s. In summary, these studies revealed that several major classes of antibiotics induced apoptotic pathways resulting in cell death of joint cells *in vitro*, and cartilage damage was confirmed in an equine model of IA injection of amikacin. Long-term evaluation of horses for progression of lameness associated with the injected joint and radiographic evidence of osteoarthritis would strengthen clinical translatability of these findings. Further induction of leukocyte apoptosis by locally injected antibiotics may serve to mask the inflammatory effects of antibiotic induced articular damage. These findings highlight the potential for off-target cytotoxic effects of high-dose antibiotics administered locally to joints or other tissue sites.

In contrast to reported findings with aminoglycosides, no differences in histologic scoring of synovium and cartilage or synovial fluid cytological parameters were observed following ceftiofur sodium (150 mg) injection in the antebrachiocarpal joint. Taken together, these studies

support the *in vitro* findings that cytotoxicity differs between antibiotic classes and therefore may be mitigated by consideration of drug selection and dose.[16] Further evaluation and comparison of antibiotic toxicity between classes *in vivo* is indicated. In addition, it may be important to note that simultaneous administration of other medications may mitigate the toxic effects of antibiotics in the joint, as would be the case in prophylactic administration of antibiotics in routine joint injections. Bolt *et al.* demonstrated *in vitro* that diluted triamcinolone and hyaluronic acid supported chondrocyte morphology in culture and protected chondrocytes from the toxic effects of LPS, amikacin and mepivacaine.[13] More recently, Mochal-King *et al.* demonstrated that an avocado/soybean/glucosamine/chondroitin (ASU + GLU + CS) supplement reduced prostaglandin production by antibiotic-exposed chondrocytes and concluded that horses routinely receiving antibiotic injections intra-articularly may benefit from administration of oral supplements.[14] These studies warrant further *in vivo* investigation.

In summary, and although further work is indicated, guidelines for dosing strategies for IA antibiotic use may be drawn from these studies to assist practitioners in clinical decision making. Little evidence exists to justify prophylactic antibiotic usage in equine joint injections, including in ambulatory practice, with the exception of use with PSGAGs.[2-5] Co-administration of other medications in the joint may mitigate the cytotoxic effects of antibiotics in joints, but warrants further evaluation in live horses.[13,14] Alternative antibiotics to the commonly injected aminoglycosides are less cytotoxic to cells of the joint *in vitro* and may be administered to achieve higher local concentrations with less risk for induction of cartilage degradation and inflammation.[15,16,22] In cases of septic arthritis, IA antibiotic selection would ideally be based on culture and sensitivity of the organism involved when available and dosed based on volume of joint injected. In lieu of a positive culture or while waiting for culture and sensitivity results to

become available, the authors do not advocate for delaying local antibiotic therapy, but suggest that antibiotic selection may be guided by bacterial isolates identified on Gram staining as well as the previously cited information on relative toxicity of different antibiotics to minimize local toxicity in the interim. Studies to further evaluate relative antibiotic toxicity *in vivo* and in the presence of osteoarthritis are warranted.

Table 1.2: Summary of *in vivo* studies investigating the effects of antibiotics on equine tissues.

***In vivo* Investigations of Cytotoxicity**						
Investigator	**Antibiotic**	**Dose**	**Horses**	**Joint**	**Outcome Parameters**	**Main Findings**
Stover *et al.* 1985	Gentamicin	150 mg	n=30, normal	radiocarpal	Synovial fluid (SF) analysis	Gentamicin injected joints had greater turbidity,
					Gross evaluation SF, synovium	higher RBC and WBC counts, higher refractive indices
					Microscopic evaluation SF, synovium	Microscopic changes in gentamicin injected joints included
						edema, leukocytic infiltration, loss of synovial lining cells
Gustafson et al. 1989	Amikacin sulfate	125 mg	n=8, infected	midcarpal	Evidence of septic arthritis	Intra-articular amikacin injected immediately after inoculating
				infected	Clinical signs	joint with *S. aureus* decreased potentiation of infection by
				(*S. aureus*)	Synovial fluid analysis	polysulfated glycosaminoglycans.
					Gross evaluation synovium	
					Histopathologic evaluation synovium	
Mills *et al.* 2000	Ceftiofur sodium	150 mg	n=6, normal	radiocarpal	Synovial fluid differential cell counts	Cytologic characteristics of synovial fluid did not differ
					Synovial fluid pH	between joints injected with ceftiofur sodium vs. saline.
					Synovial fluid total protein concentration	Minimal gross or microscopic differences were noted.
					Synovial fluid mucin precipitate quality	
Lescun *et al.* 2002	Gentamicin	continuous	n=6, normal	tarsocrural	Histologic scoring cartilage, synovium	No significant differences in histologic scoring of synovial
		infusion 5 days			Gross evaluationjoints	membranes between GM- and BES- treated joints.
						Loss of synovial intimal cells were noted in gentamicin
						treated joints.
Sanchez Teran	Amikacin sulfate	500 mg	n=5, normal	midcarpal	Serum and synovial SAA	Repeated IA administration amikacin caused increased levels
***et al.* 2012**		every 48h			Synovial fluid TNCC and differential counts	TP and NCC in synovial fluid.
		5 times			Synovial fluid total protein concentration	Synovial SAA concenrations did not increase in either group
						following repeated amikacin or arthrocentesis alone.
						Synovial SAA may be more reliable marker than TP and NCC
						to evaluate joint for sepsis previously treated with amikacin.
Newman *et al.* 2013	Tobramycin	240mg IA	n=6, normal	midcarpal	Synovial fluid TNCC, TP, cytology	Tobramycin resulted in mild chemical synovitis evidenced by
					Histopathologic scoring	increased cell count and total protein.
Perez-Nogues	Cefovecin sodium	240mg IA	n=6, normal	radiocarpal	Synovial fluid pH, TNCC, TP	Cefovecin sodium resulted in significant increases in TP concentrations
***et al.* 2017**						at 5 minutes and TNCC at 30 minutes following injection.
Pezzanite *et al.* 2020	Amikacin sulfate	31.25 mg	n=6, normal	tarsocrural	Synovial fluid TNCC, TP, WBC differential	No differences were seen in SF parameters between treatments.
		125 mg			Biomarkers collagen degradation (C2C, C12C)	Amikacin induced dose-dependent differences in biomarkers of
		500 mg			Biomarkers inflammation (23 cytokines)	inflammation (TNFα, IL1-β) and cartilage degradation (C2C, C12C)
Pharmacokinetic Evaluations						
Investigator	**Antibiotic**	**Dose**	**Horses**	**Joint**	**Outcome Parameters**	**Main Findings**
Lloyd *et al.* 1988	Gentamicin	150 mg IA	n=6, normal	radiocarpal	Synovial fluid concentrations gentamicin	Mean peak synovial fluid concentrations after IA administration
	(buffered or not)				over 24h	were not different between buffered vs. unbuffered.
Mills *et al.* 2000	Ceftiofur sodium	150 mg IA	n=6, normal	radiocarpal	Serum and synovial fluid ceftiofur sodium	Synovial concentrations remained >MIC over 24h
					levels at 12 time points over 24h	Synovial half-life after IA injection was 5.1 hours.
Werner *et al.* 2003	Gentamicin	1 g IA vs. RLP	n=12, normal	metacarpo-	Bone, synovial fluid and serum	Mean peak synovial fluid concentrations were higher when
				phalangeal	gentamicin levels (1,4,8,12,24h)	administered IA vs. regional limb perfusion (RLP).
					when administered IA versus RLP	No differences in serum or bone gentamicin concentrations
						were seen with gentamicin administration IA versus RLP.
Taintor *et al.* 2006	Amikacin sulfate	500mg IA	n=6, normal	radiocarpal	Amikacin synovial fluid levels every 24h	Amikacin concentrations were decreased in inflamed joints
			vs. inflamed	normal or	over 120h	compared to normal joints.
				inflamed		Mean synovial fluid concentrations remained >MIC for sensitive
				(endotoxin)		organisms in normal joints 96h, inflamed joints 48h.
Newman *et al.* 2013	Tobramycin	240mg IA	n=6, normal	midcarpal	Tobramycin synovial fluid levels	Tobramycin persisted up to 48h in synovial fluid at 14.8ug/mL
					(0,0.5,1,2,4,24h)	Synovial fluid concentrations were 3056 ug/mL at 30 minutes
						following administration.
Perez-Nogues	Cefovecin sodium	240mg IA	n=6, normal	radiocarpal	Cefovecin sodium synovial fluid, plasma levels	Duration cefovecin above MIC 1ug/mL was 28.8 +/- 2.58 h in SF and
***et al.* 2017**					(0,5,15,30min and 1,2,4,8,12,24,36,48,72h)	16.0 +/-2.86h in plasma
Pezzanite *et al.* 2020	Amikacin sulfate	31.25 mg	n=3, normal	tarsocrural	Synovial fluid amikacin levels	Amikacin concentrations reached >10x MIC and remained
		125 mg			(0,0.5,1,2,4,8,24)	>MIC for all doses injected for at least 24h.
		500 mg				

Abbreviations: Intra-articular (IA); Minimum inhibitory concentration (MIC); Serum amyloid A (SAA); Synovial fluid (SF); Total nucleated cell count (TNCC), Total protein (TP).

1.4 Review of intra-articular antibiotic administration in people

Evaluating the use of intra-articular antibiotics in veterinary practice may lead to more frequent local antibiotic use in people. Intra-articular antibiotic use has gained more recent attention in human orthopedic practice, particularly in the treatment of implant infections following prosthetic arthroplasty.[29-34] Periprosthetic joint infection can be a devastating complication of joint replacement in humans leading to expensive and prolonged treatments, although the overall incidence remains relatively low at approximately 0.3-1.7% for total knee replacements (TKA) and 0.8-1.9% for total hip replacements (THA).[35-37] Antibiotic bone cements are also used to achieve high local concentrations in fracture repairs, which may involve intra-articular tissues.[38]

In vitro studies using human tissues - *In vitro* investigations of antibiotics with human skeletal cells for local delivery systems have reported that concentrations achieved clinically with antibiotic bone cements may cause skeletal cell toxicity and actually delay or prevent tissue and fracture healing.[38] Chondrocytes and osteoblasts were treated with ciprofloxacin, vancomycin, and tobramycin and assessed for changes in cellular morphology, proliferation, and numbers based on lactate dehydrogenase ratios.[38] Ciprofloxacin induced changes in cell membrane, spread and extension, and all three antibiotics investigated induced dose-dependent decreases in cellular proliferation (ciprofloxacin at doses >100 μg/mL; vancomycin and tobramycin at doses >2000 μg/mL). Osteoblasts released significantly increased lactate dehydrogenase ratios when exposed to all three antibiotics, indicating tissue damage. Taken together, these findings demonstrate the potential for local cellular toxicity with antibiotics and suggest that achieving a balance between reaching microbicidal levels while minimizing toxicity is critical for cell survival and function.

Dogan *et al.* further assessed the cytotoxicity of three antibiotics (vancomycin, teicoplanin, linezolid) commonly used *in vitro* against *Staphylococcus aureus* to chondrocytes obtained from human gonarthrosis patients.[39] In this study, dosing of antibiotics was based on the practiced minimum bactericidal values for each antibiotic with *S. aureus* (vancomycin 16 mg/L, teicoplanin 64 mg/L, and linezolid 32 mg/L), representing much lower concentrations than those achieved with doses commonly used in equine practice. At the single antibiotic dose assessed for each drug, no differences in cell viability following antibiotic exposure were noted based on MTT assay.[39] These findings provide further evidence to guide dosing strategies in veterinary practice, demonstrating that antibiotic doses may be significantly reduced compared to that commonly reported clinically in horses to decrease skeletal cell toxicity while achieving relevant levels following injection.[39]

Clinical studies in human patients - Clinical reports of direct intra-articular injection or local delivery of antibiotics via implantable pumps or catheters have been described following total knee and hip arthroplasties (TKA and THA, respectively) in humans.[29-34,40,41] Direct infusion of antibiotics with pumps or indwelling catheters have been used successfully in several case studies to salvage acutely and chronically infected total joint arthroplasties.[32,40,41] Davenport *et al.* reported obtaining antibiotic concentrations in joints of 8 to 10x MIC in all cases treated. Successful resolution of infection in acutely infected total joint arthroplasties (9 hips, 13 knees) was achieved in 17/20 (85%) patients for at least 30 months following treatment.[41] Antibiotic selection and dose were based on organism identification and culture and sensitivity.[41] Perry *et al.* reported treatment of an additional twelve patients with acutely infected arthroplasties (symptoms present less than ten weeks) with amikacin delivered intra-articularly using an implantable pump.[32] Intra-articular levels of amikacin obtained from eight patients ranged in

concentration from 150 to 1688 µg/mL, while systemic levels remained below 10 µg/mL, and resolution of infection was achieved in 10/12 patients (83%).[32] These initial retrospective reports of efficacy prompted further evaluation to optimize timing and dosing of intra-articular antibiotic use in treating infected arthroplasties.

The pharmacokinetics of vancomycin injected intra-articularly (500 mg every 24 hours) to treat five patients with established TKA infections of greater than three months were assessed by Peppers *et al.* for six weeks following using Hickman catheters inserted intra-articularly.[34] Vancomycin reached levels well above the minimum inhibitory concentration (MIC) effective against MRSA (2-4µg/mL) with similar elimination rate from synovial fluid between patients and an average half-life of 3.4 hours, indicating daily injection appears to be effective to achieve and maintain therapeutic levels in infected TKA, although seeking data from additional patients was warranted. Vancomycin concentrations in synovial fluid following direct intra-articular (IA) versus intravenous (IV) infusion were further compared by Roy *et al*., demonstrating that while both routes of injection achieved therapeutic levels in synovial fluid, IA delivery resulted in peak levels several orders of magnitude higher and remained above MIC for at least 24 hours, in contrast to synovial fluid levels achieved with IV dosing, which would likely become subtherapeutic after 6 hours.[33] The authors concluded that the addition of IA antibiotics to treatment protocols offer advantages over IV administration or local antibiotic-impregnated cement in maintaining therapeutic doses for prolonged periods to treat localized infection. The same group of investigators further reported the use of direct infusion of antibiotics to treat one-stage revision TKA and THA, as well as reinfected failed two-stage TKA revisions with multi-drug resistant organisms.[29-31] One stage revision and six weeks of intra-articular vancomycin injections controlled infection in 17/18 (94%) patients with MRSA infected TKA and 20/21

(95%) patients with chronically infected THA.[29,31] Finally, 17/18 patients undergoing revision of failed two-stage revision TKA, where amputation of the limb is often considered the only alternative, experienced control of infection following six weeks of vancomycin infusion, with substantial improvement in mean Knee Society scores.[42]

These case studies demonstrated efficacy of intra-articular antibiotic use in the treatment of recalcitrant drug-resistant septic arthritis, particularly those involving implant biofilms following total joint replacements, using doses based on MIC of pathogens identified. Of importance to note is that human studies to date investigating intra-articular antibiotic use have centered around post-arthroplasty TKA/THA cases following removal of cartilage from the joint, in contrast to veterinary practice where the native cartilage is intact. These findings, demonstrating efficacy of lower doses than those typically used in equine veterinary practice even in the face of orthopedic implants, provide further justification for consideration and optimization of dosing strategies in veterinary practice.

1.5 Risks of intra-articular injections

Despite the demonstrated benefits described in treatment of septic arthritis, a balanced review of intra-articular antibiotic usage includes several potential risks. First, the full extent of antibiotic toxicity to synovial and articular cartilage tissues and long-term possibility for increased development or exacerbation of osteoarthritis following IA injection has not been fully determined despite transient cytotoxicity to joint tissues being well-documented in horses in multiple studies.[17-19,22] An additional consideration is the relative cost of intra-articular versus prolonged oral or systemic antibiotics. Although in many cases, the addition of the IA route of administration may shorten treatment time by achieving higher concentrations than would be

possible via other routes, this course of treatment may also result in increased cost to the client overall and more intensive management with hospitalization compared to oral medications alone administered by the owner at home. Furthermore, although the risk of infection following a single IA injection is admittedly low, repeated injections for treatment of septic arthritis may also carry an increased risk of re-introduction of infection and prolonged inflammation.

Finally, the rising incidence of multi-drug resistant infections is increasingly documented globally as an emerging threat in both equine[43-53] and human clinical practice, with orthopedic and joint surgery being affected similarly to other specialties.[54-58] These trends prompt re-evaluation of antibiotic protocols in general, including local injection or use in intra-synovial lavage, as inappropriate dosing may further induce greater development of resistance with potentially significant detrimental ramifications for both horses and staff in equine veterinary hospitals.[53] Multi-drug resistant bacterial isolates have been shown to be more frequently cultured from horses previously undergoing procedures or hospitalization and those treated with antibiotics.[50,53] While the prophylactic use of antibiotics is widely practiced, their overuse and misuse threaten to undermine their therapeutic value and continued successful implementation.[46,49] It is acknowledged that the development of septic arthritis may in some instances necessitate euthanasia of the horse or result in reduced athletic function, which is used as rationale by some practitioners for prophylactic antibiotic use. However, an individualized therapeutic approach would consider trends and reported resistance patterns in pathogens most frequently encountered in different species affected by septic arthritis and antimicrobial susceptibilities to improve clinical outcomes through a multidisciplinary approach.[56] *Staphylococcus* and *Streptococcus spp.* are most commonly associated with septic arthritis in humans.[55-57] In equine patients, *Enterobacteriaceae spp.* are more commonly cultured from joint

infections resulting from penetrating wounds while Staphylococcus *spp.* are more frequently identified in cases of iatrogenic septic arthritis following joint injection.[59] In one recent retrospective evaluation of septic synovial structures in horses, multi-drug resistance and presence of gram-negative bacteria species were associated with decreased survival rates.[60] The increasing importance of methicillin-resistant *staphylococci*, vancomycin-resistant *enterococci*, and multidrug resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa* have been reported across species and are of growing concern in treating bacterial infections across species.[45-46,48,49,51,54,55,58] These findings should prompt balanced consideration of the risks and benefits of IA antibiotic selection and dosing strategies with treatments tailored to individual cases and ideally based on antibiotic sensitivities.

1.6 Supplemental intra-articular therapies to antibiotics to treat joint infection

The increasing incidence of antibiotic resistance has driven investigation of novel biological therapies with antimicrobial properties, including platelet rich plasma lysate and mesenchymal stromal cells.[60-65] Recent literature describes formation of free-floating biofilm aggregates by *Staphylococcus aureus* and other gram-positive and -negative isolates that are most frequently reported to be involved in human septic arthritis (*Streptococcus zooepidemicus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) in synovial fluid, rendering them more tolerant to antibiotic therapy compared to their planktonic form.[61] The equine model of septic arthritis and availability of equine synovial fluid for use in *in vitro* assays makes the horse a valuable translational model to study the host-pathogen interactions in human septic arthritis as well, compared to the more difficult to source synovial fluid from healthy human individuals.[61,62] The antimicrobial and immunomodulatory activity of several potential biological therapy candidates

to improve outcomes in treatment of septic arthritis as supplemental treatments to antibiotics are further described.

Platelet lysate - Autologous platelet rich plasma (PRP) has been described previously in the veterinary and human literature, most commonly for the treatment of musculoskeletal disorders including tendon and ligament injuries and osteoarthritis and more recently in the context of treating bacterial infections.[66-70] Platelet rich plasma lysate (PRP-L) represents an acellular alternative to PRP, making allogeneic use an option and containing platelet bioactive factors including antimicrobial peptides such as defensin.[71] Gilbertie *et al.* demonstrated that lysis of platelets and pooling of lysates from multiple horses enhanced antimicrobial activity, indicating that removal of the cellular components of PRP may mitigate the ability of bacteria such as *S. aureus* to exploit platelets as virulence factors.[72] Pooled PRP-L from multiple donors exhibited synergism with amikacin against biofilm aggregates in synovial fluid *in vitro*, with greater activity against gram positive bacterial isolates.[61] Fractionation of the pooled PRP-L product identified the bioactive component to be low in molecular weight (<10kDa) and cationic. *In vivo* studies evaluating the platelet lysate product in an induced model of S. aureus tarsocrural septic arthritis demonstrated reduced quantitative bacterial counts in synovial fluid, and lower pain scores and levels of inflammatory proteins in horses treated with PRP-L and amikacin versus amikacin alone.[73]

Mesenchymal stromal cells - Mesenchymal stromal cells (MSC) exert potent immunomodulatory and antibacterial activities, making them attractive as biological therapies for diverse conditions, including musculoskeletal injuries, wound healing, and bacterial infections.[63,65,74-79] Equine MSC were initially demonstrated to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* through antimicrobial peptide secretion and depolarization of

cell membranes.[64] Their application in the treatment of infection is gaining increasing attention and methods to optimize antibacterial activity have been further explored. Cortes-Araya *et al.* compared tissue sources for MSC culture (endometrium, adipose, and bone marrow), demonstrating that MSC from all three origins expressed both direct and indirect antimicrobial properties, expressing monocyte chemoattractant protein-1, IL-6, IL-8, toll-like receptor (TLR)-4 and chemokine ligand-5.[65] Exposure of MSC to TLR-4 agonist lipopolysaccharide (LPS) induced increased expression of cytokines by MSC of all tissue origins, indicating that immunomodulatory properties of MSC may be further upregulated in the presence of bacterial components in infection *in vivo.*[65]

The concept of immune activation has been further investigated by exposing MSC to toll-like and nod-like receptor agonists to enhance antibacterial properties prior to *in vivo* injection,[63,74] with demonstrated increased bacterial killing and secretion of immunomodulatory cytokines with both human and equine cells *in vitro.*[63,74] Immune activated cellular therapy (MSC co-cultured with TLR-3 ligand polyinosinic:polycytidylic acid (pIC)) in addition to antibiotic therapy resulted in synergistic enhanced clearance of bacteria in murine models of implant infection and pet dogs with spontaneous multi-drug resistant infections.[74-76] Case controlled studies investigating the use of pIC activated MSC in the treatment of equine multi-drug resistant *Staphylococcal* septic arthritis are ongoing by this group of investigators at the time of this report. Finally, packaging of conditioned medium from MSC in core-shell hydrogel microcapsules was recently described and may represent a viable method to deliver bioactive factors secreted by MSC integral to tissue healing while avoiding the legislative constraints of cellular products.[77]

In summary, integration of biological approaches such as platelet lysate and mesenchymal stromal cell therapy may offer adjunctive therapies to antibiotics to improve clinical outcomes, particularly in the treatment of multi-drug resistant septic arthritis, without adding to the potential local cytotoxicity with intra-articular antibiotic injections or contributing to antibiotic resistances.

1.7 Conclusions

Antibiotic usage in equine veterinary practice deserves reconsideration in general due to trends in multidrug resistance. Intra-articular antibiotics have been used off-label in horses for decades without definition of appropriate doses or demonstration of efficacy in preventing infection when used prophylactically. While achieving high local concentrations may be advantageous in the treatment of septic arthritis while minimizing side effects of systemic antibiotic administration, more recent data suggests that if antibiotics are used intra-articularly, consideration of selection and dose may minimize potential cytotoxicity to the joint. Additional work, including both experimental studies and large-scale, multicenter, randomized clinical trials are needed to completely address the knowledge gaps still remaining regarding local antibiotic use. In summary, injection of intra-articular antibiotics carries pros and cons that should be weighed by clinicians when using this route of administration.

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CHAPTER 2: Amikacin induces rapid, dose-dependent apoptotic cell death in equine chondrocytes and synovial cells *in vitro* [ii]

2.1 Summary

Equine veterinarians frequently inject aminoglycoside antibiotics intra-articularly, either to treat septic arthritis or for prophylaxis with other medications when injecting joints for osteoarthritis. Although aminoglycosides have been demonstrated to be toxic to equine mesenchymal stem cells (MSC), their effects on resident joint cells have not been previously investigated. Moreover, safe and effective intra-articular doses have not been defined. The objectives of this study were to determine effects of concentration, duration of exposure, pH, and the presence of synovial fluid on the cytotoxic effects of amikacin on equine chondrocytes, synoviocytes, and bone-marrow and adipose-derived MSC. Four cell types were harvested from three donor horses and plated in triplicate wells for 48h prior to the addition of amikacin. The effects of amikacin on cell viability were assessed for different exposure times, concentrations, and with pH buffered or unbuffered in media, as well as in the presence of synovial fluid. Cell metabolism/viability was assessed by colorimetric MTT assay. Cell proliferation was assessed by live cell imaging. Cell viability was assessed using trypan blue and dimeric cyanine nucleic acid stain (yoyo-1). To determine the mechanism of cell death, apoptosis was evaluated using Annexin V and 7AAD staining with flow cytometric quantification. Induction of apoptotic cell death pathways was assessed using caspase3 expression. Amikacin was demonstrated to be cytotoxic to equine joint cells and MSC in a rapid, dose-dependent, pH-independent manner, which occurs primarily by

[ii] This chapter includes the complete published manuscript: Pezzanite L, Chow L, Soontararak S, Phillips J, Goodrich L, Dow S. Amikacin induces rapid dose-dependent apoptotic cell death in equine chondrocytes and synovial cells in vitro. Equine Vet J. 2020;52:715-724. This article was reproduced with permission from John Wiley and Sons, Inc., Hoboken, NJ.

apoptosis. Amikacin cytotoxicity was not mitigated by the addition of synovial fluid *in vitro*. Further studies are necessary to determine whether these *in vitro* results predict joint injury in live animal models. Amikacin at clinically applied doses induces rapid, pronounced cell death of equine joint cells.

2.2 Introduction

Equine veterinarians frequently inject antibiotics intra-articularly (IA), either in the treatment of septic arthritis or prophylactically when injecting other medications in osteoarthritis. The incidence of septic arthritis following joint injection in horses is <0.1% (range 0.0002-0.092%) across three large retrospective studies.[1-3] Concurrent administration of antibiotics with injections was not identified in these studies as a factor that reduced the risk of development of joint infection. Despite these findings, prophylactic use of intra-articular antibiotics remains prevalent among practitioners. In one recent survey of equine veterinarians, 46% of practitioners reported combining antimicrobial agents with any IA therapy, and an additional 32% of practitioners used antibiotics in some situations, most commonly when injecting polysulfated glycosaminoglycans or corticosteroids.[4]

Aminoglycoside antibiotics, such as amikacin, are commonly used IA due to their broad spectrum of antimicrobial activity. However, aminoglycosides as a class exert direct cytotoxicity against mammalian cells *in vitro*, including cultured human and equine bone-marrow-derived mesenchymal stem cells (MSC) and equine chondrocytes.[5-10] Aminoglycosides are toxic to cultured MSC, over a wide range of concentrations.[6,8,9] Incubation of bone-marrow-derived MSC with other classes of antimicrobials (e.g. penicillins) did not exert the same direct cytotoxicity to MSC.[8] Based on these data, mixing of aminoglycoside antimicrobials with cultured MSC prior to

therapeutic use was not recommended.[8] However, aminoglycosides are still frequently injected following treatment with other intra-articular medications,[4] ostensibly for the purpose of preventing iatrogenic joint infections, though the rarity of this event does not justify routine prophylaxis.

The primary focus of this study was to evaluate the toxic effects of amikacin[a] on joint cells and to compare that to the toxicity of amikacin for MSC. Specifically, the objectives were to evaluate the effects of amikacin concentration, time of exposure and pH on viability, proliferation and metabolism of native joint cells (chondrocytes and synovial cells) and to compare relative toxicity to MSC (bone-marrow (BMD-MSC) and adipose derived (AD-MSC) by determining the inhibitory concentration 50, or concentration of amikacin at which 50% of cells were viable for each of the four cell lines assessed. Secondary objectives were to determine whether synovial fluid mitigated the cytotoxic effects of amikacin, and to define the mechanisms by which amikacin induces cytotoxicity. We hypothesized that amikacin is toxic to normal joint cells at concentrations achieved in joint injections and at the same concentrations where toxicity to MSC have been observed.

2.3 Materials and Methods

Tissue collection and cell culture. Tissues (chondrocytes, synovial cells, adipose and bone marrow derived MSC) were harvested post-mortem from each of 3 donor horses, aged 2 to 4 years old, euthanized for other reasons and without evidence of osteoarthritis. Synovial fluid was collected aseptically and frozen at -80°C for future use. Following isolation and culture, cells were frozen after the first passage at concentrations of $5x10^6$cells/ml and stored in liquid nitrogen until ready for use.

To isolate MSC, the sternum and tail-head of donor horses were clipped and aseptically prepared. Bone marrow aspirate (5 mL) was obtained from the sternum using jamshidi into a syringe containing 1ml heparin (10,000 U). Adipose tissue (3 to 5 grams) was obtained from the tail-head with scalpel blade and placed in a 50ml conical tube containing phosphate-buffered saline (PBS) with penicillin/streptomycin and antimycotics. To obtain synovial cells and chondrocytes, the femoropatellar joints were aseptically prepared and tissues obtained using scalpel blade. Cartilage and synovial tissues were maintained on ice in PBS with antibiotics/antimycotics until digestion.

Cartilage samples were minced and digested overnight in complete supplemented Dulbecco's Modified Eagle's Medium (DMEM) (10% FBS, antibiotic/antimycotic, 1M HEPES) with type 2 collagenase[b] (0.75 mg/ml or 7.5 mg collagenase per gram 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 (70 uM and 40 uM), centrifuged at 400g for 10 minutes and cells counted. Cells were frozen in 95% fetal bovine serum, 5% dimethyl sulfoxide at $10x10^6$cells/ml and stored in liquid nitrogen until use.

Synovial samples were digested as described for chondrocytes for 4 hours at 37° C with 5% CO_2, then filtered, centrifuged at 400g for 10 minutes, and counted. Synoviocytes were plated in complete DMEM media at 10,000 to 15,000 cells per cm^2, maintained until uniform in appearance at second passage, then frozen at $5x10^6$cells/ml in liquid nitrogen until use.

Adipose samples were rinsed twice in PBS, minced and placed in 50ml conical tube in complete DMEM media with type 1 collagenase[b] (1 mg/mL in complete DMEM media, 10 mL per gram tissue digested). Samples were digested shaking 6 hours at 37°C. Tissues were passed through 70 uM cell strainer, centrifuged at 400g 10 minutes and counted. Adipose MSC were

plated in complete DMEM media at $3.5x10^3$ per cm^2 until confluent then frozen at $5x10^6$ cells/mL in liquid nitrogen until use.

Bone marrow aspirates were centrifuged by ficoll[c] density separation at 400xg 18 minutes to pellet red cells as previously described.[11,12] Bone-marrow-derived MSC were plated at 10,000 cells/cm^2 and expanded in culture for five to seven days then frozen at $5x10^6$ cells/mL in liquid nitrogen until use.

Cells were thawed quickly in a 37°C water bath and recovered for 48 hours under standard incubation conditions (37 C with 5% CO_2). For all experiments, cells were plated in triplicate in complete DMEM media on 96 well plates at 30,000 cells/well for 48 hours prior to the addition of amikacin. The effect of amikacin was assessed for different exposure times, concentrations in media, with pH buffered or unbuffered, and in the presence of synovial fluid or not. Plates were stored in the incubator (37°C with 5% CO_2) following addition of amikacin for all experiments.

Effect of amikacin dose and media pH on cell viability and metabolic activity. Cell viability based on permeability was assessed for four cell types at multiple concentrations by counting cells that exclude trypan blue by automated cell counter[d] to determine percentage of live cells following amikacin exposure for 24 hours. Dose response for each concentration was normalized to control, and the data was transformed to "normalized dose response vs. log10(concentration)" at which point the IC50 was estimated by nonlinear regression implemented in GraphPad Prism8[e].

The effect of amikacin (25 mg/mL, unbuffered, in complete DMEM media) on four cell types from three donors was assessed for different exposure times (1,2,3,6,12 hours) using colorimetric MTT assay[f] (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at

baseline and each of the time points evaluated. The MTT assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells. MTT stock solution[f] (10 µl, 5 mg/mL) is added into treatment wells containing 200 µl solution on a 96-well plate. Wells are mixed thoroughly and incubated for 3 hours. At that time, MTT solubilization solution[g] is added and wells are vigorously pipetted to break up formazan crystals. The cellular reduction involves pyridine nucleotide cofactors NADH and NADPH. The formazan crystals formed are solubilized and the resulting colored solution is quantified using a scanning multi-well spectrophotometer at 570 nm [h].

Colorimetric MTT assay was used to assess cell viability by cell metabolic activity following incubation for 24h in buffered or un-buffered media. The pH was measured at 37°C and 5% CO_2 culture conditions to minimize variables that may confound pH (temperature, % CO_2) using pH probe[i].

Mitochondrial mass was assessed for four cell types following exposure to amikacin (25 mg/ml in complete DMEM media) for four hours using Mitotracker[j] green fluorescent mitochondrial staining. Visualization of fluorescence staining was done using Olympus IX83 spinning disk confocal microscope.

Effect of amikacin dose and media pH on cell proliferation. The effect of amikacin[a] on four cell types from three donors was assessed at different concentrations in complete DMEM (25 to 0.003 mg/mL in complete DMEM media) and with media pH buffered or unbuffered. Live cell imaging (Incucyte®[k]) was performed every three hours over 24 hours to assess cell proliferation by confluence.

Effect of presence of synovial fluid on cell metabolism. Synovial fluid was obtained immediately post-mortem from horses without evidence of osteoarthritis and euthanized for

reasons unrelated to the present study. Synovial fluid was centrifuged at 1,000g for 10 minutes. The synovial fluid supernatant was aliquoted in 100 µl aliquots and frozen at -80°C for use at a later time. Synovial cells from three donors were plated in triplicate wells at 30,000 cells/well on a 96 well plate in 200 µl complete DMEM media for 48 hours. At that time, media was removed and replaced with one of four treatments (200 µl/well). Control treatments consisted of synovial fluid alone (200 µl SF) and complete DMEM media alone (200 µl media). Treatments consisted of synovial fluid with 25 mg/ml amikacin (20 µl amikacin in 180 µl synovial fluid) and complete media with 25 mg/ml amikacin (20 µl amikacin in 180 µl media). At two hours following addition of treatments to wells, viability of cells was evaluated by MTT colorimetric assay.

Effect of reagent grade on viability. The effect of aminoglycoside (amikacin, gentamicin) formulation (clinical or reagent grade) on viability of synovial cells from three donor horses each in triplicate was evaluated. Synovial cells were incubated with clinical grade amikacin[a], reagent grade amikacin[l], and reagent grade gentamicin[m], at 25mg/ml in media for 24 hours and viability was assessed by trypan blue dye exclusion.

Effect of amikacin on cartilage and synovial explant tissues. Equine cartilage and synovial explant tissues were obtained from the stifle joints of three horses without evidence of osteoarthritis euthanized for reasons unrelated to the study. Full-thickness cartilage was obtained by shaving the lateral trochlear ridge of the distal femur under aseptic conditions as previously described.[10] Explant tissues were pooled, minced to approximate 2mm thickness, and then incubated at 37C 5% CO_2 in complete DMEM with or without amikacin (5 or 25mg/ml) for 48 hours (synovium) or 72 hours (cartilage).[10] Explant tissues were transferred to 8-chamber cover glass slides (Thermo Fisher) and stained with LIVE/DEAD ™ Cell Imaging Kit (Thermo Fisher) according to manufacturers' instructions. Explants were imaged 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. Image J was used to count the live/ dead pixels of each separated z stack image.

Mechanism of cell death. Synovial cells from three donors were assessed for occurrence of apoptosis after amikacin treatment (25mg/ml in complete DMEM media) using Annexin V and 7AAD with flow cytometric quantification, as previously described.[13] Annexin-V-Fluos Staining Kit[n] was used to identify apoptotic, necrotic, and viable cells. Live cells were identified as Annexin V negative, PI negative. Cells undergoing early apoptosis were identified as Annexin V positive, PI negative. Cells undergoing late apoptosis were identified as Annexin V positive, PI positive. Cells undergoing necrosis were identified as Annexin V negative, PI positive.

Synovial cells from three donors were assessed for apoptosis using human/mouse active anti-caspase 3 antibody. Visualization of fluorescence staining was done using Olympus IX83 spinning disk confocal microscope, with exposure normalized to concentration matched isotype polyclonal rabbit IgG.

Data Analysis. Two-way ANOVA was performed to assess effect of cell type and amikacin concentration (**Figure 2.1A**) and exposure time to amikacin (**Figure 2.1B**) on cell viability in complete DMEM media. A three-way ANOVA was performed in complete DMEM media to examine effect of amikacin concentration, pH buffering, and cell type on cell viability using MTT assay (**Figure 2.2**). Three-way ANOVA was performed to further analyze amikacin concentration, time of exposure and pH buffering on cell proliferation determined by percent confluence using live cell imaging (**Figure 2.3**). Tukey's multiple comparisons tests were performed to compare metabolism between cells exposed to different amikacin concentrations. Cell viability in the presence of synovial fluid or media alone was analyzed by one-way

ANOVA. Percent viability relative to baseline for cells in the presence of synovial fluid vs. media alone was compared by unpaired t-test. For each test, the three technical replicates were averaged for each donor, and the resulting three averages used for the remaining analysis. Three-way ANOVAs was performed in R version 3.6[o]. All other analyses were done using GraphPad Prism8 Software[e]. Significance was assessed at $p<0.05$.

2.4 Results

Amikacin at clinically relevant concentrations is rapidly toxic to normal cells. The cytotoxic effects of amikacin were independent of cell type, with dose-dependent killing of chondrocytes, synovial cells, AD-MSC and BMD-MSC. When assessing cell viability for the 4 cell types at multiple amikacin concentrations using a trypan blue dye exclusion following amikacin exposure for 24 hours, the amikacin dose at which approximately 50% of the cells were dead (i.e. inhibitory concentration 50 or IC50) was determined for each cell type (synovial cells 0.7993mg/ml, AD-MSC 0.7848mg/ml, BMD-MSC 0.3311mg/ml, chondrocytes 0.3132mg/ml) (**Figure 2.1A**). Concentration ($p<0.0001$) and cell type ($p=0.0003$) were found to be significant factors in cell viability when amikacin dose was titrated from 0.003 to 25mg/ml and viability assessed by trypan blue dye exclusion.

Amikacin at a concentration of 25 mg/ml, which is the calculated concentration achieved in joints of horses following typical intra-articular doses of amikacin, was rapidly cytotoxic to all 4 cell types evaluated (**Figure 2.1B**). Cell viability was rapidly eliminated within one hour of high-concentration amikacin exposure and did not increase with time of exposure beyond the one-hour time point.

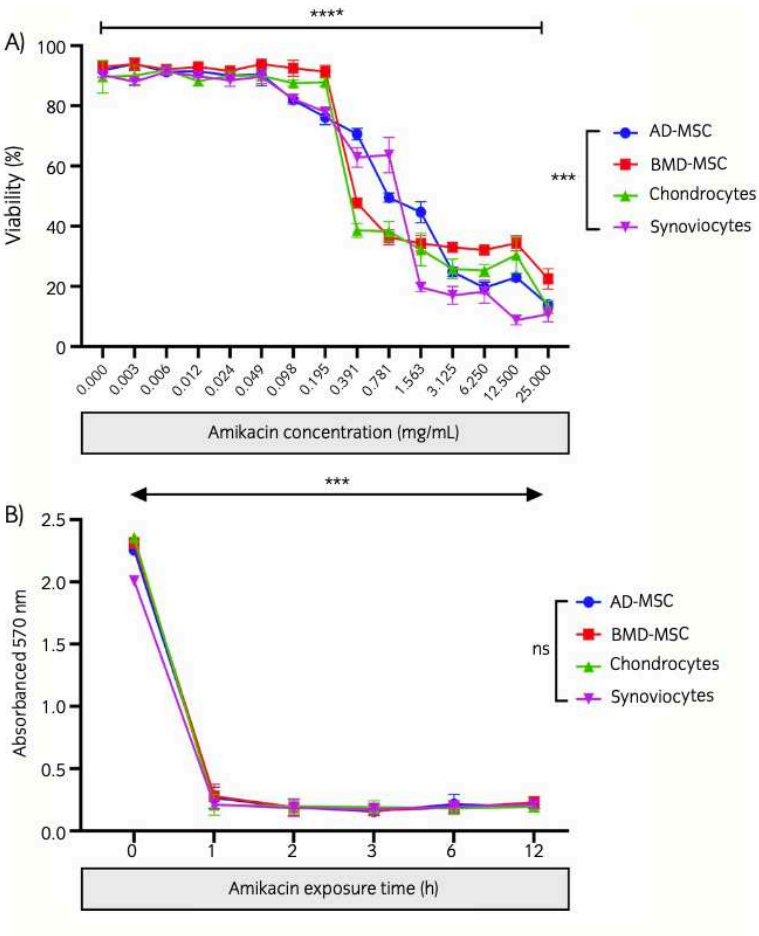

Figure 2.1: Effect of amikacin on cell viability and metabolism. (1A) Cell viability was assessed for four cell types from three donor horses over a dose titration of amikacin using trypan blue dye exclusion staining to determine percentage of live cells following amikacin exposure for 24 hours. X-axis represents amikacin concentration, y-axis represents percentage live cells. **(1B)** Amikacin at a concentration of 25mg/ml was added to four different cell types. Exposure time shown on x axis, MTT colorimetric assay absorbance shown on y-axis. Color legend for four different cell types shown on right. All experiments were performed in duplicate with comparable results.

Amikacin exposure decreases cellular metabolism in a pH-independent, concentration-dependent manner. The addition of amikacin to media significantly reduced pH of media in comparison to baseline ($p<0.001$). Mean pH of media before and after amikacin addition was 7.59 and 6.81, respectively. Media supplemented with NaOH in the buffered pH group had mean pH 7.60, which was not significantly different from baseline ($p=0.4$).

The effect of amikacin concentration and media pH (buffered or non-buffered) on cell metabolism of four cell types was assessed by MTT assay following exposure to amikacin for 24 hours (**Figure 2.2**). The effects of amikacin concentration and media pH for each of the four cell types was assessed individually and compared between cell types. When analyzed by three-way ANOVA across all four cell types, there was a significant effect for amikacin concentration on cell viability ($p<0.001$). Buffering of media pH and cell type did not significantly affect cell viability ($p=0.3$, $p>0.9$, respectively). There was not a significant interaction between concentration and buffering of media pH on cell viability ($p=0.5$) across the four cell lines.

When each cell type was evaluated individually, amikacin concentration significantly affected cell viability (AD-MSC $p=0.021$, BMD-MSC $p=0.030$, chondrocytes $p=0.009$, synovial cells $p=0.01$) and pH buffering did not significantly affect cell viability (AD-MSC $p=0.6$, BMD-MSC $p>0.9$, chondrocytes $p=0.479$, synovial cells $p=0.6$).

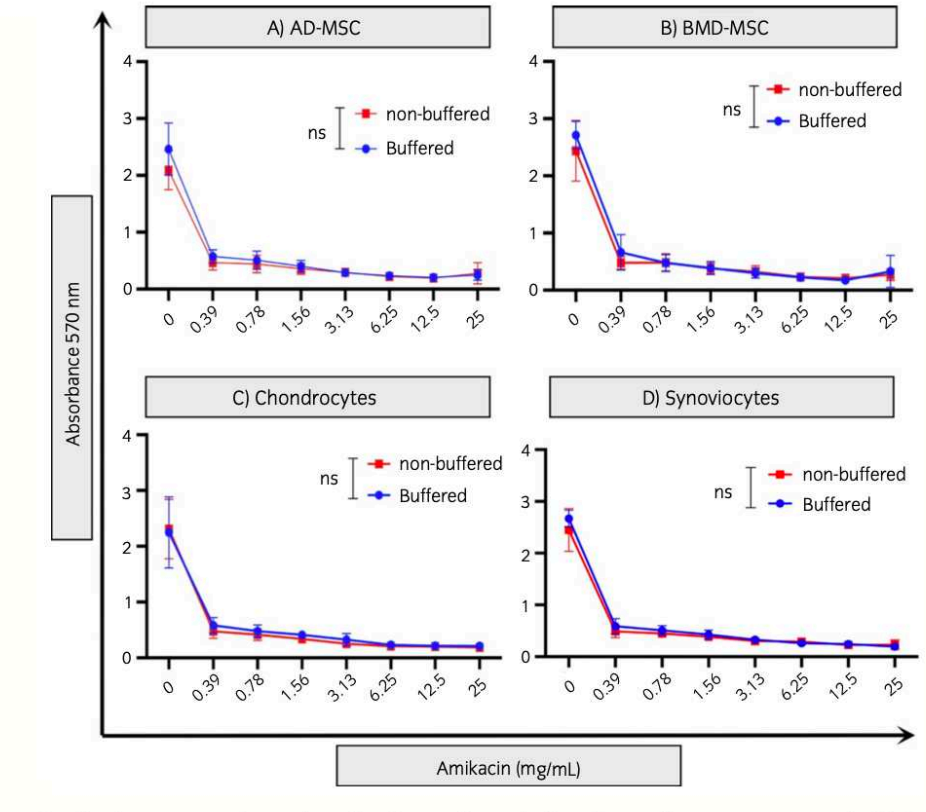

Figure 2.2: Effect of amikacin concentration and media pH on cell metabolism. Four cell types from three donors horses were treated with increasing doses on amikacin, shown on x-axis. The y-axis represents colorimetric MTT assay absorbance (570nm). Red line represents unbuffered cell culture conditions; blue represents buffered culture conditions (pH 7.4). All experiments were performed in duplicate with comparable results.

The effect of amikacin on mitochondrial mass was assessed visually under fluorescent microscopy using MitoTracker green fluorescent mitochondrial stain, amikacin treated cells demonstrated reduced mitochondrial mass, indicating reduced metabolism. **(Figure 2.3)**

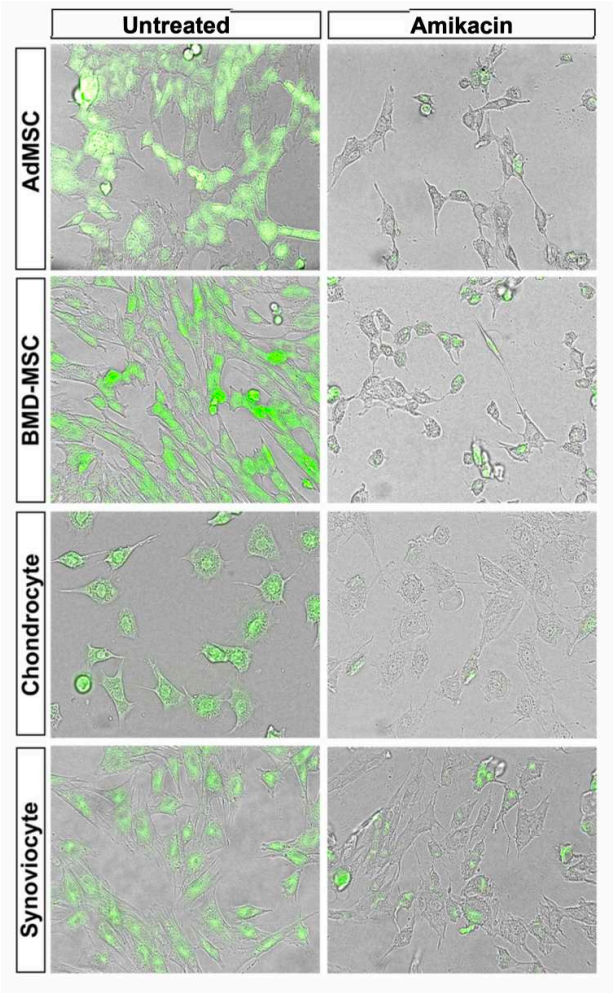

Figure 2.3: Effect of amikacin on mitochondrial mass by visual assessment under microscopy. Four cell types were visualized under florescent microscope at 10x magnification after staining with MitoTracker green fluorescent mitochondrial stain. Right column shows decrease in staining following four hours exposure to 25mg/mL amikacin. Green color represents mitochondrial mass. Figures are representative of results seen in three donor horses.

Amikacin exposure at clinically relevant doses suppresses cell proliferation in a pH-independent manner. A three-way ANOVA was performed on four cell types from three donor horses to examine effect of amikacin concentration, exposure time and pH buffering on cell proliferation determined by percent confluence using live cell imaging (Incucyte®) (**Figure 2.4**). The final ANOVA model was determined by stepwise removal of non-significant terms from the full model including all interactions. As the four cell types inherently proliferate at different rates, the data was first evaluated without cell type as a factor. There was not a significant effect of pH buffering ($p=0.6$) on cell proliferation, but both time of exposure to amikacin and amikacin concentration were significant factors ($p<0.001$). The final model also demonstrated a significant interaction between concentration and exposure time ($p<0.001$), with increasing concentrations showing decreased effect due to time of exposure to amikacin.

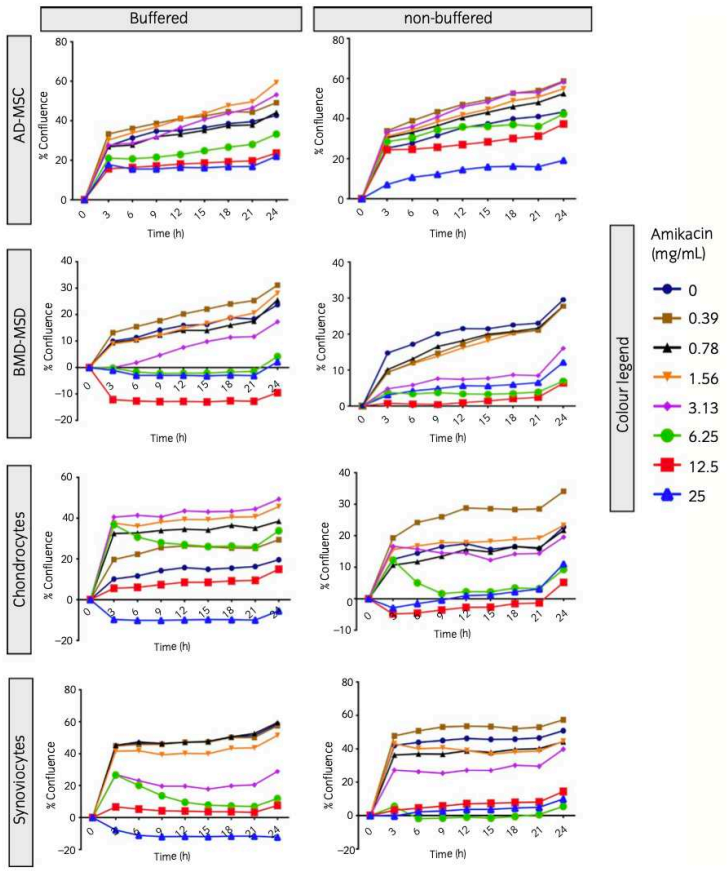

Figure 2.4: Effect of amikacin concentration and media pH on cell proliferation over time. Proliferation of four cell types from three donors horses as assessed by live cell imaging. The x-axis shows time in culture and y-axis shows percent confluence. Color bars on right indicate amikacin concentrations used. Experiments were performed in duplicate with comparable results.

***Synovial fluid does not alter amikacin toxicity*.** When normalized to starting values for cell metabolism for synovial cells cultured in synovial fluid alone or media alone, there was no difference in reduction in metabolism between cells cultured in synovial fluid or media on unpaired t-test ($p=0.4$) (**Figure 2.5A**).

Cytotoxicity of clinical grade amikacin product equivalent to that of research grade amikacin. The preceding results generated with clinical grade amikacin raised the possibility that the observed cytoxicity might be mediated by another compound (e.g. preservatives) in the clinical material. Therefore, the cytotoxicity studies were repeated using reagent-grade amikacin[n]. When assessed by two-way ANOVA for type of amikacin (reagent grade *vs* clinical) and concentration, there was a significant effect for both type ($p=0.02$) and concentration ($p<0.001$). When clinical amikacin was compared to reagent at each concentration, there were only significant differences at 0.390625mg/ml ($p=0.001$) and 3.125mg/ml ($p=0.05$), with clinical grade being more cytotoxic. When clinical amikacin was compared to chemical grade gentamicin[o], there was only significant differences at concentration 0.390625mg/ml ($p<0.001$) (**Figure 2.5B**).

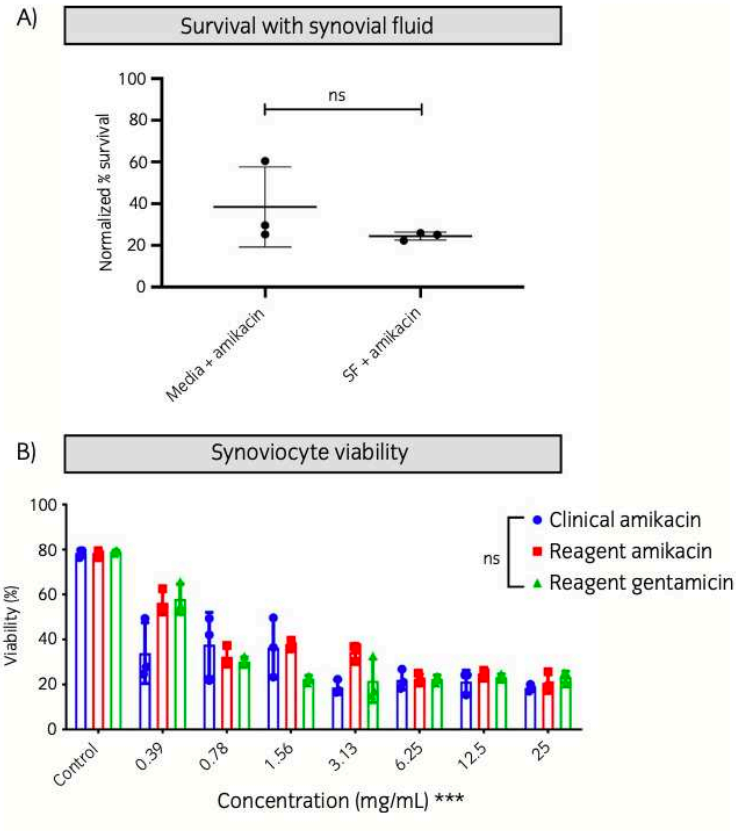

Figure 2.5: Effect of synovial fluid and reagent grade on synovial cell viability. (2.5A) Viability of synoviocytes from three donor horses with or without synovial fluid after the addition of amikacin as determined by MTT assay. Y-axis shows % survival calculated by the absorbance of synoviocytes + amikacin divided by the untreated control. Significance calculated with non-parametric Mann-Whitney test p=0.2. **(2.5B)** Viability of synoviocytes determined by trypan blue, with % live cells on y axis. X axis shows increasing concentrations of clinical amikacin (blue), reagent grade amikacin (red) or reagent grade gentamicin (green). Significance determined by 2way ANOVA, with p<0.0001 for concentration effect, and p=0.07 (ns) for reagent vs. clinical grade antibiotics. Experiments performed in duplicate with comparable results.

Amikacin reduces viability in cartilage and synovial explant tissues at clinically relevant doses. Viability of synovial explant tissues was significantly reduced following 48 hours culture with amikacin (p<0.0001, p<0.0001, p=0.005; 0 vs 5mg/ml; 0 vs 25mg/ml, 5 vs 25mg/ml, respectively). Viability of cartilage explant tissues was significantly reduced following

72 hours culture with amikacin ($p<0.001$, $p<0.001$, $p=0.04$; 0 vs 5mg/ml, 0 vs 25mg/ml, 5 vs 25mg/ml, respectively) (**Figure 2.6**).

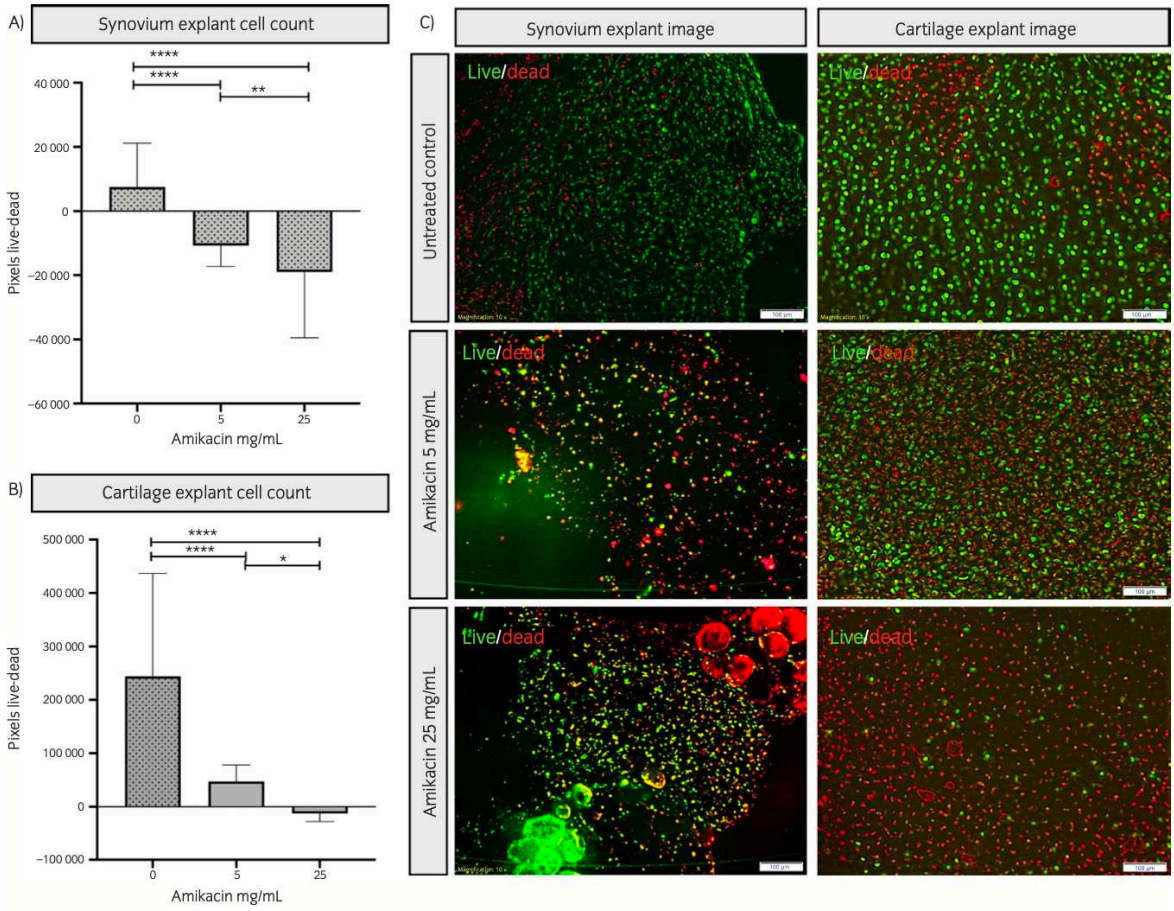

Figure 2.6: Viability of equine synovium and cartilage explants after incubation with amikacin. 5A) Live minus Dead pixel counts from n=3 horse donors from z stack synovial explant images after 48 hours of incubation with amikacin, calculated using image J. Bar graphs show counts from increasing doses of Amikacin (x axis). **5B)** Live minus Dead pixel counts from n=3 z stack cartilage explant images after 72 hours of incubation with amikacin, calculated using image J. Bar graphs show counts from increasing doses of Amikacin (x axis). **5C)** Merged z stack images of equine explants treated with amikacin for 48 hours (synovium) or 72 hours (cartilage). Red staining shows dead cells, green shows intact, live cells.

Amikacin induces cell death by apoptosis. To determine mechanism of cell death, synovial cells were assessed for occurrence of apoptosis using Annexin V and 7-AAD staining

with flow cytometric quantification over time following amikacin (25 mg/mL) addition to media. Cells underwent late apoptosis over time, using ethanol as a positive control for apoptosis. To further confirm mechanism of cell death as apoptosis, four cell types were assessed for upregulation of caspase3 expression following amikacin exposure at multiple time points and concentrations of amikacin. Cells demonstrated concentration-dependent upregulation of caspase expression beginning at 30 minutes post-exposure, consistent with induction of final common apoptotic pathways (**Figure 2.7, 2.8)**

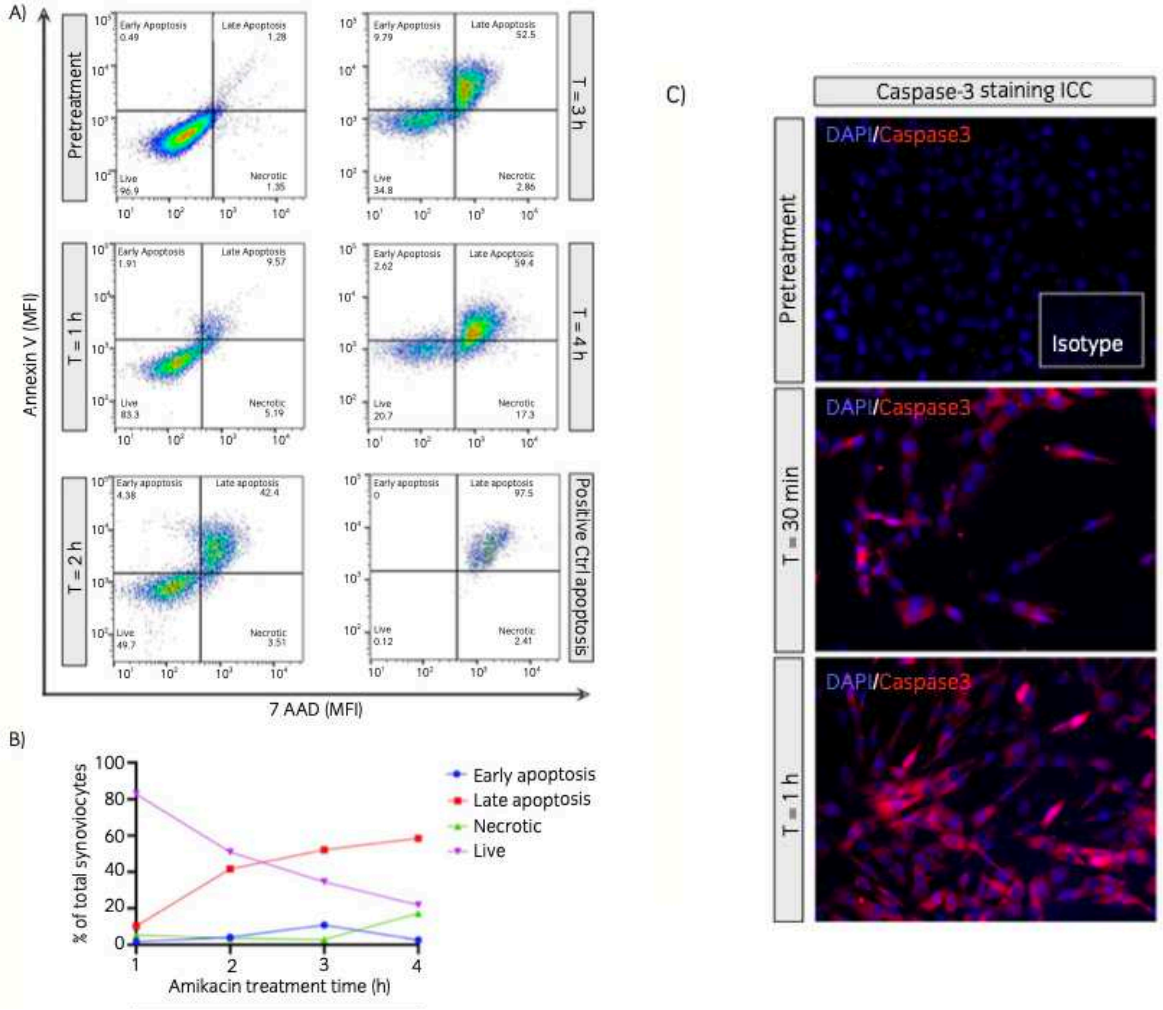

Figure 2.7: Mechanism of cell death. **(7A)** Synoviocytes were assessed for apoptosis over time using AnnexinV and 7AAD staining with flow cytometric quantification. In each panel, x-axis represents 7AAD florescence intensity, which penetrates necrotic cell membranes. The y-axis represents AnnexinV florescence intensity, which stains apoptotic cells. Double positive cells (upper right quadrant) represent late apoptotic cells which are positive for both 7AAD and Annexin as seen in "positive control" for apoptosis in last panel. **(7B)** Numerical representation of early (blue), late (red) apoptotic cells, as well as necrotic (green) and live cells (purple). Each dot shows mean and SD (bars) of technical replicates. Y axis represents percentage of cells. **(7C)** Caspase 3 staining (red) of synovial cells after exposure to 12.5 mg/mL amikacin.

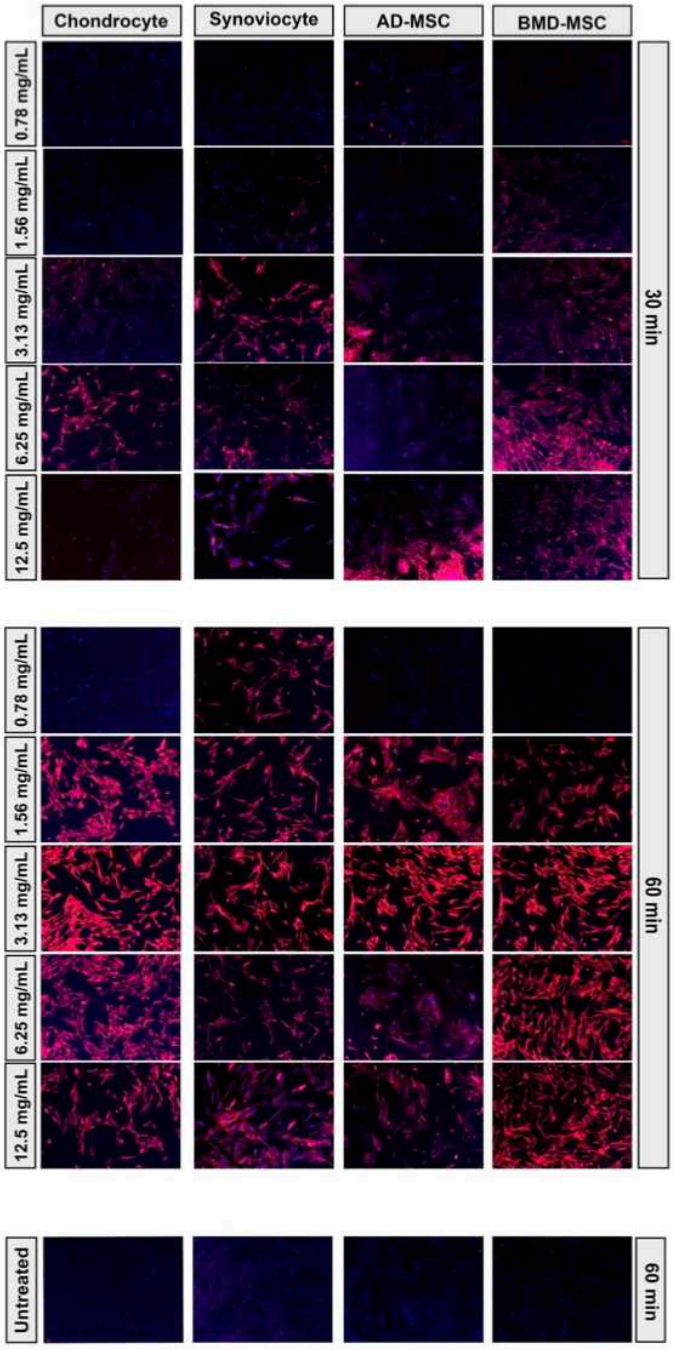

Figure 2.8: Caspase expression following increasing doses of amikacin in four cell types. Caspase staining of chondrocytes, synovial cells, adipose derived MSC (AD-MSC), and bone marrow derived MSC (BMD-MSC) from three donor horses. Cells were fixed after 30 minutes or one hour of amikacin exposure then stained with anti caspase3 antibody as described in methods. Positive staining shown in red.

2.5 Discussion

Intra-articular injections of amikacin are common in equine clinical practice. However, it is not clear that this practice can be justified based on prophylaxis of joint infections, given the extremely low overall incidence of iatrogenic septic arthritis in horses. For example, prior studies have failed to demonstrate a reduction in incidence of septic arthritis following prophylactic use of antimicrobials in joint injections has not been demonstrated.[1-3] Moreover, the extremely low incidence of iatrogenic septic arthritis also argues against any benefit from prophylactic administration of amikacin.[1-3] Our study findings support our hypothesis to establish the inherent cytotoxicity of amikacin to the cells integral to the synovium and cartilage of the joint in horses as well as to MSC. Amikacin cytotoxicity develops in a rapid, dose-dependent, pH-independent manner, mediated by induction of apoptosis. Importantly, the cytotoxic effects of amikacin were not mitigated by synovial fluid. Furthermore, the concentrations of amikacin evaluated in this study were adjusted to more closely simulate concentrations that cells are exposed to in the joints of horses injected with amikacin, which lends further clinical relevance to the results obtained.[8] Intra-articular doses of 250 to 500 mg amikacin per joint have been previously reported,[8,14] which would yield 25 to 50mg/ml final concentration in joint fluid depending on the joint injected. Based on our data, these doses easily exceed concentrations that are markedly cytotoxic to joint cells. In summary, current doses of intra-articular amikacin commonly used by equine veterinarians have the potential to cause iatrogenic harm to normal joint cells.

Our study results agree with previous studies demonstrating the concentration-dependent detrimental effects of aminoglycosides on cultured cells.[6,8] Amikacin has been demonstrated to negatively impact viability and gene expression of cultured equine and human BM-MSCs even at concentrations 200 times less than those used in clinical doses.[6] These doses rapidly kill within one hour of exposure and suppress proliferation of native joint cells for at least 24 hours. Given the relatively slow distribution of pharmaceuticals out of synovial fluid following injection and rapid cytotoxic action of aminoglycosides, joint cells and stem cells are likely to be exposed for prolonged periods to high concentrations of amikacin.[15-17] Taintor *et al.* reported that when 500mg amikacin is administered into normal or inflamed antebrachiocarpal joints, the concentration in that joint was maintained above the MIC (4µg/ml) of amikacin reported for most common equine pathogens for 72 or 48 hours, respectively. Therefore, determination of intra-articular amikacin concentrations by clinicians would ideally be based upon the known MIC of the targeted bacterial pathogens, as well as consideration of joint volume. Selection of amikacin should also be based upon results of bacterial culture and sensitivity in cases of confirmed septic arthritis if positive culture is available.

The cytotoxicity of amikacin in this study was not associated with the acidic pH of media induced by the addition of amikacin to cell culture media, which is consistent with previous reports.[8] The addition of amikacin to cell culture media resulted in lower pH and buffering amikacin with NaOH to physiologic pH did not improve cell metabolism or proliferation. These results were not surprising as other cell types have been demonstrated to tolerate a relatively wide range of pH in the short-term, while storage of cells long-term is improved in mildly acidic solutions.[18,19] *In vivo*, gentamicin sulfate, but not amikacin sulfate, has been shown to induce a transient chemical synovitis in joints of clinically normal horses, which was attributed to be

related to a low pH as well as to a direct chemical effect.[12,20] In addition, the effect of amikacin in the presence of synovial fluid was investigated to more closely mimic the clinical scenario. The detrimental effects of amikacin on synovial cells were not mitigated by the presence of synovial fluid. Finally, when clinical and reagent grade formulations of amikacin and gentamicin were evaluated, there were differences in viability between products only at the lowest concentration evaluated. These results indicate that cell death due to aminoglycoside exposure was dose-dependent by a mechanism that reduced cell metabolism, and not the result of pH reduction or product formulation.

The mechanism of cell death following amikacin exposure was further investigated using annexin and 7AAD staining and caspase expression and found to be the result of induction of apoptosis. Aminoglycosides are known to induce toxicity in bacterial (prokaryotic) cells through binding to the 30S ribosomal subunit.[7] Furthermore, in some instances, mechanism of toxicity for mammalian (eukaryotic) cells is very similar to or identical with that for bacterial cells.[7] Aminoglycoside antibiotics are known to be nephrotoxic, ototoxic, and capable of inducing neuromuscular blockade at concentrated doses in cells of mammalian species, when efflux from cells is compromised.[7] Antimicrobial classes other than aminoglycosides, that kill bacteria through different mechanisms of action, may be more suitable to the intra-articular environment or if co-injected with MSC.

Limitations of this study include the *in vitro* nature of experimental design and relatively low number of equine cultured cell lines investigated (n=3). The methods described here could be further developed to include evaluation of the effect of amikacin on cells in a low oxygen environment with agitation in order to more closely mimic the in vivo joint environment. Furthermore, controls with equivalent volumes of saline added to each well instead of amikacin

would lend further credence to the results obtained. Further studies investigating the effects of amikacin on cartilage and synovial tissue in explant tissues, as well as careful dose titration studies in live horses are indicated to determine suitable doses for intra-articular use that would minimize the detrimental effects of aminoglycosides. The penetration of amikacin into equine articular *cartilage in vivo* is not known, and therefore the cytotoxic effects of amikacin may be less clinically relevant following *in vivo* injection. Amikacin was the first antimicrobial investigated as it is the antibiotic most commonly used IA at our institution. However, it is recognized that antibiotic usage varies by region. Other antimicrobials may be investigated in future studies to broaden the scope of this work and to determine if other classes of drugs are less detrimental to equine joint cells and therefore more suitable for intra-articular injection.

Further investigation of the effect of amikacin on the equine joint in both cartilage and synovial explant tissues as well as in live horses in both normal and effusive states to assess collagen degradation and cartilage matrix synthesis would lend further credence to these results. In diseased joints with increased synovial effusion, concentrations of amikacin administered intra-articularly have been shown to be significantly lower compared to that in normal joints.[11] In contrast, concentrations of antimicrobials administered parenterally reached higher concentration in inflamed than in normal joints[21,22] which has been attributed to an increase in disposition of parenterally administered drugs into inflamed joints due to increased vascularization associated with synovial inflammation.[22,23] The decrease in concentration of amikacin administered IA to inflamed joints may be the result of increased vascularity of the synovial membrane.[14] In addition, medications such as triamcinolone and hyaluronic acid, which may be injected at the same time as amikacin when treating osteoarthritis, have been shown to have a mild protective effect in chondrocyte explants against the potential toxicity induced by amikacin.[10] In summary,

while amikacin has been demonstrated here to be unequivocally cytotoxic to MSC, chondrocytes and synoviocytes *in vitro*, the effect of antibiotics on native joint cells *in vivo* may vary for reasons other than dose administered, depending on the condition of joint (i.e. effusion) or co-administration of other pharmaceuticals at the time of injection.

The results of this study demonstrate that amikacin exerts a rapid dose-dependent toxic effect on normal joint cells, as well as on MSC. While the authors are cautious to extrapolate *in vitro* findings to the *in vivo* joint environment, it is recommended that amikacin doses administered prophylactically intra-articularly be reduced or eliminated pending further in vivo dose titration and toxicity studies. Other classes of antimicrobials may be investigated that are less toxic and more suitable for intra-articular use than aminoglycosides.

Manufacturers' addresses

[a] Amikacin sulfate (250mg/ml). Teva Pharmaceuticals USA, INC. North Wales, PA, USA 19454

[b.] Collagenase type I and II. Worthington Biochemical Corporation, Lakewood, NJ, USA, 08701.

[c.] Ficoll-Paque TM PLUS. GE Healthcare Bio-Sciences AB SE-751 84 Uppsala, Sweden.

[d]. Automated cell counter, Nexcelom. Bioscience Cellometer Auto T4. Lawrence, MA, USA, 01843

[e]. GraphPad Software Prism8. San Diego, CA, USA, 92108.

[f]MTT (3-[4,5-diimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) labeling reagent. Sigma Aldrich. St. Louis, MO, USA, 63178.

[g]. MTT solubilization solution. Sigma Aldrich. St. Louis, MO, USA, 63178.

[h]. Synergy HT Plate Reader. Biotek Instruments, Inc. Winooski, VT, USA, 05405-0998.

[i] Corning pinnacle 540 pH meter. Corning Incorporated. Corning, NY, USA 14831.

[j] Mitotracker™ Green FM Invitrogen, ThermoFisher Scientific. Waltham, MA USA, 02451.

[k] Incucyte® S3 Live-Cell Analysis System. Essen Bioscience Inc. Ann Arbor, MI, USA, 48108.

[l]Amikacin sulfate salt. Sigma Aldrich. St. Louis, MO, USA, 63178

[m]Gentamicin sulfate salt. Sigma Aldrich. St. Louis, MO, USA, 63178.

[n]Annexin-V-Fluos Staining Kit, Roche. Mannheim, Germany.

[o]R package version 3.6.0 (2019-04-26) R Foundation. Vienna, Austria.

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CHAPTER 3: Use of *in vitro* assays to identify antibiotics that are cytotoxic to normal equine chondrocytes and synovial cells[iii]

3.1 Summary

Intra-articular (IA) antibiotic usage is prevalent in equine practice. However, recent emergence of antimicrobial resistance prompts re-evaluation of antibiotic prophylaxis. Furthermore, many commonly used antibiotics exert direct cytotoxicity to equine cells, and appropriate IA doses have not been defined. The objective of this study was to screen antibiotics for cytotoxicity against equine joint cells in both monolayer culture and explant tissues as an initial *in vitro* assessment of safety. 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 visualized using Olympus IX83 spinning disk confocal microscope. Percentage of live versus dead cells was quantified. Antibiotics from different antimicrobial classes expressed dose-dependent but variable cytotoxicity to equine joint cells in vitro. Aminoglycosides and doxycycline had the lowest IC50 (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. Further studies are

[iii]This chapter includes the complete published manuscript: Pezzanite L, Chow L, Piquini G, Griffenhagen G, Ramirez D, Dow S, Goodrich L. Use of *in vitro* assays to identify antibiotics that are cytotoxic to normal equine chondrocytes and synovial cells. *Equine Vet J.* 2021;53:579-589. This article was reproduced with permission from John Wiley and Sons, Inc., Hoboken, NJ.

necessary to extrapolate these *in vitro* data results to the *in vivo* joint environment. In conclusion, 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 minimize damage to native articular cartilage and synovial cells when intra-articular antibiotics are used in equine practice.

3.2 Introduction

Intra-articular antibiotic usage is prevalent in equine clinical practice .[1,2] Over 70% of equine practitioners report including antibiotics in at least some situations when injecting joints prophylactically in the treatment of osteoarthritis, with 46.2% using antibiotics in every injection.[1] In one recent report describing the risk of synovial sepsis following prophylactic joint injection in equine ambulatory practice, practitioners reported using amikacin sulphate in 93.4% of joints injected, most commonly with corticosteroids (89.3%) and hyaluronate (83.5%).[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]

In instances of confirmed septic arthritis, achieving high concentrations of antibiotics in the joint may be considered necessary to resolve the infection to allow survival of the patient. However, all intra-articular administration of antibiotics, whether to treat established septic

arthritis or with the goal to prevent joint infection following injection, is considered "off-label" usage. Although there are anecdotal reports of IA antibiotic doses used by equine practitioners,[7,8] the appropriate dose to maximize efficacy while minimizing 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. Development of evidence-based strategies in selection and dosing of antibiotics may help guide clinical decision-making in these situations.

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 edema, 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 hypothesized 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.

3.3 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), euthanized 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 $10x10^6$cells/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 $5x10^6$cells/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 (**Figure 3.1, 3.2**). 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 visualization 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]

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 normalizing dose response for each concentration to control, transforming data to normalized 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 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 $P<0.05$.

3.4 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 3.1 and 3.2**).

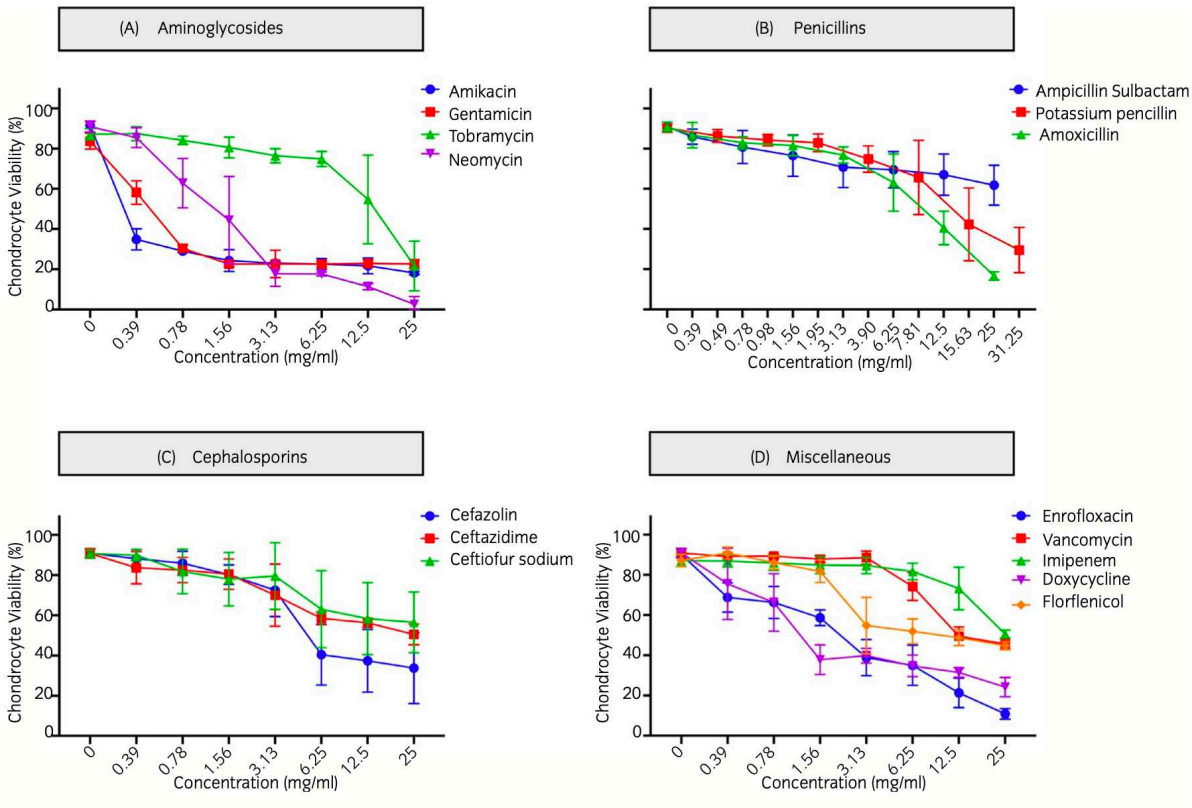

Figure 3.1: Effect of antibiotics on chondrocyte cell viability in monolayer culture. Cell viability (mean +/- SD) was assessed in chondrocytes from each of three donor horses, each in triplicate, using trypan blue exclusion staining to determine percentage of live cells following

antibiotic exposure for 24 hours. X-axis represents antibiotic concentration, y-axis represents percentage live cells.

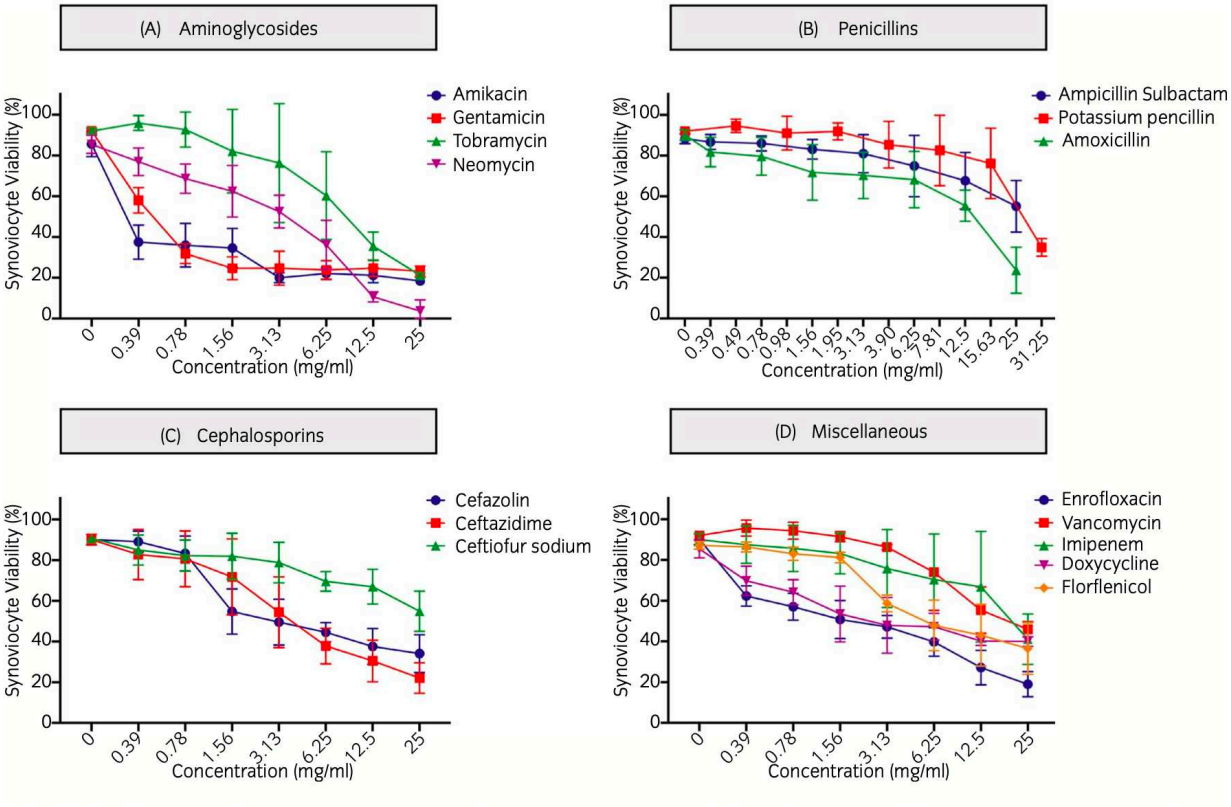

Figure 3.2: Effect of antibiotics on synovial cell viability in monolayer culture. Cell viability (mean +/- SD) was assessed in synovial cells from each of 3 donor horses, each in triplicate, using trypan blue exclusion staining to determine percentage of live cells following antibiotic exposure for 24 hours. X-axis represents antibiotic concentration, y-axis represents percentage live cells.

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 3.1**).

Table 3.1: Determination of half maximal inhibitory concentration (IC50). 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 normalized to control, and the data was transformed to "normalized dose response vs. log10(concentration)" at which point the half maximal inhibitory concentration (IC50) was estimated by nonlinear regression implemented in GraphPad Prism8[P]. In instances where the IC50 fell outside the range of concentrations evaluated, or the data were not distributed in sigmoidal fashion following normalization 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

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 3.2**).

Table 3.2: Statistical comparison of viability of equine synovial cells and chondrocytes between antibiotics and amikacin at each antibiotic concentration evaluated. Overall, the antibiotic used (synovial cells p<0.0001, chondrocytes p<0.0001) was a significant factor to cell viability following antibiotic exposure for both cell types when antibiotics were titrated from 25 to 0.39mg/mL. Specific comparisons in viability between each antibiotic to amikacin at each concentration are outlined below.

Antibiotic	Synovial Cells															
	Antibiotic Concentration (mg/mL)															
	0		0.39		0.78		1.56		3.12		6.25		12.5		25	
	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
Aminoglycosides																
gentamicin	-6.456	0.735	-20.544	<0.001	4.133	0.947	9.961	0.267	-4.733	0.91	-1.756	0.999	-3.478	0.974	-4.744	0.909
neomycin	0.167	1	-39.456	<0.001	-32.689	<0.001	-27.817	<0.001	-32.500	<0.001	-14.022	0.031	10.544	0.208	14.756	0.019
tobramycin	-6.367	0.746	-58.444	<0.001	-56.744	<0.001	-47.506	<0.001	-56.344	<0.001	-38.222	<0.001	-14.300	0.026	-2.744	0.991
Penicillins																
amoxicillin	-4.489	0.927	-44.267	<0.001	-43.689	<0.001	-37.139	<0.001	-50.322	<0.001	-46.100	<0.001	-34.178	<0.001	-5.244	0.869
ampicillin-sulbactam	-2.667	0.992	-49.233	<0.001	-50.056	<0.001	-48.517	<0.001	-61.000	<0.001	-52.778	<0.001	-46.422	<0.001	-36.678	<0.001
potassium-penicillin	-6.367	0.746	-57.133	<0.001	-55.067	<0.001	-57.272	<0.001	-65.411	<0.001	-60.444	<0.001	-54.978	<0.001	-16.500	0.005
Cephalosporins																
cefazolin	-4.489	0.927	-51.644	<0.001	-47.322	<0.001	-20.139	<0.001	-29.578	<0.001	-22.489	<0.001	-16.411	0.006	-15.689	0.01
ceftazidime	-4.489	0.927	-45.289	<0.001	-44.700	<0.001	-37.194	<0.001	-34.456	<0.001	-15.733	0.01	-9.300	0.344	-3.678	0.967
ceftiofur sodium	-5.078	0.883	-47.522	<0.001	-46.267	<0.001	-47.339	<0.001	-58.856	<0.001	-47.478	<0.001	-45.800	<0.001	-36.478	<0.001
Miscellaneous																
doxycycline	0.167	1	-32.267	<0.001	-28.189	<0.001	-18.883	<0.001	-27.933	<0.001	-25.156	<0.001	-18.978	<0.001	-21.522	<0.001
enrofloxacin	-4.489	0.927	-24.800	<0.001	-21.067	<0.001	-16.150	0.007	-27.222	<0.001	-17.678	0.002	-5.944	0.797	-0.578	1
florfenicol	-1.556	0.999	-48.922	<0.001	-47.089	<0.001	-46.617	<0.001	-38.711	<0.001	-25.789	<0.001	-21.867	<0.001	-18.067	0.002
imipenem	-4.367	0.934	-50.033	<0.001	-49.722	<0.001	-48.539	<0.001	-55.867	<0.001	-48.267	<0.001	-45.633	<0.001	-22.700	<0.001
vancomycin	-6.367	0.746	-58.144	<0.001	-58.389	<0.001	-56.861	<0.001	-66.344	<0.001	-51.889	<0.001	-34.200	<0.001	-27.578	<0.001

Antibiotic	Chondrocytes															
	Antibiotic Concentration (mg/mL)															
	0		0.39		0.78		1.56		3.12		6.25		12.5		25	
	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value	estimate	p-value
Aminoglycosides																
gentamicin	7.811	0.33	-23.311	<0.001	-1.322	0.999	1.778	0.997	0.311	1	0.011	1	-1.222	0.999	-4.411	0.862
neomycin	3.300	0.955	-50.600	<0.001	-33.722	<0.001	-20.111	<0.001	5.211	0.757	4.933	0.797	10.356	0.079	15.656	<0.001
tobramycin	4.344	0.869	-52.567	<0.001	-55.033	<0.001	-56.222	<0.001	-53.478	<0.001	-52.233	<0.001	-33.000	<0.001	-3.456	0.946
Penicillins																
amoxicillin	0.633	1	-51.867	<0.001	-53.822	<0.001	-57.178	<0.001	-53.811	<0.001	-40.544	<0.001	-18.844	<0.001	1.556	0.998
ampicillin-sulbactam	0.478	1	-51.078	<0.001	-51.700	<0.001	-52.178	<0.001	-47.800	<0.001	-46.878	<0.001	-45.311	<0.001	-43.589	<0.001
potassium-penicillin	1.322	0.999	-51.467	<0.001	-55.156	<0.001	-58.567	<0.001	-51.867	<0.001	-43.089	<0.001	-20.589	<0.001	-11.300	0.04
Cephalosporins																
cefazolin	0.478	1	-53.400	<0.001	-56.911	<0.001	-55.933	<0.001	-49.556	<0.001	-17.867	<0.001	-15.678	<0.001	-15.556	<0.001
ceftazidime	0.633	1	-48.878	<0.001	-53.533	<0.001	-56.200	<0.001	-47.089	<0.001	-35.922	<0.001	-34.611	<0.001	-32.322	<0.001
ceftiofur sodium	0.744	1	-55.133	<0.001	-52.789	<0.001	-53.689	<0.001	-56.622	<0.001	-40.489	<0.001	-36.678	<0.001	-38.322	<0.001
Miscellaneous																
doxycycline	0.478	1	-40.700	<0.001	-37.233	<0.001	-13.478	0.007	-16.867	<0.001	-12.156	0.021	-9.733	0.118	-5.978	0.634
enrofloxacin	1.322	0.999	-33.956	<0.001	-37.267	<0.001	-34.344	<0.001	-15.878	<0.001	-12.422	0.016	0.411	1	7.356	0.4
florfenicol	4.278	0.877	-56.056	<0.001	-57.044	<0.001	-57.456	<0.001	-31.856	<0.001	-29.344	<0.001	-27.078	<0.001	-26.644	<0.001
imipenem	4.433	0.86	-52.078	<0.001	-56.878	<0.001	-60.556	<0.001	-61.756	<0.001	-59.156	<0.001	-51.522	<0.001	-32.344	<0.001
vancomycin	0.633	1	-54.400	<0.001	-60.278	<0.001	-63.544	<0.001	-65.600	<0.001	-51.678	<0.001	-27.767	<0.001	-27.367	<0.001

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 3.3)** 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).

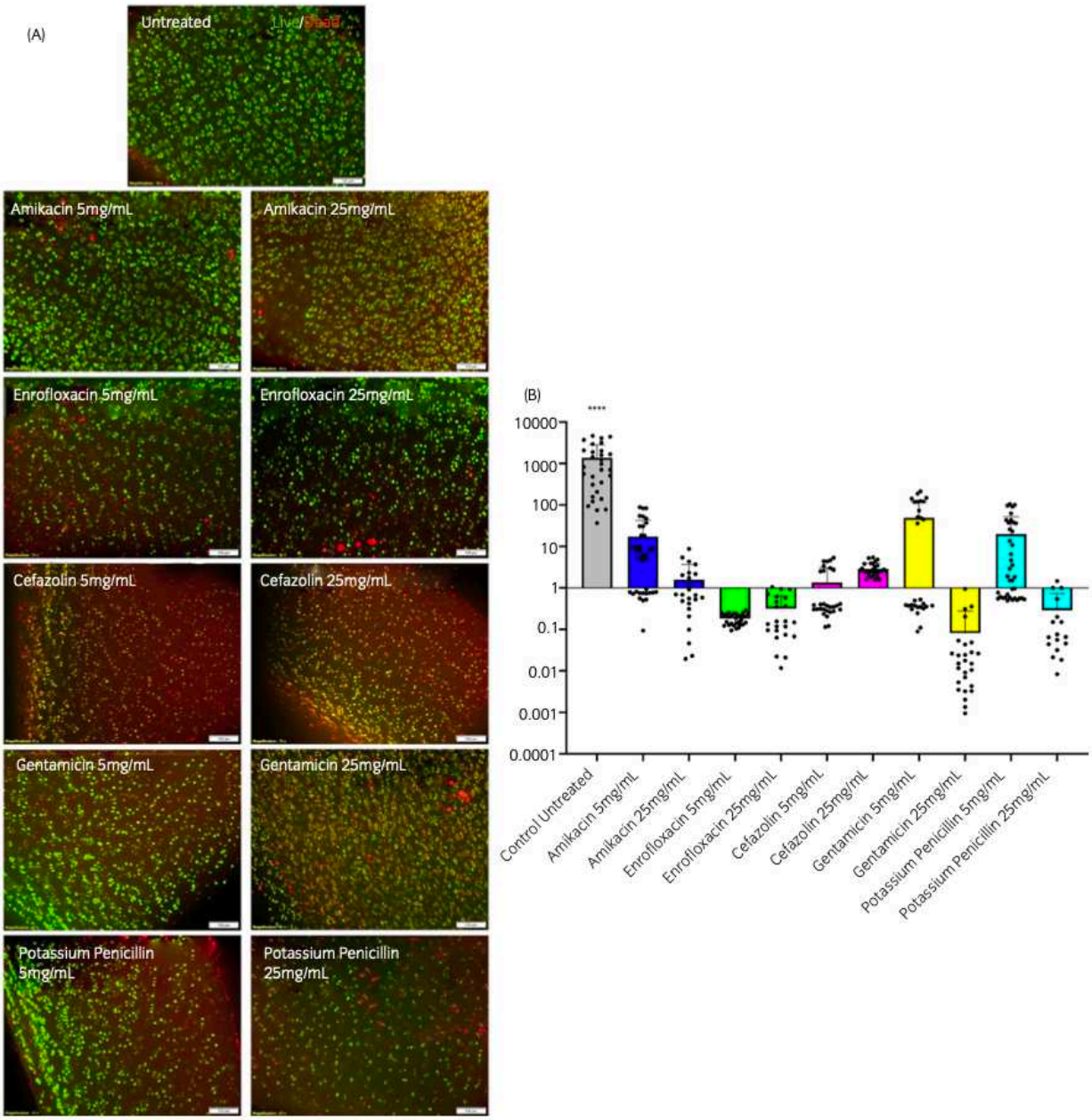

Figure 3.3: Viability of equine cartilage explants following incubation with antibiotics. Cartilage explant tissues were cultured in complete DMEM media supplemented with 10% fetal bovine serum or media supplemented with one of five antibiotics at either 25 or 5mg/mL. **3A)** Merged z-stack images of 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).

3.5 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 analyzed 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 vascularization 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 be 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 at 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 minimizing 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 orthopedic 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 maximize efficacy and minimize 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 minimize 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 minimize damage to native joint cells and maximize efficacy when antibiotics are administered intra-articularly.

Manufacturers' addresses

[a]Amikacin sulfate (250mg/ml). Teva Pharmaceuticals INC. North Wales, PA USA 19454

[b]Gentamicin sulfate. Sigma Life Science. St. Louis, MO USA 63103.

[c]Neomycin trisulfate salt hydrate. Sigma Life Science. St. Louis, MO USA 63103.

[d]Tobramycin sulfate salt. Sigma Life Science. St. Louis, MO USA 63103.

[e]Ampicillin sulbactam salt. Meitheal Pharmaceuticals. Chicago, IL USA 60631.

[f]Penicillin G potassium, USP. Pfizer Inc. New York City, NY USA 10017.

[g]Amoxicillin trihydrate. Sigma Life Science. St. Louis, MO USA 63103.

[h]Cefazolin salt. Sigma Life Science. St. Louis, MO USA 63103.

[i]Ceftazidime salt. Sigma Life Science. St. Louis, MO USA 63103.

[j]Ceftiofur sodium. Zoetis Inc., Kalamazoo, MI USA 49007.

[k]Enrofloxacin (22.7mg/ml). Bayer Healthcare LLC. Shawnee Mission, Kansas, USA 66201.

[l]Vancomycin. Sigma Life Science. St. Louis, MO USA 63103.

[m]Imipenem. Sigma Life Science. St. Louis, MO USA 63103.

[n]Doxycycline. Sigma Life Science. St. Louis, MO USA 63103.

[o]Florfenicol. Sigma Life Science. St. Louis, MO USA 63103.

[p]LIVE/DEAD ™ Cell Imaging Kit. Thermo Fisher. Waltham, MA 02451

[q]Olympus cellSens software. Olympus Life Sciences. Tokyo, Japan.

[r]GraphPad Software Prism8. San Diego, CA 92108.

[s]R package version 3.6.0 (2019-04-26). R Foundation. Vienna, Austria.

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CHAPTER 4: Evaluation of intra-articular amikacin administration in an equine non-inflammatory joint model to identify effective bactericidal concentrations while minimizing cytotoxicity[iv]

4.1 Summary

Septic arthritis causes significant morbidity and mortality in veterinary and human clinical practice and is increasingly complicated by multidrug-resistant infections. Intra-articular (IA) antibiotic administration achieves high local drug concentrations but is considered off-label usage and appropriate doses have not been defined. Using an equine joint model, we investigated effects of amikacin injected at three different doses (500, 125, 31.25 mg) on immune and cartilage responses in tibiotarsal joints. Synovial fluid (SF) was sampled at multiple time points over 24 hours, cell counts determined, and amikacin concentrations measured by liquid chromatography mass spectrometry. Cytokine concentrations and collagen degradation products in SF were measured by ELISA and multiplex immunoassays. Mean amikacin concentrations in SF were ≥MIC (0.004 mg/ml) for most common equine joint pathogens at all time points tested to 24 hours for all three amikacin doses evaluated. Inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1β) increased significantly in SF in the highest amikacin dose group, despite the fact that increases in SF cell counts were not observed. Similarly, biomarkers of cartilage type II collagen cleavage (C2C, C12C) were increased in SF following amikacin injection. Mechanistically, we further demonstrated using *in vitro* studies

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[iv] This chapter includes the complete published manuscript: Pezzanite L, Chow L, Hendrickson D, Gustafson DL, AR Moore, Stoneback J, Griffenhagen GM, Piquini G, Phillips J, Lunghofer P, Dow S, and Goodrich LR. Evaluation of intra-articular amikacin administration in an equine non-inflammatory joint model to identify effective bactericidal concentrations while minimizing cytotoxicity. *Front. Vet. Sci.* doi:10.3389/fvets.2021.676774. This article was reproduced with permission from Frontiers Media, Seattle, WA.

that chondrocytes and synoviocytes killed by exposure to amikacin underwent apoptotic cell death and were phagocytosed by macrophages in a non-inflammatory process resembling efferocytosis. Neutrophils and T-cells were susceptible to amikacin cytotoxicity at clinically relevant doses, which may result in blunting of cellular inflammatory responses in SF and account for the lack of increase in total nucleated cell counts following amikacin injection. In summary, decisions on whether to inject cytotoxic antibiotics such as aminoglycosides intra-articularly and what doses to use should take into account the potential harm that antibiotics may cause and consider lower doses than those previously reported in equine practice.

4.2 Introduction

Septic arthritis causes significant morbidity and mortality in both equine and human clinical practice.[1,2] Case fatality rates in humans are 11-15% in monoarticular disease but increase to 50% when multiple joints are involved.[1,3-6] Reduced functional outcomes and osteomyelitis are reported in 24% and 8% of human survivors, respectively.[7] Risk factors for developing septic arthritis in people include joint prosthesis, rheumatoid or osteo-arthritis, skin disease, previous intra-articular corticosteroid injection and comorbidities such as diabetes.[1,8-10] Regardless of age group or risk factors present, *Staphylococcus aureus* is the most common causative agent in humans, followed by other gram-positive bacteria.[7,9,11] The incidence of septic arthritis in people is reportedly increasing which has been attributed to a progressively aged population, higher number of invasive orthopedic procedures being undertaken, more frequent orthopedic-related infections, and increased use of immunosuppressive therapies.[1,8] Therefore, investigation of therapies to improve outcomes in septic arthritis is indicated.

Current treatments for septic arthritis in both human and equine patients include lavage with debridement of purulent material via arthroscopy or less commonly arthrotomy, antibiotics administered systemically and by regional perfusion, or rarely serial synovial fluid aspirations in patients too unstable to undergo general anesthesia.[1,2] However, unlike the case in human orthopedic practice, antibiotics have also been administered intra-articularly (IA) in equine veterinary medicine as an adjunctive therapy to treat septic arthritis for decades.[13-17] This route of administration has more recently gained attention in human medical practice, particularly following prosthetic arthroplasty, in an attempt to address the issue of chronic biofilm infections with an increasing incidence of drug-resistant bacteria through achieving high local antibiotic concentrations.[18-22] However, intra-articular antibiotic administration is "off-label" in all species and appropriate intra-articular doses have not been defined nor potential cytotoxicity with this route of administration investigated for any of the major classes of antibiotics. Previous studies have demonstrated the potential for antibiotic cytotoxicity *in vitro* on joint cells from humans and veterinary species.[18,19,23-25] Moreover, despite widespread use of intra-articular antibiotics in equine practices, studies in horses have revealed variable toxicity following IA antibiotic administration depending on the antibiotic and dose selected.[26-33] However, no previous studies have compared antibiotic cytotoxicity or performed *in vivo* dose titration studies to assess the impact of antibiotics administered IA in any species on joint inflammation and cartilage degradation.

Therefore, we conducted a series of preliminary *in vitro* investigations to better define the capacity of different antibiotic classes to exert cytotoxic effects on chondrocytes and synovial cells from horses and dogs.[23,24] We demonstrated that amikacin, the antibiotic most commonly used intra-articularly in horses,[13-15] was the most cytotoxic of antibiotics evaluated to

chondrocytes, and rapidly induced apoptotic cell death.[24] We built on that work in this study, using a combination of *in vitro* assays and an *in vivo* equine model of intra-articular antibiotic administration to evaluate the potential impact of amikacin doses used clinically in equine practice on overall joint health. We hypothesized that intra-articular administration of amikacin at conventional doses would induce inflammation and cartilage injury. We determined the pharmacokinetics of amikacin administered at multiple doses, and quantified biomarkers of inflammation and cartilage damage in synovial fluid. To further assess macrophage responses to joint cells killed by amikacin, we generated equine monocyte-derived macrophage cocultures and assessed macrophage secretion of inflammatory cytokines following engulfment of amikacin-killed cells in a process known as efferocytosis. The overall goal of this work was to identify amikacin doses that could achieve effective bacterial inhibitory concentrations for common joint pathogens while minimizing induction of cytotoxicity and inflammation in the joint.

4.3 Materials and Methods

Horses – The use of six healthy 4 to 12-year-old Quarter Horses (four geldings, 2 mares) was approved by the Colorado State University (CSU) Institutional Animal Care and Use Committee (protocol #19-9058A). Horses were determined healthy by physical examination and lameness evaluation by board-certified veterinary surgeons (LG, LP) and radiographic examination (four-view) of both tarsi. All horses were sound in the hindlimbs at the trot and lacked radiographic evidence of osteoarthritis of the tarsal joints prior to study enrollment.

Study design – This study was implemented with a randomized incomplete block design, with the random assignments being first leg treated (left or right), with two of the four treatments

administered to each horse, and then order that the treatments were administered. A random number generator, random.org, was used to randomize both initially treated leg as well as which of the two treatments the horses received. Each horse received either amikacin at one of three doses, (amikacin sulfate (250 mg/mL); Teva Pharmaceuticals, INC) or lactated Ringer's solution (LRS) intra-articularly (**Figure 4.1**).

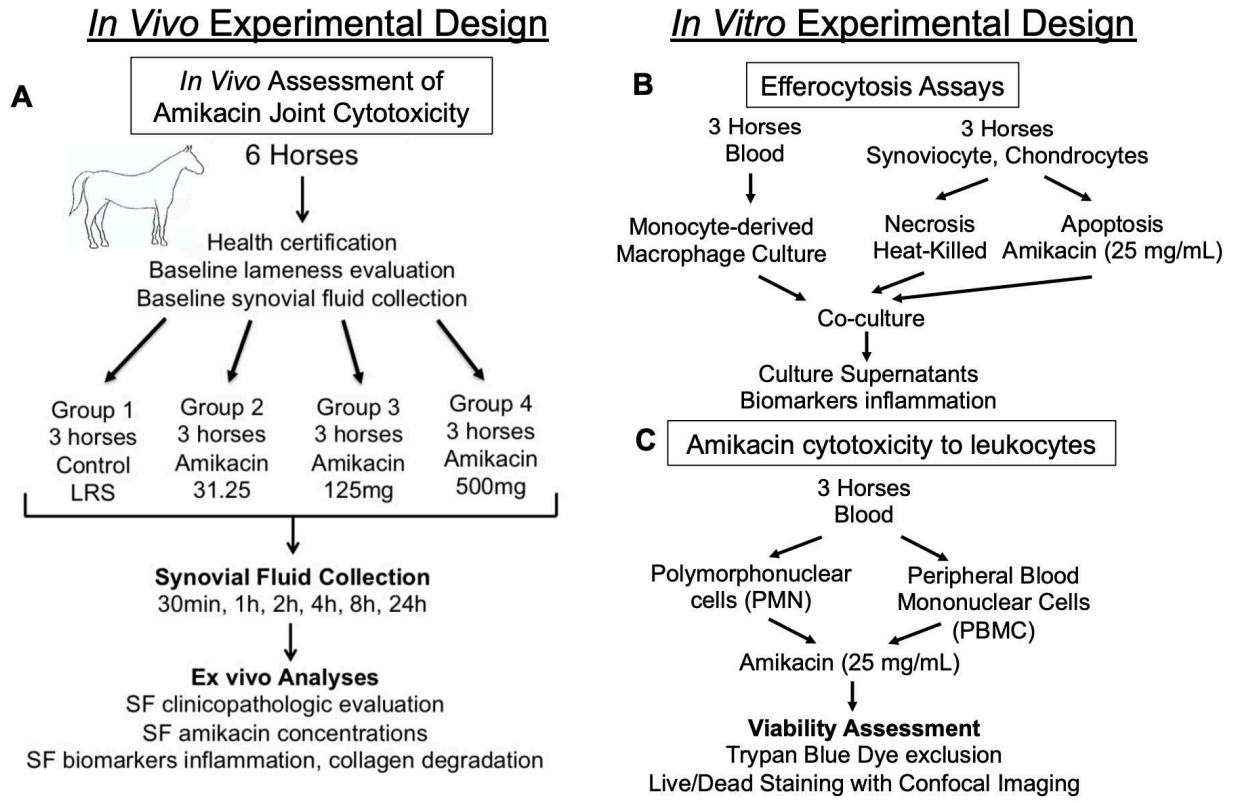

Figure 4.1: Diagram outlining the experimental design. Schematic overview of the study design for the A) *in vivo* assessment of cytotoxicity of amikacin on the joint of horses, B) *in vitro* evaluation of macrophage efferocytosis of joint cells killed by amikacin, and C) *in vitro* assessment of amikacin cytotoxicity to leukocytes. Abbreviations: Synovial Fluid (SF).

The treatment groups were allocated as follows: Group one, amikacin (31.25 mg, diluted in LRS to a total volume of 2ml); Group two, amikacin (125 mg, dilution in LRS to a total volume of 2 mL); Group three, amikacin (500 mg); Group four, equivalent volume of LRS (2

mL) as control group. Each horse was initially assigned to receive one treatment in either the left or right tibiotarsal joint. After a two-week wash-out period, the contralateral joint was injected with a second, randomly assigned treatment. Randomization of treatment allocation and order was performed by one individual (LP) who was unblinded during the experiment; all other collaborators were blinded to treatment throughout data collection and analysis. Thus, six horses were administered two treatments each, but no two horses receive the same pair of treatments, and each group included three horses total (n=3). The sample size of three horses per treatment allocation was determined based on previous literature.[14]

Horses were sedated with a combination of xylazine (0.2-1 mg/kg IV) or detomidine (0.01-0.02 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV) to effect. The tibiotarsal joint was aseptically prepared with chlorhexidine gluconate (4%, VetOne, MWI, Boise, ID, 83705) followed by 70% ethanol, and injected as described above. The highest dose was selected based on previous reports by equine practitioners of use of up to 500 mg amikacin per joint and lower titrated doses (125 mg, 31.25 mg) were also evaluated.[25,34]

Synovial fluid sample collection – Synovial fluid (SF) samples (1-2 mL) were aspirated from the injected tibiotarsal joint at baseline and 0.5, 1, 2, 4, 8, and 24 hours post-drug administration. Prior to each sample collection, the tibiotarsal joint was aseptically prepared and horses were sedated as described above. SF was aspirated with a sterile 20-gauge needle into vials and either submitted immediately for clinicopathogical fluid analysis or aliquoted and stored at -80°C until later use in immunoassays or amikacin concentration determination via mass spectrometry for pharmacokinetic analysis.

Synovial fluid clinicopathological parameters – SF samples were submitted to the CSU Clinical Pathology Laboratory for evaluation by a board-certified clinical pathologist (ARM) for

fluid analysis including total nucleated cell count (Hematrue, Heska Corp, Loveland CO, USA), refractometric total protein, preparation of Wright-Giemsa stained (Aero-spray, Logan, UT, USA) direct smears for determination of a manual leukocyte differential, and subjective grading of the glycosaminoglycans (adequate or disrupted) and quantity of red blood cells (within normal limits or excessive).

Amikacin concentration determination - Extraction and analysis of amikacin from equine SF samples was performed using high-pressure liquid chromatography/tandem mass spectrometry (LC/MS/MS) system. The system consisted of a 50 mm C_{18} column (2.1 mm i.d.) with a Phenomenex C18 Filter Frit Guard Cartridge on the Shimadzu HPLC system coupled to the 3200 A-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Inc., Foster City, CA) with a flow rate of 750 µL/min. The instrument was operated in multiple reaction monitoring (MRM) positive ion mode. LC gradient conditions (ion-pairing chromatography) were 10mM heptafluorobutyric acid with 10mM ammonium hydroxide in milli-Q water (Mobile Phase A) and 5mM heptafluorobutyric acid with 5mM ammonium hydroxide in 10:90 milli-Q water: Acetonitrile (Mobile Phase B).

Amikacin stock solution (100 mg/mL) in 10 mM heptafluorobutyric acid with 10mM ammonium hydroxide diluted in milli-Q water was prepared. A standard curve (10, 25, 50, 75, 100, 250, 500, 750, 1,000, 2,500, 5000, and 10,000) of amikacin (µg/mL) in Milli-Q water was prepared. A 1 mg/mL solution of amikacin in 10 mM ammonium hydroxide in milli-Q water was prepared, and 40 mL of Final Dilution Solution (FDS) (50/50) Mobile Phase B/Milli-Q with 2,500 ng/mL amikacin was used as an internal standard. Blank equine synovial fluid (45µl) was added to 1.5 mL micro centrifuge tubes as dilution standards, vortexed for 1 minute and centrifuged for 5 minutes at 14,000 RPM. Standard dilutions (1:100, 1:1,000, 1:10,000) in milli-

Q water were prepared by transferring 10 µL of each standard to 990 µL of Milli-Q water in 1.5 mL microcentrifuge tubes. Six QC samples (3x25, 3x100, and 3x750 µg/mL) were prepared, vortexed for 5 minutes and centrifuged for 1 minute at 8,000g. Standard samples (125µL) were transferred to sample vials and analyzed.

To analyze amikacin concentrations in SF samples, dilutions (1:100, 1:1000, and 1:10000) were prepared using Milli-Q in 1.5 mL microcentrifuge tubes. Samples were vortex mixed for 5 minutes, centrifuged for 1 minutes at 8,000g and transferred to auto sample vials for analysis.

Determination of biomarkers of cartilage metabolism – Competitive ELISAs, previously validated for equine samples (IBEX Pharma, Quebec, Canada) were used to measure concentrations of biomarkers C2C (biomarker of Type II collagen degradation) and C12C (biomarkers of Type I (soft tissue) and Type II (cartilage) collagen degradation) in SF from all treatment groups collected at time points 0, 8, and 24h as previously described.[35]

Determination of biomarkers of joint inflammation (cytokines, collectin) – Concentrations of C reactive protein (CRP), a widely-accepted inflammatory marker, were measured using a competitive ELISA, previously validated for use in equine samples (Immunology Consultant Laboratories, Portland, Oregon, 97224) from all treatment groups at time points 0, 8, and 24h. Fluorescent bead-based multiplex assay (Milliplex MAP Equine Cytokine/Chemokine Magnetic Beads Multiplex Assay, Millipore Sigma, Burlington, MA, 01803) was used to quantify the concentrations of 23 analytes (Eotaxin/CCL11, FGF-2. Fractalkine/CS3CL1, G-CSF, GM-CSF, GRO, IFN, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17 α, IL-18, IP-10, MCP-1, RANTES/CCL5 and TNF-α) in SF from all time points.

In vitro culture of equine macrophages - To generate macrophages, equine peripheral blood mononuclear cells were isolated from whole blood of three horses (Quarter Horses, aged two to three years, on mare and two geldings) via density gradient centrifugation (Ficoll-Paque TM plus, GE Healthcare Bio-Sciences) and cultured in macrophage medium (Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, non-essential amino acids, and penicillin/streptomycin antibiotics; SigmaAldrich) with human M-CSF (PeproTech, Rocky Hill, NJ USA 80553) at 25 ng/mL to stimulate differentiation into macrophages in three to five days, as previously described.[36]

Efferocytosis and cytokine suppression assays – Equine synoviocytes and chondrocytes were collected postmortem and isolated from three different horses euthanized for reasons unrelated to the study (Quarter Horses; aged two to three years; two geldings and one mare) and expanded in culture as previously described.[23] The synoviocytes and chondrocytes were killed rapidly at passage one by exposure to either amikacin (25 mg/ml; diluted in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin antibiotics; representing apoptotic cell death) or heat (HK; 50ºC water bath; representing necrotic cell death) and then co-cultured at 1:1 ratio with equine monocyte-derived macrophages in monolayer culture for two hours. After two hours in culture with dead cells to allow time for phagocytosis, macrophages were washed in phosphate buffered saline and allowed to culture in growth media for an additional 18 hours. Supernatants were collected at that time for evaluation of cytokines on a limited panel multiplex bead assay (IL1-ß, IL-6, TNF- α) (MilliporeSigma, Burlington, MA) and TGF-ß1 ELISA (R&D Systems, Minneapolis, MN, 55413).

Amikacin cytotoxicity on polymorphonuclear cells and peripheral blood mononuclear cells – Three different horses (Quarter Horses, aged two to three years, one gelding and two

mares) donated blood for isolation of peripheral blood mononuclear cells (PBMC) and polymorphonuclear cells (PMNC), and the cytotoxicity of amikacin on each cell line was assessed. The PBMC were isolated via density gradient centrifugation (Ficoll-Paque TM plus, GE Healthcare Bio-Sciences) at 400 g for 30 minutes. The PMNC were isolated using the Lympholyte-Poly (Cedarlane, Peterborough, United Kingdom) separation gradient according to manufacturer's instructions. The PBMC and PMN from the three horses were exposed to amikacin over a range of concentrations in complete growth medium (25, 12.5, 6.25, 3.125, 1.56, 0.78 0.39 mg/mL vs control) in triplicate for 24 hours or one hour, respectively. Live/dead visualization of PBMC following amikacin exposure was further performed using the LIVE/DEAD Viability and Counting Kit (Thermo Fisher Scientific) according to manufacturer's instructions and visualized on an Olympus IC83 confocal microscope. Ratios of live to dead cells were calculated by imaging total area of each channel using ImageJ software.[37]

Data Analysis and Pharmacokinetic Modeling – Statistical comparisons between the four treatment groups used ANOVA, followed by Tukey's adjustment for multiple comparisons. Data and residuals were visually assessed for normality. All data points collected were included in the final analysis. Significant differences between clinicopathological parameters and biomarkers were evaluated between baseline and at each time point using a two-way ANOVA with repeated measures. The appearance of glycosaminoglycans, granulated small monocytes or excessive red blood cells over the range of amikacin concentrations and time were analyzed using logistic regression (function *'glm'* from the base STATS package). Biomarkers were normalized to baseline for analysis due to variability between baseline values between individual horses.

The half maximal inhibitory concentration (IC50), or concentration of antibiotic at which 50% of cells (PBMC, PMNC) were viable, was determined by normalizing dose response for each concentration to control, transforming data to normalized dose response vs. log10 (concentration) and estimating using IC50 nonlinear regression by fitting the data to a three-parameter sigmoid function (implemented as "log(inhibitor) vs. dose response"). In instances where the IC50 was calculated to be 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 not be determined based on the concentrations assessed.

Pharmacokinetic parameters for each individual dose level were calculated by non-compartmental analysis using the PKNCA package for R.[38] AVOVA and IC50 calculations were performed using Prism software v8.4.1 (GraphPad Software Inc., La Jolla, CA). Logistic regression and pharmacokinetic analyses were performed using R v4.0.0 ("Arbor Day", R Foundation for Statistical Computing, Vienna, Austria.) Significance was set at $p \leq 0.05$.

4.4 Results

Defining amikacin pharmacokinetics following intra-articular administration-

Synovial fluid amikacin concentrations were detectable for all doses injected at all time points evaluated and the mean remained ≥MIC for most common equine pathogens (>4 μg/ml) at 24 hours (**Figure 4.2**). Pharmacokinetic data (c_{max}, half-life, AUC_{last} and AUC_{inf_pred}) are reported in **Table 4.1**. Amikacin values at each time point (mean +/- s.d.) are reported in **Table 4.2**.

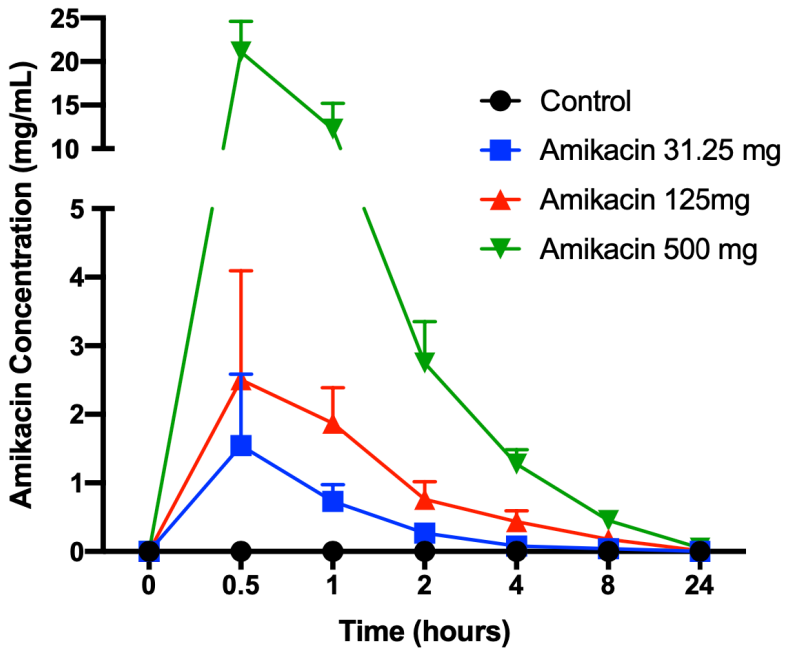

Figure 4.2: Synovial fluid amikacin concentrations over time following single intra-articular administration of amikacin at three concentrations. Synovial fluid amikacin concentrations (n=3 horses per group) were determined by high-pressure liquid chromatography / mass spectrometry and expressed as mean (+/- SD).

Table 4.1: Mean (+/-SD) synovial fluid amikacin concentrations over 24 hours following a single intra-articular administration of amikacin at one of three doses (500, 125, or 31.25mg) or control (LRS), n=3 horses per group.

	Treatment							
	Control		Amikacin 31.25 mg		Amikacin 125 mg		Amikacin 500 mg	
	Mean (mg/ml)	SD	Mean (mg/ml)	SD	Mean (mg/ml)	SD	Mean (mg/ml)	SD
Time (h)								
0	0.000	0.000	0	0	0.000	0.000	0.000	0.000
0.5	0.000	0.000	1.545	1.041	2.503	1.590	21.100	3.500
1	0.000	0.000	0.732	0.244	1.870	0.517	12.233	2.967
2	0.000	0.000	0.266	0.113	0.761	0.257	2.750	0.601
4	0.000	0.000	0.078	0.010	0.435	0.157	1.273	0.211
8	0.000	0.000	0.040	0.012	0.177	0.120	0.450	0.141
24	0.000	0.000	0.004	0.001	0.012	0.005	0.047	0.032

Table 4.2: Pharmacokinetic parameters evaluated in synovial fluid following a single intra-articular administration of amikacin at one of three doses to six Quarter Horses. All values were generated using noncompartmental analysis. C_{max}, maximum measured concentration (µg/mL); half-life (hours); AUC_{last}, area under the synovial fluid concentration curve from time 0 to the last measurable concentration; $AUC_{0\text{-}inf}$, area under the synovial fluid concentration curve extrapolated to infinity (µg/ml).

Amikacin dose (mg)	c_{max} (ug/ml)	Half-life (h)	AUC_{last} (ug/ml)	AUC_{inf_pred} (ug/ml)
31.25	1,680	4.58	2,210	2,230
125	3,230	3.79	6,120	6,180
500	20,900	4.33	29,300	29,600

Impact of intra-articular amikacin on biomarkers of cartilage metabolism and health– Immunoassay revealed dose-dependent increases in C2C levels, which were increased in horses treated with 125 mg amikacin at 8h vs. baseline (p=0.02), horses receiving 500 mg amikacin at 24h hours vs. baseline (p=0.01) and at 24h vs. 8h (p=0.05). When evaluated at each time point, C2C levels had high magnitude of change between treatment groups at 8h for 31.25 mg vs. 125 mg (p=0.002) and 125 mg vs. 500 mg (p=0.005), and at 24h for 31.25 mg vs. 500 mg (p=0.03) and 125 mg vs. 500 mg (p=0.04) (**Figure 4.3**). C12C levels increased in horses treated with 500 mg amikacin at 24h vs. baseline (p=0.0006) and at 24h vs. 8h (p=0.006). When evaluated at each time point, C12C was increased with 500 mg amikacin vs. control at 24h (p=0.002) (**Figure 4.3**).

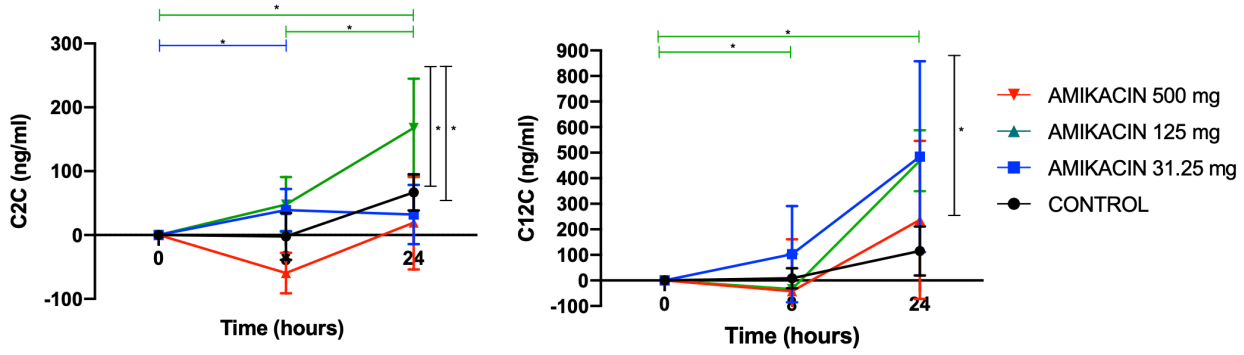

Figure 4.3: Concentrations of biomarkers of collagen degradation following single intra-articular administration of amikacin at three doses compared to control. Competitive enzyme-linked immunosorbent assays (ELISAs), previously validated for use in equine synovial fluid, were used to measure the biomarker concentrations of neo-epitope C2C (biomarker of Type II collagen degradation) and C12C (biomarker of Type I (soft tissue) and Type II (cartilage) collagen degradation) in synovial fluid of n=3 horses per group. Results are expressed as mean +/- SD, normalized to baseline.

Glycosaminoglycan content was not different between groups (p=0.95) but was disrupted with increasing frequency over time with repeated arthrocentesis in all treatment groups (p<0.001).

Impact of intra-articular amikacin administration on cytokine and collection concentrations in SF- Cytokine multiplex analysis was used to assess the impact of amikacin on SF cytokine concentrations over time (**Table 4.3, Figures 4.4**). CRP concentrations increased in horses treated with 500 mg amikacin compared to all other treatments at 8h and 24h, which did not reach statistical significance (concentration effect overall, p=0.08) (**Figure 4.4**). Multiplex assay documented detectable levels for ten of 23 cytokines (IL-1ß, FGF, G-CSF, IL-10, TNF-α, IL-6, IL-8, IL-18, IP-10, or MCP-1). Five cytokines (IL-1ß, FGF, G-CSF, IL-10, TNF-α) had different levels between time points for each treatment group evaluated or between treatment groups at different time points (**Table 4.3, Figure 4.4)**. IL-1ß was higher across time points when 125 mg was injected vs. control (p=0.0008) and 500 mg vs. control (p<0.0001). TNF-α levels were higher in samples with 500 mg amikacin vs. control (p=0.03). Levels of IL-10 were higher in 31.25 mg amikacin vs. control (p=0.007). No differences between treatment groups were observed in levels of IL-6, IL-8, IL-18, IP-10, or MCP-1.

Table 4.3: Statistical analysis of cytokine levels in synovial fluid following amikacin treatment at various doses or control (lactated Ringer's solution). Cytokine levels (IL-1ß, TNF-α, IL-6, IL-10, IL-18) were compared across time points between treatment groups and control by two-way ANOVA with repeated measures. Significance was assessed at *p≤0.05.

Treatment	IL1-β		TNF-α		IL-6		IL-10		IL-18	
Control vs. amikacin 31.25 mg	ns	0.10	ns	0.78	ns	0.35	*	0.007	ns	0.18
Control vs. amikacin 125 mg	*	0.0008	ns	0.43	ns	0.62	ns	0.53	ns	0.46
Control vs. amikacin 500 mg	*	<0.0001	*	0.03	ns	0.81	ns	0.54	ns	0.18

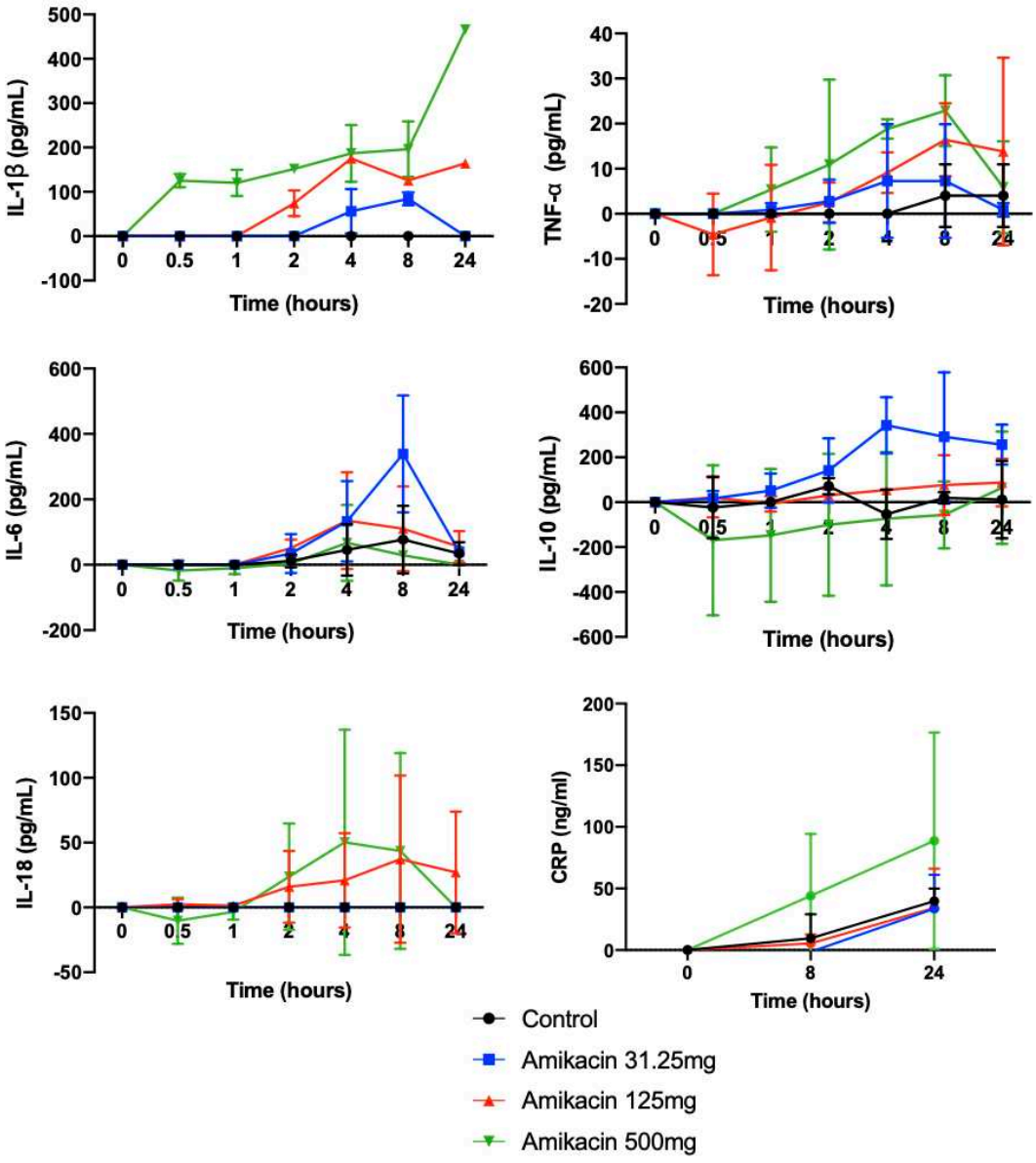

Figure 4.4: Concentrations of biomarkers of inflammation in synovial fluid of joints injected with amikacin. The concentrations of 23 analytes (Eotaxin/CCL11, FGF-2. Fractalkine/CS3CL1, G-CSF, GM-CSF, GRO, IFN, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12 (p70), IL-13, IL-17a, IL-18, IP-10, MCP-1, RANTES/CCL5 and TNFα) were quantified in synovial fluid samples from all treatment groups at all time points samples using fluorescent bead-based multiplex assay. Measurable cytokine levels were detectable for ten of 23 cytokines (IL1β, FGF, G-CSF, IL-10, TNFα, IL-6, IL-8, IL-18, IP-10, or MCP-1). Cytokine levels were compared between treatment groups at each time point and between time points for each treatment group, as well as for overall effect of treatment and time by two-way analysis of variance. Significant differences were seen across time points for IL1-β for control vs. amikacin 125mg ($p<0.0008$) and control vs. 500mg ($p<0.0001$), TNF-α for control vs. 500mg ($p=0.0281$), and IL-10 for control vs amikacin 31.25mg ($p=0.0066$). Five cytokines (IL1β, FGF, G-CSF, IL-10, TNFα) additionally had significantly different levels between time

points for each treatment group evaluated or between treatment groups at different time points. No significant differences between treatment groups were observed in levels of IL-6, IL-8, IL-18, IP-10, or MCP-1. An ELISA was also used to determine concentrations of inflammatory C reactive protein (CRP) in synovial fluid. Three joints were included per treatment group. Cytokine levels were compared between three treatment groups using a 2-way ANOVA. Results are expressed as mean +/- SD, normalized to baseline.

Synovial fluid clinicopathological parameters –No differences were found between treatment groups and controls with respect to total nucleated cell counts, total protein and red blood cell count at each time point evaluated (p=0.35, p=0.70, p=0.14, respectively) (**Figure 4.5**). However, differences were seen between time points with repeated synoviocenteses (total nucleated cell count p=0.0002, total protein p=0.001, red blood cell count p=0.02). There was no difference between amikacin treatments for each of the four cell types (neutrophils p=0.14, monocytes p=0.50, eosinophils p=0.89, basophils p=0.14), but there were differences for time with repeated synoviocenteses for each cell line (neutrophils p<0.0001, monocytes p<0.0001, eosinophils p=0.02, basophils p=0.03) (**Figure 4.5**). Presence of granulated small monocytes and excessive red blood cells was not different between treatment groups (*p*=0.41, p=0.05 respectively). Presence of granulated small monocytes did not differ over time (p=0.74). Presence of excessive red blood cells increased over time (p=0.002).

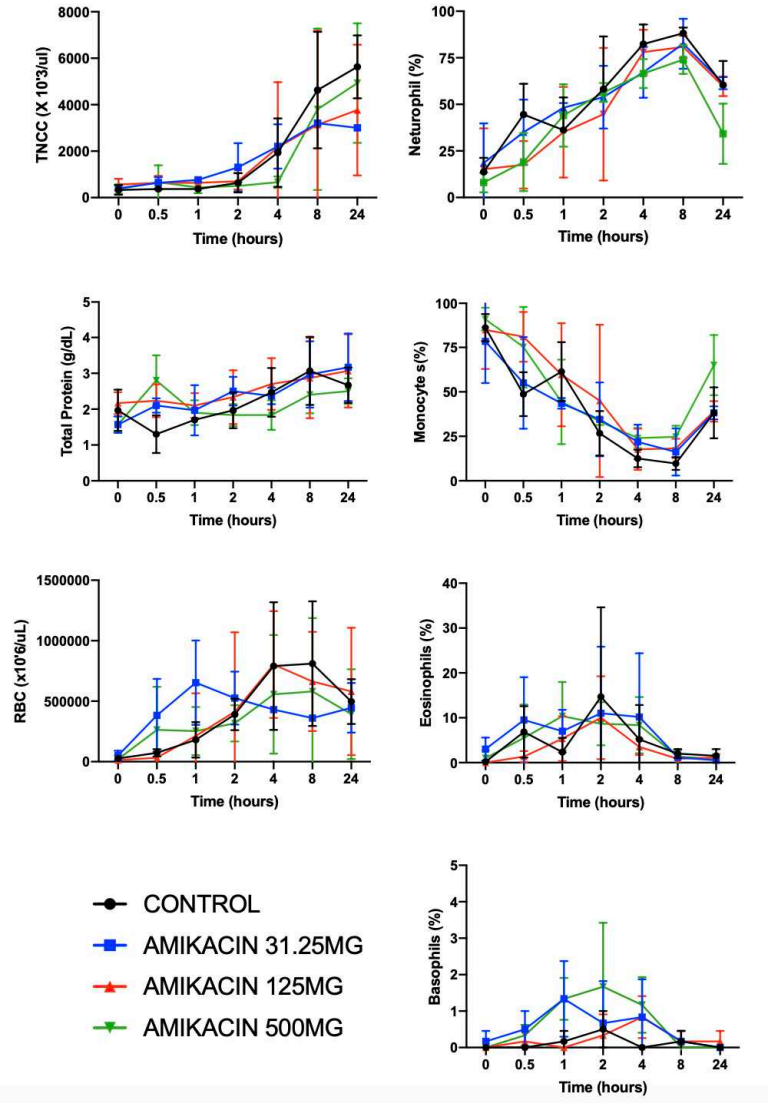

Figure 4.5: Clinicopathologic parameters for synovial fluid following single intra-articular administration of amikacin in three treatment groups. Fluid analysis on synovial fluid samples obtained from three horses per group was performed on all samples for total nucleated cell count, total protein, and red blood cells count and leukocyte differential (y-axes are not equivalent across graphs). There was significantly increased neutrophils (%) and decreased monocytes (%) with repeated arthrocenteses over time. Results are expressed as mean +/- SD.

Mechanisms of amikacin cytotoxicity and impact on cell clearance by macrophages -

Macrophages engulfing amikacin-killed cells demonstrated reduced release of pro-inflammatory cytokines (IL-1ß, TNF- α , IL-6) and a concomitant increased production of anti-inflammatory cytokines (TGF- ß) (**Figure 4.6**). For example, secretion of TGF-ß1 was increased following

efferocytosis of amikacin killed synovial lining cells, compared to necrotic cells (p=0.01) Similarly, amikacin-killed chondrocytes also induced more TGF- ß than heat-killed chondrocytes (p=0.03). In contrast, we observed that less IL-1ß was produced by macrophages incubated with amikacin-killed cells than by macrophages incubated with heat-killed cells (p=0.006). This was interpreted as evidence of efferocytosis of apoptotic cells, as previously described (39). Similar responses were noted for TNF- α (p<0.0001) and IL-6 (p<0.0001) production between amikacin versus heat-killed cells. These findings indicate that cell death induced by amikacin is inherently anti-inflammatory, compared to pathways associated with necrosis. These findings *in vitro* help explain why SF from amikacin-treated horses manifested a relatively benign response to ongoing cellular death and tissue injury locally within the joint.

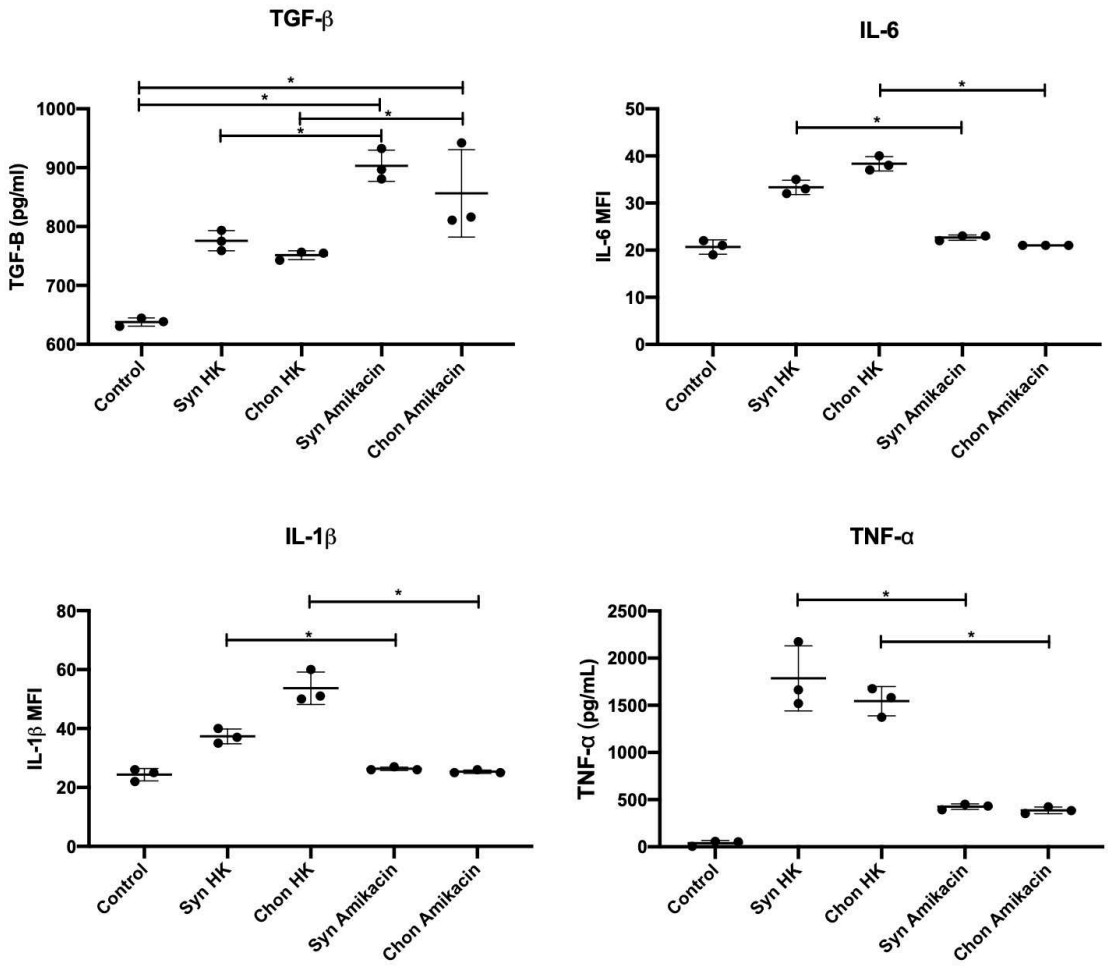

Figure 4.6: Efferocytosis and cytokine suppression assays quantifying IL1-ß, IL-6, TNF-α, and TGF-ß1 in macrophage culture media after incubation with equine synoviocytes and chondrocytes killed by induced apoptosis (amikacin 25 mg/mL) or necrosis (heat killed; HK). Data was evaluated by 2-way ANOVA. Significance was assessed at *p≤0.05.

Relative susceptibility of leukocytes and joint cells to amikacin cytotoxicity. The preceding findings suggested another potential mechanism to dampen joint responses to amikacin toxicity. The SF white blood cell counts were not increased in amikacin-treated horses relative to control, despite evidence of inflammation and cartilage damage (**Figures 4.3,4.4,4.5**). This could be explained for example if leukocytes, especially neutrophils, were particularly susceptible to amikacin toxicity. Therefore, we determined the mean cytotoxicity-inducing concentrations for purified populations of equine neutrophils and lymphocytes. The IC50 was 6.62 mg/mL for lymphocytes and between 0.78 and 1.56 mg/mL for neutrophils, demonstrating that lymphocyte toxicity would be induced at concentrations achieved clinically following amikacin injection. Live/dead staining of PBMC following amikacin exposure demonstrated dose-dependent cytotoxicity of amikacin exposed PBMC at 4h vs. control (**Figure 4.7**). Thus, these findings suggest that the absence of a significant rise in SF leukocytes following amikacin injection may be attributed in part to direct and rapid leukocyte killing *in situ* by high concentrations of amikacin, followed by rapid clearance by synovial macrophages.

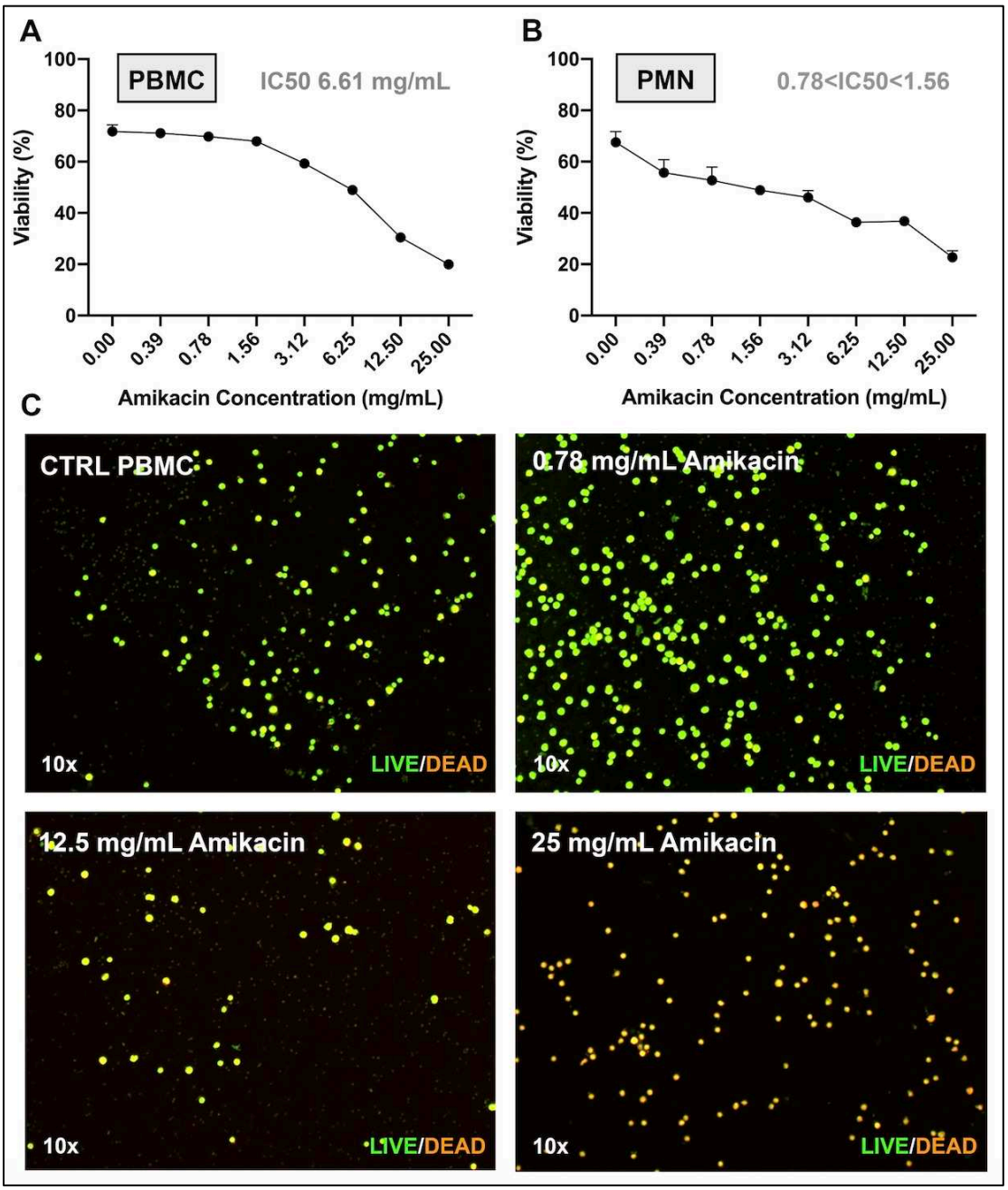

Figure 4.7: Effect of amikacin on neutrophils and peripheral blood mononuclear cells at clinically relevant doses. The cytotoxicity of amikacin on **(7A)** peripheral blood mononuclear cells (PBMC) and **(7B)** polymorphonuclear cells (PMN) derived from whole blood of three horse donors each in triplicate and assessed by trypan blue dye exclusion over a range of amikacin concentrations The inhibitory concentration 50 (IC50), or concentration at which 50% of the cells were dead is denoted. **(7C)** Live/dead visualization of PBMC following amikacin exposure over a range of doses reached clinically (0.78, 12.5, 25mg/ml) performed using the LIVE/DEAD Viability and Counting Kit (Thermo Fisher Scientific).

4.5. Discussion

Intra-articular antibiotics have been used for decades in equine practice and have been more recently reported in humans to treat orthopedic device related infections following arthroplasty.[20-22] As all intra-articular antibiotic use is 'off-label', appropriate doses have not been determined and potential cytotoxicity by this route of administration has not been fully assessed. Amikacin sulfate is the antibiotic most frequently used in joints in equine clinical practice, and previous work has further demonstrated it to be cytotoxic to equine cartilage *in vitro*.[23,24] For these reasons, we evaluated the cytotoxicity of three doses of amikacin administered in the tarsocrural joint of horses towards the goal of providing dosing guidelines for practitioners, with translational relevance to physicians who may also inject antibiotics in joints. Intra-articular administration of amikacin at doses currently used in horses induced both chondrotoxicity and inflammation in this model, supporting our initial hypothesis. Pharmacokinetic analysis of synovial fluid indicated that lower amikacin doses than those frequently used in equine practice could achieve effective MIC concentrations, while minimizing potential toxicity. We further investigated the mechanisms by which amikacin-induced inflammation was resolved within the joint using *in vitro* co-cultures of equine monocyte-derived macrophages with chondrocytes and synoviocytes and demonstrated it to be through a process resembling efferocytosis, further suppressing inflammation.

Amikacin sulfate is injected prophylactically in equine joint injections and to treat septic arthritis prior to receipt of bacterial culture and sensitivity due to its broad spectrum of activity against both Gram positive and negative bacterial isolates including *Staphylococcus*, *Escherichia*, *Enterobacter* and *Pseudomonas*.[40] This study was the first to evaluate an *in vivo* dose titration for intra-articular amikacin administration in horses, building on previous

veterinary literature regarding amikacin pharmacokinetics. Sedrish *et al.* reported SF amikacin concentrations remained ≥MIC (4 μg/ml) for 24 hours when 500 mg was injected into a normal equine radiocarpal joint.[13] Taintor *et al.* further demonstrated that joint inflammation accelerated amikacin distribution from the joint, as amikacin (500 mg, radiocarpal joint) remained ≥MIC in normal joints for 72h but only 48h in endotoxin-inflamed joints, which was attributed to differential antibiotic movement from the joint with increased synovial vascularity in inflammation.[15] In this study, mean amikacin concentrations in synovial fluid reached levels well over 100x MIC and mean levels were sustained at or above the established MIC for most common equine pathogens (4 μg/ml) for at least 24 hours following injection of all amikacin doses assessed (31.25, 125, 500 mg).[41] While it is recognized that select equine organisms have been reported to have higher MIC (e.g. coagulase positive *Staphylococcus sp.* 8 μg/ml, *Pseudomonas aeruginosa* 64 μg/ml, *Streptococcus zooepidemicus* 128 μg/ml), these findings would suggest that lower amikacin doses than those commonly used by equine practitioners (125 to 500 mg) may be utilized to both reach and sustain therapeutic levels, although further evaluation in inflamed or infected joints is warranted.[15,34]

The degree of inflammation induced by amikacin injection and subsequent mechanisms of resolution of inflammation warrant further discussion. Clinicopathological analysis of synovial fluid samples revealed alterations in differential cellular composition over time with repeated synoviocenteses, but no difference between treatment groups. This may be attributed to the frequency of SF sampling, resulting in elevated total nucleated cell count and total protein as well as altered leukocyte differential, which could have overwhelmed any individual treatment effect. However, amikacin injection elicited dose-dependent increases in pro-inflammatory cytokines TNF-α and IL1-β in the joint, indicating an inflammatory response. Concentrations of

IL-10 were also elevated in the synovial fluid of horses injected with the lowest dose of amikacin; as IL-10 secretion is typically suppressed by IL-1ß, this may be attributed to less significant IL-1ß downregulation of IL-10 at lower amikacin concentrations.

We explored this further to determine the mechanism by which amikacin-induced inflammation was resolved using *in vitro* assays to demonstrate phagocytosis and clearance of joint cells killed by amikacin via equine macrophages. Dead and dying cells are rapidly cleared in the body by tissue macrophages through a process known as efferocytosis, and the mechanism of cell death (e.g. apoptosis, necrosis) significantly impacts how macrophages respond to these phagocytosed cells (39). In general, macrophage phagocytosis of apoptotic cells leads to suppression of inflammation, while engulfment of necrotic cells provides a strong inflammatory stimulus.[42] Therefore, we conducted *in vitro* experiments to elucidate the mechanisms by which amikacin kills joint cells, and then examined effects on macrophage activation responses following engulfment of dead and dying cells. Stimulation of anti-inflammatory cytokine production (TGF-β) and suppression of pro-inflammatory cytokine secretion (TNF-α, IL1-β, IL-6) was observed in this model, compared to cells that died by necrotic pathways, consistent with previous reports.[39,42] Efferocytosis has been described as an important step to resolve inflammation and restore normal tissue function, with macrophages playing an integral role in maintenance of joint homeostasis.[39,42] Differential cytotoxicity of amikacin to cell types was also demonstrated, with leukocytes overall, and particularly neutrophils, being sensitive to amikacin-induced cell death at concentrations that would be easily achieved in synovial fluid with doses commonly used in equine practice. The susceptibility to antibiotic killing of leukocytes likely accounts for the lack of differences observed in clinicopathological parameters of synovial fluid and could mask the inflammatory effects of antibiotic-induced cytotoxicity. These findings

indicate that particular attention should be paid to the off-target cytotoxic effects of certain antibiotics when used for local treatment of infections due to the potential for inducing tissue injury and immune suppression.

Limitations to study design include small horse sample size, evaluation in normal versus inflamed joints, lack of synovial fluid collection at later time points for biomarker analysis to investigate when inflammatory cytokine concentrations returned to baseline, and lack of histopathologic evaluation of synovial tissues. Greater sample sizes and additional sampling of synovial fluid past 24 hours were not performed due to financial constraints but may have revealed continued increases in biomarkers of collagen degradation and potentially statistically significant elevation in CRP values, as has been previously reported.[35] Furthermore, synovial fluid sampling at later time points and evaluation of histopathology of synovial tissues may have provided further information as to whether intra-articular amikacin administration resulted in long-term joint damage or was only associated with a transient increase in pro-inflammatory cytokines and cartilage degradation products. The timing and frequency of synovial fluid sampling were performed to emphasize pharmacokinetic analysis of amikacin concentrations in synovial fluid, where a greater number of early time points allowed for determinations of synovial fluid concentration inflection points and the single 24-hour time points allowed calculation of ß and the terminal half-life slope, as has been previously described.[14,35] Synovial fluid sampling may have transiently affected other parameters evaluated (e.g. clinicopathological parameters), as has been previously reported,[43] but the study design which included a control sample should have mitigated this potential complication. Comparison of elevations in inflammatory biomarkers and cartilage degradation products to long-term histopathological findings may have provided some clarity as to whether the detrimental effects associated with

amikacin injection reported here are temporary or enduring, as cytological evaluation of synovial fluid has been previously reported to be more sensitive than histology.[44] Further *in vivo* evaluation of antibiotic distribution and cytotoxicity when co-administered with other medications intra-articularly or in inflamed or infected joints is warranted. In investigating the use of amikacin in treatment of infected joints, evaluation of additional parameters not reported here such as synovial fluid pH, glucose and lactate levels, may be important in monitoring response to therapy.[45,46] This work will serve as a platform from which further investigations of intra-articular antibiotic use will build.

The horse represents a valuable large animal preclinical model for human joint disease, including treatment of septic arthritis.[47-53] The joint volume and cartilage thickness of horses more closely approximates that of human cartilage compared to other animal models, and the larger volume of synovial fluid within equine joints allows for greater ease of synovial fluid sampling for analysis of increased outcome parameters.[47-51] Equine articular cartilage is subject to loading forces of similar or greater magnitude than human cartilage, which may have important implications when evaluating intra-articular therapies.[47-49] Formation of bacterial biofilm aggregates in equine synovial fluid as a substitute for human synovial fluid was recently described *in vitro,* and clinical evidence of septic arthritis as a disease process in horses is well documented, providing further evidence to support the use of the equine preclinical model as both a translational and clinically relevant model for human joint disease.[53,54]

This study demonstrated that concentrations of amikacin administered intra-articularly in horses reached and sustained therapeutic levels for most common equine pathogens for all doses assessed, while eliciting increased production of cartilage degradation products and pro-inflammatory biomarkers. Effects of amikacin on equine cartilage *in situ* and in the presence of

inflammation and sepsis warrant further investigation, but these findings indicate that amikacin can penetrate cartilage to elicit dose-dependent cytotoxicity *in vivo*. In conclusion, the use of intra-articular antibiotics has the potential to augment current treatment regimens for septic arthritis but induces joint inflammation and cartilage degradation at higher doses *in vivo*. Decisions on whether to inject cytotoxic antibiotics such as aminoglycosides intra-articularly and what doses to use should take into account the potential harm that antibiotics may cause and consider lower doses than those previously reported in equine practice.

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CHAPTER 5: Toll-like receptor activation of equine mesenchymal stromal cells to enhance antibacterial activity and immunomodulatory cytokine secretion[v]

5.1 Summary

The objective of this study was to evaluate effects of Toll-like and Nucleotide binding oligomerization domain (NOD)-like receptor (TLR, NLR) ligand stimulation of equine mesenchymal stromal cells (MSC) on antibacterial and immunomodulatory properties *in vitro*. Equine bone marrow-derived MSC from three horses were stimulated *in vitro* with TLR (polyinosinic:polycytidylic acid [pIC], lipopolysaccharide [LPS]) and NLR agonists (γ-D-Glu-mDAP [IE-DAP]) for 2h, and plated at $1x10^5$ cells/well for 24h. MSC-conditioned media (MSC-CM) were collected and assessed for antimicrobial peptide cathelicidin/LL-37 production, bactericidal action against multidrug-resistant planktonic and biofilm *S. aureus* and neutrophil phagocytosis. Bacterial growth was measured by plating bacteria and counting viable colonies, reading culture absorbance, and live-dead staining with confocal microscopy imaging. Following initial comparison of activating stimuli, TLR3-agonist pIC protocols (cell density during activation and plating, culture time, %serum) were further optimized for bactericidal activity and secretion of interleukin-8 (IL-8), monocyte-chemoattractant-protein (MCP-1), and cathelicidin/LL37. MSC stimulation with pIC, LPS, and IE-DAP resulted in increased cathelicidin/LL37. PIC and IE-DAP activation reduced planktonic bacterial colony counts. PIC stimulation ($2×10^6$ cells/ml, 2h, 10 μg/mL) suppressed biofilm formation, enhanced neutrophil

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bacterial phagocytosis, and increased MCP-1 secretion. MSC culture in 10 vs 1% serum reduced detectable cathelicidin/LL37 levels and suppressed bactericidal activity. TLR-3 pIC MSC activation was most effective to enhance antibacterial and cytokine responses. MSC antimicrobial properties were affected by FBS concentration. *In vitro* TLR-3 activation of equine MSC is a simple approach to enhance antibacterial properties of MSC towards improving treatment of antibiotic-resistant infections.

5.2 Introduction

Antimicrobial resistance presents a serious challenge to treatment of postoperative infections in both human and veterinary surgery.[1-11] Prevalence and risk factors for development of infection after orthopedic surgery in equine patients have been previously described, with infection rates ranging from 28 to 80% in equine long bone fracture repair or arthrodesis.[12-15] Curtiss *et al.* recently reported surgical site infections occurred in 14% in horses undergoing orthopedic internal fixation, with only 45.5% surviving to discharge after developing infection.[16] Failure to respond to treatment was attributed in part to changes in bacterial isolation patterns and susceptibilities over time, with an increasing incidence of multi-drug resistance.[16] Similarly, in human orthopedic and trauma surgery, postoperative infection occurs in over 30% of cases, with treatment success rates varying between 57 and 88%.[17-23]

Infections with multi-drug resistant bacterial strains negatively impact clinical outcomes and significantly increase healthcare expenditure.[16,24] Current approaches to treat equine orthopedic infection include surgical debridement and systemic and local antimicrobials with the goals to reduce pain associated with inflammation, restore function of infected joints, and consolidate fractures while minimizing osteomyelitis.[2] However, pressure from excessive antibiotic usage has driven increases in the incidence of multidrug-resistant bacteria. The rapid

development of resistance to pharmacologic drugs necessitates advancement of novel strategies to combat infection. Evolving treatments to improve outcomes in chronically infected non-healing tissues include implant surface coatings, local antibiotic release systems, and biological therapies.[25-31].

Mesenchymal stromal cells (MSC) possess antimicrobial and immunomodulatory properties and are particularly attractive as adjunctive therapies to antibiotics as they are not subject to development of resistance.[29,31-48] MSC are vital participants in normal tissue repair processes, promoting healing through angiogenesis, epithelialization, collagen deposition, granulation tissue formation, and release of inflammatory mediators.[29,38-48] Direct effects are mediated by antimicrobial peptide production and release, resulting in depolarization of bacterial cell membranes and inhibition of bacterial growth.[29,38,39] Indirect antimicrobial actions involve activation and recruitment of immune effector cells, as MSC express genes for production of the immunomodulatory and chemoattractant cytokines interleukin 6 (IL-6), interleukin 8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1).[31,35,38,40-47] In addition to bacterial clearance, immunomodulation exerted by MSC may provide further advantages over traditional antibiotic therapy to reduce inflammation associated with infection and decrease healing time, pain and sequelae associated with infection.[48-50]

One mechanism by which MSC play a role in the inflammatory response to infection is through expression of Toll-like (TLR) and Nucleotide binding oligomerization domain (NOD)-like receptors (NLR).[48-50] Activation of these receptors may be exploited to enhance the antimicrobial and immunomodulatory properties of MSC prior to injection. Receptor ligands present on bacteria induce MSC to produce anti-inflammatory mediators, resulting in a MSC phenotype that promotes healing.[48-50] For example, exposure to bacterial TLR-4 ligand

lipopolysaccharide (LPS) has been demonstrated to induce immunomodulatory cytokine expression in equine MSC.[31] In addition, TLR-3 ligand activation of human and murine MSC before injection in mice prolonged neutrophil survival and increased chemokine release stimulating MSC migration, resulting in greater antimicrobial peptide production.[40,51-59] Furthermore, murine and canine stromal cells cultured with TLR-3 ligand polyinosinic:polycytidylic acid (pIC) demonstrated synergism with antibiotics to improve infection control in a mouse model of chronic implant infection and in dogs with naturally occurring wounds *in vivo* compared to antibiotics alone.[51,52] These data provide justification to further investigate and compare methods of equine MSC immune activation to enhance bacterial clearance and immune modulation towards the ultimate goal of improved treatment of multi-drug resistant postoperative infections.[51,52]

The objectives of this study were to evaluate how TLR and NLR receptor activation of equine MSC affects direct inhibition of planktonic and biofilm bacterial growth and quantitative expression of immunomodulatory cytokines and antimicrobial peptides. We hypothesized that activation of MSC would increase secretion of cytokines involved in recruitment of innate immune effector cells, enhance bacterial phagocytosis and upregulate antimicrobial peptide expression, resulting in an overall increased bactericidal effect.

5.3 Materials and Methods

Horses – Bone marrow aspirate for stromal cell culture was harvested immediately postmortem from each of three donor horses (Quarter horses; two mares, one gelding; aged three to five years), subjected to euthanasia by intravenous pentobarbital injection for reasons

unrelated to the study. Institutional Animal Care and Use Committee (protocol #1101) approval was obtained for tissue collection.

Tissue collection **–** The sternum of donor horses was clipped and aseptically prepared in routine fashion. Bone marrow aspirate (5 mL) was obtained from one site between the fourth to sixth sternebrae using a Jamshidi needle into a syringe containing 1 mL heparin (1×10^4 U). Bone marrow aspirates were centrifuged by ficoll (Ficoll-Paque^{TM} Plus; GE Healthcare Bio-Sciences) density centrifugation at 400g at 18ºC for 18 minutes to pellet red cells as previously described.[60,61] Bone marrow derived MSC were plated at 1×10^4 cells/cm^2 and expanded in culture (37˚C, 5% CO_2, 95% humidity) to 80% confluence for approximately seven days in complete growth medium (Dulbecco's Modified Eagle's Medium (DMEM) with 1000 mg/L glucose, 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), 1M HEPES). Cells were detached from flasks by trypsinization, then frozen at $5x10^6$ cells/mL in freeze media (90% FBS, 10% dimethyl sulfoxide [DMSO]) in liquid nitrogen vapor phase until further use. Cells were thawed quickly in a 37˚C water bath, recovered and expanded in the above described complete growth medium for at least 48 hours under standard incubation conditions (37˚C, 5% CO_2, 95% humidity) prior to re-plating to generate conditioned medium. All MSC used for *in vitro* studies are routinely evaluated for surface phenotype, and found to be $CD44^+CD90^+$, and $CD34^-CD45^-$, using equine cross-reactive antibodies as previously described,[62] and in accordance with International Society for Cellular Therapy (ISCT) minimal criteria for defining mesenchymal stromal cells.[63]

Generation of MSC conditioned medium (MSC-CM) **–** To generate conditioned medium, MSC were culture expanded to passage two to four in growth medium (complete Dulbecco's Modified Eagle's Medium (DMEM) with 1000 mg/L glucose (10% fetal bovine

serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), 1M HEPES)), washed three times in PBS, and transitioned to antibiotic-free growth medium (DMEM with 1000 mg/L glucose, 1M HEPES, and variable FBS percentage [10%, 5%, 2.5% or 1%]). To compare methods of MSC activation, MSC were detached from flasks by trypsinization, resuspended at 2×10^6 cells/mL in MSC growth medium, and activated in suspension in 15 mL filter top Bio-Reaction tubes (CellTreat Scientific Products, Pepperell, MA, USA) incubated at 37˚C in 5% CO_2 in 95% humidity to examine the relative effects of activation on bactericidal activity and secretion of antimicrobial peptides.

MSC were treated with the following stimulants at concentrations reported in previous publications[64,65]: 1) TLR-3 agonist poly-inosinic, polycytidylic acid (pIC) 10 µg/mL (InVivoGen, San Diego, CA); 2) TLR-4 agonist lipopolysaccharide (10 ng/mL) (Sigma-Aldrich); 3) NOD-1 agonist γ-D-Glu-m IE-DAP (10 µg/mL). LPS was reconstituted at 1 mg/mL in PBS according to manufacturer's instructions (Sigma Aldrich, St. Louis, MO, USA), frozen at -80ºC in microcentrifuge tubes until use in assays, then thawed and diluted to 10 ng/mL for MSC activation as previously described.[64] MSC were incubated in antibiotic-free growth medium for 2 hours at 37˚C in a 5% CO_2 incubator at 95% humidity with agitation (i.e. multiple gentle inversions of tube containing cell suspension) every 30 minutes, then washed twice with PBS and plated in 24-well plates at 1×10^6 cells/well in antibiotic-free growth medium. Supernatants were collected 24 hours later, centrifuged twice for 7 minutes at 300g at 18ºC to remove cellular debris, and frozen in 120 µL aliquots at -80˚C for later use in assays. Relative stimulatory activity of MSC-CM following activation was assessed in bacterial killing and biofilm suppression assays and for cathelicidin/LL37 antimicrobial peptide production as described below.

Based on results of the initial TLR and NLR comparison, TLR-3 agonist pIC demonstrated greatest reduction in numbers of both planktonic and biofilm bacteria. To optimize activation conditions of TLR-3 agonist pIC with equine MSC, studies to determine what culture conditions resulted in secreted factors associated with immunomodulation (IL-8, MCP-1) were conducted. MSC were activated with 10 μg/mL pIC for 2 hours. Factors evaluated included cell density during activation (0.5, 1, 2×10^6), cells/well plating density on 24-well plate (0.5, 1, 2×10^5), and culture time following activation (24 h, 48 h, 72 h) to generate MSC-CM with maximal levels of immunomodulatory cytokines IL-8 and MCP-1. The MSC-CM were harvested from the wells, centrifuged twice for 7 minutes at 300g at 18°C to remove cellular debris, and frozen in 120 μL aliquots at -80˚C for later use in immunoassays.

Determination of biomarkers of inflammation **–** Competitive ELISAs, previously validated for use in equine samples, were used to measure the biomarker concentrations of IL-8 (Equine IL-8 / Interleukin 8 Sandwich ELISA, Lifespan Biosciences, Seattle, WA, USA) and MCP-1 (Equine MCP-1/CCL2 ELISA, RaybioTech, Peachtree Corners, GA, USA) in MSC-CM following various activation methods. The pIC-activation method (10 μg/ml, 2h incubation, 2×10^6 cells/ml, plated 1×10^5 cells/well on 24-well plate for 24h) that resulted in the highest levels of IL-8 and MCP-1 was used for the remaining studies.

Quantitative immunoassay for antimicrobial peptide expression **–** A competitive ELISA, previously validated for cathelicidin/LL37 expression in human samples and cross-reactive in equine samples (human cathelicidin/LL37 Sandwich ELISA kit, Lifespan Biosciences, Seattle, WA, USA), was used to determine levels in equine MSC-CM following various activation methods. Culture conditions assessed included activation (pIC, LPS, IE-DAP) or resting and FBS concentration 10%, 5%, 2.5% or 1%).

Bacterial culture – The human methicillin resistant *Staphylococcus aureus* (MRSA) strain USA300 used in bacterial killing and biofilm assays was generously provided by H. Schweizer (Colorado State University). The bacterial culture and sensitivity of this isolate is supplied in **Figure 5.1**. Bacteria were expanded in LB broth and frozen at -80ºC in 20% glycerol until further use. Overnight bacterial cultures were grown in antibiotic-free MSC growth medium (DMEM with 1000 mg/L glucose, 10% or 1% FBS, 1M HEPES) before use in assays. On the day of the experiment, bacterial sub-cultures were grown to log phase in MSC medium (OD600 of 0.6, corresponding to 7.5 Log10 CFU/mL) then used immediately.

S. aureus planktonic bacterial killing assays – To assess the ability of MSC-CM to directly kill bacteria, MSC-CM (activated or resting MSC-CM, with serum concentration in antibiotic-free growth medium, 10% or 1%) were inoculated with actively dividing log phase multi-drug resistant *S. aureus*. Antibiotic-free MSC-CM or MSC growth medium was plated at 200 µL per well on a 96-well plate, and 500 bacteria at log phase (O.D. 0.6) were added per well. Plates were incubated shaking at 100 rpm at 37˚C for 16 hours. Negative control wells containing antibiotic free MSC growth medium without bacterial inoculation were also included. Following incubation with bacteria, medium was transferred to 1.5 mL tubes, vortexed to evenly distribute bacteria, diluted tenfold, plated on Luria-Bertani (LB) agar plates (100 µL/quadrant) and incubated at 37˚C for 18 hours. Colony-forming units (CFU) were counted manually.

Antimicrobial	Interpretation	MIC
Amikacin	Susceptible	<=16.0
Amoxicillin/Clavulanic Acid	Resistant	8.0
Ampicillin	Resistant	8.0
Cefazolin	Resistant	4.0
Cefovecin	Resistant	>8.0
Cefpodoxime	Resistant	>8.0
Ceftiofur	Intermediate	4.0
Cephalothin	Resistant	<=2.0
Chloramphenicol	Susceptible	<=8.0
Ciprofloxacin	Resistant	>4.0
Clindamycin	Susceptible	<=0.5
Doxycycline	Intermediate	0.25
Enrofloxacin	Resistant	4.0
Erythromycin	Susceptible	0.5
Gentamicin	Susceptible	<=2.0
Imipenem	Resistant	<=1.0
Marbofloxacin	Resistant	>4.0
Minocycline	Resistant	<=0.5
Moxifloxacin	Intermediate	1.0
Nitrofurantoin	Susceptible	<=16.0
Oflaxacin	Resistant	>1.0
Oxacillin	Resistant	>2.0
Oxytetracycline	Susceptible	<=0.5
Penicillin	Resistant	2.0
Pradofloxacin	Resistant	2.0
Rifampin	Susceptible	<=1.0
Tetracyline	Susceptible	<=0.25
Tobramycin	Susceptible	<=4.0
Trimethoprim/Sulfamethoxazole	Susceptible	<=2.0
Vancomycin	Susceptible	<=1.0

Figure 5.1: Bacterial culture and sensitivity of the human MRSA strain of *S. aureus* (USA300) isolate used in bacterial killing and biofilm assays, demonstrating multiple resistances to antibiotics commonly used in equine clinical practice.

S. aureus biofilm assays **–** Multidrug resistant *S. aureus* (strain USA300) was grown to log-phase (O.D. 0.6) in antibiotic-free growth medium (1% FBS), then diluted to an optical density reading (O.D. 600 nm) of 0.1. To determine whether MSC-CM could disrupt fully formed biofilms, round slide coverslips were rinsed in 100% ethanol, placed in the bottom of wells of 24-well plates and allowed to airdry. Then 350 µL of *S. aureus* (log phase O.D. 0.6 diluted to O.D. 0.1 in antibiotic-free growth medium containing 1% serum) was added to each

well and incubated at 37˚C at 95% humidity for 72 hours. The media were removed and 400 µL MSC-CM (resting or pIC activated) or control antibiotic-free growth medium was added and allowed to incubate for 24 hours at 37˚C. Medium was aspirated, and wells were washed twice with PBS. Live/dead visualization of biofilms was performed using the LIVE/DEAD BacLight Bacterial Viability and Counting Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Visualization of fluorescent staining was done confocal microscopy (Olympus IC83 spinning disk microscope). Green represents live bacterial clusters, while red represents dead bacteria stained with propidium iodide. Merged channels are shown as yellow, where live and dead bacterial cells overlap. Ratios of live to dead cells were calculated by imaging total area of each channel using ImageJ software as previously described.[66-68]

To further determine whether MSC-CM could inhibit biofilm formation, 200 µL bacteria (log phase O.D. 0.6 diluted to O.D. 0.1 in antibiotic-free growth medium containing 1% FBS) were aliquoted per well on a 96-well flat bottom cell-culture plate (Thermo Fisher Scientific). At 24 and 48 hours, 100 µL of medium was removed and replaced with MSC-CM (resting or pIC activated) or control growth medium. At 72 hours, nonadherent bacteria were removed by washing with PBS, and remaining biofilms were stained with 0.05% crystal violet solution (Sigma-Aldrich). Retained crystal violet was then dissolved with 95% ethanol and O.D. readings were obtained using a microplate reader, at wavelength of 570 nm.

Neutrophil bacterial phagocytosis assay – Neutrophils were isolated from the blood of three different healthy donor horses (Quarter horses; two geldings, one stallion; all three years of age) using the Lympholyte-Poly (Cedarlane, Peterborough, United Kingdom) separation gradient according to manufacturer's instructions. Briefly, blood was collected using a 20-gauge needle and syringe into standard EDTA anticoagulant-containing blood tubes. Whole anticoagulated

blood (6 mL) was layered on top of 6 mL Lympholyte-Poly solution in a 15 mL conical tube, then spun at 18ºC at 1800g for 35 minutes. Following centrifugation, erythrocytes pellet to the bottom of the conical tube and two discrete leukocyte bands become visible, separating mononuclear (upper) from polymorphonuclear (lower) layers. The polymorphonuclear cell band was harvested using a 1000 uL pipette into a separate conical tube and diluted with equal volume 0.45% NaCl, then diluted in twice volume 0.9% NaCl. Cells were washed and pelleted by centrifugation at 18ºC at 400g for 10 minutes. The polymorphonuclear cell pellet was then resuspended in antibiotic free growth medium (10% FBS) and counted using an automated cell counter (Nexcelom; Bioscience Cellometer Auto T4).

Quantitative phagocytosis over time was performed using the IncuCyte Zoom system software as previously described (Essen BioScience Inc., Ann Arbor, Michigan).[64] Log phase *S. aureus* cultures were fixed and stained using pHrodo Red Phagocytosis Particle Labeling Kit (Thermo Fisher Scientific) according to manufacturer's instructions. Neutrophils ($5x10^5$) were incubated on 24-well plate wells, with either MSC growth medium, MSC-CM or pIC-MSC-CM, and *S. aureus* was added at MOI 25:1 (bacteria to cells). Live cell imaging (nine images captured per well) was performed at 6 hours using 10x objective and analyzed using IncuCyte® S3 Live-Cell Analysis System software (Essen BioScience, Inc.).

Data Analysis **–**All data were visually assessed for normality and were judged to be normally distributed. The measurement of CFUs during the log phase of bacterial growth was measured out in logCFU because this allows a linear mapping of optical density to bacterial counts. Each set of data was modeled individually using a linear mixed model (function lmer from the lme4[69] and lmerTest[70] packages) with treatment as a fixed effect and donor as a random effect to account for differences in donor cell lines. Model assumptions of homoscedastisity and

normality of error distribution were verified by analysis of QQ plots and fitted vs residual values and model fit was judged to be appropriate. Differences between groups were evaluated using differences in estimated marginal means (function emmeans from the emmeans[71] package), with *p*-values adjusted using Tukey's adjustment for multiple comparisons. All statistical analyses were performed using R for Mac (R version 4.0.0 "Arbor Day", Vienna, Austria)[72]. Graphical analyses and graph generation were performed using Prism software v8.4.1 (GraphPad Software Inc., La Jolla, CA). For all analyses, statistical significance was assessed as $p \leq 0.05$.

5.4 Results

Comparison of MSC activation techniques on antimicrobial peptide secretion and bactericidal activity. To compare activation of MSC through different major innate immune pathways (TLR3 agonist pIC, TLR4 agonist LPS, NOD1 agonist IE-DAP), the direct bactericidal activity against planktonic and biofilm *S. aureus* bacteria, as well as expression of antimicrobial peptides were assessed. Both activated and non-activated MSC-CM had elevated cathelicidin/LL-37 levels compared to control medium (**Figure 5.2A**): pIC ($p<0.0001$), LPS ($p<0.0001$), IE-DAP ($p<0.0001$), non-activated MSC-CM ($p=0.017$). Stimulation with all agonists also resulted in increased cathelicidin/LL-37 compared to non-activated MSC-CM: pIC ($p=0.02$), LPS ($p=0.004$), and IE-DAP ($p=0.023$). Only pIC ($p=0.004$) and IE-DAP ($p=0.027$) promoted an increase in bactericidal activity evidenced by reduced viable colony counts in planktonic bacterial killing assay compared to control (**Figure 5.2B**). Non-activated and all stimulated MSC-CM treatments (LPS, pIC, IE-DAP) decreased biofilm formation by preventing attachment as evidenced by lower absorbance compared to control ($P<0.0001$ for all treatments). Only pIC resulted in further inhibition of biofilm formation compared to non-activated MSC-CM

(p=0.001) (**Figure 5.2C**). Based on results of the initial TLR and NLR receptor comparison, optimal culture conditions for pIC-activation were investigated further.

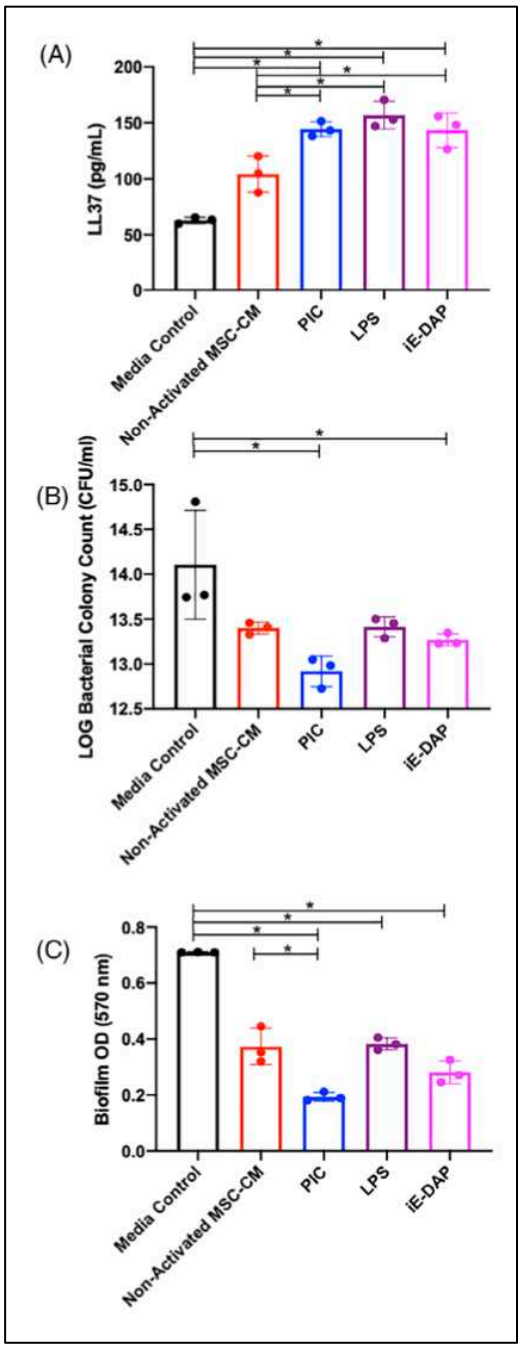

Figure 5.2: ***Mesenchymal stromal cell (MSC) activation stimulates antimicrobial peptide production and bactericidal activity against planktonic S. aureus bacteria and newly forming biofilms.*** MSC were treated with either 1) Toll-Like Receptor (TLR)-3 agonist poly-inosinic, polycytidylic acid (pIC) (10 μg/mL); 2) TLR-4 agonist lipopolysaccharide (LPS) (10 ng/mL); or 3) Nucleotide-binding oligomerization domain-like receptor (NLR)-1 agonist γ-D-Glu-m IE-DAP (10 μg/mL). MSC were incubated for 2 hours, then plated on 24-well plates at $1x10^5$ cells/well, and mesenchymal stromal cell conditioned medium (MSC-CM) collected 24 hours later. Activation of MSC-CM was compared relative to control for **5.2A)** cathelicidin/LL-37 antimicrobial peptide production via ELISA immunoassay, **5.2B)** planktonic *S.aureus* killing in bacterial killing assays shown in log (CFU/mL) and **5.2C)** suppression of biofilm formation by crystal violet staining indicated by OD520 absorption values. MSC stimulation with pIC, LPS, and IE-DAP resulted in increased cathelicidin/LL-37 compared to non-activated MSC and control media. Only pIC and IE-DAP induced reduced planktonic bacterial colony counts, and suppressed biofilm formation compared to non-activated MSC-CM. Graphs show means and standard deviations of three biological replicates with significance assessed at $p<0.05$ (indicated by *).

MSC pIC-activation enhances immunomodulatory cytokine production. MSC activation with pIC at cell density in solution of 2×10^6 cells/mL for 2 hours, followed by plating cells at all densities evaluated (5×10^4, 1×10^5, 2×10^5 cells/well) 24h resulted in elevated MCP-1 compared to non-activated MSC-CM ($p<0.0001$) (**Figure 5.3A**). Levels of IL-8 did not differ between activated and non-activated protocols at plating densities evaluated (p-0.317, p=0.316, p=0.317 for plating densities 5×10^4, 1×10^5, 2×10^5 cells/well, respectively) (**Figure 5.3B**). The activation protocol (2×10^6 cells/mL for 2 hours followed by plating at1×10^5) was used for the remaining pIC studies.

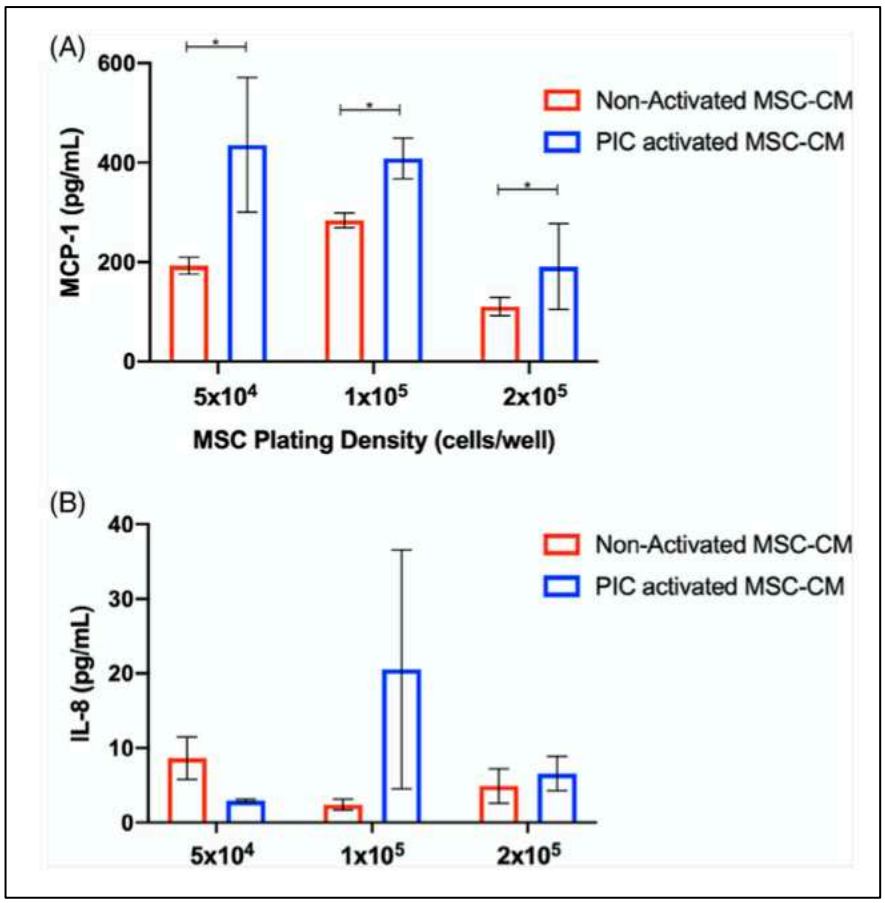

Figure 5.3: ***Mesenchymal stromal cell (MSC) activation by Toll-Like Receptor-3 (TLR-3) polyinosinic:polycytidylic acid (pIC) pathway enhances cytokine production.*** MSC were incubated with TLR-3 pIC (10 µg/mL) for 2 hours and plated in 24-well plates at either 0.5, 1, or 2×10^5 cells/well for 24 hours. Competitive ELISA immunoassays were used to measure biomarker concentrations of MCP-1 (**3A**) and IL-8 (**3B**) in supernatants. MCP-1 was increased in pIC-activated compared to resting MSC-CM at all plating densities evaluated. IL-8 levels did not differ between pIC- and resting MSC-CM plated at the same cell densities. Graphs show means and standard deviations of three biological replicates with significance assessed at $p<0.05$ (indicated by *).

MSC pIC-activation in low-serum growth medium enhances antimicrobial peptide expression. To determine whether antimicrobial peptide (AMP) expression following pIC-activation is affected by reducing serum concentration in MSC growth medium, we examined expression of the peptide cathelicidin/LL-37 using immunoassays. All MSC-CM and pIC activated treatments had increased cathelicidin/LL-37 levels compared to control medium

(p<0.001). These studies revealed that pIC-activation resulted in increased cathelicidin/LL-37 production, which was apparent when serum concentration in medium was reduced to 1% (p=0.011) and 2.5% (p=0.05) (**Figure 5.4**).

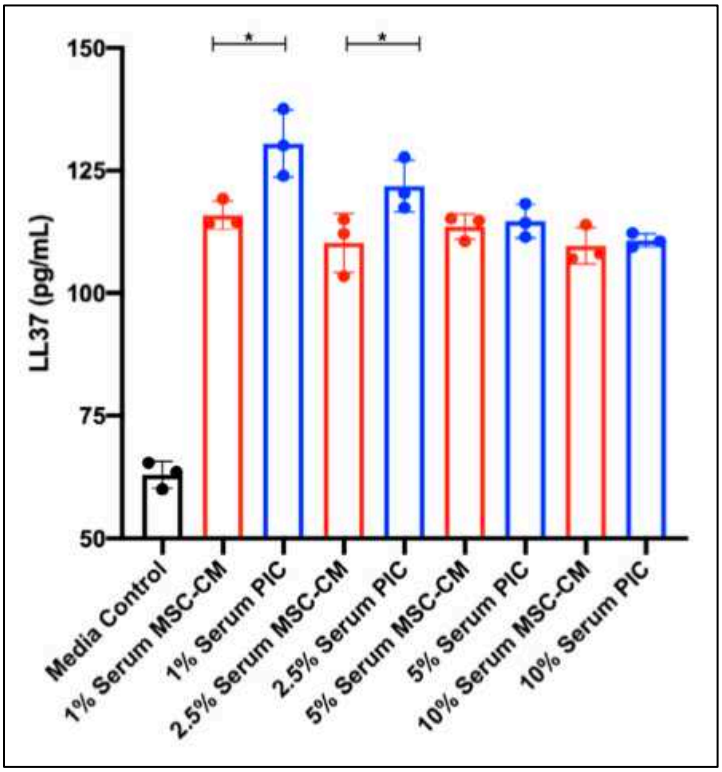

Figure 5.4: Polyinosinic:polycytidylic acid (*pIC)-activation enhances antimicrobial peptide production and increasing serum concentration binds and inactivates antimicrobial peptides.* Equine mesenchymal stromal cells (MSC) were stimulated with pIC (10µg/mL, 2 hours, $2x10^6$ cell/ml) for 24h in DMEM media supplemented with varying concentrations of fetal bovine serum (10%, 5%, 2.5%, 1% or 0%). Conditioned medium was collected after 24 hours and assessed for cathelicidin/LL-37 antimicrobial peptide levels via ELISA immunoassay. Cathelicin/LL-37 levels were elevated in pIC activated versus resting MSC-CM when FBS concentration was reduced to 2.5 and 1%. Graphs show means and standard deviations of three biological replicates with significance assessed at $p<0.05$ (indicated by *).

pIC-activation enhances bactericidal factors produced by MSC. Bacterial killing assays were performed to determine whether activation or culture conditions (i.e. serum reduction) affected bactericidal activity against *S. aureus*. Incubation with MSC-CM resulted in up to 3-log CFU/mL reduction of bacterial growth, which was enhanced by pIC-activation and serum

reduction (**Figure 5.5**). In 10% serum growth medium, bacterial colony counts were significantly reduced in both non-activated (p=0.049) and pIC-activated (p=0.015) compared to control, but no difference was seen between pIC activated or non-activated MSC-CM (p=0.376) (**Figure 5.5A**). In 1% serum growth medium, bacterial colony counts were significantly reduced in both non-activated (p<0.001) and pIC-activated (p<0.001) compared to control, and colony counts were lower in pIC-activated wells compared to non-activated (p=0.022) (**Figure 5.5B**).

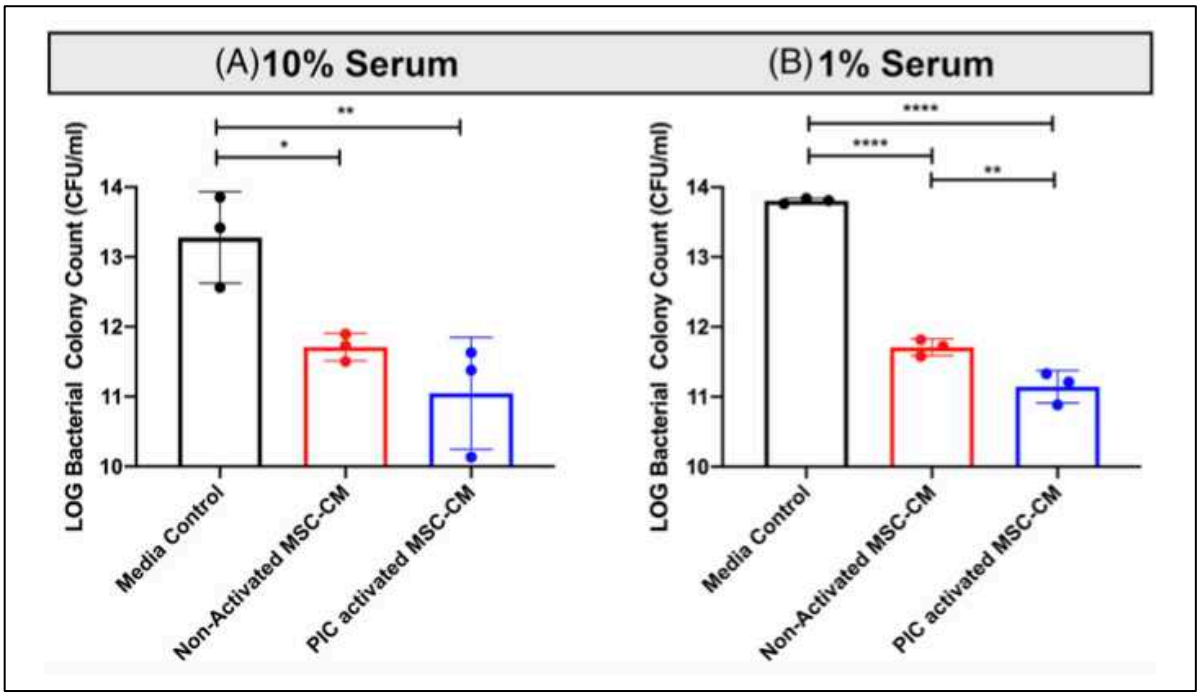

Figure 5.5: ***Bactericidal activity of mesenchymal stromal cell conditioned medium (MSC-CM) on multidrug resistant S.aureus was enhanced by activation with polyinosinic:polycytidylic acid (pIC) at reduced serum concentrations in culture media.*** To assess the ability of MSC-CM to directly kill bacteria, MSC-CM (activated or resting MSC-CM, with FBS concentration in antibiotic-free growth medium 10% (**5.5A**), or 1% (**5.5B**)) were inoculated with actively dividing log phase multi-drug resistant *S. aureus*. MSC-CM or growth media was plated at 200 µL per well on a 96-well plate, and 500 bacteria at log phase (O.D. 0.6) were added per well. Co-culture plates were incubated shaking at 100 rpm at 37˚C for 16 hours. Negative control wells containing antibiotic free DMEM without bacterial inoculation were also included. Following incubation with bacteria, media was transferred to 1.5 mL tubes, vortexed to evenly distribute bacteria, diluted tenfold, plated on LB agar plates (100 µL/quadrant) and incubated at 37˚C for 18 hours. Bacterial growth shown on y-axis was measured by plating bacteria and counting viable colony forming units, expressed in log CFU/mL. Graphs show means and standard deviations of three biological replicates with significance assessed at $p<0.05$ (indicated by *).

MSC secreted factors penetrate established biofilms to exert killing effect. Equine MSC-CM were observed to disrupt established biofilms, as evidenced by the decrease in the live/dead fluorescence ratio exhibited by bacteria following overnight exposure (p= 0.001). (**Figure 5.6**). There was no difference in the decrease in live/dead ratio between intact biofilms exposed to MSC-CM and pIC-activated MSC-CM (p= 0.79).

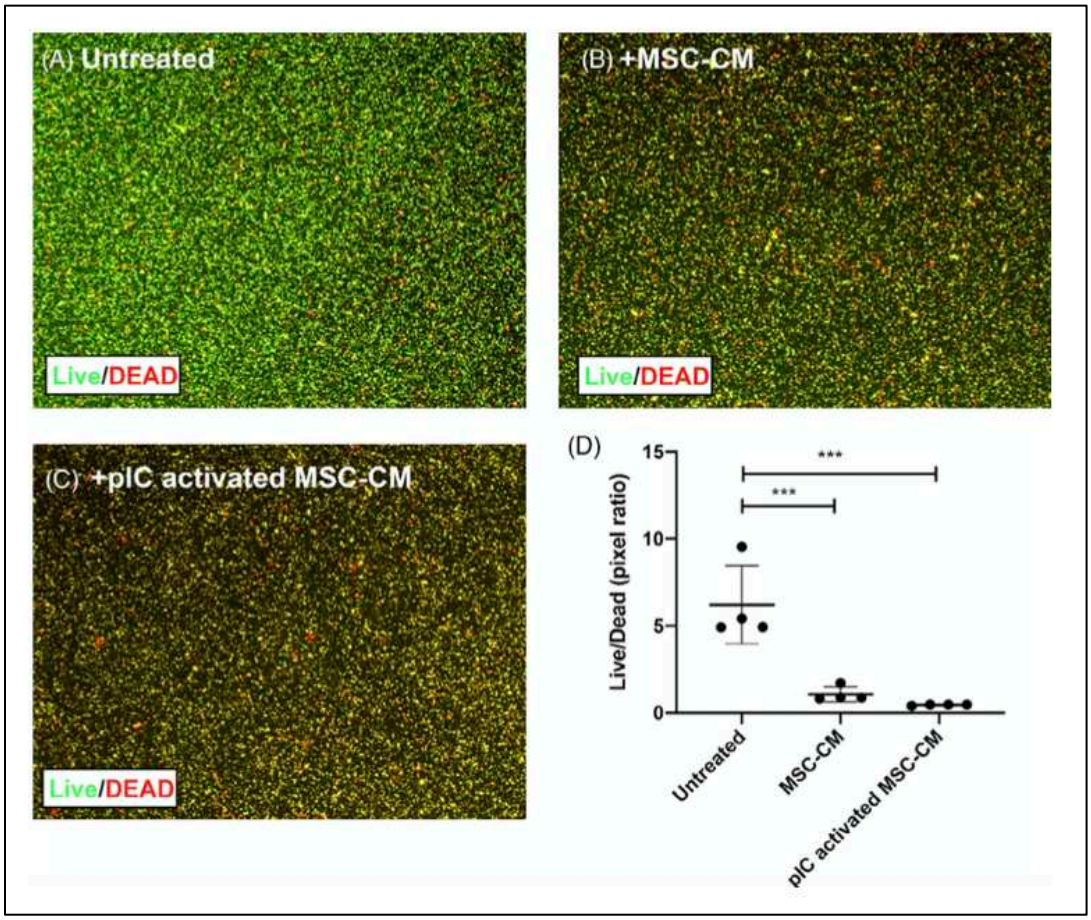

Figure 5.6: Mesenchymal stromal cell conditioned medium (*MSC-CM) and polyinosinic:polycytidylic (pIC)-activated MSC-CM have robust bactericidal activity against S. aureus biofilms.* *S. aureus* bacteria grown as biofilms were incubated with **6A)** control medium, **6B)** MSC-CM, or **6C)** pIC activated MSC-CM, then stained with LIVE/DEAD BacLight kit to identify live and dead bacterial colonies, as revealed by immunohistochemical staining and evaluation by confocal microscopy. Green (SYTO9) represents live bacterial clusters, while red clusters represent dead bacteria stained with propidium iodide. Merged channels show yellow color as red and green overlap. **6D)** Bars depict the ratio of live vs dead bacteria in biofilms, quantified using ImageJ software, at four discrete locations per image with $p<0.05$ indicated by asterisks (*).

Activated MSC-CM stimulates neutrophil phagocytosis. Neutrophils incubated with MSC-CM from pIC-activated MSC exhibited increased bacterial phagocytosis compared to those incubated with growth medium (p=0.009) or non-activated MSC-CM (p=0.029) (**Figure 5.7**). Exposure to non-activated MSC-CM did not increase phagocytosis compared to growth medium (p=0.65).

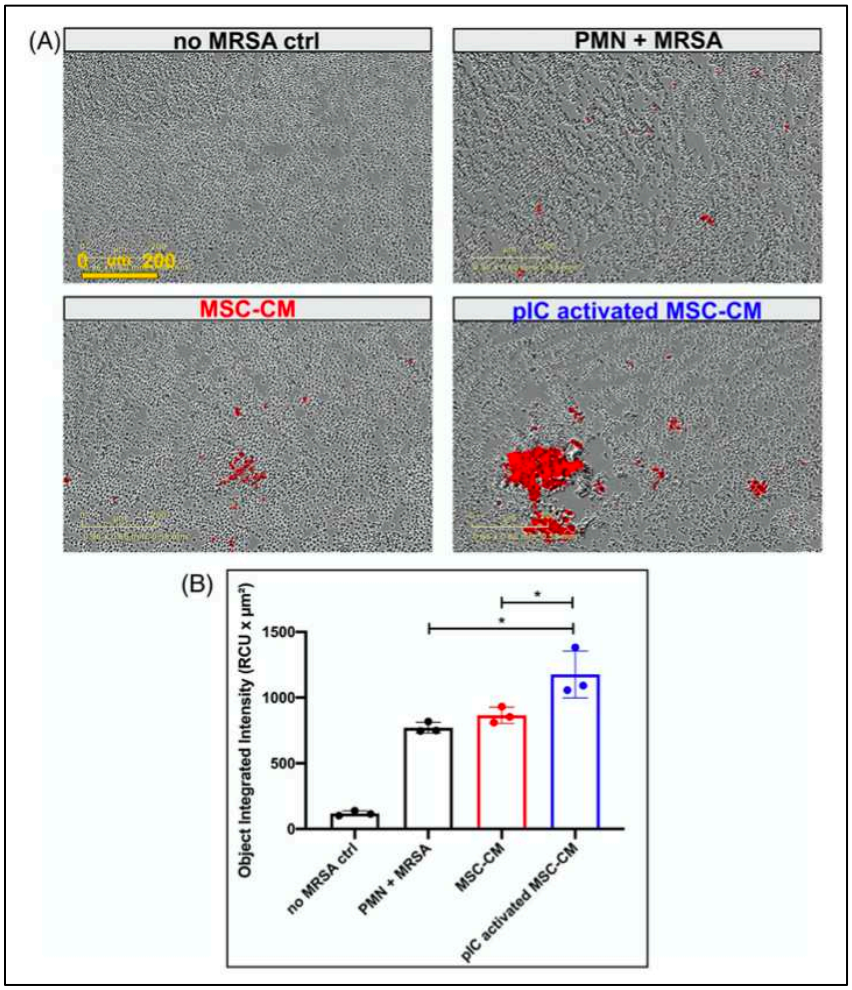

Figure 5.7: Mesenchymal stromal cell conditioned medium (*MSC-CM) and polyinosinic:polycytidylic (pIC)-activated MSC-CM significantly enhance equine neutrophil phagocytosis of bacteria.* Equine neutrophils were incubated with MSC-CM to assess effect on bacterial phagocytosis of *S. aureus* labeled with pH red dye. **7A)** Representative photos obtained from the IncuCyte ZOOM system from neutrophils incubated for 6h following addition of labeled *S. aureus*, for each of the four conditions tested. Red indicates phagocytosed bacteria. **7B)** The x-axis depicts condition of neutrophils with bacteria, while the Y axis depicts phagocytosed bacteria, quantitated as the average µM per well. Significant differences between groups ($p<0.05$) are noted with asterisks(*).

5.5 Discussion

These studies expanded upon the previously reported understanding of the direct antibacterial properties of equine MSC[26,28] and demonstrated new paracrine mechanisms by which equine MSC elicit cytokine secretion to activate the immune response to infection. MSC activation with all TLR and NLR stimuli evaluated resulted in increased antimicrobial peptide expression, but only TLR-3 pIC stimulation resulted in reduced planktonic bacterial colony counts and suppression of biofilm formation, supporting our hypothesis. Activation methods with TLR-3 pIC were evaluated further and optimized to upregulate secretion of immunomodulatory cytokine MCP-1 and antimicrobial peptides, which became apparent *in vitro* when AMPs were not bound in growth media that included lower (1 %) serum concentrations. This work notably demonstrated the potential for equine MSC to induce greater bacterial phagocytosis by neutrophils and disrupt newly forming and pre-existing biofilms, which were enhanced in newly forming biofilms by TLR-activation, and of which there is little previous literature.[26,62,73]

This study investigated the effect of serum concentration in MSC-CM on cathelicidin/LL37 production and bactericidal activity, providing new insight by demonstrating that FBS levels in culture are critical to detection of unbound, and therefore active, antimicrobial peptides (AMPs) when investigated *in vitro*. The ability of MSC to produce AMPs is one of the aspects of their antibacterial properties most thoroughly investigated.[26,38,39,64,74,75] Multiple studies have demonstrated spontaneous production of AMPs by human and animal MSCs.[26,38,39,64] In a mouse model of bacterial pneumonia, gene expression and protein production of human AMP cathelicidin hCAP-18/LL37 were upregulated following bacterial challenge and found to be partially responsible for the antimicrobial activity of hMSC in this

model.[38] Recently, Harman *et al.* demonstrated that equine blood-derived MSCs inhibit bacterial growth (*E.coli, S. aureus)*, and that MSC-CM depolarizes bacterial cell membranes. Four specific AMPs produced by eqMSCs were identified (cathelicidin/LL37, elafin, lipocalin, cystatin C), with AMP gene and protein expression determined by RT-PCR and Western blot analysis, respectively.[26] However, there have been conflicting reports as to whether activation with cytokines or TLR ligands cause increased AMP production or bactericidal activity by MSC.[57,64,74] This work contributed to clarifying discrepancies between these previous studies, [57,64,74] demonstrating that reduction of serum in MSC-CM to 1% and TLR/NLR activation resulted in greater measurable levels of cathelicidin/LL37 and enhanced bactericidal activity against planktonic *MRSA* and in newly forming biofilms. The difference in AMP production and overall bactericidal ability between MSC-CM with different serum concentrations is attributed to binding of the small AMPs by FBS resulting in lower detectable levels by ELISA,[76] as has been previously described with other peptides.[77] While AMP production is a constitutive property of MSC, these findings suggest that the effective or unbound population may be enhanced through TLR-activation and medium serum reduction for increased bactericidal activity and prevention of biofilm formation.

This study built off previous work to further demonstrate that activation of equine MSC with pIC triggered increased secretion of immunomodulatory cytokines and neutrophil phagocytosis. MSC interact with the innate immune system to generate an immune response,[44,58] and activating MSC with stimuli increases their immunomodulatory properties.[77-79] Increased levels of MCP-1 are associated with paracrine recruitment of innate immune cells and neutrophil phagocytosis of bacteria, an effect that could help in eradication of chronic infections *in vivo*.[51,52,64] The findings of this study are consistent with Brandau *et al*., where LPS-stimulated

MSCs contributed to elimination of infection via upregulation of genes associated with phagocytosis in neutrophils.[33] Upregulation of immunomodulatory cytokine production in the face of TLR-activation suggest that MSC recruit and activate granulocytes through cytokine secretion, and both cell types subsequently work in concert to combat bacterial infection.[33] MSC pIC-activation induced greater neutrophil response to bacteria, enhancing phagocytosis, an effect that may be of additional benefit in eradication of chronic infection *in vivo*. These findings reinforce the concept that immune modulation and recruitment likely play a key role in the antimicrobial activity of MSC *in vivo.*

Limitations of this study warrant further discussion, including the *in vitro* nature of study design, number of cell lines, range of cell passages used, and inclusion of fetal bovine serum in cell culture media. The *in vitro* experimental design described, using a relatively low number of individual equine cultured cell lines (n=3), was sufficient to see treatment differences. However, it is acknowledged that these results may not be extrapolated as population estimates and should be reinforced by further *in vivo* studies using a larger number of horses. Previous work from this laboratory has demonstrated that MSC retain comparable antimicrobial activity to passage ten and maintain their antimicrobial potency with TLR-stimulation following cryopreservation (R. Impastato and L. Chow, unpublished data). Therefore, MSCs were used in the experiments described here within a range of low passages commonly used in clinical cases and after only a single freeze-thaw cycle. The inclusion of FBS in culture media necessitates additional consideration, as recent studies have investigated alternatives to xenogeneic growth factors as media supplements.[79-84] However, this group has generated data demonstrating that equine MSC cultured in alternate growth sources do not maintain equivalent antimicrobial peptide production and bacterial killing capacity *in vitro* (Pezzanite *et al*., manuscript in review). For this reason,

MSC investigated for use as anti-infective cellular therapies have been maintained in FBS cell culture for optimal activity. Furthermore, at the time of this writing, FBS is currently used in culture media of MSC indicated for use in equine clinical patients at this institution in general due to lack of detrimental effects observed with its use as well as the increased cost associated with xenogene-free culture.

Further investigation of the effect of immune activated cellular therapy locally in states of infection to assess treatment effect on cytokine production, bacterial load, and histology *in vivo* is warranted and would lend further credence to these results. The findings reported here serve to support additional case-controlled studies investigating the local use of TLR3-pIC activated MSC in equine infections, particularly septic arthritis or implant associated infections, which are currently ongoing at the time of this writing. Further studies are necessary to provide clinical recommendations regarding dosing, timing and route of administration of TLR-activated MSC in clinical patients and are beyond the scope of the work presented here.

In summary, equine immune activated MSC have enhanced direct and indirect antimicrobial properties to stimulate the host immune response to infection. In this study, TLR and NLR activation of equine MSC, particularly TLR3-pIC, improved killing of antibiotic-resistant bacteria and disruption of biofilm formation by stimulating increased secretion of antimicrobial peptides and immunomodulatory cytokines and neutrophil phagocytosis of bacteria. This technology represents a novel therapy to enhance currently available techniques to combat infections with multi-drug resistant bacterial isolates in equine surgery.

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CHAPTER 6: Impact of three different serum sources on functional properties of equine mesenchymal stromal cells[vi]

6.1 Summary

Culture and expansion of equine mesenchymal stromal cells (MSC) is routinely performed using fetal bovine serum (FBS) as a source of growth factors, nutrients and extracellular matrix proteins. However, the desire to minimize introduction of xenogeneic bovine proteins or pathogens and to standardize cellular products intended for clinical application has driven evaluation of alternatives to FBS. Replacement of FBS in culture for several days before administration has been proposed to reduce antigenicity and potentially prolong survival after injection. However, the functional consequences of MSC culture in different serum types have not been fully evaluated. The objective of this study was to compare the immunomodulatory and antibacterial properties of MSC cultured in three serum sources: FBS, autologous or allogeneic equine serum. We hypothesized that continuous culture in FBS would generate MSC with improved functionality compared to equine serum and that there would not be important differences between MSC cultured in autologous versus allogeneic equine serum. To address these questions, MSC from three healthy donor horses were expanded in medium with FBS, then switched to culture in FBS, autologous, or allogeneic equine serum for 72h. The impact of this 72h culture period in different sera on cell viability, cell doubling time, cell morphology, bactericidal capability, chondrogenic differentiation, and production of cytokines and antimicrobial peptides was assessed. Altering serum source did not affect cell viability or

[vi] This chapter includes the complete published manuscript: Pezzanite L, Chow L, Griffenhagen G, Dow S, Goodrich L. Impact of three different serum sources on functional properties of equine mesenchymal stromal cells. *Front. Vet. Sci.* doi.org/10.3389/fvets.2021.634064. This article was reproduced with permission from Frontiers Media, Seattle, WA.

morphology. However, cells cultured in FBS had shorter cell doubling times and secreted more IL-4, IL-5, IL-17, RANTES, GM-CSF, FGF-2, Eotaxin, and antimicrobial peptide cathelicidin/LL-37 than cells cultured in either source of equine serum. Cells cultured in FBS also exhibited greater spontaneous bactericidal activity. Notably, significant differences in any of these parameters were not observed when autologous versus allogeneic equine serum was used for cell culture. Chondrogenic differentiation was not different between different serum sources. These results indicate that MSC culture in FBS will generate more functional cells based on a number of parameters, and that the theoretical risks of FBS use in MSC culture should be weighed against the loss of MSC function likely to be incurred from culture in equine serum.

6.2 Introduction

Mesenchymal stromal cells (MSC) derived from bone marrow, adipose or blood tissues exert potent immunomodulatory and antibacterial activities, which renders them attractive as biological therapies for diverse conditions, including musculoskeletal injuries, wound healing, and bacterial infections.[1-12] *In vitro* cell expansion of MSC is required to obtain enough cells for clinical use, and a number of previous studies have evaluated the impact of tissue culture medium and serum source on relevant MSC properties.[13] Culture and expansion of equine MSC is routinely performed using fetal bovine serum (FBS) as a source of growth factors, nutrients and extracellular matrix proteins. However, the use of FBS to expand equine MSC has been linked to potential hypersensitivity reactions, and the risk of introduction of viral or prion pathogens.[14-17] The International Society for Cellular Therapy and several regulatory agencies have responded with position statements recommending the avoidance of FBS in MSC culture

for clinical applications when possible and has called for a consensus on serum replacements in cell culture media.[16-20]

Alternatives to FBS supplementation include autologous or allogeneic serum, purified recombinant or synthetic proteins, platelet lysate, and defined serum-free medium.[16,21-24] With regards to defined serum-free medium, multiple different commercial formulations are available, but all suffer from high cost, which renders their use for clinical studies cost-prohibitive in some situations. Therefore, species-matched serum (autologous or allogeneic) with conventional cell culture medium, has been proposed as an alternative to FBS. The impact of serum source on MSC properties has been most fully explored with human MSC, with conflicting results in terms of proliferation and differentiation, but few studies have described serum-free culture of MSC from large animal veterinary species.[13,25-44] When comparing culture of human MSC in FBS, human serum or platelet lysate, Aldahmash *et al.* and Perez-Ilzarbe *et al.* found no difference in morphology or capacity for differentiation and proliferation growth rates, while Kuznetsov *et al.* reported FBS media culture resulted in greater proliferation and enhanced bone formation following *in vivo* transplantation.[38,39,43] Schubert *et al.* compared serum-free culture of human and equine MSC, demonstrating that culture of equine, but not human MSC, in serum-free conditions resulted in altered morphology and variable proliferation and surface immunophenotype, which seemed to depend on the media lot.[13] These findings emphasize that media formulations are specific for cell types and culture procedures, and that development of media conditions should be optimized for MSCs from the animal species of interest. However, to date, the relative effects of FBS versus equine autologous or allogeneic serum on the functional properties of equine MSC have not been evaluated.

Therefore, the aims of this study were to compare relevant functional properties of equine MSC cultured in medium with either FBS, equine autologous, or equine allogeneic serum. The functional properties evaluated included cell viability, proliferation rate, morphology, concentration of cytokines and antimicrobial peptides in MSC-conditioned medium, bactericidal activity and chondrogenic differentiation potential. We hypothesized that cells would have greater functionality, particularly antibacterial activity, when cultured in FBS containing medium, and that there would be no significant differences in functionality when culture in autologous and allogeneic equine serum was compared.

6.3 Materials and Methods

Horses – Schematic overview of study design and methods used is provided in **Figure 6.1**. Six healthy 2 to 3-year-old Quarter Horse research horses (three geldings, three mares) were tissue donors in this study. All horses were part of the university-owned herd at Colorado State University, and studies were approved by the Institutional Animal Care and Use Committee (protocol #1101). All horses were determined healthy by physical examination and bloodwork (complete blood count, diagnostic panel). Three horses were used as donors of bone marrow aspirate and autologous serum, and three different horses were used as donors of allogeneic serum.

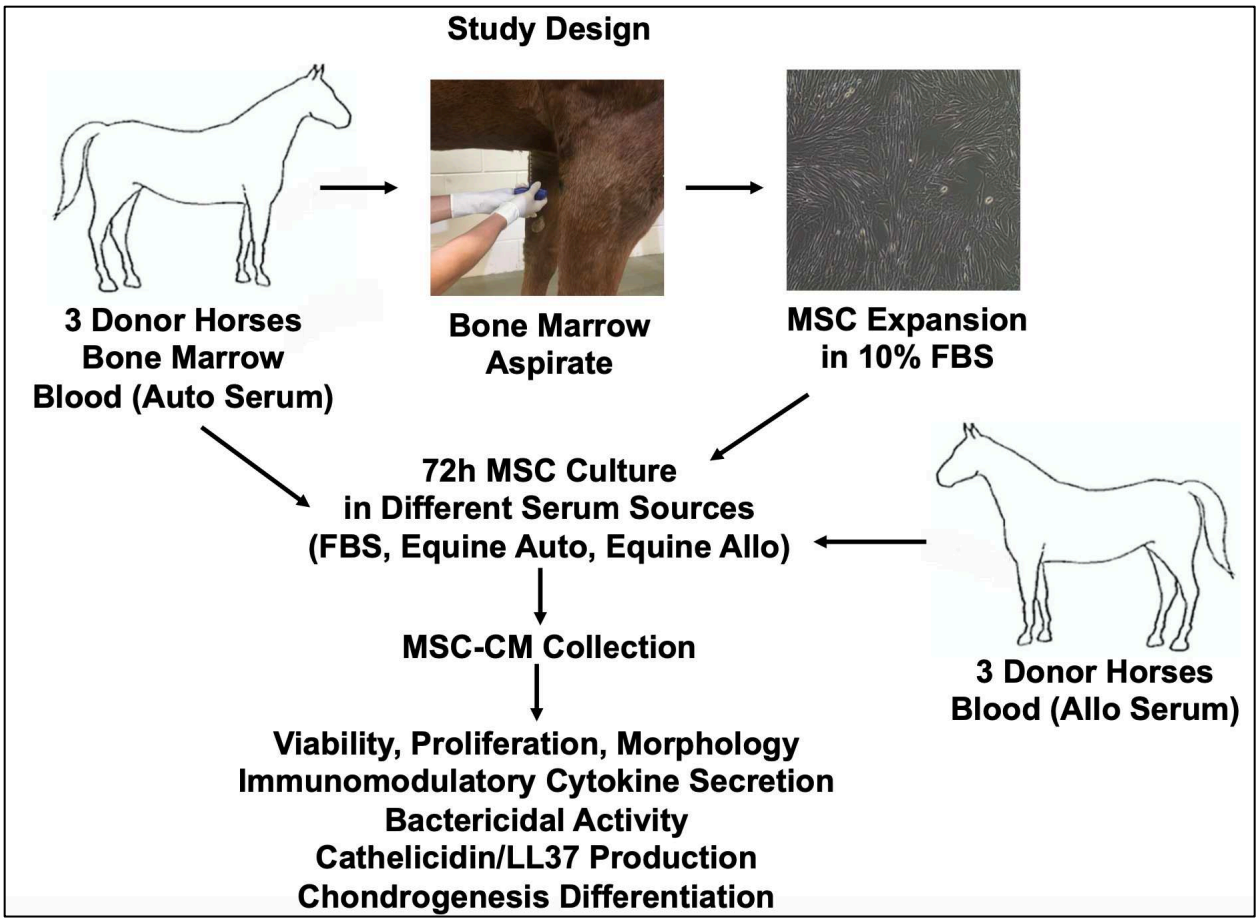


Figure 6.1: Schematic overview of study design and methods used. Abbreviations: Allogeneic (Allo), Autologous (Auto), Fetal bovine serum (FBS).

Tissue collection and cell culture – To collect bone marrow aspirate, the sternum of three donor horses (Quarter horses, two geldings and one mare, ages 2.5, 2.5 and 3 years) was clipped and aseptically prepared in routine fashion. Bone marrow aspirate (5 mL) was obtained using 11-gauge jamshidi into a syringe containing 1 mL heparin (10,000 IU). Bone marrow aspirate was plated on 75 cm^2 plates in complete supplemented growth medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL), 1 mol/L HEPES). The MSC were expanded in culture and then frozen at $5x10^6$/mL in freeze media (90% FBS, 10% DMSO) in liquid nitrogen vapor phase until further use. All MSC used for *in vitro* studies were evaluated for surface phenotype and found to

be CD44+CD90+ and CD34-CD45-, using equine cross-reactive antibodies as previously described (45) and in accordance with International Society for Cellular Therapy (ISCT) minimal criteria for defining mesenchymal stromal cells.[46]

To obtain equine serum for use in cell culture, whole blood was collected from all six donor horses (three autologous and three allogeneic to MSC cultured) into red-top blood tubes lacking anticoagulant. Blood tubes were spun at 1800 rcf for 10 minutes, and serum was removed, heat-inactivated (heated 30 minutes at 56°C with mixing), sterile filtered, aliquoted in 1 mL aliquots, and frozen at -80ºC for later use. Serum from the same three donors as were used for bone marrow aspirate donated 'autologous serum' in all experiments. Pooled serum from three additional donors (Quarter horses, one gelding and two mares, ages 2, 3 and 3 years) was used as 'allogeneic serum' in all experiments.

Viability, Proliferation and Morphology – Cells were thawed quickly in a 37ºC water bath, recovered and expanded for at least 48 hours under standard incubation conditions (37ºC with 5% CO2) in complete growth media with 10% FBS supplementation. Cells were then transitioned to growth media containing either FBS, autologous or allogeneic equine serum, as subsequently described. Cells were used between passage one to three for all experiments described. Plates and flasks containing MSC were stored in the incubator (37ºC with 5% CO2) following serum treatment for all experiments.

To assess cell viability following culture in media containing various serum sources, MSC from three individual horse donors were plated at 50,000 cells/well on 24-well plates in growth media containing either 10% FBS, autologous or allogeneic equine serum. Cell viability was assessed using trypan blue dye exclusion staining to determine percentage of live cells following 24, 48, and 72 hours in culture using an automated cell counter (Nexcelom; Bioscience

Cellometer Auto T4). Experiments were performed in duplicate for all three donors, with each donor assessed in triplicate.

To assess cell proliferation following culture in media containing different serum sources, MSC from three individual horse donors were plated at 100,000 cells/well on 6-well plates in growth media containing either 10% FBS, autologous or allogeneic equine serum. Cells were trypsinized and counted at 24, 48, and 72 hours following plating using an automated cell counter (Nexcelom; Bioscience Cellometer Auto T4). Population doubling time was calculated for each of three cell lines cultured in different serum sources over 72 hours, as previously reported.[47] Experiments were repeated in duplicate for all three donor horses, each in triplicate.

Morphology of MSC plated in different serum sources (10% serum, either FBS, autologous or allogeneic equine serum) at 100,000 cells/well on 6-well plates was documented and assessed over 72 hours. Images were obtained of cells in culture using imaging software (Olympus; CellSens, Tokyo, Japan).

Cytokine Secretion **–** Equine MSC from three donor horses were cultured in growth media with either 10% FBS, autologous or allogeneic equine serum for 72 hours, then plated at 100,000 cells/well for 24 hours on 24-well plates in media containing their respective serum sources. Supernatants were collected at 24 hours and fluorescent bead-based multiplex assay (Milliplex MAP Equine Cytokine/Chemokine Magnetic Beads Multiplex Assay, Millipore Sigma, Burlington, MA, 01803) was used to quantify the concentrations of 23 analytes (Eotaxin/CCL11, FGF-2. Fractalkine/CS3CL1, G-CSF, GM-CSF, GRO, IFN, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17a, IL-18, IP-10, MCP-1, RANTES/CCL5 and TNFα) in conditioned media.

Antimicrobial Peptide (Cathelicidin/LL37) Expression – Equine MSC from three donor horses were maintained in flasks in growth media containing either 10% FBS or autologous or allogeneic equine serum for 72 hours. Cells were then trypsinized, counted, and either stimulated or not with toll-like receptor ligand 3 polyinosinic:polycytidylic acid (pIC) at 10 μg/mL in DMEM media at $2x10^6$ cells/mL as previously reported,[48] then plated at 100,000 cells/well on 24-well plates for 24 hours in media containing their respective serum sources at either 10 or 1%. Conditioned media was collected and assessed for quantitative antimicrobial peptide cathelicidin/LL-37 by competitive ELISA, previously validated for expression in human samples and cross-reactive to equine (Human LL37/Cathelicidin Sandwich ELISA kit, Lifespan Biosciences, Seattle, WA, 98121), to determine relative levels in conditioned media following activation or resting in different serum concentrations from different sources.

Bacterial Killing Activity – Equine MSC form three donor horses were maintained in flasks in growth media containing either 10% FBS or autologous or allogeneic equine serum for 72 hours. Cells were then trypsinized, counted, and plated at 100,000 cells/well on 24-well plates for 24 hours in media containing their respective serum sources (10%). MSC conditioned medium (MSC-CM) was collected at that time and frozen at -80°C for use in bacterial killing assays.

The human multi-drug resistant strain of *S. aureus* (USA300) was kindly provided by H. Schweizer (Colorado State University). The bacterial culture and sensitivity of this isolate is supplied previously (Figure 5.1). Bacteria were expanded in Luria-Bertani (LB) broth (BD Falcon) and frozen in 20% glycerol until further use. Overnight bacterial cultures were grown in MSC growth media (10% FBS) without antibiotics prior to use in assays. On the day of the

bacterial killing assay experiment, bacterial sub-cultures were grown to log phase in MSC media (OD600 of 0.6, corresponding to 7.5 Log10 CFU/mL) then used immediately.

To determine if serum source in cell culture impacted the ability of MSC conditioned medium (MSC-CM) to directly kill bacteria, MSC-CM from the three horse donors cultured in different serum sources (10%) were inoculated with actively dividing log phase MRSA. The antibiotic-free MSC-CM or antibiotic-free MSC growth media (positive control) were plated at 200 µl/well on a 96-well plate, and 500 bacteria in log phase (OD 0.6) were added per well. Co-culture plates were incubated shaking at 100 rpm at 37ºC for 16 hours. Negative control wells containing antibiotic-free DMEM without bacterial inoculation were also included. Following incubation with bacteria, media were transferred to 1.5 mL conical tubes, vortexed to evenly distribute bacteria, diluted ten-fold, plated on LB agar plates (100 µL/quadrant) and incubated at 37ºC for 18 hours. Colony-forming units (CFU) were counted manually. Experiments were performed in duplicate for three horses, each in triplicate.

Chondrogenic Differentiation – Equine MSC were assessed for chondrogenic differentiation potential following culture for 72 hours in either 10% FBS, autologous or allogeneic equine serum by Alcian Blue staining (ThermoFisher Scientific StemPro® Chondrogenesis Differentiation Kit, Waltham, MA). Briefly, following culture in respective serum sources for 72 hours, cells were trypsinized, washed in PBS and the cell pellets resuspended in chondrogenesis medium (StemPro® osteocyte/chondrocyte differentiation basal medium and StemPro® chondrogenesis supplement (9:1), penicillin (100 U/mL) and streptomycin (100 µg/mL)) to generate a cell solution of 1.6 x 10^6 cells / 100 µL and then seeded in 5-µL droplets on six-well plates. Cells were maintained on plates for two hours in 37ºC incubator at 5% CO2 and then chondrogenesis media was added to the plates to cover the pellets.

Media was changed every three days and pellets maintained in culture in the 37ºC incubator at 5% CO2 for a total of 28 days, then evaluated for Alcian Blue staining visually by microscopy (Olympus SC30 microscope, Tokyo, Japan).

Statistical Analysis – Raw data were plotted and visually assessed for normality prior to statistical analysis. Data from the cell proliferation assay were log transformed to improve raw distribution and model fit, while other data were judged to be normally distributed. Data were modeled individually using a linear mixed model (function lmer from the lme4[49] and lmerTest[50] packages) with donor as a random effect to account for differences in donor cell lines. For the bacterial killing assay, proliferation was modeled as doubling time, and for the multiple cytokine secretion assays media type was modeled as the sole fixed effect. For the antimicrobial peptide expression assay, type of media, pIC activation status, and media concentration were modeled with a 3-way interaction between factors in order to allow for a difference in slope of the fitted line at different media percentages and activation states. The difference in cathelicidin/LL-37 expression was then determined at each of the 4 combinations of pIC activation (yes/no) and media percentage (1 or 10%).

For both the cell viability assay and proliferation determined by automated cell count, the model was fitted with the fixed effect of media type, time as a continuous factor, and a type/time interaction. The cell proliferation data were log transformed to improve model fit as previously noted. Differences between media types were then evaluated at each time point using estimated marginal means.

Model assumptions of homoscedastisity and normality of error distribution were verified by analysis of QQ plots and fitted vs residual values and model fit was judged to be appropriate. Differences between groups were evaluated using differences in estimated marginal means

(function emmeans from the emmeans[51] package), with *p*-values adjusted using Tukey's adjustment for multiple comparisons or Dunnet's Test where appropriate. All statistical analyses were performed using R for Mac (R version 4.0.0 "Arbor Day" and 3.6.0 "Planting of a Tree")[52]. Graphical analyses and graph generation were performed using Prism software v8.4.1 (GraphPad Software Inc., La Jolla, CA). For all analyses, statistical significance was assessed as $p<0.05$.

6.4 Results

Cell Viability, Proliferation and Morphology – Viability of MSC in culture was not affected by serum source (10%) after 24, 48 or 72 hours in culture (**Figure 6.2A**). However, proliferation of equine MSC cultured in FBS was faster compared to those cultured in autologous or allogeneic equine serum at both 48 and 72 hours ($p<0.001$) and faster than those cultured in allogeneic (but not autologous) serum at 24 hours ($p=0.002$) (**Figure 6.2B**). Population doubling time (mean +/- SD, across three MSC cell lines) was determined to be 21.21 +/- 4.7 hours in FBS, 28 +/- 6.9 hours in autologous equine serum, and 29.86 +/- 7.9 in allogeneic equine serum. Population doubling times for each of the three MSC cell lines are reported in **Figure 6.3**. When analyzed via automated cell count, there were a significantly greater number of cells in culture at 48 and 72 hours in FBS media compared to autologous and allogeneic equine serum ($p<0.001$ for all). There was also a greater number of cells cultured in FBS vs. allogeneic serum containing media at 24 hours ($p=0.002$). Examination of equine MSC cultured in different serum sources did not reveal observable differences in cell morphology over 72 hours culture, although proliferation as determined by visual assessment was more rapid in cells cultured in FBS. Representative images documenting cell morphology after 72 hours in culture are shown in **Figure 6.2C**.

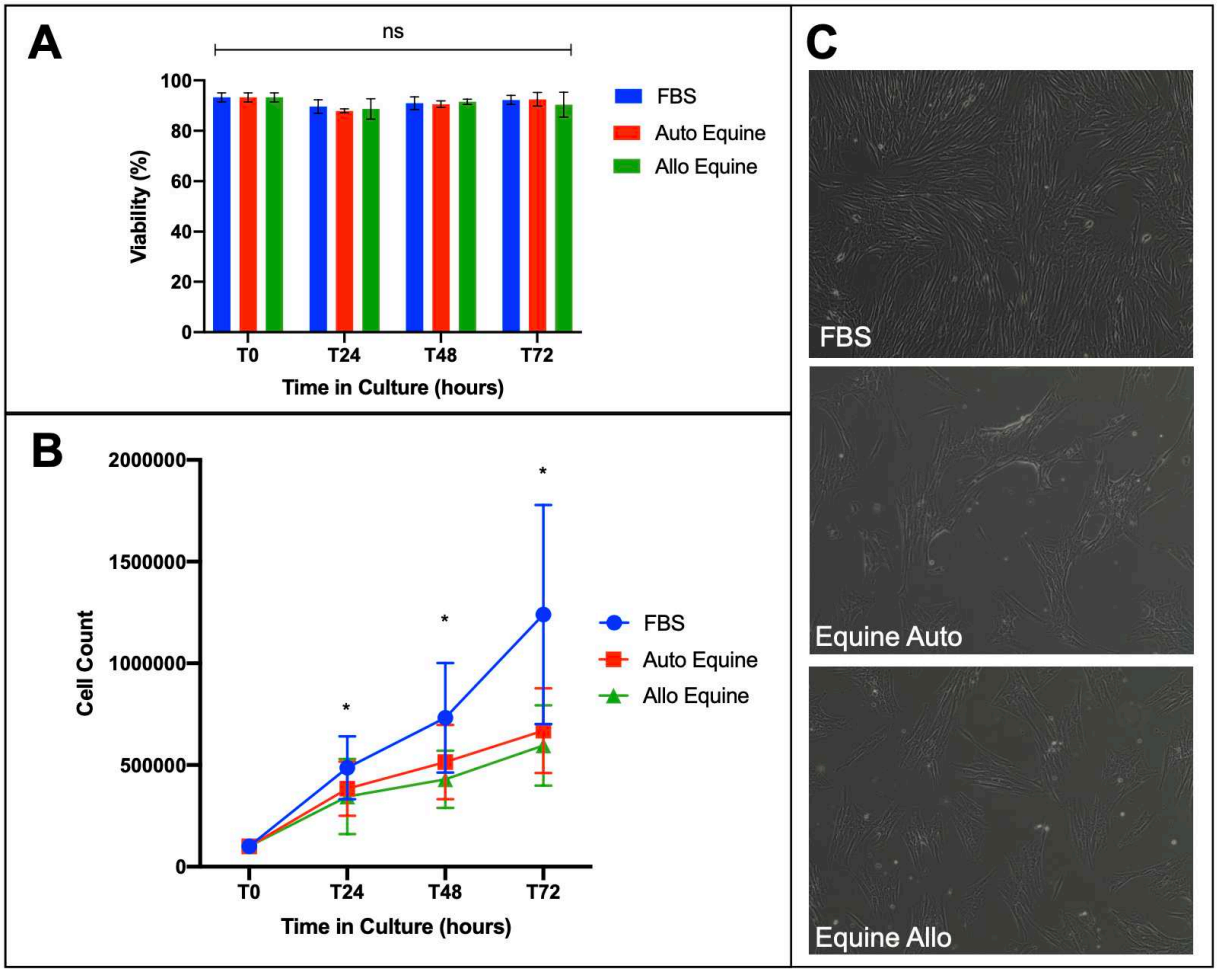
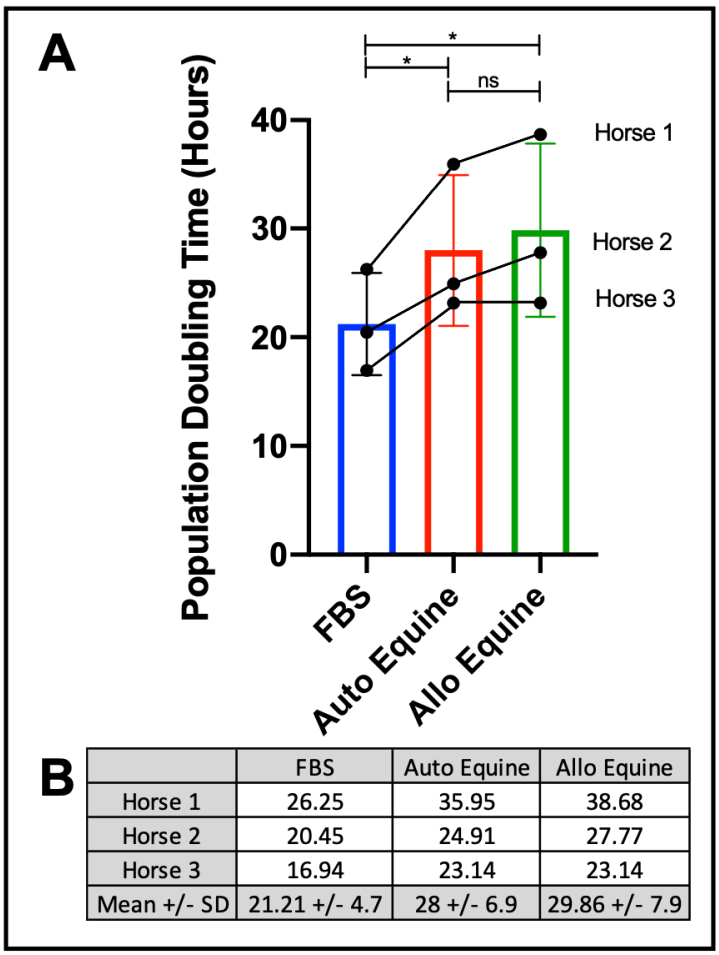


Figure 6.2: Effect of serum source on cell viability, proliferation, and morphology of equine MSC in culture. A) Viability of equine MSC from n=3 donor horses plated at 50,000 cells/well on a 24-well plate in either 10% FBS, autologous or allogeneic equine serum was assessed via trypan blue dye exclusion over 72 hours in culture, demonstrating no difference in viability between culture conditions. **B)** Proliferation of MSC plated on 6-well plates at 100,000 cells/well was assessed by quantitative cell count at baseline and over 72 hours in culture, demonstrating that cells cultured in 10% FBS proliferated at a faster rate compared to those in either autologous and allogeneic equine serum. **C)** Morphology of MSC plated in different serum sources on 6-well plates at 100,000 cells/well was assessed visual via microscopy over 72 hours. All MSC demonstrated characteristic fibroblastic morphology, although cells cultured in FBS proliferated faster by visual assessment. Bars are mean and standard deviation of three biological replicates. All experiments were performed in duplicate, with comparable results.



B

	FBS	Auto Equine	Allo Equine
Horse 1	26.25	35.95	38.68
Horse 2	20.45	24.91	27.77
Horse 3	16.94	23.14	23.14
Mean +/- SD	21.21 +/- 4.7	28 +/- 6.9	29.86 +/- 7.9

Figure 6.3: Determination of population doubling time (hours). MSC from each of three donors (n=3) were plated at 100,000 cells/well on 6-well plates and cultured in different serum sources for 72 hours. Population doubling time was determined for each cell line individually and overall, as described in methods, and presented graphically (**A**) and numerically (**B**).

Cytokine Secretion – MSC conditioned medium from cells cultured in FBS (10%) contained higher levels of seven cytokines (IL-4, IL-5, IL-17A, GM-CSF, Eotaxin, RANTES, and FGF-2) compared to autologous or allogeneic serum (**Figure 6.4, Tables 6.1 and 6.2**). Levels of five cytokines were below the detection limit of the multiplex assay (IL-2, IL-12, IL-18, IFN-y, and MCP-1). There were no statistical differences between serum treatment groups for the remaining eleven biomarkers assessed (IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-13, IP-10,

TNFα, GRO, G-CSF, and Fractalkine). Cytokine levels in control media were below the detection limit of the multiplex assay.

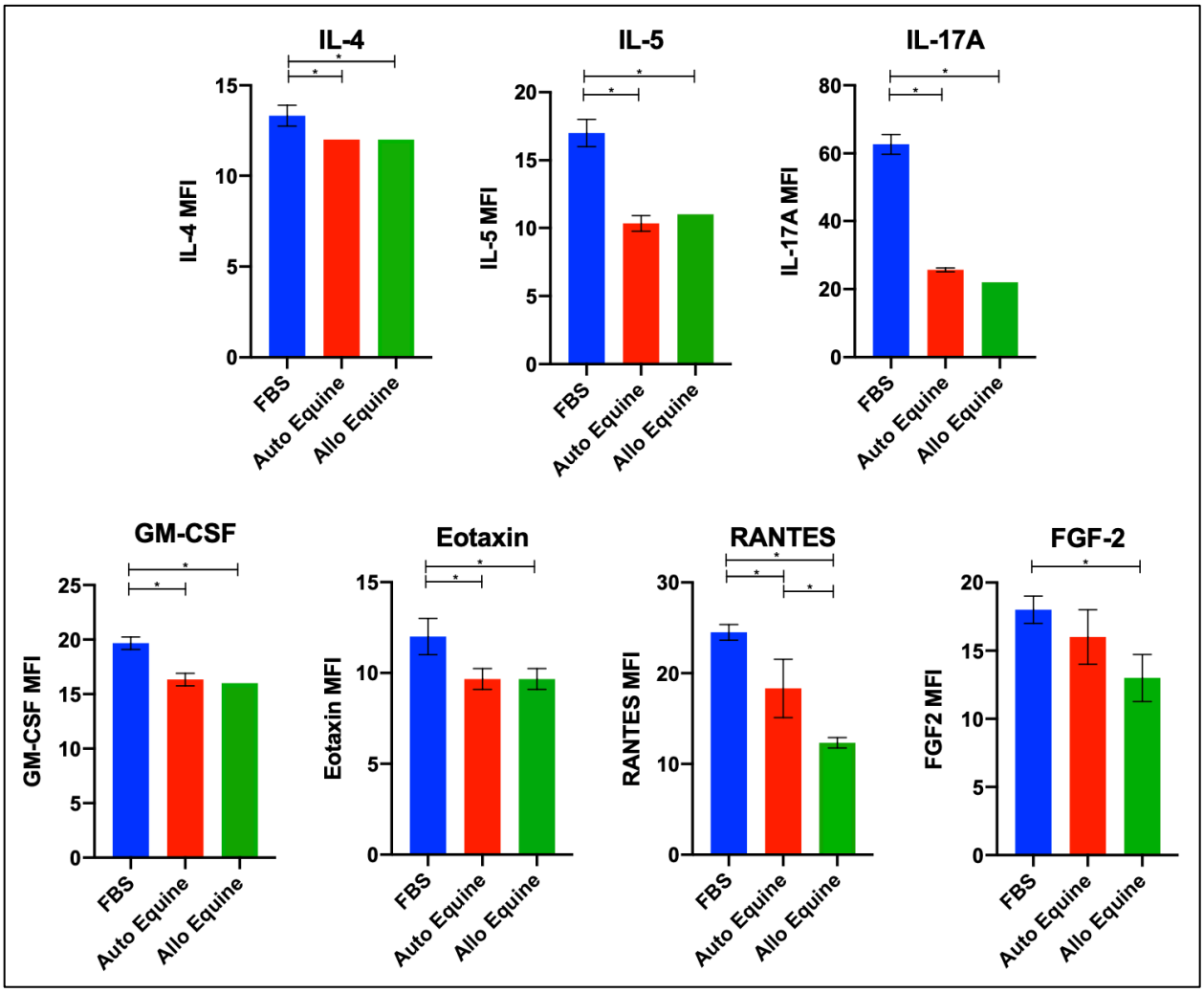


Figure 6.4: Effect of serum source in cell culture on MSC cytokine secretion. Equine MSC from three donor horses were cultured in media using different serum sources (10%; FBS, autologous or allogeneic equine serum) for 72 hours then plated for 24h on 24 well plates at 100,000 cells/well in their respective serum sources. Supernatants were collected after 24 hours for evaluation of cytokines on a 23-cytokine fluorescent bead-based multiplex assay. Cells cultured in FBS produced higher levels of seven cytokines (IL-4, IL-5, IL-17A, GM-CSF, Eotaxin, RANTES, and FGF-2) compared to autologous or allogeneic serum. Levels of five cytokines were below the detection limit of the multiplex assay (IL-2, IL-12, IL-18, IFNy, and MCP-1). For the remaining eleven biomarkers assessed (IL-1a, IL-1B, IL-6, IL-8, IL-10, IL-13, IP-10, TNFa, GRO, G-CSF, and Fractalkine) there were no statistical differences between serum treatment groups in levels of cytokines secreted. Bars are mean and standard deviation of three biological replicates.

Table 6.1: Measurable cytokine levels (MFI mean +/- SD) in MSC conditioned media containing either 10% FBS, autologous or allogeneic equine serum.

	IL-1α	IL-1β	IL-4	IL-5	IL-6	IL-8	IL-10	IL-13	IL-17A
FBS	11.3 +/– 1.5	24 +/– 2	13.3 +/– 0.6	17 +/– 1	20.3 +/– 2.5	63.6 +/– 54.5	19 +/– 0	27.3 +/– 21.4	62.6 +/– 2.9
Auto equine	9.8 +/– 0.8	23 +/– 0	12 +/– 0	10.3 +/– 0.6	22.3 +/– 4.5	63 +/– 21.3	20 +/– 1	17.3 +/– 2.3	25 +/– 0.6
Allo equine	10 +/– 0	23.3 +/– 0.6	12 +/– 0	11 +/– 0	24.7 +/– 1.5	59.7 +/– 6.8	20 +/– 1	16 +/– 1	22 +/– 0
Treatment	**FGF-2**	**Eotaxin**	**G-CSF**	**GM-CSF**	**Fractalkine**	**IP-10**	**GRO**	**TNF-α**	**RANTES**
FBS	18 +/– 1	12 +/– 1	8.7 +/– 2.1	19.7 +/– 0.6	10 +/– 0	17.3 +/– 2.9	79 +/–107.7	13.3 +/– 0.6	24.5 +/– 0.9
Auto equine	16 +/2	9.6 +/– 0.6	7.6 +/– 0.6	16.3 +/– 0.6	9 +/– 1	15.3 +/– 0.6	8.5 +/– 1.3	16.3 +/– 3.2	18.3 +/– 3.2
Allo equine	13 +/– 1.7	9.6 +/–0.6	7.6 +/– 0.6	16 +/– 0	8.3 +/– 0.6	18.7 +/– 0.6	10.3 +/– 4.2	15.7 +/– 0.6	12.3 +/– 0.6

Table 6.2: Statistical analysis of measurable cytokine levels in MSC conditioned media containing either 10% FBS, autologous or allogeneic equine serum.

Treatment	IL-1α		IL-1β		IL-4		IL-5		IL-6		IL-8		IL-10		IL-13		IL-17A	
FBS vs. auto	ns	0.23	ns	0.59	**	0.01	****	<0.0001	ns	0.72	ns	0.2	ns	0.36	ns	0.61	****	<0.0001
FBS vs. Allo	ns	0.3	ns	0.78	**	0.01	****	<0.0001	ns	0.28	ns	0.16	ns	0.36	ns	0.54	****	<0.0001
Auto vs. Allo	ns	0.98	ns	0.93	ns	>0.99	ns	0.48	ns	0.65	ns	0.98	ns	>0.99	ns	0.99	ns	0.09
Treatment	**FGF-2**		**Eotaxin**		**G-CSF**		**GM-CSF**		**Fractalkine**		**IP-10**		**GRO**		**TNF-α**		**RANTES**	
FBS vs. auto	ns	0.36	*	0.02	ns	0.63	***	0.0003	ns	0.24	ns	0.39	ns	0.41	ns	0.21	*	0.02
FBS vs. Allo	*	0.02	*	0.02	ns	0.63	***	0.0002	ns	0.06	ns	0.64	ns	0.42	ns	0.36	***	0.0006
Auto vs. Allo	ns	0.14	ns	>0.99	ns	>0.99	ns	0.68	ns	0.49	ns	0.12	ns	0.99	ns	0.91	*	0.02

*Statistical significance was indicated as *P ≤ 05, **P ≤ 01, ***P ≤ 001, ****P ≤ 0001.*

Cathelicidin/LL37 secretion – Antimicrobial peptide (cathelicidin/LL-37) expression was higher in MSC-CM from pIC-activated cells cultured in 1% FBS compared to those cultured in autologous or allogeneic equine serum (FBS vs. autologous, p=0.01; FBS vs. allogeneic, p=0.04). No differences in cathelicidin/LL-37 secretion were found between serum treatment groups for cells cultured in either 10 or 1% non-pIC-activated or 10% pIC-activated culture conditions (**Figure 6.5**).

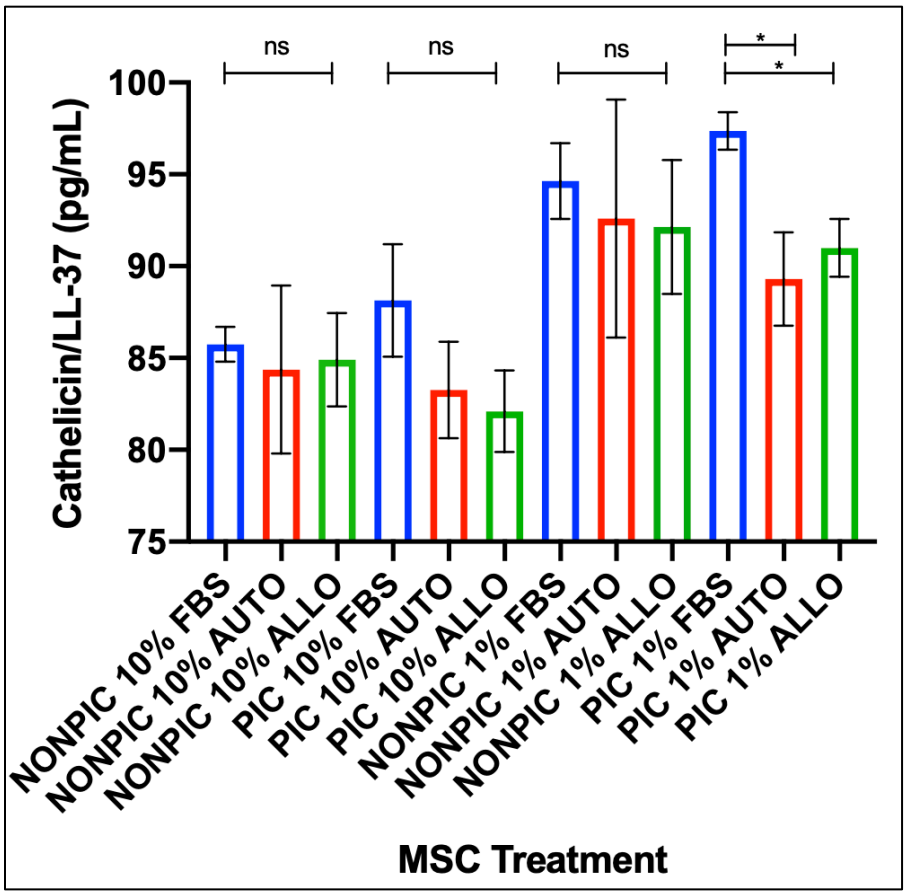


Figure 6.5: Effect of TLR-agonist priming and serum concentration in culture on antimicrobial peptide secretion. Equine MSC were plated on 75cm^2 plates for 72 hours in different serum sources (FBS, autologous or allogeneic equine serum), then trypsinized and stimulated with pIC (10µg/mL, 2 hours, 2x10^6 cell/ml) and plated for 24h in DMEM media supplemented with varying concentrations (10 or 1%) of different serum sources (fetal bovine serum, autologous or allogeneic equine serum) on 24-well plates at 100,000 cells/well. Conditioned medium was collected after 24 hours in culture and assessed for quantitative cathelicidin/LL-37 antimicrobial peptide production via ELISA. In 1% serum culture following pIC activation, LL37 production was significantly higher from MSC in FBS cultured media compared to autologous or allogeneic equine serum. Bars are mean and standard deviation of three biological replicates.

Bacterial Killing Activity – Bacterial growth was inhibited by the MSC-CM cultured in all serum sources compared to control (FBS p=0.001, autologous p=0.01, allogeneic p=0.02), and to the greatest extent with FBS-cultured cells. Bacterial growth was further inhibited by the MSC-CM from FBS-cultured cells compared to those in autologous (p=0.02) and allogeneic

equine serum (p=0.04). No differences in bacterial inhibition were seen between autologous or allogeneic MSC-CM treatments (p=0.94) (**Figure 6.6**).

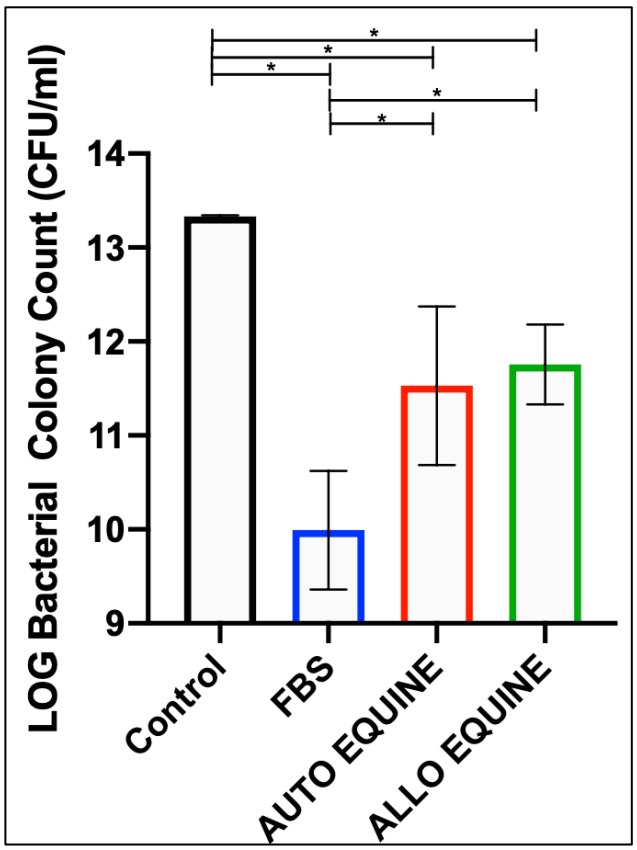


Figure 6.6: Effect of serum source in culture media on bactericidal activity of MSC-CM on multidrug resistant *S. aureus*. Multi-drug resistant *S. aureus* was cultured with MSC-CM cultured in media with different serum sources (10% FBS, autologous or allogeneic equine serum) for 16h and plated on LB agar quadrant plates for 18h. Bacterial growth shown on y-axis was measured by plating bacteria and counting viable colonies. Bacterial growth was reduced by all MSC-CM treatments compared to control and were also further reduced in MSC-CM from FBS-cultured cells compared to autologous or allogeneic equine serum. Bars are mean and standard deviation of three biological replicates. All experiments were performed in duplicate, with comparable results.

Chondrogenic Differentiation and Gene Expression – Chondrogenic differentiation was not inhibited by treatment with media with different serum sources for 72 hours prior to differentiation as evidenced by Alcian Blue expression at 28 days (**Figure 6.7**).

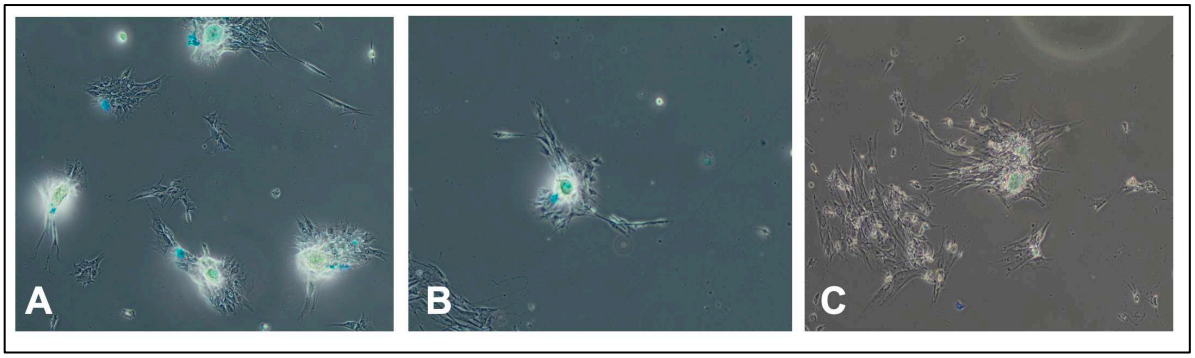


Figure 6.7: Effect of serum source in culture on chondrogenesis differentiation of equine MSC. Equine MSC were cultured in 75cm^2 flasks for 72 hours in media containing either 10% FBS, autologous or allogeneic equine serum, then trypsinized and re-plated in chondrogenesis differentiation media for 28 days. Media was changed every 3 days. At that time, cells were examined for Alcian blue expression indicating chondrogenic differentiation via microscopy. Serum source ((A) FBS, (B) autologous equine serum, or (C) allogeneic equine serum) for 72 hours did not inhibit chondrogenic differentiation.

6.5 Discussion

Mesenchymal stromal cells are increasingly used in equine clinical practice, particularly in the treatment of musculoskeletal disorders, although more recent literature has demonstrated their potential efficacy in other disease processes such as immune-mediated disorders and infection.[1-12] Cell culture in defined, serum-free medium has been proposed as an alternative to FBS to reduce antigenicity associated with the proteins themselves and potentially increase longevity of MSC following injection which may improve treatment effect.[44] However, serum free medium as an alternative is cost-prohibitive in some situations for routine clinical use. Little is known however regarding the relative merits of each serum source with respect to MSC function. In our study, we found that FBS culture resulted in faster proliferation with shorter

population doubling times and significantly greater secretion of key cytokines and antimicrobial peptides, which may be relevant to stromal cell function following clinical application in inflammation or infection, and supported our original hypothesis. These findings should prompt equine practitioners using MSC in their clinical practice to weigh the relative benefits of different serum sources in cell culture prior to administration.

In this study, we reported that equine MSC viability was not reduced following 72 hours in culture with different serum sources, although proliferation was significantly faster resulting in a greater number of cells in FBS vs. equine serum culture. Chondrogenic differentiation was not suppressed by culture in alternative serum sources to FBS, although full trilineage differentiation was not explored. Previous studies have reported differing results in terms of MSC morphology and capacity for proliferation and differentiation following culture in different media, which may be species dependent.[13,38,43] For instance, culture of equine MSC in serum-free conditions was demonstrated to result in expression of a more variable surface phenotype in equine MSC with lower percentages of CD90+ cells and small subpopulations of CD14+, CD34+, CD45+ or MHCII+ cells.[13] In contrast, human MSC maintained a more consistent morphology with CD29+, CD90+ and CD105+ immunophenotyped following serum-free culture.[13] Furthermore, morphology of equine but not human MSC were altered with increased detachment of multilayers and cellular aggregation noted.[13] These studies, taken together, highlight that variability in cell response to serum-free culture in terms of viability, proliferation, differentiation and morphology depends on a number of factors and, importantly, should be optimized for the species of interest.

Concentrations of seven biomarkers (IL-4, IL-5, IL-17A, GM-CSF, Eotaxin, RANTES, FGF-2) were found to be increased in supernatants collected from MSC cultured with FBS

versus autologous or allogeneic equine serum. Lack of statistical significance in levels of several other cytokines (e.g. IL-8, IL-13, GRO) may be explained in part, due to small number of horse cell lines (n=3) and the large variability between cell lines evaluated. Various studies have demonstrated therapeutic potential for IL-4 in osteoarthritis.[53-58] IL-4 receptors are expressed on chondrocytes and synovial cells,[54,55] and IL-4 signaling has been shown to alter mechano-transduction in chondrocytes associated with turnover of matrix in OA.[56] In addition, IL-4 inhibits chondrocyte apoptosis and cartilage breakdown and reduces synovial inflammation by antagonizing TNF-α-induced production of prostaglandin E2 by synovial fibroblasts in OA.[59] Furthermore, genetic variation in IL-4R genes increase susceptibility of individuals to OA.[57] Finally, IL-4 induces production of IL-1 receptor antagonist, which may have important implications when MSC are injected in treatment of osteoarthritis where IL-1 is frequently elevated.[60]

Several of the other cytokines elevated in FBS-cultured MSC-CM have also been implicated to have prognostic value in the degree of severity and progression of osteoarthritis. Significant concentration differences in IL-5 in synovial fluid were noted between subjects with little or no arthritis compared to those with advanced arthritis based on the ICRS scale.[61] When synovial fluid samples from patients undergoing knee arthroscopy were evaluated for biomarker levels, RANTES (regulated upon activation, normal T cell expressed and secreted), in addition to vascular endothelial growth factor and platelet-derived growth factor, was one of the strongest predictors of postoperative improvement at final follow-up regardless of the degree of cartilage injury at time of surgery.[62] RANTES has also been reported as a mediator of acute and chronic inflammation, with recruitment of macrophages, mast cells, and eosinophils, as well as prostaglandin E2 (PGE2) generation demonstrated following RANTES injection in skin of

experimental rodent models.[63] IL-17 promotes recruitment of both neutrophils and monocytes by inducing various chemokines,[64] which has been suggested to mediate inflammation in human rheumatoid arthritis (RA).[65]

Interestingly, FGF-2 has been suggested to have both a chondroprotective role in cartilage metabolism and a catabolic effect on articular cartilage homeostasis, and may play a role in cartilage regeneration and repair.[66-68] The conflicting roles of FGF-2 have been suggested to be dependent upon differences in the balance of FGF receptors (FGFRs) within the tissue of interest which may be species-dependent.[68,69] For example, FGF-2 induces catabolic and anti-anabolic effects in human articular cartilage,[67,68] but exerts an overall anabolic effect in murine cartilage.[66] At the time of this writing, the reasons for species specificity remain unknown but may potentially be accounted for by differences in receptor levels between species and may be further clarified when species specific FGFR profiles, including equine, are published.[69] Increased eotaxin levels in the synovial fluid of individuals with osteoarthritis have been reported,[70] and correlated with disease severity.[71] Eotaxin production by chondrocytes was further demonstrated to be induced by stimulation of chondrocytes with IL-1B or TNF-a.[70] In contrast however, high serum levels of eotaxin are associated with less radiographic progression in early rheumatoid arthritis patients, suggesting a counter-regulatory role.[72] Finally, granulocyte-macrophage colony-stimulating factor (GM-CSF), elevated in MSC-CM of FBS-cultured stromal cells, has been described as a growth factor that induces proliferation and differentiation of bone marrow myeloid progenitor cells, and therefore may exert an important effect to encourage migration of myeloid cells in inflammation, stimulating renewal of macrophages and granulocytes and survival of targeted cells.[73] Both protective and pathogenic roles for GM-CSF

in inflammatory and autoimmune diseases have been described, demonstrating multiple roles for GM-CSF and potential therapeutic strategies that may exploit its role in inflammation.[73]

Equine MSC possess antimicrobial and immunomodulatory properties and their application in the treatment of bacterial infections is gaining increasing attention.[3,4] MSC are normal participants in tissue repair processes, promoting healing through epithelialization, angiogenesis, granulation tissue formation, collagen deposition and release of inflammatory mediators.[74-84] Direct antibacterial effects mediated by secretion of antimicrobial peptides such as cathelicidin/LL-37,[74,75] and indirect effects through activation and recruitment of immune effector cells as MSC express genes for production of immunomodulatory and chemoattractant cytokines including IL-6, IL-8, and MCP-1.[74,75,76,83,85] In this study, we demonstrated that cathelicidin/LL-37 production and bactericidal ability to *S. aureus in vitro* was reduced in MSC-CM from cells cultured in autologous or allogeneic equine serum compared to FBS. These findings may have important implications towards optimizing MSC antibacterial activity when used to treat infections, and the relative antimicrobial capacity of equine MSC cultured in different serum sources warrants further investigation *in vivo*.

Limitations of this study include the *in vitro* nature of design, relatively low number of individual equine cell lines assessed (n=3), short period of time in culture with alternate serum sources (72 hours) and the fact the all MSCs were initially cultured in the presence of FBS. Culture of MSC in their respective media from the beginning of the study could have yielded additional information. It is also acknowledged as a limitation that conditions for MSC cultured in autologous or allogeneic equine sera were changed, versus those in FBS remained the same throughout the study. However, the study was performed as such to replicate clinical scenarios where equine practitioners replace the FBS as the serum source several days prior to clinical

application. In addition, further *in vivo* evaluation and comparison of safety and efficacy of MSC that were cultured in different serum sources is warranted. Secretion of additional various cytokines and growth factors that may play a role in joint inflammation and degeneration where MSC are applied in the treatment of osteoarthritis such as TGF-ß, PDGF, or aggrecanases were not quantified in this study. Additional immunophenotypic evaluation for surface markers of MSC and comparison of surface marker expression prior to and following culture in different serum sources could have been performed and may have added additional value to the study design. In assessing chondrogenic differentiation, the time period of 72 hours in culture is relatively short prior to 28 days of culture in chondrogenesis differentiation assays, and it is acknowledged that although the chondrogenesis differentiation media was serum-free, a potential effect of different sera sources could have been masked by the length of time in culture following removal from the alternate sera sources investigated. The study did not assess the effect of serum donor age on composition of sera; as fetal bovine serum has demonstrated differences compared to adult bovine serum, the inclusion of and comparison to fetal horse serum here could have also strengthened study design. Finally, this study was limited in comparing FBS to only autologous or allogeneic equine serum, and further studies may also evaluate alternative xenogene-free options to FBS in culture media including platelet lysate and commercially available serum substitutes. Batch-to-batch variability and transmission of species-specific viruses are also a risk with allogeneic equine serum use in culture (as they are with FBS). Therefore, recombinant products represent the only sustainable alternative to FBS, although the price of commercially available media may be prohibitive in some circumstances.

In conclusion, this study demonstrated that MSC cultured in medium with FBS were more functionally active than MSC cultured in equine serum. However, no difference was found

in MSC cultured in autologous serum compared to cells cultured in allogeneic equine serum. To ascertain whether these *in vitro* effects translate to clinically significant differences in MSC efficacy, randomized trials comparing the effectiveness of MSC cultured in FBS to MSC cultured in equine serum need to be conducted. Until such trials are completed, it is important to consider not just serum effects on cell viability and proliferation, but also on the intended MSC effector cell functions for the given disease indication when opting for cell serum sources.

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CHAPTER 7: TLR-activated allogeneic mesenchymal stromal cell therapy reduces bioburden and biomarkers of inflammation in an equine model of multidrug resistant *Staphylococcal* septic arthritis

7.1 Summary

Septic arthritis causes significant morbidity and mortality in human and veterinary patients and is increasingly complicated by the rising incidence of multidrug resistance. Mesenchymal stromal cells (MSC) possess antimicrobial and immunomodulatory properties through antimicrobial peptide secretion and paracrine recruitment of immune effector cells, which are enhanced by stimulation with toll-like-receptor (TLR) agonists. Objectives of this study were to determine if intra-articular (IA) administration of TLR3-activated bone-marrow-derived equine MSCs with antibiotics improved clinical outcomes and reduced bacterial bioburden and pro-inflammatory cytokine levels in an equine model of septic arthritis compared to antibiotics alone. Eight horses were inoculated in one randomly assigned tibiotarsal joint with multidrug resistant *Staphylococcus aureus* ($1x10^4$ CFU). Bone-marrow-derived mesenchymal stromal cells (MSC) from three healthy adult donor horses were activated in culture with TLR-3 agonist polyinosinic:polycytidylic (pIC) acid (10 µg/mL) at $2x10^6$ MSC/mL for 2 hours, washed three times in phosphate buffered saline (PBS), and resuspended in 1 mL PBS for injection. Recipient horses received either IA TLR-MSC and vancomycin (100 mg) or vancomycin alone on days 1, 4 and 7 following infection. Inflammation/pain scoring, complete blood counts, synovial fluid (SF) analyses, quantitative bacterial counts, inflammatory biomarkers, and macroscopic scoring were assessed. Pain/inflammation scores (p=0.0002), quantitative bacterial counts (SF D4 p=0.03, D7 p=0.02; synovium p=0.003) and peripheral blood neutrophil counts (D4 p=0.03, D7 p=0.06) were lower in TLR3-MSC-treated horses. SF (D7) from treated horses had lower total nucleated cell counts (p=0.09), total protein (p=0.08), and lactate (p=<0.0001) and higher

glucose (p=0.009). SF IL-6 (p=0.02) and IL-18 (p=0.02) was lower in treated horses. In summary, intra-articular TLR-3-MSC injection inhibited bacterial growth and improved clinical parameters in multi-drug resistant septic arthritis. Study limitations included small horse sample size, lack of data collection at later time points, and lack of an additional control group receiving non-TLR activated MSC therapy. Further evaluation of imaging findings and histology of joint tissues are indicated. Equine TLR-MSC therapy improved outcomes in antibiotic-resistant joint infections.

7.2 Introduction

The rapid development of antibiotic resistance to pharmacologic drugs in human and veterinary medicine necessitates advancement of novel therapeutic strategies to treat infection. Biological therapies such as mesenchymal stromal cells (MSC) are attractive as they possess antimicrobial and immunomodulatory properties.[1] Direct effects of MSC are mediated by antimicrobial peptide production and release,[2-5] while indirect antimicrobial actions are facilitated by the immunomodulatory effect of MSCs on innate immune cells including recruitment of monocytes and neutrophils to the site of infection.[2,6-16] One mechanism by which MSC play a role in response to inflammation associated with infection is through expression of toll-like receptors (TLR). Previous studies have demonstrated that the antimicrobial and immunomodulatory properties of MSC, including antimicrobial peptide secretion, direct inhibition of bacterial growth, secretion of immunomodulatory cytokines, and neutrophil phagocytosis of bacteria, can be enhanced by stimulation with TLR ligands *in vitro*.[2,8,16] In a murine model of chronic implant infection, MSC cultured with TLR-3 ligand polyinosinic:polycytidylic acid (pIC) demonstrated synergism with antibiotics to effectively

eliminate infection, with improved activity over resting MSC with or without antibiotics, or antibiotics alone. These initial studies in rodent models prompted further investigation of TLR-activated MSC therapy to treat multidrug resistant septic arthritis in horses. The ability of MSCs to enhance immune function of the host and act synergistically with current therapeutic options is attractive to circumvent the rapid development of resistance inherent in use of new pharmacologic agents.

The investigation of novel therapies to treat infection in large animal disease models is critical to assessing efficacy of treatments for humans that have largely been confined to laboratory rodents with induced disease.[16] The equine preclinical model of septic arthritis is a translational and clinically relevant model for human joint disease, with greater similarity in joint volume, cartilage thickness and articular cartilage loading forces to that of humans than small animal laboratory species.[17-23] In addition, the large joint volume of horses allows for collection of greater volumes of synovial fluid for analysis of an increased number of outcome parameters in assessing response to treatment.[17-20,22,23] Development of septic arthritis as a naturally occurring disease process in horses is well-documented,[24] and bacterial biofilm aggregate formation in equine synovial fluid has also recently been described as a substitute for human synovial fluid, providing further evidence for the equine model of septic arthritis for human disease.[23] Equine MSC have been shown to depolarize the cell membranes and inhibit growth of both Gram positive and negative bacteria, partially through secretion of four antimicrobial peptides,[4] and this antibacterial activity was upregulated by TLR3 pIC ligand activation,[8] further supporting the use of an equine model to investigate the antimicrobial effects of MSC further *in vivo*.

Therefore, we conducted a series of studies to determine if intra-articular administration of TLR3-pIC activated bone marrow derived MSC and antibiotics would improve clinical outcomes and reduce bacterial bioburden and biomarkers of joint inflammation in an equine model of septic arthritis compared to antibiotics alone. We hypothesized that immune activated MSC therapy with antibiotics would result in more rapid resolution of bacterial infection and synovial markers of inflammation compared to horses treated with antibiotics alone, resulting in improved clinical parameters, as assessed by lameness evaluation and inflammation scoring. Our notable findings were that treatment of multi-drug resistant *Staphylococcus aureus* septic arthritis with immune activated equine MSC therapy and vancomycin significantly reduced quantitative bacterial cultures of synovial fluid and synovium and lowered levels of pro-inflammatory cytokines, with improved overall pain scores.

7.3 Materials and Methods

Study design – Horses were randomized by leg to receive either treatment or control using a random number generator, random.org. The sample size of four horses per group was determined based on previous literature[25] and following interim analysis of data demonstrating significant differences with four horses per treatment group. Randomization of treatment allocation was performed by one individual (LP) who was unblinded during the study; all other collaborators were blinded to treatment throughout data collection and analysis.

Study design is summarized in **Figure 7.1**. Horses were enrolled in four cohorts of two horses per cohort. On day zero of each cohort, an intravenous jugular catheter was placed and horses were injected intra-articularly in one randomly assigned tibiotarsal joint with $1x10^4$ *Staphylococcus aureus* bacteria. Horses then received either intra-articular treatment consisting

of $20x10^6$ pooled allogeneic MSC from three donor horses in 1 mL phosphate buffered saline (PBS) and 100 mg vancomycin or control (1 mL PBS and 100 mg vancomycin) on days 1, 4 and 7 following infection.

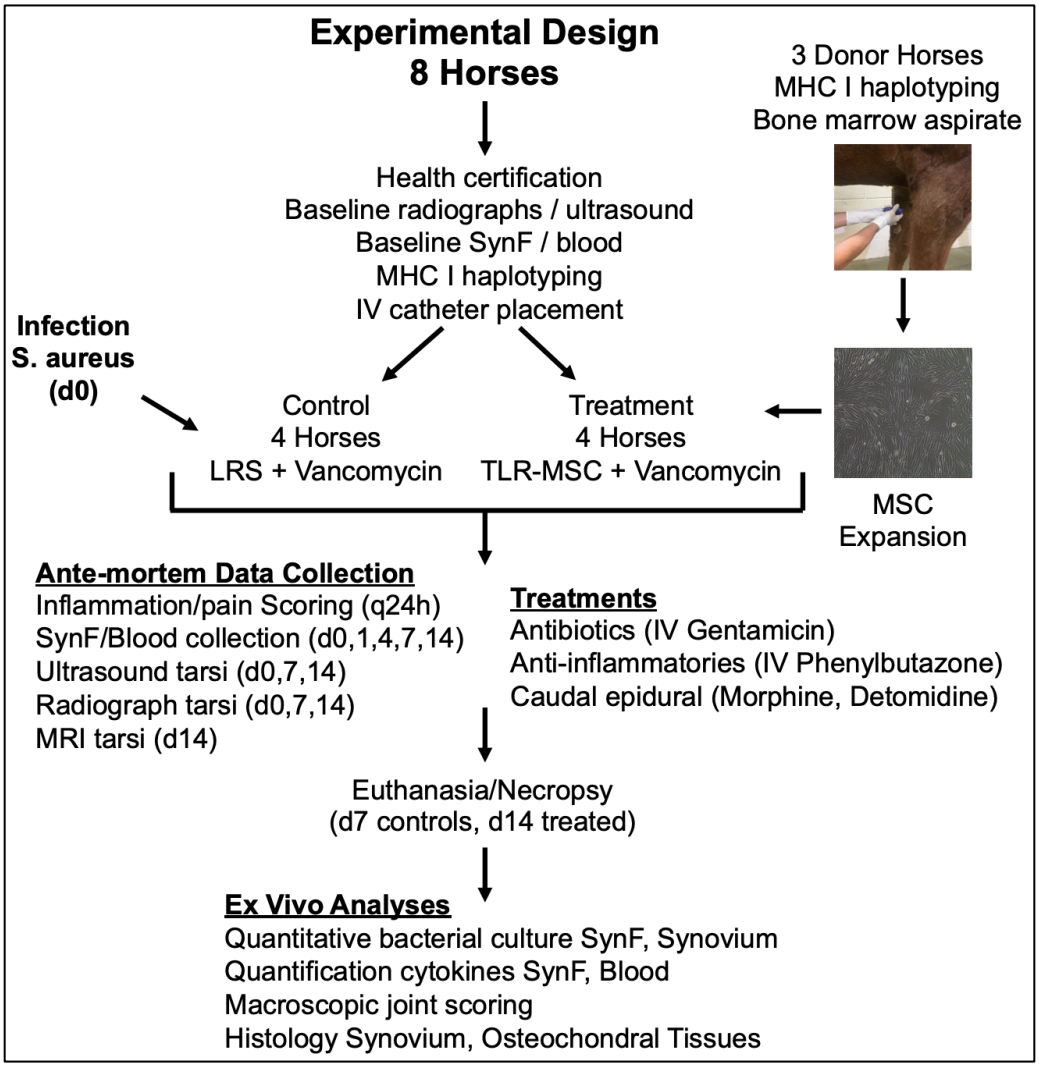

Figure 7.1: Schematic overview of the study design for the *in vivo* model of infectious arthritis in horses. Horses (n=8) were quarantined 14 days and acclimated to stalls for 5 to 7 days in cohorts of 2 prior to the beginning of each cohort. Horses were determined to be systemically healthy based on physical examination, lameness evaluation, negative salmonellosis testing, and normal complete blood count before study enrollment. On day 0 of each cohort, each horse was inoculated in one randomly assigned tarsocrural joint with *S. aureus*. On Day 1, 4, and 7 horses were treated with saline or TLR3 activated MSC intra-articularly and vancomycin. All horses received caudal epidurals with morphine (30 mg) and detomidine (6 mg) on day 0, systemic antibiotics (gentamicin 6.6mg/kg IV every 24 hours until d7 (end-term) for controls and d10 for treated horses) and nonsteroidal-anti-inflammatories (phenylbutazone 2.2 mg/kg IV every 12 hours until euthanasia). Pain and inflammation scoring were performed daily. Synovial

fluid and blood samples were obtained on days 0,1,4,7 and 14. Radiographs and ultrasound were performed at baseline and days 7 and 14. Magnetic resonance imaging was performed at end-term (d7 for controls and d14 for treated horses). *Ex vivo* analyses consisting of macroscopic scoring of joint tissues, histopathology, biomarker analysis, bacterial identification and quantitative bacterial counts on synovial fluid and synovium were performed.

For intra-articular infection and treatment, horses were sedated with a combination of detomidine (0.01-0.02 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV) to effect. The tibiotarsal joint was aseptically prepared with chlorhexidine gluconate (4%, VetOne, MWI, Boise, ID, 83705) followed by 70% ethanol. Vancomycin dose was determined in a pilot study based on assessment of antibiotic levels in synovial fluid by immunoassay over 72 hours following injection of 100 mg vancomycin in the tarsocrural joint of one normal horse, in combination with determination of cytotoxicity of vancomycin on equine bone-marrow derived mesenchymal stromal cells which would be co-administered (**Figure 7.2**).

For pain management, all horses received a caudal epidural consisting of detomidine (6 mg) and morphine (30 mg) followed by 20 mL saline at the time of infection. Additional caudal epidurals were performed in control horses on days five and six following infection if deemed necessary based on pain scoring and clinical assessment. Horses were maintained on nonsteroidal anti-inflammatories (phenylbutazone 2.2 mg/kg intravenously (IV) every 12 hours) for the duration of the study, with the first dose administered at the time of infection. Pain and inflammation scoring were monitored daily. Horses were administered systemic antibiotics (gentamicin 6.6 mg/kg IV every 24 hours) beginning 24 hours following infection and continuing to end-term for control horses (d7) or through day 10 for treated horses. Synovial fluid (4 mL) and blood samples were collected at baseline and on days 1, 4, 7 and 14 following infection. Horses assigned to control were humanely euthanized on day 7. Horses assigned to

receive treatment were continued until day 14 endpoint, after discontinuing systemic antibiotics on day 10.

Animals – Use of eight healthy three to four-year-old horses (three stallions, three geldings, two mares) as treatment recipients was approved by the Colorado State University (CSU) Institutional Animal Care and Use Committee (protocol #977). Horses were determined to be systemically healthy by physical examination, complete blood count, negative fecal testing for salmonellosis, lameness evaluation by board-certified veterinary surgeons (LG, LP) and radiographs (four-view) of both tarsi. All horses were sound in the hindlimbs at the trot and lacked radiographic evidence of tarsal osteoarthritis prior to study enrollment. Horses were quarantined for at least fourteen days or until meeting above criteria for study enrollment and acclimated to stalls for five to seven days prior to enrollment.

Three different three-year-old Quarter Horses (one mare, two geldings) determined to be healthy by physical examination and bloodwork (complete blood count, diagnostic panel) donated bone marrow aspirate for mesenchymal stromal cell culture and expansion. Institutional Animal Care and Use Committee (protocol #1101) approval was obtained for tissue collection.

MHC haplotype analysis – The MHC haplotype of each donor and recipient horse was determined. DNA was extracted from whole blood using a commercially available kit (Qiagen, Valencia, CA 91355) and amplified using multiplex fluorescent polymerase chain reaction with known primers for 12 microsatellite loci within the MHC region as previously described.[26] DNA PCR fragments were submitted to the Cornell University BioResource Center (BRC) and electrophoresed on an ABI 3700 instrument. Fragment analysis files were analyzed using GeneMarker software (SoftGenetics, State College, Pennsylvania). Haplotypes were reported

when matched to previously characterized haplotypes, and unknown haplotypes were reported when no individuals with the same haplotype were previously identified (**Table 7.1**)

Table 7.1: Microsatellite haplotype data for MSC donor and treatment recipient horses.

		Intra-MHC Microsatellite Alleles											
Horse	Microsatellite haplotype	Class I				Class III		Class II					
MSC Donor Horses		UMNJH-38	COR110	305-93	CZM002	ABGe9019	UMNe65	ABGe9030	EQMHC1	COR112	COR113	UM011	COR114
A	A9a	156	217	0	247	307	255	215	190	264	272	169	255
	A5a	156	221	0	261	299	257	212	190	254	260	172	243
B	A5a	156	221	0	261	299	257	212	190	254	260	172	243
	A10a	156	221	0	259	312	261	207	190	237	264	180	243
C	A5a	156	221	0	261	299	257	212	190	254	260	172	243
	A3b	163	207	0	251	312	261	211	192	262	268	176	247
Treatment Recipient Horses													
Control													
A	Not phased	156	211	342	249	301	259	205	192	250	266	169	245
	Not phased	156	221	345	232	310	263	217	199	254	266	170	249
B	A5a	156	221	340	261	299	257	212	190	254	260	172	243
	Novel	161	211	341	255	314	259	219	190	262	270	184	245
C	Not phased	156	215	345	253	312	257	221	180	252	274	171	243
	Not phased	156	211	345	259	299	261	215	190	260	266	169	249
D	Novel	161	211	341	255	312	261	207	190	237	264	180	243
	A3b	163	207	343	251	312	261	211	192	262	268	176	247
Treatment (MSC Recipients)													
E	A3b	163	207	343	251	312	259	211	192	262	268	176	247
	Novel	156	215	345	251	312	259	221	180	252	274	171	243
F	COR188	156	221	342	230	318	257	219	190	254	270	172	249
	Novel	156	219	347	230	316	263	215	190	237	268	176	247
G	Not Phased	156	207	340	230	314	257	215	190	262	260	172	243
	Not Phased	156	207	346	261	316	259	215	196	268	268	176	247
H	A2	156	211	343	249	301	259	209	192	262	268	174	234
	A5a	156	221	340	261	299	257	212	190	254	260	172	243

Bacterial culture **–** The human-derived methicillin resistant *Staphylococcus aureus* (MRSA) strain USA300 used in bacterial killing and biofilm assays was kindly provided by H. Schweizer (Colorado State University). The bacterial culture and sensitivity of this isolate is supplied previously (Figure 5.1). Bacteria were initially expanded in LB broth and frozen at -80ºC in 20% glycerol until further use. Overnight bacterial cultures were grown in antibiotic-free MSC growth medium (DMEM with 1000 mg/L glucose, 10% or 1% FBS, 1M HEPES) the day before synovial injection. On the day of injection for each cohort, bacterial sub-cultures were grown to log phase in antibiotic-free MSC medium (OD600 of 0.6, corresponding to 7.5 Log10 CFU/mL) then used immediately. Bacterial inoculum ($1x10^4$ CFU) was calculated based on the

optical density and previously determined growth curve equation and then resuspended in a syringe containing 1 mL phosphate buffered saline (PBS) for injection. Bacterial inoculum was maintained on ice for transport from the laboratory to recipient horses for injection. A pressure bandage was placed over the injection site for approximately one hour following injection to minimize extravasation of bacterial inoculum into the periarticular tissues.

Mesenchymal stromal cell culture - For bone marrow aspirate, the donor horses were sedated with a combination of detomidine (0.01-0.02 mg/kg IV) and butorphanol tartrate (0.01 mg/kg IV) to effect. The sternum of donor horses was clipped and aseptically prepared with chlorhexidine gluconate (4%, VetOne, MWI, Boise, ID, 83705) followed by 70% ethanol in routine fashion. Bone marrow aspirate (5 mL) was obtained from a single site between the fourth to sixth sternebrae using a Jamshidi needle into a sterile syringe containing 1 mL heparin (1×10^4 U). Bone marrow aspirates were plated and expanded in culture (37˚C, 5% CO_2, 95% humidity) to 80% confluence for approximately ten days in complete growth medium (Dulbecco's Modified Eagle's Medium (DMEM) with 1000 mg/L glucose, 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL), 1M HEPES). Cells were detached from flasks by trypsinization, then frozen at $5x10^6$ cells/mL in freeze media (90% FBS, 10% dimethyl sulfoxide [DMSO]) in liquid nitrogen vapor phase until further use.

Cells from the three donor horses were thawed quickly in a 37°C water bath and recovered in complete growth medium for 72 hours under standard incubation conditions (37˚C, 5% CO_2, 95% humidity) prior to each cell injection, which occurred on days 1, 4 and 7 following intra-articular bacterial infection. Prior to injection, MSC were trypsinized and counted. A total of 20 x 10^6 MSC pooled from three donors were stimulated with TLR-3 agonist polyinosinic, polycytidylic acid (pIC) at 10 µg/mL (InVivoGen, San Diego, CA) suspended in complete

growth media at a concentration of $2x10^6$ MSC/mL and incubated at 37˚C, 5% CO_2, 95% humidity. MSC were used between passage one to five for all injections. All MSC used for *in vivo* studies are routinely evaluated for surface phenotype, and found to be $CD44^+CD90^+$, and $CD34^-CD45^-$, using equine cross-reactive antibodies as previously described[27] and in accordance with International Society for Cellular Therapy (ISCT) minimal criteria for defining mesenchymal stromal cells.[28]

Clinical observations **–** Horses were examined twice daily for comfort, physical examination parameters, signs of infection including pain on palpation of the injected joint, swelling, heat, lameness or other signs of sepsis (change in general demeanor etc). Photographs and videos of each horse were recorded daily in the morning prior to administration of nonsteroidal anti-inflammatory medications for blinded grading of lameness, periarticular swelling and distal limb edema. Thermography images of the injected tibiotarsal joint and contralateral control joint were recorded and relative heat signatures determined subjectively. Joint circumference of the tibiotarsal joint was measured daily using a flexible tape measurer. To standard joint circumference, each joint was marked at the level it was measured for consistency. Pain and inflammation scoring was determined by two board-certified surgeons, blinded to treatment, based on grading five parameters (physical examination, lameness evaluation, distal limb edema, synovial swelling, synovial heat), on a scale of 0 to 3 (0 normal, 1 mild, 2 moderate, 3 marked) for a maximum total score of 15.

Synovial fluid collection **–** Synovial fluid (SF) samples (4 mL) were aspirated from the injected tibiotarsal joint at baseline (day 0) and at 1, 4, 7, and 14 days (for treated horses) following infection. Prior to each sample collection, the tibiotarsal joint was aseptically prepared and horses were sedated as described above. SF was aspirated with a sterile 18-gauge needle and

extension set into vials and either submitted for fluid analysis immediately or aliquoted and stored at -80°C until later use in immunoassays.

Clinicopathological parameters – SF samples were submitted to the CSU Clinical Pathology Laboratory for evaluation by a board-certified clinical pathologist (ARM) for fluid analysis including total nucleated cell count (Hematrue, Heska Corp, Loveland CO, USA), refractometric total protein, preparation of Wright-Giemsa stained (Aero-spray, Logan, UT, USA) direct smears for determination of a manual leukocyte differential, and subjective grading of the glycosaminoglycans (adequate or disrupted) and quantity of red blood cells (within normal limits or excessive). Blood samples were submitted in EDTA containing tubes for complete blood count and leukocyte differential. Whole blood samples obtained in EDTA containing tubes were centrifuged at 3000 rpm for 10 minutes, after which plasma was aliquoted and frozen at -80°C for use in immunoassays at a later time. Synovial fluid lactate levels were determined by handheld reader (Lactate Plus Portable Lactate Reader, Nova Biomedical, Maltham, MA 02454). Synovial fluid glucose levels were determined by handheld reader (AlphaTrak 2 Blood Glucose Monitoring System, Zoetis, Lincoln, NE 68521).

Euthanasia and Necropsy - At the endpoint of the study (d7 for control and d14 for treated horses), horses were euthanized using a lethal dose of intravenous pentobarbital (1ml / 5kg) administered via indwelling catheter in the jugular vein. The hindlimbs were removed and magnetic resonance imaging of both tarsi was performed. The hindlimbs were then aseptically prepared and final synovial fluid samples collected. The tibiotarsal joints were dissected aseptically and photographed for evaluation of macroscopic morphology. Assessment of macroscopic observations was performed based on photographs of injected and contralateral limbs by two board-certified equine surgeons blinded to treatment. Each of four parameters

(synovial proliferation, subcutaneous thickening/edema, vascularity, and cartilage erosion were consensus graded on a scale of 0 to 4 (0=normal, 1=slight abnormalities, 2=mild, 3=moderate, 4=severe) for a maximal total score of 16. Synovial membrane samples were collected from four different sites within the joint (dorsomedial, dorsolateral, palmaromedial, and palmarolateral). Four osteochondral samples were taken from both the lateral and medial trochlea of the talus from the dorsal and plantar aspects.

Determination of biomarkers of joint inflammation – Fluorescent bead-based multiplex assay (Milliplex MAP Equine Cytokine/Chemokine Magnetic Beads Multiplex Assay, Millipore Sigma, Burlington, MA, 01803) was used to quantify the concentrations of 23 analytes (Eotaxin/CCL11, FGF-2. Fractalkine/CS3CL1, G-CSF, GM-CSF, GRO, IFN, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8/CXCL8, IL-10, IL-12, IL-13, IL-17a, IL-18, IP-10, MCP-1, RANTES/CCL5 and TNF-α) in SF and plasma from all time points (d0, 1, 4, 7, 14). Quantification of serum amyloid A (SAA) in SF and plasma was performed using a handheld SAA test reader (StableLab, Epona Biotech Limited, Sligo, Ireland) according to manufacturer's instructions.

Determination of vancomycin dose and levels in synovial fluid – Vancomycin dose was determined in an initial pilot investigation evaluating vancomycin toxicity on mesenchymal stromal cells which would be co-administered and duration of time that vancomycin levels remained above MIC for the bacterial isolate used (1μg/mL). Cell viability (mean +/- SD) of MSC from the three bone marrow aspirate donor horses described previously was assessed, each in triplicate, using trypan blue dye exclusion staining to determine percentage of live cells following vancomycin exposure for 24 hours. Dose response for each concentration was normalized to control, and the data transformed to 'normalized dose response vs.

log10(concentration)' at which point the half maximal inhibitory concentration (IC50) was estimated by nonlinear regression implemented in GraphPad Prism8 (GraphPad Software Prism8). The duration of time that vancomycin remained above MIC for the bacterial isolate used was assessed by injecting 100 mg vancomycin in the tibiotarsal joint of a single pilot horse (three-year-old Quarter Horse mare). Synovial fluid samples were obtained at times 0, 1, 4, 8, 24, 48, 72 hours following administration and vancomycin concentrations determined using competitive ELISA (BioVision, Milpitas, CA, USA 95035). Finally, concentrations of vancomycin were measured in SF of the tibiotarsal joint of treated and control horses on days 0, 1, 4, 7 and 14 using the same competitive ELISA.

Diagnostic imaging – Ultrasound images of the injected and contralateral tarsi were obtained at days 0, 7, and 14 (treated horses). Images were scored for degree of distention, degree of synovial thickening, degree of fibrinous loculation, and degree of vascularity as visualized with power Doppler were scored on a scale of 0 to 3 (0 = normal, 1= mild, 2 = moderate, 3 = markedly abnormal). Character of synovial effusion and presence of hyperechoic foci were scored on a scale of 0 or 1 (0 = absent/anechoic, 1 = present/echogenic). Radiographic images of the injected and contralateral tarsi (four-view_ were obtained at days 0,7, and 14 (treated horses). Radiographs were evaluated for osteoarthritis on a scale of 0 to 4 (0 = normal, 4 = severe change) for osteophyte formation, boney proliferation at the joint capsule attachment, subchondral bone lysis and subchondral bone sclerosis. Magnetic resonance imaging of the injected and control tarsi was performed immediately postmortem at end-term. All imaging findings were recorded for future evaluation by a board-certified veterinary radiologist.

Quantitative and qualitative bacterial cultures –Samples of end-term synovial fluid and synovial tissue were sent to the Clinical Microbiology Laboratory at Colorado State University

for bacterial identification and sensitivity pattern in blood culture vials (BDTM BactecTM Media, Fisher Scientific, Waltham, MA, 02451). Qualitative bacterial sensitivity patterns were compared to that obtained initially for the injected bacterial isolate (**Supplemental Document 7.1**). Quantitative bacterial cultures were performed of synovial fluid at d0, 1, 4, 7, and 14 and of synovium at end-term as previously described.[30] Briefly, to generate quantitative cultures, synovial fluid aliquots were centrifuged at 8000g for 5 minutes and the supernatant was removed. The bacterial pellet was washed three times with PBS, resuspended in 1 mL PBS and incubated with 0.05 mg/mL hyaluronidase on a shaker at 120rpm at 37°C to disperse any aggregated bacteria. Samples were then centrifuged again at 8000g for 5 minutes and reconstituted in PBS for serial dilution. Synovium obtained aseptically at end-term was weighed and incubated with 1.5mg/mL type 2 collagenase and 0.05 mg/mL hyaluronidase for one hour then pushed through a tissue strainer. Bacterial load was determined using serial dilutions in PBS and plate counting of CFU, and reported as CFU per mL of synovial fluid or per gram of synovial tissue.

Histologic evaluation of osteochondral and synovial tissues – Osteochondral and synovial tissues collected at end-term were fixed in neutral-buffered 10% formalin and zinc fixative. Samples were processed for histologic examination with Saf-O fast green for qualitative assessment of GAG and PG content, hematoxylin and eosin staining to assess for inflammatory changes. Assessment of histology specimens were saved for future scoring according to the modified OARSI histology initiative for osteoarthritis in horses developed for septic arthritis.[31] Additional immunohistochemistry staining will be performed for elastase as a marker of neutrophils, IBA1 as a marker for macrophage infiltration, and CD204 as a marker for M2-oriented macrophages.

Data analysis **–**Vancomycin cytotoxicity on equine bone marrow derived mesenchymal stromal cells was assessed by determination of half maximal inhibitory concentration (IC50), or concentration of antibiotic at which 50% of cells were viable, by normalizing dose response vs log10 (concentration) and estimating IC50 by nonlinear regression in GraphPad Prism v8.4.1 (GraphPad Software Inc.) by fitting the data to a three-parameter sigmoid function (implemented as "log(inhibitor) vs dose response."

Normality was assessed via Shapiro-Wilk tests as well as distribution of diagnostic plots. The effect of treatment and time were evaluated using two-way analysis of variance with Tukey's adjustment for multiple comparisons test to compare inflammation/pain scores, cytokine levels in synovial fluid and plasma, quantitative bacterial cultures of synovial fluid over time, and systemic clinicopathological parameters and biomarkers of inflammation between treated and control horses. Unpaired t-tests were used to compare quantitative bacterial counts in synovium at end-term and total macroscopic scores of joints. Analyses were performed using GraphPad Prism v8.4.1 (GraphPad Software, Inc.), with significance assessed at $p < 0.05$.

7.4 Results

Vancomycin dosing and concentrations - Vancomycin dose was determined initially in a pilot investigation of vancomycin cytotoxicity on MSC and evaluation of the duration of time following. The inhibitory concentration 50 (IC50), or concentration of vancomycin at which 50% MSC cell death occurs was determined to be 3.658 mg/mL (**Figure 7.2A**). Vancomycin concentrations in synovial fluid when 100 mg was injected into the tibiotarsal joint of a single pilot horse were found to remain well above MIC (1 μg/mL) for the bacterial isolate injected, providing further support for the dose and dosing interval (d 1, 4 and 7) of vancomycin

performed (**Figures 7.2B, C**). Vancomycin levels in synovial fluid were determined and remained above 1 µg/mL in both treatment and control horses at each of the time points following administration (days 4, 7, 14). Vancomycin levels in synovial fluid were variable, which may be attributed to differences in effusion, but were not significantly different between treated versus control groups at any time point (D0 p=0.95, D1 p=0.86, D4 p=0.74, D7 p=0.98).

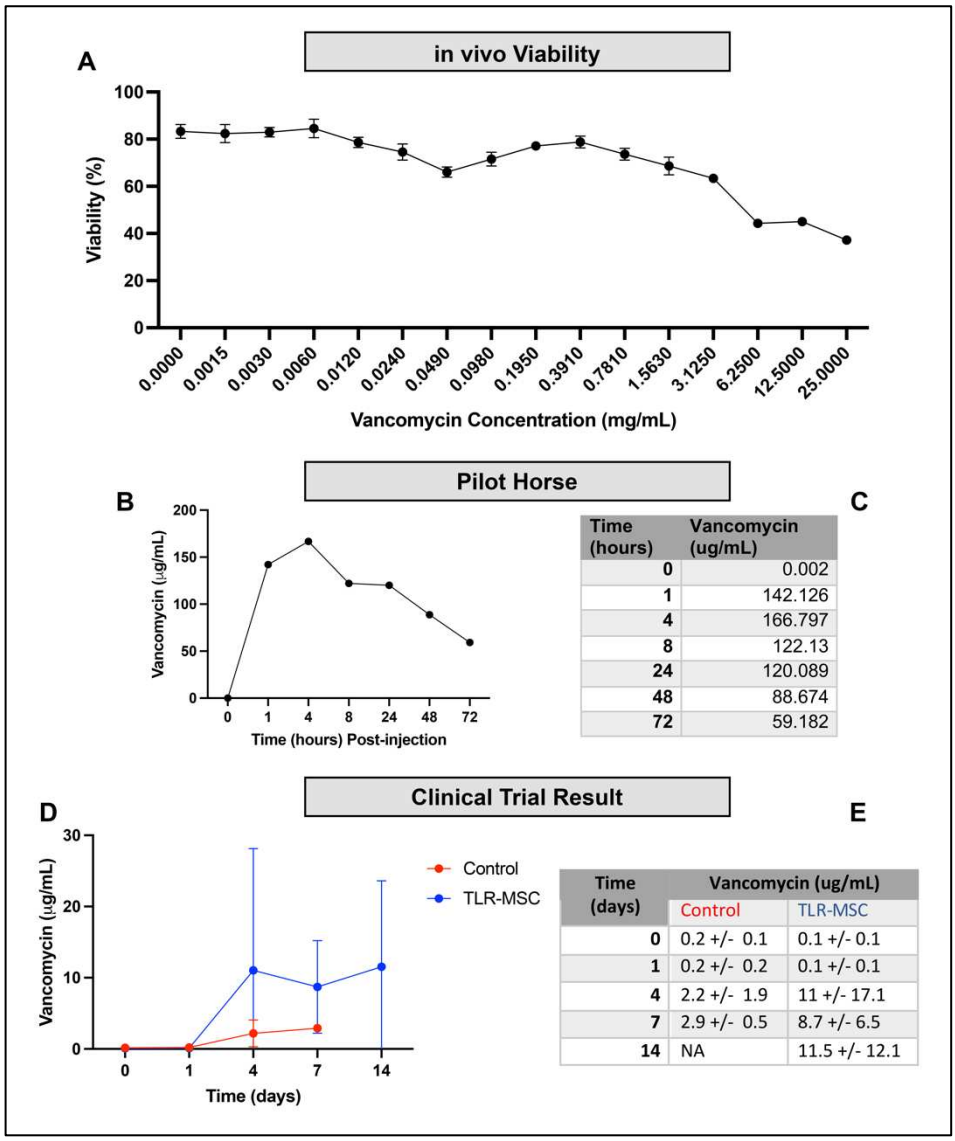

Figure 7.2: Pilot data towards determination of vancomycin dose and concentrations in synovial fluid in treated versus control horses. A) Effect of vancomycin on viability of equine bone marrow-derived mesenchymal stromal cells in monolayer culture. Cell viability (mean +/- SD) was assessed in MSC from each of three donor horses, each in triplicate, using trypan blue

dye exclusion staining to determine percentage of live cells following antibiotic exposure for 24 hours. X-axis represents antibiotic concentration; y-axis represents percentage of live cells. Dose response for each concentration was normalized to control, and the data transformed to 'normalized dose response vs. log10(concentration)' at which point the half maximal inhibitory concentration (IC50) was estimated by nonlinear regression implemented in GraphPad Prism8 (GraphPad Software Prism8). **B)** Vancomycin (100 mg) was injected in the tibiotarsal joint of a 3-year-old Quarter Horse mare and synoviocenteses performed at baseline and 1, 4, 8, 24, 48, and 72 hours following injection to obtain synovial fluid samples (1 to 2 mL). Vancomycin concentrations in synovial fluid were assessed via immunoassay (BioVision, Milpitas, CA, USA 95035)), and **C)** remained above MIC for the targeted pathogen (1µg/ml) at 72 hours following injection. **D)** Vancomycin levels in synovial fluid were determined and **E)** remained above 1 µg/mL in both treatment and control horses at each of the time points following administration (days 4, 7, 14). Vancomycin were not significantly different between treated versus control groups at any time point.

Pain scoring - Pain/inflammation scores were lower in treated horses across time points

(p=0.0002) and at multiple individual time points (D3,4, p=0.02; D5,6,7, p=0.04) (**Figure 7.3**).

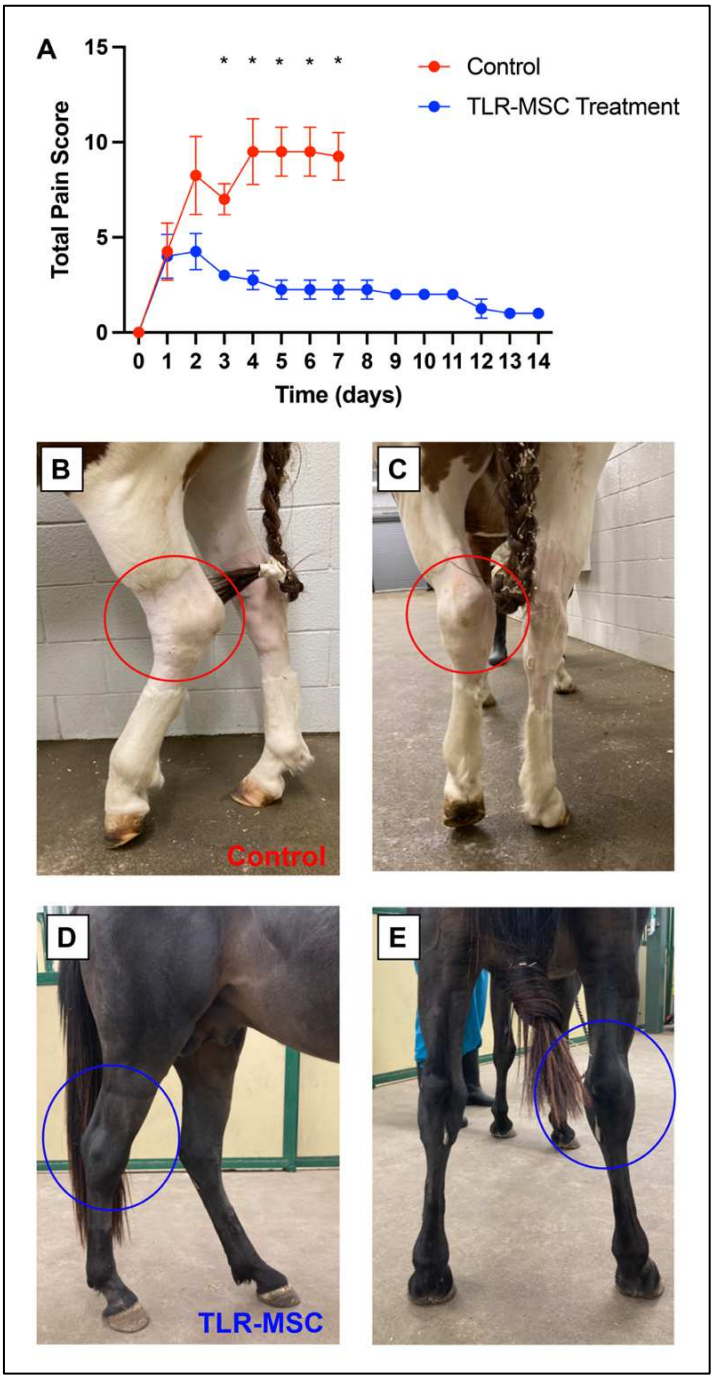

Figure 7.3: Pain/inflammation scoring. A) Overall pain/inflammation scores were determined by grading a total of five parameters (physical examination including temperature, pulse rate, and respiratory rate, lameness evaluation, distal limb edema, joint circumference, synovial heat) on a scale of 0 to 3 (0=normal, 3=marked) for a maximum score of 15. Representative images shown for control **(B,C)** and treated **(D,E)** horses at end-term (day 7 or 14, respectively).

Quantitative bacterial counts **-** Quantitative bacterial counts of SF (D4 p=0.03, D7 p=0.02) (**Figure 7.4A**) and end-term synovium (p=0.003) (**Figure 7.4B**) were lower in TLR MSC-treated horses. Representative images of quantitative culture quad plates of synovial fluid from control versus treated horses at day 7 shown. (**Figure 7.4C**). Qualitative bacterial cultures were negative in all four TLR-MSC treated horses. Differences in bacterial susceptibility from original sensitivities reported were noted in end-term cultures from two control horses.

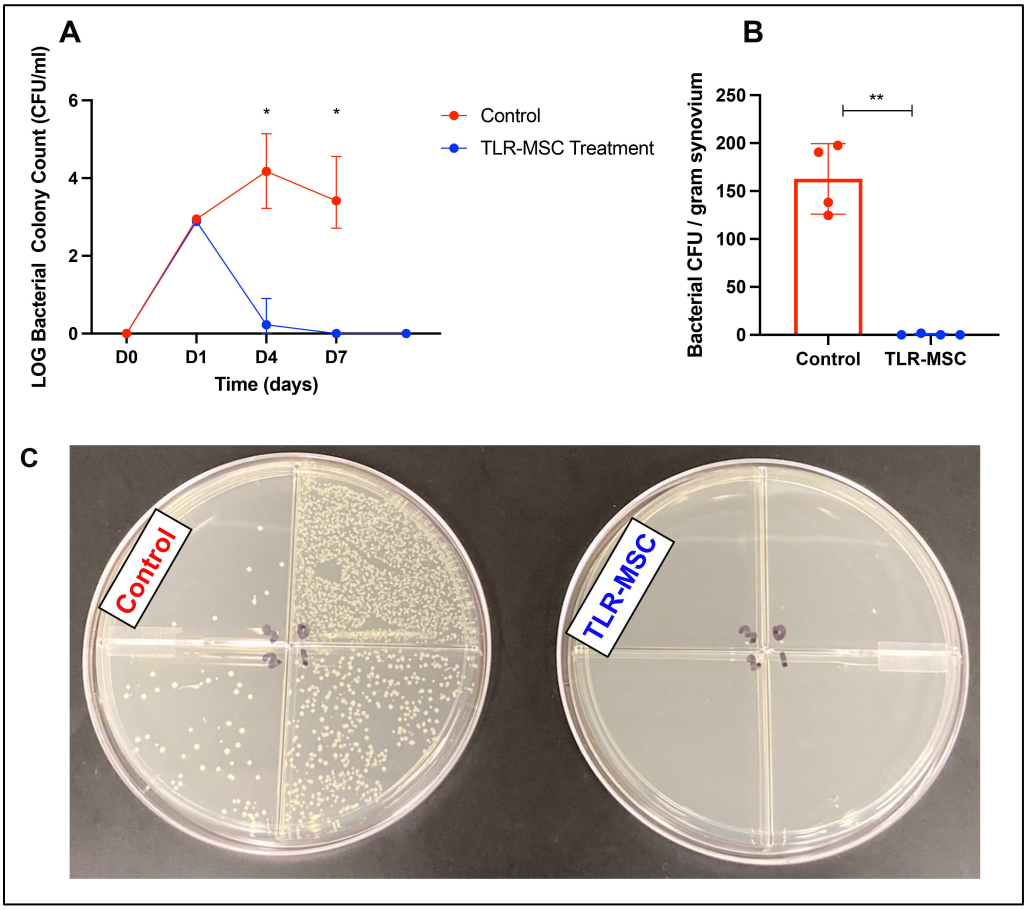

Figure 7.4 Quantitative bacterial culture of synovial fluid and synovium. Quantitative bacterial cultures were performed of **A)** SF at d0, 1, 4, 7, and 14 and of **B)** synovium at end-term. Briefly, to generate quantitative cultures, synovial fluid aliquots were centrifuged at 8000g for 5 minutes and the supernatant was removed. The bacterial pellet was washed three times with PBS, resuspended in 1 mL PBS and incubated with 0.05 mg/mL hyaluronidase on a shaker at 120rpm at 37°C for 10 minutes to disperse any aggregated bacteria. Samples were then centrifuged again for 5 minutes and reconstituted in PBS for serial dilution. Synovium obtained aseptically at end-term was weighed and incubated with 1.5mg/mL type 2 collagenase and 0.05mg/mL hyaluronidase for one hour then pushed through a tissue strainer. Bacterial load was

then determined using serial dilutions in PBS and plate counting of CFU and reported as CFU/mL synovial fluid or per gram synovial tissue. **C)** Representative images of quantitative culture quad-plates from control versus treated synovial fluid at day 7 shown.

Clinicopathologic evaluation of peripheral blood - Complete blood count revealed lower peripheral neutrophil levels in treated horses (D4 p=0.03; D7 p=0.06). Serum amyloid A was lower in plasma of treated horses on D4 (p=0.01) and D7 (p=0.02). (**Figure 7.5**).

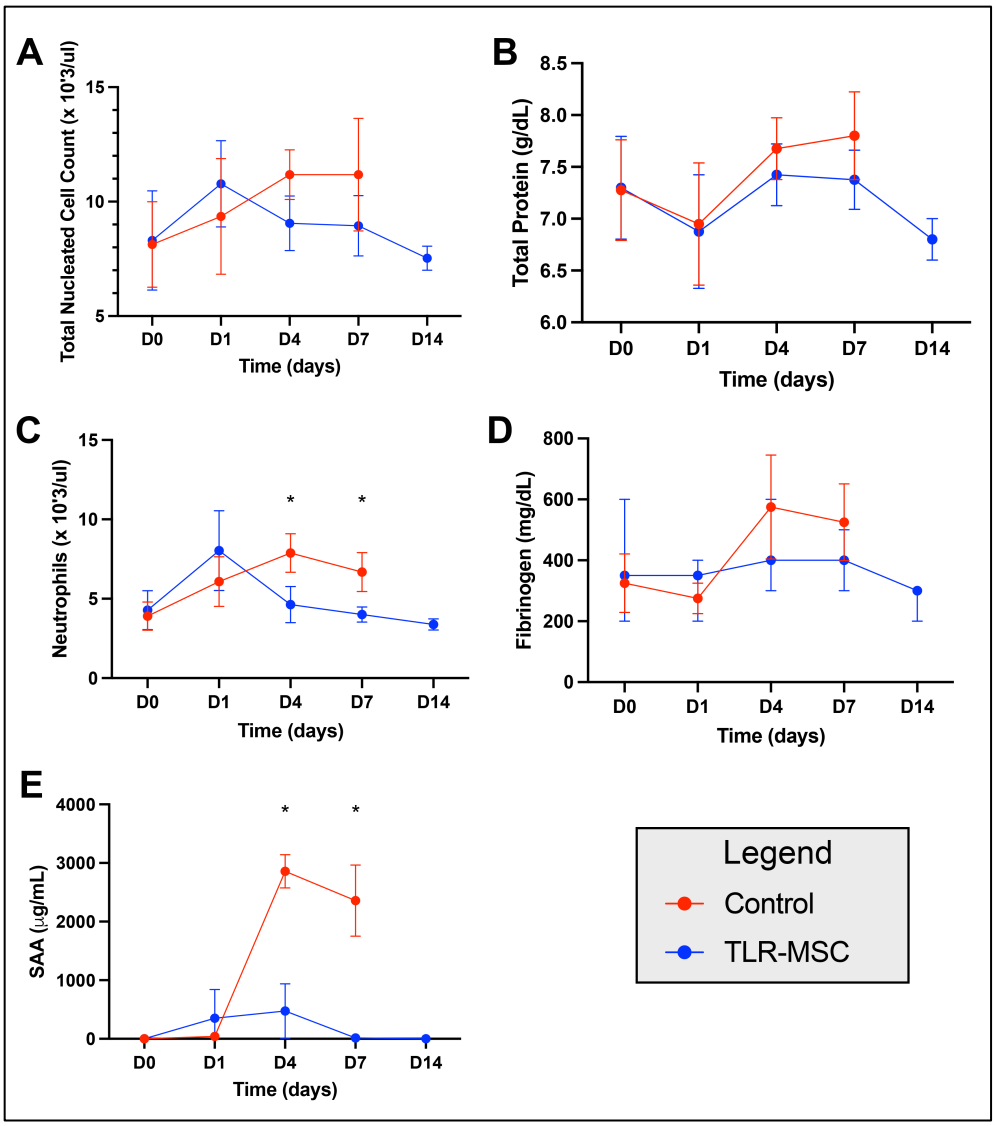

Figure 7.5: Systemic clinicopathologic parameters and biomarkers of inflammation. A-D) Blood samples were submitted on days 0,1,4,7, and 14 in EDTA-containing tubes for complete blood count and leukocyte differential. **E)** Quantification of serum amyloid (SAA) in plasma was performed using a handheld SAA test reader (StableLab, Epona Biotech Limited, Sligo, Ireland) according to manufacturer's instructions.

Clinicopathologic evaluation of synovial fluid - SF analysis revealed lower total nucleated cell counts (p=0.09), total protein (p=0.08), and lactate (p=<0.0001), and higher glucose levels (p=0.009) in treated versus control horses at D7. (**Figure 7.6**). When converted to absolute values, no differences in relative proportions of neutrophils, large or small mononuclear cells, eosinophils or basophils were noted. There was no difference in subjective glycosaminoglycan content or presence of excess red blood cells.

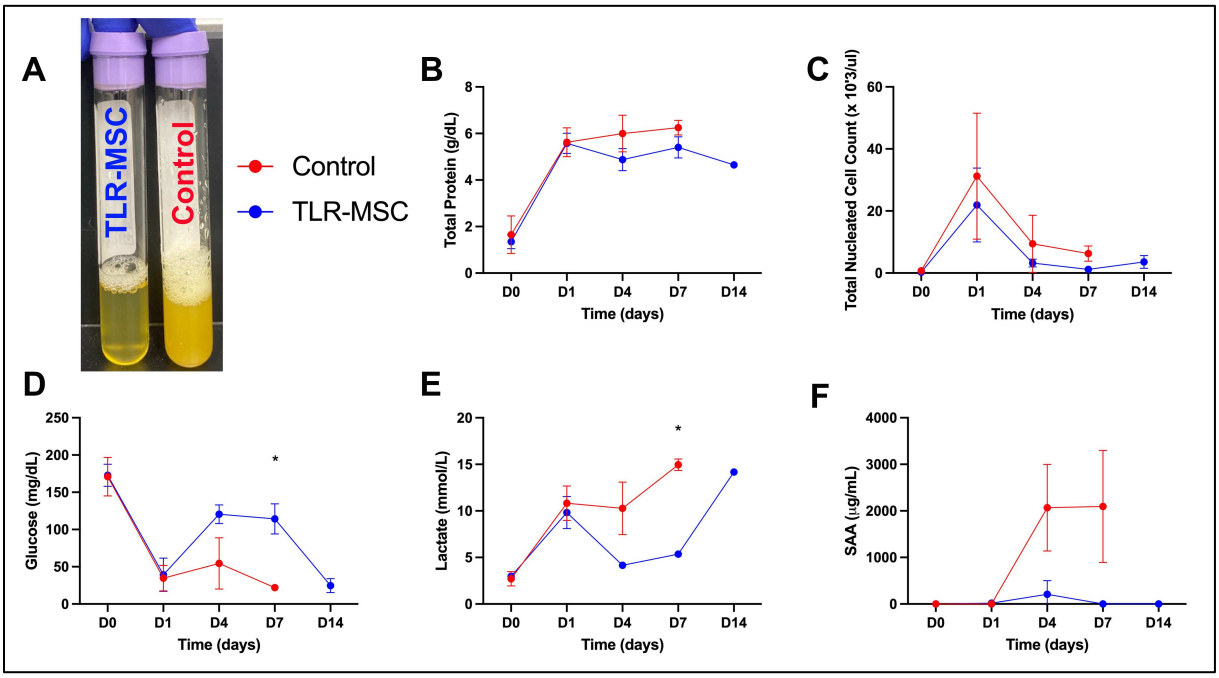

Figure 7.6: Evaluation of clinicopathologic levels in synovial fluid. Synovial fluid (SF) samples (4 mL) were aspirated from the infected tibiotarsal joint at baseline (day 0) and at days 1,4,7, and 14. **A)** Representative macroscopic images of synovial fluid obtained at day 4 from treated versus control horses. SF samples were assessed for **B)** refractometric total protein and **C)** total nucleated cell count (Hematrue, Heska Corp, Loveland, CO, USA) **D)** glucose by handheld reader (AlphaTrak 2 Blood Glucose Monitoring System, Zoetis, Lincoln, NE 68521), **E)** lactate by handheld reader (Lactate Plus Portable Lactate Reader, Nova Biomedical, Waltham, MA 02454), and **F)** serum amyloid A (SAA) by handheld reader (StableLab, Epona Biotech Limited, Sligo, Ireland).

Biomarker analysis - Multiplex biomarker analysis of synovial fluid revealed lower IL-6 levels across time points (p=0.02) and at individual time points (D4 p=0.03, D7 p=0.11) and lower IL-18 levels across time points in treated horses (p=0.02). (**Figure 7.7**). No differences were seen in analytes assessed in plasma between treated and control horses.

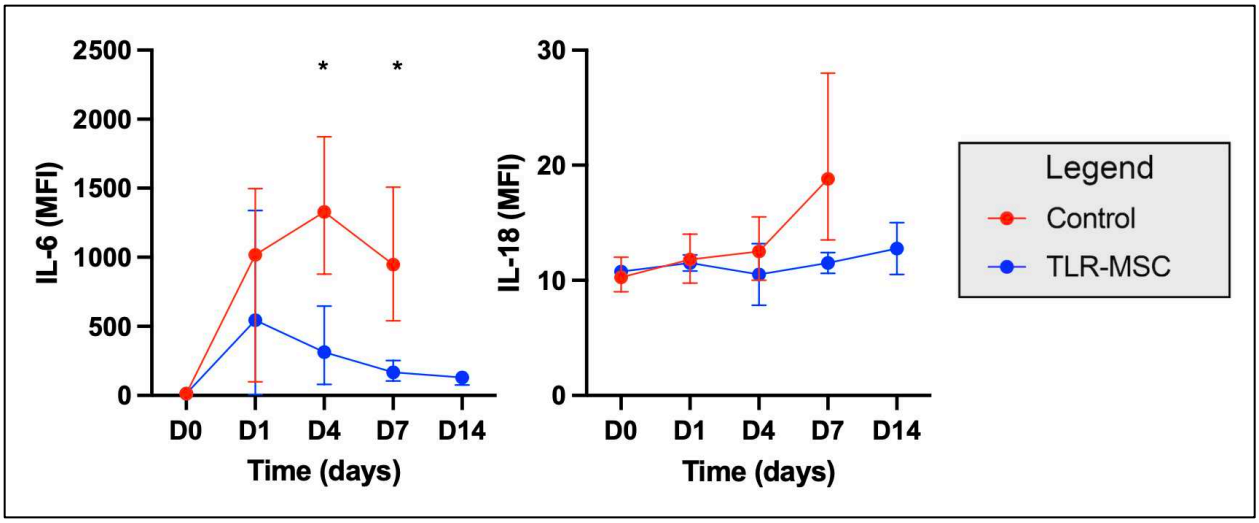

Figure 7.7: Synovial fluid biomarkers of inflammation. Fluorescent bead-based multiplex assay (Milliplex MAP Equine Cytokine/Chemokine Magnetic Beads Multiplex Assay, Millipore Sigma, Burlington, MA, 01803) was used to quantify concentrations of 23 analytes in SF.

Macroscopic joint scoring - Overall macroscopic scores at end-term, accounting for synovial proliferation, subcutaneous thickening/edema, vascularity and cartilage erosion, were lower in treated versus control horses (p=0.003) (**Figure 7.8**).

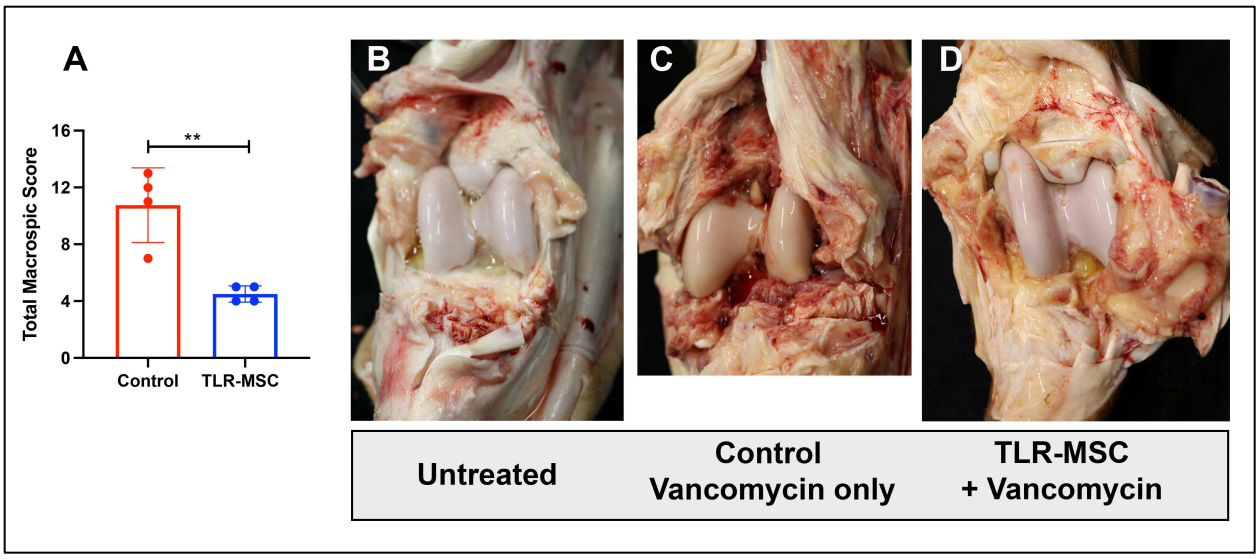

Figure 7.8: Macroscopic joint scoring at end-term. Assessment of macroscopic observations was performed by consensus scoring of two-board certified equine surgeons using a modified scoring system for septic arthritis. **A)** Total score reported as mean +/- SD, **B)** noninjected control limb, **C)** infected and control limb treated with vancomycin, and **D)** infected and TLR-MSC + vancomycin treated limb.

At the time of this writing, histologic evaluation and imaging scoring of radiographs, ultrasound, and magnetic resonance imaging are pending.

7.5. Discussion

Septic arthritis represents a potentially life-threatening condition in humans and veterinary species, which can further result in reduced functional outcomes and osteomyelitis in survivors.[32-38] Mesenchymal stromal cells exert potent antibacterial and immunomodulatory activity, making them attractive as biological agents in treating a variety of disease conditions, including synovial infection.[4,5] Recently, we further investigated *in vitro* culture techniques to stimulate equine MSC with toll-like receptor agonists to maximize secretion of antimicrobial peptides and immunomodulatory cytokines resulting in enhanced antibacterial activity.[8] In this study of horses with acute *S. aureus* infectious arthritis, TLR-MSC treatment, in combination

with conventional antibiotic therapy, resulted in lower quantitative bacterial concentrations in synovial fluid and tissues, and improvement in clinical parameters compared to antibiotics alone, supporting our initial hypothesis. These studies expanded upon the previously reported understanding of the anti-infective properties of equine MSCs to demonstrate efficacy to treat localized synovial infection in a large animal model.

Staphylococcus aureus, as well as other Gram positive and negative bacterial isolates, forms free-floating biofilm aggregates in synovial fluid,[23,39-41] which have been shown to persist despite high concentrations of antimicrobials.[42] Historically, difficulty in isolating and culturing bacteria from synovial fluid of patients with infectious arthritis has been reported.[43-45] Quantitative culture techniques described here were based on novel experimental methods recently described and validated to accurately quantify *S. aureus* biofloat aggregates in synovial fluid as CFU per millileter, utilizing enzymatic digestion to break up aggregates for effective and consistent determination of bacterial load.[30,42] Mesenchymal stromal cells have been previously described to express anti-biofilm activity, and eradication of infection presumably confined in large part to floating biofilm aggregates was demonstrated here, further validating anti-infective cellular therapy as a promising option to reduce bacterial load in joint infection.[8,16]

The results of this study support immunomodulation with TLR-MSC treatment. Treated horses displayed lower concentrations of IL-18, IL-6 and SAA. Interleukin-18 has been reported as an important mediator of both the innate and adaptive immune responses, with multiple reports implicating its role in progression of rheumatoid arthritis,[46-47] where synovial IL-18 expression correlated with concentrations of TNFa and IL-1B.[48-49] Interleukin-6 has also been previously reported to be elevated in osteoarthritic joints and was initially primarily considered to be a catabolic cytokine,[50-52] although more recent studies have presented evidence for an

immunomodulatory role with pro-chondrogenic properties as well.[53,54] Finally, serum amyloid A has been reportedly increased in horses with septic arthritis but not synovitis, consistent with the findings of this work.[55] MSC have been previously reported to interact with the host innate immune response, although further studies are indicated to determine specific mechanisms of action in this model.[7,10]

Macroscopic evaluation of joints at end-term was performed based on a novel septic synovitis grading system adapted from previous reports of osteoarthritis scoring,[31] and rheumatoid arthritis inflammation.[55] Notably, fibrinous synovial proliferation accumulation was greater with increased subcutaneous thickening in control horses, resulting in greater overall macroscopic scores in those patients. These findings are in accordance with previous reports of *S. aureus* bacterial species using the coagulation system of the host to accumulate fibrinous exudate and build fibrin-based biofloats.[56-59]

Lack of consistency in cellular products and quality is a current challenge in effective implementation of cell-based therapies in clinical trials.[60] The use of a pooled product from multiple donors was implemented in this study in an attempt to overcome variability in antimicrobial potency observed between donors *in vitro.*[8] Major histocompatibility classes were determined for horses in this study, with two of four MSC recipients being partially matched and two being completely mismatched to the donor haplotypes. Further investigation of the effect of partial or full MHC incompatibility between donor and recipient horses on antibacterial efficacy with allogeneic MSC therapy is warranted to determine if matching MHC haplotype results in prolonged cell duration in the synovial space and therefore potentially enhanced antimicrobial effect. In addition, although not performed in this experimental case-controlled study, horses

used for donation of tissues for MSC culture for clinical cases must be screened for viral diseases which can be transmitted through biological therapies.[61-62]

The inclusion of fetal bovine serum (FBS) in culture media necessitates additional discussion. The use of FBS to expand equine MSCs has been associated with potential hypersensitivity reactions and the risk of introduction of viral or prion pathogens,[63-66] prompting recent investigations to alternatives to xenogeneic growth factors as media supplements.[67-72] Furthermore, several regulatory agencies including the International Society for Cellular Therapy have recommended use of alternatives to FBS in culture media when possible and called for a consensus on serum replacements in cell culture media.[65,66,73,74] However, based on data generated by this group of investigators demonstrating that equine MSCs cultured in conditions using alternate growth sources of autologous and allogeneic equine serum exhibited diminished antibacterial capacity and antimicrobial peptide secretion, MSC were maintained in FBS throughout culture and expansion for optimal antibacterial activity. Further comparison of antimicrobial properties of MSC cultured in FBS to additional alternative growth sources is indicated to determine whether replacement of xenogeneic proteins with other supplements is possible while maintaining antibacterial effect. In summary, the potential risks of FBS use in culture media must be weighed against loss of antibacterial function with use of alternate serum sources when applying TLR-activated MSC therapy clinically in the future.

Limitations of study design include small horse sample size, lack of data collection at later time points, and lack of control group receiving non-stimulated MSC therapy for comparison. Greater sample sizes and continuation of the study past the time points assessed were not performed due to the severity of model induction and pain observed with initial synovial infection and model progression, particularly in control horses. Furthermore, the horse

number (n=8) evaluated was sufficient to detect significant differences in multiple outcome parameters assessed. The comparison of TLR-activated MSC and antibiotics to antibiotics alone was based on previous data in a mouse model of implant infection, demonstrating optimal antibacterial effect with TLR activation of MSC in combination with antibiotic therapy.[16] However, it is acknowledged that study design here may have been improved with inclusion of additional control groups (e.g. non-TLR-activated MSC therapy alone or in combination with antibiotics, TLR-activated MSC therapy alone, no treatment), but this was not performed due to the severity of the model and desire to reduce horse number. Further comparison of imaging findings and histopathological evaluation of synovial tissues (osteochondral and synovium) are pending at the time of writing, and will add to the body of evidence surrounding use of TLR activated MSC therapy in treatment of bacterial infections. Future investigation of cell delivery techniques including scaffolds to improve cell engraftment and microparticle release devices may prolong the antibacterial effect observed compared to injection of single cell suspensions.

In summary, TLR-3 activated equine MSC therapy in combination with antibiotics reduced bacterial bioburden and improved clinical outcomes in treatment of antibiotic-resistant equine joint infections compared to antibiotics alone. *In vitro* TLR activation of MSC prior to injection represents a relatively simple method to enhance antimicrobial properties of MSC towards improved infection control. This anti-infective cellular technology represents a novel therapeutic strategy to enhance currently available techniques to treat multidrug resistant infection.

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CHAPTER 8: Concluding Remarks and Future Directions

Septic arthritis causes significant morbidity and mortality in veterinary and human clinical practice and is increasingly complicated by multidrug-resistant infections. Antibiotics have been injected intra-articularly by equine veterinarians for decades with the rationale to achieve high local drug concentrations in treatment of synovial infections while minimizing side effects associated with systemic antibiotic administration. This route of administration has gained increasing attention more recently in human orthopedic practice, particularly in treatment of infection following prosthetic arthroplasty. However, as this route of administration is 'off-label,' appropriate doses to achieve effective bactericidal concentrations while reducing cytotoxicity to joint tissues have not been established. The studies reported here demonstrated that antibiotics from different drug classes express dose-dependent and variable cytotoxicity to equine joint cells *in vitro*, with aminoglycosides and doxycycline exhibiting the greatest cytotoxicity of antibiotics assessed. Based on this observed cytotoxicity and reported frequency of use in equine practice, the cytotoxicity of amikacin sulfate was investigated further using a non-inflammatory equine joint model. Inflammatory cytokines and biomarkers of cartilage type II cleavage were increased in synovial fluid following amikacin injection at doses commonly used in equine clinical practice. These findings indicate that decisions on use of antibiotics intra-articularly in treatment of septic arthritis should take into account the potential harm that antibiotics may cause and consider alternative drug classes to aminoglycosides or lower doses than those previously reported in equine practice.

Further studies are indicated to assess the potential long-term effects of local antibiotic administration, including histopathologic evaluation of joint tissues, as well as comparison of cytotoxicity when antibiotics are administered in inflamed versus normal joints, and when co-administered with other medications. Synovial fluid sampling at later time points and evaluation of histopathology of synovial tissues may provide further information as to whether the pro-inflammatory and chondrotoxic effects noted with intra-articular amikacin usage are transient or result in more permanent tissue damage which may contribute to progression of osteoarthritis. In addition to treatment of septic arthritis, antibiotics are at times administered intra-articularly for prophylaxis when other medications such as local anesthetics, corticosteroids, and viscosity supplements are injected in diagnosis or treatment of osteoarthritis. Future studies will include further evaluation of whether simultaneous administration with other medications mitigates antibiotic cytotoxicity *in vivo.* Further investigation of pharmacokinetic distribution of antibiotics when administered locally by multiple routes (e.g. intra-articularly as well as by regional limb perfusion) is also indicated.

Specifically, the interaction between antibiotics used in equine practice and corticosteroids will be evaluated further. Co-administration of antibiotics and corticosteroids has previously been demonstrated to result in precipitation and aggregate formation, which may reduce activity or either or both drugs and result in further irritation to surrounding tissues.[1,2] However, Bolt *et al.* demonstrated *in vitro* that diluted triamcinolone supported chondrocyte morphology in culture and protected chondrocytes to some degree from the toxic effects of amikacin, mepivacaine, and lipopolysaccharide (LPS).[3] Therefore, further investigation of combinations of corticosteroids and antibiotics *in vitro* and *in vivo* in the context of equine joint injections is warranted. Antibiotics commonly used in joint injections in horses (amikacin,

gentamicin) as well as alternative antibiotics with greater safety profiles (e.g. ampicillin sulbactam, imipenem, ceftiofur sodium) will be assessed in combination with corticosteroids used in equine joints (e.g. triamcinolone acetonide, methylprednisolone acetate, and betamethasone sulfate). *In vitro* assays will include initial assessment for precipitation and aggregate formation between antibiotic combinations via standard and dark field microscopy over a range of concentrations for antibiotics in media and synovial (25 to 0.39 mg/mL) based on estimation of concentrations achieved in joint fluid and doses of corticosteroids reportedly commonly injected per joint (triamcinolone acetonide 6 to 18 mg, methylprednisolone acetate 40 to 120 mg, betamethasone sulfate 3 to 18 mg).[4] Antibiotic-corticosteroid combinations resulting in precipitation will be further assessed *in vitro* to determine effect on viability, and metabolism of chondrocyte and synoviocyte co-cultures in media and synovial fluid. Viability will be assessed via trypan blue dye exclusion of and MTT colorimetric assay, mitochondrial metabolism will be assessed for Mitotracker green fluorescent staining, and membrane integrity of joint cells and viability will be assessed via dimeric cyanine nucleic acid staining (YOYO-1) assay. Finally, the effect of co-administration of medications on cytotoxicity of amikacin will be assessed in equine joints in vivo. Antibiotics (e.g. amikacin 125-500mg) and corticosteroids (triamcinolone acetonide 6-18mg, methylprednisolone acetate 40-120mg) most commonly used in equine practice will be injected simultaneously in the tarsocrural joint of horses and serial synovial fluid samples will be performed over 96 hours. Synovial fluid will be assessed for clinicopathological parameters, levels of amikacin to determine if pharmacokinetic distribution is altered by co-administration, and biomarkers of inflammation (23-cytokine analysis via multiplex immunoassay, C reactive protein via ELISA immunoassay) and cartilage degradation (C2C, C12C via ELISA immunoassay). These studies will further practitioners' understanding of

interactions of drugs commonly administered simultaneously with the overall goal of improving evidenced based strategies for selection and dosing of drugs injected intra-articularly in horses.

In the work described herein, immune activated cellular therapies were investigated further as adjunctive treatments to antibiotics in treatment of multidrug resistant infections, including those with a biofilm component such as is seen in synovial infection. Mesenchymal stromal cells (MSC) have been demonstrated to possess antimicrobial and immunomodulatory properties, which may be upregulated through stimulation of toll-like receptors (TLR) expressed in the inflammatory response to infection. These studies reported optimization of *in vitro* culture techniques for equine MSC with TLR and Nod-like receptor (NLR) ligands to maximize antimicrobial peptide expression and immunomodulatory cytokine secretion, and TLR-3 ligand pIC (10μg/mL, $2x10^6$ cells/mL, 2 hours) was identified to move forward with for further *in vivo* evaluations of antibacterial activity. The effect of serum source in MSC culture on antimicrobial properties of activated cells was further evaluated, and MSC culture in fetal bovine serum versus autologous or allogeneic equine serum generated more functional cells based on several parameters including antimicrobial peptide secretion, greater spontaneous bactericidal activity and immunomodulatory cytokine secretion, indicating that the proposed risks of FBS use in MSC culture should be weighed against the loss of MSC function likely to be incurred from culture in equine serum. Finally, these *in vitro* culture techniques were applied towards use of TLR-3 pIC activated MSC therapy in an equine model of *Staphylococcal* septic arthritis. Horses treated with TLR-MSC and vancomycin had lower inflammation/pain scores, quantitative bacterial cultures in synovia fluid and synovium, more rapid normalization of synovial fluid parameters, and lower levels of pro-inflammatory cytokines in synovial fluid than horses treated with vancomycin alone. These findings demonstrate that local administration of TLR-activated

MSC therapy represents a promising adjunctive strategy to mitigate inflammation and infection in multidrug resistant septic arthritis.

Future studies will build on this work to explore additional aspects of MSC culture techniques to enhance antibacterial activity while potentially reducing use of xenogeneic proteins in culture media. Culture and expansion of MSC has routinely been performed with fetal bovine serum (FBS), but more recent evidence linking FBS usage to introduction of prion or viral pathogens and hypersensitivity reactions have prompted recommendations by regulatory bodies to recommend avoidance of FBS in cell culture when possible.[5-9] In addition to the autologous and allogeneic equine serum investigated here, additional alternatives to FBS for media supplementation in cell culture include platelet lysate, purified recombinant or synthetic proteins, and defined serum-free commercial formulations.[5,10-13] In future studies, the immunomodulatory and antibacterial properties of MSC cultured in these three alternate serum sources will be compared. Specifically, the impact of 72h and prolonged periods of cell culture (7 to 14 days) on cell viability, doubling time, morphology, bactericidal capability and production of immunomodulatory cytokines and antimicrobial peptides will be assessed. These studies will expand the field to determine optimal serum source in cell culture conditions to enhance bactericidal and immunomodulatory active of equine MSC and potentially identify suitable alternatives to FBS to reduce use of xenogeneic proteins in cell culture while maintaining antibacterial activity.

Finally, in this work, we confirmed the antimicrobial properties of equine MSC in a large animal model of septic arthritis and demonstrated that those properties can be enhanced with *in vitro* techniques prior to clinical application. We have characterized several mechanisms by which TLR-activated MSC exert antimicrobial action and modulate inflammation, which may

have various applications in treatment of orthopedic infection. Future studies will include further exploration of the mechanisms by which TLR activation of MSC enhances their antimicrobial properties, for example, through evaluation of gene clustering and cell type classification using single cell RNA sequencing technology. Moving forward, further development of strategies for other routes of administration or sustained or slow release of immune activated cellular therapy will be investigated further, which including use of cellular scaffolds with microparticle release devices.

In summary, the studies described here will contribute to clinicians' understanding of the pros and cons of local antibiotic usage in treatment of septic synovitis, and further introduces evidence for use of TLR-activated cellular therapy as an adjunctive treatment for infection, particularly those involving multidrug resistant bacterial species and biofilms. These findings may be broadly applicable to treatment of infection in musculoskeletal disease and following placement of orthopedic devices in human and veterinary surgery.

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