PHARMACOKINETICS AND PHARMACODYNAMICS OF ENROFLOXACIN AND LOW-DOSE AMIKACIN AFTER REGIONAL INTRAVENOUS LIMB PERFUSION IN STANDING HORSES

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Alberto Parra-Sanchez

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THESIS ABSTRACT

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Objective - To evaluate the tisular pharmacokinetics/dynamics of enrofloxacin and low-dose amikacin after regional intravenous limb perfusion (RILP) in standing horses.

Animals – Fourteen adult horses

Procedures – Enrofloxacin (1.5mg/kg, 7 horses) or 250 mg of amikacin (7 horses) were randomly infused by RILP in the front limb of standing horses. Capillary ultrafiltration devices were implanted in the subcutaneous tissue (ST) and bone marrow (BM) of the third metacarpal bone for collection of interstitial fluid. Samples were obtained from the serum, synovial fluid of the radiocarpal joint, interstitial fluid of ST and BM, prior to tourniquet release (time 0) and at 0.5, 1, 4, 8, 12, 24, and 36 hours. Concentrations of amikacin were detected by fluorescence polarization immunoassay and enrofloxacin concentrations were detected by high pressure liquid chromatography.

Pharmacokinetic/pharmacodynamics analysis of tissue concentrations were measured and calculated using an MIC ≥ 16 µg/ml for amikacin and ≥0.5 µg/ml for enrofloxacin.

Results – **C**apillary ultrafiltration probes were effective in the collection of interstitial fluid samples for analysis. Three horses developed vasculitis after RILP with enrofloxacin. For both antimicrobials, the synovial fluid sample at time 0 attained the highest concentration (enrofloxacin - (Median (Range) 13.22 (0.254 - 167.9) µg/ml), (amikacin 26.2, (5.78 - 50.0) µg/ml). For enrofloxacin, therapeutic tissue concentrations above the MIC were maintained for approximately 24 hours in the ST and synovial fluid, and for 36 hours for the BM. For amikacin, therapeutic tissue concentrations above the MIC90 were not detected after perfusion. Pharmacodynamic variables for enrofloxacin indicated effective therapeutic concentrations for all the tissues.

Conclusions and Clinical Relevance – Administration of 1.5 mg/kg of enrofloxacin by RILP should be considered as an alternative for treatment of equine orthopedic infections. However, care must be taken during administration. A higher dose of amikacin (>250 mg) is recommended for effective tissue concentrations after RILP in standing horses.

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INTRODUCTION

Treatment of septic arthritis and osteomyelitis in horses is difficult, tedious, and expensive. Orthopedic infection can end a horse's athletic career and life.[1] Successful treatment depends on many factors, including virulence of the causative agent, competence of the host defense mechanism, efficacy of a chosen antimicrobial drug, and importantly, tissue levels achieved after antimicrobial administration. Thus, resolution of an infection requires in part delivery of an appropriate antimicrobial to target tissues in concentrations sufficiently above the minimum inhibitory concentration (MIC) for the particular organism for an appropriate period of time, as well as adjunct medical and surgical therapies.[2] Generally systemic antimicrobial therapy is anticipated to result in delivery of therapeutic concentrations of the chosen drug to infected tissues. However, as result of decreased antimicrobial delivery, changes in local blood supply associated with soft tissue sepsis or traumatic injury may negatively impact the efficacy. Decreased tissue concentration of antimicrobials, damaged blood supply in septic tissues that isolates bacteria from the host defenses contribute to therapeutic failure and development of antimicrobial resistance.[2,3]

Regional intravenous limb perfusion (RILP) with antimicrobial agents has been described as an excellent adjunctive therapy for the treatment of septic conditions in the distal limb of horses.[4] This technique delivers high concentrations of antimicrobials to the

synovial and osseous structures of the distal limb. Aminoglycosides are the most common antibiotic administered by regional limb perfusion because the most common pathogens responsible for orthopedic infections in horses are targeted. Further, regional therapy decreases the risk of nephrotoxicity associated with systemic use of these agents.[5] The dose of amikacin routinely administered by RILP in clinical practice range from 500 mg to 2 gram of antibiotic. The perfusate is generally diluted with physiological saline solution to a total of 20 to 60 mL of solution.[6] Prior investigation demonstrated that a dose as low as 125 mg achieved high antimicrobial concentration in the synovial fluid of the fetlock joint following RILP in anesthetized horses.[7] However, whether or not potentially effective concentrations will be delivered to the bone or soft tissue is not clear. It is our intention to determine if a lower dose of amikacin than usually administered results in potentially effective concentrations of amikacin after RILP in standing horses.

Although scientific supports exist for RILP administration of aminoglycosides, the increasing incidence of multi-drug antimicrobial resistance such as *Methicillin* resistant *Staphylococcus aureus* mandates the need for alternative antimicrobial drugs. Furthermore, novel antimicrobial drug delivery systems such as RILP must be validated with alternative antimicrobials with demonstrated efficacy against infectious organisms.[8,9] Other antimicrobials agents have been administered to horses by RILP, including vancomycin, cefotaxime, and ampicillin.[9] However, further investigations are essential to understand the pharmacokinetic of other antimicrobial agents as alternative for regional limb perfusion therapy.

Enrofloxacin, a fluoroquinolone approved for use in animals, is characterized by a sensitivity spectrum and distribution pattern desirable for treatment of orthopedic infections in the distal limb of horses.[10] The pharmacokinetic of enrofloxacin have been reported in horses after oral and parenteral administration.[11,12] However, enrofloxacin has not been studied after RILP in part of a potential association with vasculitis.[13] Furthermore, high concentrations of enrofloxacin may be destructive to chondrocytes or synovial structures particularly in young horses.[14,15]

Judicious antimicrobial therapy needs to be supported by studies that demonstrate drug concentration achieved with recommended doses. However, such studies are limited by methods selected to collect the interstitial fluid which can influence the concentration of drug measured and estimates of pharmacokinetic parameters.[16] Tissue samples are usually collected by tissue biopsies or tissue cages in the standing horse, at surgery, or after euthanasia. Each method is characterized by limitations. For example, homogenization of tissue samples can overestimate lipophilic drug concentration and underestimate hydrophilic drug concentration due to the combination of interstitial fluid, and blood through the homogenization process.[17,18]

Capillary ultrafiltration is a novel sampling method for collecting low molecular weight substances (i.e. interstitial fluid) in living biological systems.[19,20] The ultrafiltration device allows the collection of protein-unbound fluid from the interstitial space that can be directly analyzed without extraction, thus, eliminating the homogenization process. Advantages of this technique for pharmacokinetic studies include minimal invasiveness, and preparation, and serviceable permanence of several weeks, allowing multiple sample collections.[20] Animal discomfort is minimal and

animals can move freely throughout the study period. Pharmacokinetics assessments of drugs have been conducted using capillary ultrafiltration probes implanted into the subcutaneous tissue of rats and dogs,[21,22] but to date this has not been investigated in the limb of horses.

The purpose of this study was to investigate regional limb perfusion for administration of enrofloxacin and a low dose of amikacin in standing horses. The specific objectives were: (1) to evaluate the pharmacokinetics of intravenous limb regional perfusion of enrofloxacin and amikacin on drug concentrations in systemic blood, synovial fluid, interstitial fluid of subcutaneous tissue and bone marrow in standing horses; (2) to investigate pharmacodynamic indices for either amikacin or enrofloxacin in these tissues using a minimum inhibitory concentration (MIC) that would cover the most common equine pathogens (MIC $\geq$ 16 μg/ml for amikacin and $\geq$0.5 μg/ml for enrofloxacin); and (3) to evaluate an ultrafiltration system for collection of interstitial tissue and bone marrow fluid for *in vivo* sampling of drugs in horses.

REVIEW OF LITERATURE

Orthopedic Infections

Equine, orthopedic infections include septic synovial structures and/or osseous infections. Infection of bones and synovial structures (i.e. joints, tendon sheaths) involves colonization and proliferation of one or more species of bacteria, causing a pathologic condition because of their presence. Colonization usually takes place throughout two different routes, hematogenous or direct inoculation. Identification of the causative agent is helpful in determining treatment and prognosis. A variety of microorganisms are generally involved in musculoskeletal equine orthopedic infections.[23] Successful treatment requires judicious use of antimicrobials, surgical drainage and debridement, a rehabilitation program, and the proper use of non steroidal anti-inflammatory drugs.

Septic Arthritis

Septic arthritis is a serious synovial condition that can result in irreversible cartilage damage, capsular fibrosis, and intra-synovial adhesions, leading to unsoundness or death if not diagnosed or treated properly

Pathophysiology

Colonization and proliferation of bacteria in synovial structures induces a severe inflammatory response known as septic arthritis. These bacteria gain access to joint cavities throughout the blood or due to direct inoculation following trauma or iatrogenic inoculation (i.e. injections, joint surgery). Hematogenous derived septic arthritis is more

commonly recognized in the neonate than in the adult horse. Septic arthritis in neonates is a common complication of generalized septicemia. Septic arthritis secondary to septicemia was recognized as a cause of death in 12.5% of foals aged 8-31 days.[24] Failure of passive transfer is the most important role in the development of septicemia, reported to be as high as 78%.[25] The most common organisms involved in septicemia and septic arthritis are bacteria found mainly in the environment. Thus, there is a high incidence for gram-negative bacteria such as *Escherichia coli, Salmonella, Pseudomonas, Enterobacter, Acinetobacter, Proteus, Klebsiella, and Citrobacter spp*.[26,27] Because these infections are spread throughout the blood stream, infection usually involves multiple joints; the most commonly involved sites are those at which bone growth is most active, such as the metaphyseal side of the physis, epiphysis and synovial structures.[28]

In adult horses, septic synovial structures are often a result of direct trauma (i.e. laceration, puncture wounds) or iatrogenic (surgery, arthrocentesis). With iatrogenic or nosocomial infections, *Staphylococcus aureus* is the most common isolate cultured.[1,27] In addition the use of certain intra-articular medications such as corticosteroids and polysulfated glycosaminoglycans potentially decrease the articular defenses and predispose to the development of infection. For example, intra-articular administration of corticosteroids has been shown to induce migration of polymorphonuclear leucocytes into the synovial space.[29] Consequently, a small number of bacteria introduced to the synovial structure during procedure may rapidly multiply and seeds in the synovial membrane, while steroid inhibit the normal anti-inflammatory response, increasing the likelihood of septic arthritis. Otherwise, septic arthritis secondary to wounding usually results in a

mixed bacterial infection. Therefore, any wound that communicates to the synovial structures is considered at a high risk for development of septic arthritis.

After bacteria settle in the synovial membrane, an inflammatory response attempts to resterilize the infected synovial structure. This process results in disruption of the blood-synovial barrier, activation of inflammatory mediators, plasmin, kinin, and coagulation pathways. Inflammatory mediators such as eicosanoids, growth factors, interleukins (IL-1, IL-6), tumor necrosis factor (TNF), and prostaglandin E_2 (PGE_2), further activate chondrocytes substances such as matrix metalloproteinases (MMP). Matrix metalloproteinases serve to increase synovial vascular permeability, attract neutrophils and monocytes to the synovial space, degrade hyaluronan within the synovial fluid and articular cartilage, and promote the formation of fibrin.[30] A vicious cycle of inflammation results in irreversible damage to the articular cartilage and degenerative joint disease

Diagnosis

Early evaluation and management of suspected synovial sepsis increases the chances for successful outcome. Recognition of clinical sings (i.e. joint effusion, fever, heat, and edema) becomes essential in the diagnosis of septic arthritis. The degree of lameness present varies with the severity of the infection, ranging from 3/5 (evident at walk) to 5/5 (non-weight bearing lameness). However, lameness may be absent in presence of open joint injuries due to the lack of synovial distention. Initial management should include a complete physical and hematological examination, synovial fluid evaluation and culture, and radiographic examination.[31] In the initial approach, arthrocentesis should be performed after aseptic preparation of the intended site of

injection. In the case of wounding the location for arthrocentesis must be away from the site of the wound. Arthrocentesis is used to either collect synovial fluid for analysis or to determine if a communication exists between the wounded area and the synovial structure. Analysis of synovial fluid must include color and clarity, total cell count and differentiation, and measurement of total protein, pH, and specific gravity. The total protein level varies with the duration of the infection. Normal values will range between 0.8 to 2.5gm/dl, and an increase in this number is indicative of an inflammatory process within the joint. During infections total solids are approximately > 3-4 gm/dl.[1,32] In the presence of infection, total cell count exceed 30,000 cell/µl with a >90% neutrophils.[33,34] Radiographic evaluation of the affected area is important to identify concomitant bone fracture or to determine the presence of osseous infection. In a case of wounding where joint involvement is not clear, a contrast radiographic study will further determine the association between a wound and synovial structure.

Osseous Infection

Osteomyelitis is infection of the cortical bone and medullary cavity whereas osteitis involves only the cortical bone.[35] Bone infection in horses can occur in a very similar manner than with septic arthritis. For example, osteomyelitis in the neonate is also secondary to septicemia. Osteomyelitis in adult horses is usually a consequence of wounding, progression of septic synovial structure or following fracture healing with or without internal fixation.

Pathophysiology

Direct trauma to areas with minimal soft tissue coverage, such as the third metacarpal/metatarsal bones, may predispose to the development of osteomyelitis.[36]

Traumatic episodes are not exclusive of areas with limited soft tissue cover. For example, puncture wounds to the foot often result in septic pedal osteitis and septic navicular bursitis.[37,38] Multiple factors may predispose to the development of osteomyelitis besides the presence of bacteria and this include soft tissue necrosis, disruption of blood supply, and contaminated environment.[39] Among the most important isolated organisms we can include *Streptococcus* spp, *Proteus* spp, *E. coli*, *Corynebacterium* and anaerobes.[1] Detrimental changes in the mineralized components of the bone are the result of the inflammatory effects in the connective tissue and consist of degeneration, resorption, and regeneration.

Osteomyelitis following fracture repair in horses is usually bacterial in origin and often results from inoculation at the time of trauma or surgery. Infection of the fracture site is attributed to contamination throughout open wounds before fracture repair, contamination during surgery, or as a consequence of surgical incisional site infection.

Diagnosis

Bone infection is often presented with variable degrees of clinical signs. Prompt identification and management becomes essential for a successful outcome. Clinical signs include increase in lameness, fever, serous to purulent discharge from the surgical site, wound dehiscence, and cellulitis. Other nonspecific diagnostic tools that suggest orthopedic infection include increased total white blood cell count, elevated fibrinogen, and radiographic signs of bone lysis.[35] Radiographs identify the presence and/or progression of osteomyelitis. Initial radiographic signs reflect bone demineralization but are not apparent until 10- 21 days post infection. Approximately 30-50 % of bone mineral must be lost before changes are noticeable in radiographs.[40,41] Osteomyelitis that follows

fracture repair is manifested as areas of radiolucency adjacent to the implants or fracture site. These osseous changes can lead to implant loosening and eventually failure of the fixation.

Treatment of Orthopedic Infections

The successful management of orthopedic infections demands differentiation between the different anatomic locations and structures involved, and adopting the most appropriated treatment options. Antibiotic ideally is based on culture and antimicrobial susceptibility. Systemic antimicrobial therapy can result in delivery of therapeutic concentrations of the drug to affected tissues. However, changes in local blood supply associated with soft tissue sepsis or traumatic injury may negatively impact the efficacy of systemic administration antimicrobial due to decreased antimicrobial delivery. Local antimicrobial delivery systems are use to deliver high antibiotic concentrations to infected tissues using lower dosage. This minimizes the cost and toxic effects of the prolonged antimicrobial therapy and is an excellent adjunctive therapy for orthopedic infections. Other treatments that are important for orthopedic infections include surgical debridement and lavage of the infected area, appropriated antimicrobial therapy, and adequate coaptation.

Systemic Antimicrobials

Systemic antimicrobial therapy should be initiated in all cases of orthopedic infection. The goal of antimicrobial therapy is to achieve antibiotic concentrations in the tissues that are effective against the organism involved. Most of the systemically administered antibiotics that have been evaluated, including trimethoprim-sulfonamides, penicillins, aminoglycosides, and some chephalosporins, have been shown to be present

and active in infected synovial fluid.[3,42-44] However, concentrations achieved in the synovial fluid may not be high enough for successful treatment.[43] In many instances systemic antimicrobial therapy alone does not reach enough concentrations at the site of infection, because the presence of chronic infections, severely damaged tissue, vasculature disruption, and fibrosis.[3]

Pharmacokinetic and Pharmacodynamics

Pharmacokinetics (PK) describes the relationship between administered dose, the observed biological fluid/tissue concentration of the drug, and time. Pharmacodynamics (PD) describes the magnitude and time course of the pharmacological effect. The primary measure of antibiotic activity is the minimum inhibitory concentration (MIC). The MIC is the lowest concentration of an antibiotic that completely inhibits the growth of a microorganism in vitro. While the MIC is a good indicator of the potency of an antibiotic, it indicates nothing about the time course of antimicrobial activity. Any calculation or expression of the MIC should include a description of the method by which the MIC was determined or reference to published method (e.g. NCCLS2 or BSAC3).[45] Pharmacokinetics parameters quantify the serum level time course of an antibiotic. The pharmacokinetic parameters that are most important for evaluating antibiotic efficacy is the peak serum level (Cmax) and the Area Under the serum concentration time Curve (AUC). The PK/PD breakpoint of an agent is determined primarily by the dosing regimen, and generally applies to all pathogens causing disease at sites where extracellular tissue levels are similar to non-protein-bound serum levels.[46] In patients, the best correlation parameters are time over MIC, and the ratio of 24-hour AUC to MIC (AUIC). Studies demonstrate that the minimum effective antimicrobial action is an area

under the inhibitory titer (AUIC) of 125, where AUIC is calculated as the 24-hour serum AUC divided by the MIC of the pathogen.[47] While these parameters quantify the serum level time course, they do not describe the killing activity of an antibiotic. Integrating the PK parameters with the MIC gives us the PK/PD parameters which quantify the activity of an antibiotic: the Cmax/MIC ratio, the T>MIC, and the AUC/MIC ratio. The Cmax/MIC ratio is simply the Cmax divided by the MIC. The T>MIC (time above MIC) is the percentage of a dosage interval in which the serum level exceeds the MIC. The 24h-AUC/MIC ratio is determined by dividing the 24-hour-AUC by the MIC. The half life ($t_{1/2}$) of any drug is defined as the time it takes for the concentration to fall to half of its previous value.[48] Pharmacodynamic properties can be used to divide antibiotics into two major classes based on their mechanism of bactericidal action: (1) concentration-dependent drugs, such as aminoglycosides and fluorquinolones, and (2) concentration-independent drugs, including the ß-lactams. Antibiotics also differ in the post-antibiotic effect (PAE) that they exert.[49] In general, concentration-dependent drugs have a more prolonged PAE than concentration-independent drugs, particularly against Gram-negative pathogens. Pharmacodynamic classifications have important implications for the planning of drug regimens.[49] For concentration-dependent drugs, peak concentrations to minimal inhibitory concentration (MIC) ratios of approximately 10 are associated with clinical success. Therefore, high drug levels should be the goal of therapy. This is best achieved by high doses taken once daily. Concentration-independent agents are most effective when the duration of serum concentrations is higher than the pathogen's MIC (time > MIC) for a significant proportion of the dosing interval. Frequent dosing or continuous infusions can increase the time > MIC. Concentrations of antibiotics that are sub-lethal

can permit the emergence of resistant pathogens. Optimization of antibiotic regimens on the basis of pharmacodynamic principles could thus significantly diminish the emergence of antibiotic resistance. Pharmacokinetic parameters (AUC, Cmax or times >MIC) that correlate best with antibacterial efficacy have been defined and used to describe antibacterial activity of various classes of antimicrobial agents.[50,51] The peak serum concentration/minimum inhibitory concentration ratio (Cmax/MIC) is a good predictor of the antibacterial activity of aminoglycosides agents.[52] For β-lactam antibiotics, the length of time that the serum concentration exceeds the MIC (T>MIC) is the most relevant index affecting bacterial activity.[53]

Antimicrobial Distribution

The distribution of a drug between tissues is dependent on permeability between tissues (between blood and tissues in particular), blood flow, perfusion rate of the tissue and the ability of the drug to bind plasma proteins and tissue.[48] Effective blood flow is essential to drug delivery. Poorly perfused areas may not reach effective drug concentrations. Distribution can be limited by binding of drugs to plasma protein, particularly albumin, for acidic drugs and $\acute{\alpha}_1$-acid glycoprotein for basic drugs. Dependent upon its route of administration and target area, every drug has to be absorbed, by diffusion, through a variety of bodily tissue.[54] Tissue is composed of cells which are included within membranes, consisting of 3 layers, 2 layers of water-soluble complex lipid molecules (phospholipid) and a layer of liquid lipid, contained within these layers. The permeability of a cell membrane, for a specific drug, depends on a ratio of its water to lipid solubility. Within the body, drugs may exist as a mixture of two interchangeable forms, either water (ionized-charged) or lipid (non-ionized) soluble. The

concentration of two forms depends on characteristics of the drug molecule (pKa, pH at which 50% of the drug is ionized) and the pH of fluid in which it is dissolved. In water soluble form, drugs cannot pass through lipid membranes, but to reach their target area, they must permeate the membranes.[48]

Lipid solubility will affect the ability of the drug to bind to plasma proteins and to cross lipid membrane barriers. Very high lipid solubility can result in a drug initially divided preferentially into highly vascular lipid-rich areas. Consequently, there is a close correlation between lipid solubility and the permeability of the cell membrane to different substances. For this reason, lipid solubility is one of the most important determinants of the pharmacokinetic characteristics of a drug.[55] The drug distribution and the concentration achieved at the site of infection depend on a number of factors, including molecular size, protein binding, and lipid solubility. Because, in most cases, specific tissue concentrations are not known, serum concentrations are used to represent the levels in the extracellular fluid space, which is the site of most bacterial infections.[55] The local environment can further enhance or delay antimicrobial penetration and activity. The antibiotic concentration achieved in the blood affects the concentration at the site of infection because simple passive diffusion appears to be the method of transport for most antibiotics.[55] The antibiotic activity after reaching the site of infection is influenced by environmental conditions. Local production of enzymes, purulent and fibrinous exudate, and pH changes can adversely affect drug action.

Local Antimicrobials

In recent years there has been great interest in novel ways to deliver antimicrobial drugs to target tissues by means of local application. The primary goal of local

antimicrobial delivery systems is to achieve high and sustained therapeutic concentrations above the minimum inhibitory concentrations (MIC). Antibiotic delivery systems include intra-articular administration, antibiotic impregnated polymethylmethacrylate (PMMA), regional intravenous limb perfusion (RILP), and intraosseous regional limb perfusion (IORLP).

Intra-articular Antimicrobials

Administration of antimicrobials directly to the affected synovial structures is currently the best therapy to treat early septic arthritis. Any antimicrobial that is used intra-articularly must be minimally irritating to the synovial structure. Intra-synovial administration of 150 mg of gentamicin to the carpal joint maintain concentrations above the MIC for most equine pathogen for up to 24 hrs.[44,56] In a study comparing amikacin concentrations in normal and experimentally inflamed joints, administration of 500 mg of amikacin intra-articular in normal joints maintained mean peak concentrations of 49.8 µg/mL for up to 72hrs whereas in inflamed joints the mean peak concentrations of 19.8 µg/mL were effective for 48hrs.[57] Another study compared synovial fluid and bone gentamicin concentrations achieved after intra-articular administration or regional intravenous perfusion (RIP) (1 g gentamicin administered simultaneously in both left and right lateral palmar veins). IA administration resulted in higher concentration of gentamicin in synovial fluid than RILP administration. Synovial fluid concentration remained above minimum inhibitory concentration (MIC) for common pathogens for over 24 hours with IA and RILP administration.[5]

Antibiotic Impregnated Polymethylmethacrylate (PMMA)

Polymethylmethacrylate is a high density plastic formed by combining a fluid monomer and a powder polymer. When an antimicrobial is added it becomes suspended in the cement as it hardens.[58] The cement can be placed before it is completely hard underneath orthopedic implants (i.e. plate luting), or can be shaped into beads or shaped into a large cylindrical implant. The antimicrobials are released from the PMMA by diffusion because the tissue fluids surrounding the material create a concentration gradient for elution of the antimicrobial from the implant. Elution of the antimicrobial from the PMMA occurs in bimodal fashion. Rapid elution occurs during the first couple of days and up to 5% of the total amount of antimicrobial may be eluted during the first 24hrs. After this period slower elution of the antimicrobial take place after weeks or months after implantation.[58-60] Several antimicrobial drugs have been shown to elute from PMMA in active concentrations, including aminoglycosides (amikacin, gentamicin), and fluorquinolones such as ciprofloxacin in human beings.[61-63] In horses, PMMA impregnated with aminoglycosides and cephazolin was used in 19 horses with open fractures, joint injuries, and chronic septic conditions. A survival rate of 60% was achieved. A combination of PMMA with a wound lavage treatment in 12 horses had a rate of survival of 92 %., including 6 that presented osteomyelitis.[64] In another study successful treatment of septic arthritis was achieved in 9 horses after surgically implanted gentamicin impregnated PMMA beads into the tarsal joint.[65]

Treatment with PMMA is not without complications. Complications include the use of too many or too large PMMA beads that may cause further soft tissue trauma and pain, failure to replace the material in non-resolving infections, and placement of the material

under tendons and articular cartilage.[2] The antimicrobial elution rate from PMMA varies with size and shape of the beads, the amount of antimicrobial, the type and forms of the antimicrobial agent (solution, powder), and amount of drainage from the wound.

Regional Perfusion

Regional perfusion of antibiotics involves perfusion of the venous system or the medullary cavity proximal to the affected area.[66] Regional limb perfusion has been used in rabbits and humans to achieve high antibiotic concentrations in poorly perfused chronically infected tissues.[66] Regional perfusion has become an important adjunctive therapy in orthopedic infections. The main advantage of these techniques is that higher concentrations of antimicrobial drugs can be achieved in tissues by the use of lower dosage of antimicrobials.

Technique Considerations

Technique for intravenous regional perfusion

Most of the initial studies on regional perfusion performed the procedure under general anesthesia.[5,7,9,67,68] However, the technique can be readily performed with a horse standing. The horse is sedated with a combination of detomidine (0.01 mg/kg) and butorphanol (0.02 mg/kg) and a tourniquet is placed proximal and distal to the targeted area and infusion site. For perfusion of the distal limb (distal metacarpus/tarsus to foot), the digital vein is used. The cephalic vein in the front limb or the saphenous vein in the hind limb can be use to perfuse the region distal to the distal third of the radius or tibia, including the carpus and tarsus, respectively. However, any other superficial vein that is exposed after the tourniquet is in place can be used. After aseptic preparation of the site, a 23-gauge butterfly catheter is inserted into the selected vein and the antibiotic solution is

slowly infused over 5-15 minutes. For larger vessels such as the cephalic or saphenous veins, a 20-gauge, 1" IV catheter can be placed. The catheter can prevent extravasation of the drug during perfusion and associated complications. After infusion, the catheter is removed, pressure is applied for a few minutes in the venipuncture site, and the tourniquet is maintained for 30 minutes. The procedure is repeated every 24 hours for 3-5 days or as needed. Regional limb perfusion offers a numerous advantages in the treatment of septic structure, but also presents some complications including damage vasculature, edema in the surrounding area, and thrombosis which can limit the number of treatments to be performed.

Technique for intraosseous regional perfusion

This technique can be performed with a horse standing or under general anesthesia.[69] After the horse is sedated and restrained in the stocks, the site is clipped and prepared as for aseptic surgery. Regional nerve blocks and local infiltration of anesthetic drugs are used to desensitize the region. A 1 cm stab incision is made through the skin, subcutaneous tissue, and periosteum on the most readily accessed aspect of the bone. The soft tissue is retracted and a 3.2 mm to 4 mm (3.2 mm in foals) unicortical hole is drilled through the cortex. A special cannulated screw is then inserted after tapping the hole to the specific screw diameter. However, the cannulated screw is not always necessary because infusion can be performed by introducing the male end of a catheter extension set into the unicortical hole. Regional perfusion is performed as described for the intravenous technique. After the procedure, the skin over the cortical hole or cannulated screw is sutured and bandaged. Some complications are identified with the use of intraosseous perfusion, besides the invasiveness of the technique, special instrumentation,

and leakage of the perfusate around the cortical screw. Extra care must be taken when dealing with an open portal to the medullary cavity because of the possibility of further infection.

Administration of 1 gm of gentamicin by regional intra-osseous perfusion of the MCIII have been shown to achieve concentrations approximately 55 times higher than the reported MIC of 4-5 μg/ml for gentamicin in the antebrachiocarpal joint.[68] One study reports the successful treatment of osteomyelitis in two horses treated by a combination of regional intra-osseous perfusion and intravenous perfusion. Intraosseous perfusion of 9 gm of ampicillin sodium was used in horse #1, and horse # 2 received 1 gm of gentamicin by regional intra-osseous perfusion once, and a second perfusion was performed using regional intravenous perfusion one week after with amikacin.[4] In another study by the same author, investigators compare RILP versus systemic dosage for the treatment of experimentally induced septic arthritis. Investigators evaluate a systemic dosage 2.2 mg/kg of gentamicin administered intravenously every 6 hours versus 1 gm of the same antimicrobial administered by regional intravenous perfusion. Regional intravenous perfusion reached concentrations in the carpal joint of 221.2 ± 71.4 μg/ml versus 7.6 ± 1.6 μg/ml after IV administration.[70] Perfusing the distal aspect of the equine limb with 125 mg of amikacin in anesthetized horses resulted in antibiotic concentrations 25-50 times the MIC of most common equine pathogens in synovial fluid samples of coffin joint, and fetlock joint. Whereas, bone concentration achieved in the third phalanx were 8.6 μg/g of tissue, and in the navicular bone reached 15.4 μg/g of tissue.[7]

Several comparisons have been made between regional intra-osseous and regional intravenous perfusion. Butt *et al* studied the differences in concentrations achieved after

intraosseous and intravenous regional perfusion with 500mg of amikacin. Samples were obtained from the distal interphalangeal joint, metacarpophalangeal joint and digital tendon sheath. The maximum concentration for both techniques reported high variability. However, regional intravenous perfusion reached higher concentration approximately, 5-15 times the MIC of 4 gm/dl. There was no difference in time to peak concentration or elimination time between the two techniques.[71] In another study, a higher dose of amikacin (1 gm) was used to compare concentration of antimicrobial in the tibiotarsal joint following perfusion of the medullary cavity at the distal portion of the tibia versus venous perfusion of the saphenous vein. Perfusion of the saphenous vein resulted in higher concentration of amikacin than intraosseous administration.[67] However, in a recent study authors concluded that administration of gentamicin by direct intra-articular injection reached higher concentrations of the antimicrobial in the synovial fluid for an extended period of time when compared with RILP.[5]

Aminoglycosides have been the antibiotic of choice for regional intravenous perfusion of the distal limb of horses, but concerns of bacteria increasing resistance to many antimicrobial including β-lactam drugs (i.e. chephalosporins), aminoglycosides, macrolides, clindamycin, tetracyclines, and fluorquinolone has prompted the use of antimicrobials more selective for resistant strains, for example, methicillin resistant *staphylococcus aureus* (MRSA) infection.[72,73] One study reported the use of Vancomycin at 300 mg dosage administered by regional intravenous perfusion. Authors did not report any complications during or after the procedure. Concentrations achieved in the metacarpophalangeal joint were higher than the minimum inhibitory concentration for MRSA organisms for up to 20 hours. The maximum concentration ranged 1.47 to 3.74

μg/mL. Intravenous regional limb perfusion provided much higher synovial fluid antimicrobial concentrations than those reported after systemic administration.[9,74] Another investigator, study the synovial fluid concentrations of Ceftiofur following systemic IV administration dosage of 4.6 ± 0.2 mg/kg compared to those achieved after administration of 2 gm by regional intravenous perfusion of the radial vein in anesthetized horses. Maximum concentration in the radiocarpal joint after regional intravenous perfusion surpassed those achieved after IV administration of the drug at each sample time. Mean peak concentration of ceftiofur in the radiocarpal joint was reported as 392.7 ± 103.29 μg/mL at 0.5 hrs, whereas systemic dosage of ceftiofur administered once daily was insufficient to achieve therapeutic concentrations.[75]

Adjuvant Therapies

Synovial Drainage

Adjunctive therapies should be instituted in conjunction to systemic antimicrobial therapy to aid in the healing process. In all cases of septic arthritis or synovitis, joint lavage and debridement is important to remove inflammatory debris and bacteria from the synovial cavity. Methods of synovial lavage include through-and-through lavage, arthroscopic/tenoscopy with or without synovectomy, open arthrotomy with or without suction drainage. The main goal of these techniques is to remove as much as possible potentially damaging inflammatory mediators and bacteria from the synovial structures, to debride osseous lesions when possible, and to decrease synovial distention, resulting in pain relief.[3,76-78] Synovial sepsis is best treated by arthroscopic surgery because provides a more thorough lavage of the affected joint/sheath, allowing visualization of the affected areas, assisting in removal of fibrin and infected synovial membrane.

Nonsteroidal Anti-inflammatory Drugs

The anti-inflammatory and analgesic benefits of nonsteroidal anti-inflammatory drugs (NSAIDs) in horses with orthopedic infections significantly improve the recovery time. Pain management is just as important as controlling and eliminating infection. The most commonly use NSAIDs include phenylbutazone and flunixin meglumine. Thus, are non selective cyclooxygenase (COX) inhibitors. Cyclooxygenase (COX) is an enzyme that is responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane). Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain. COX converts arachidonic acid (AA, an ω-6 essential fatty acid) to prostaglandin H_2 (PGH_2), the precursor of the series-2 prostanoids. The enzyme contains two active sites: a heme with peroxidase activity, responsible for the reduction of PGG_2 to PGH_2, and a cyclooxygenase site, where arachidonic acid is converted into the hydroperoxy endoperoxide prostaglandin G_2 (PGG_2). The reaction proceeds through H atom abstraction from arachidonic acid by a tyrosine radical generated by the peroxidase active site. Two O_2 molecules then react with the arachidonic acid radical, yielding PGG_2.[79] The classical COX inhibitors are not selective (i.e. they inhibit all types of COX), and the main adverse effects of their use are gastric ulceration. It is believed that this may be due to the "dual-insult" of NSAIDs - direct irritation of the gastric mucosa (many NSAIDs are acids), and inhibition of prostaglandin synthesis by COX-1. Prostaglandins have a protective role in the gastrointestinal tract, preventing acid-insult to the mucosa.[79]

Tissue Sample Techniques

Several *in vivo* techniques have been developed for the collection and study of the physiology and composition of interstitial fluid in animals as well as for the study of pharmacokinetics and pharmacodinamics of antimicrobials. Sample collection techniques include tissue chambers, tissue biopsy, microdialysis, and ultrafiltration probes.

Tissue Chambers

Tissue chambers implanted subcutaneously have been used for the study of pharmacokinetics of antimicrobials in horses.[80] Tissue chambers are hollow perforated devises of different shapes and materials implanted in the subcutaneous space of animals. After implantations the chambers are surrounded and partially filled with highly vascularized granulation tissue.[81] Eventually the compartment of the tissue chamber becomes filled with fluid similar to interstitial fluid.[82] The fluid accumulation in the chamber provides a suitable method for collecting fluid that is similar in composition to interstitial fluid, in a sequential manner, with minimal stress to animals.

Tissue Biopsy

Tissue biopsies are performed in the horse either standing using local anesthesia or under general anesthesia in the case of deeper tissue or bone. Repeated biopsies of specific areas may not be a suitable option in some cases. However, special attention should be paid to pain and discomfort when using a biopsy method. Samples required a further preparation for determination of the antimicrobial concentration in tissues.

Microdialysis

Microdialysis is based on diffusion through a semipermeable membrane located at the tip of a microdialysis catheter. The catheter is constantly flushed with a fluid, such as 0.9%

sodium chloride. The membrane allows a diffusive exchange of drug molecules and other substances between extracellular fluid and perfusion fluid, thus enabling a dynamic measurement of drug concentrations in the dialysate. Dialysate concentrations are directly proportional to tissue concentrations for which the proportionality factor is the recovery of the respective substance. Because proteins do not cross the membrane, the free (i.e, protein unbound) and pharmacologically active fraction of a drug is recorded.

Capillary Ultrafiltration

Capillary ultrafiltration is a sampling method convenient for low molecular weight substances in living biological systems. By application of a negative pressure across a hydrophilic membrane capillary, small molecules are actively pulled across the membrane and collected.[21] The pressure gradient across a semipermeable membrane causes (water) and small analytes to pass through the membrane, leaving large substances behind.[20] The probe size and physical characteristics consists of one or more, hollow dialysis fibers connected to a single microbore nonpermeable outflow tube. The outflow tube is connected to the source of negative pressure, either a roller pump or a vacutainer tube. The size and configuration of the probes will depend on the site to be sampled and range from one to three fibers 2-12 cm long.[21] The vacuum pressure applied to the probe causes a net loss from the tissue sampled. Recovery can be affected *in vivo* studies by factors not necessarily related to the hydrophilic capillaries physical characteristics, but physiological and anatomical changes such as body temperature and the formation of fibrous layer around the probe. Flow rate is determined by fiber surface and perfusion of the tissue. Capillaries removes extracellular fluid at rate of 50 (nL/mn)/mm of membrane.

Ultrafiltration probes collected extracellular fluids from the interstitial region driven by a constant vacuum pressure. Extracellular fluid collected from any compartment is representative of the physiological activity of the tissue.[19] Therefore, blood concentrations of antimicrobials, electrolytes, and other metabolites may not reflect those in other sites of interest.[83]

Pharmacokinetic studies of antimicrobial concentrations in the interstitial fluid are important, since most bacterial infections are extracellular, a therapeutic regimen that optimizes concentrations at the infection site (i.e. interstitial fluid) would be better for predicting clinical success. Sample collection of extracellular fluid from the interstitial space using ultrafiltration probes offers numerous advantages including sample ready to be analyzed, constant sample collection, samples more representative of interstitial fluid. Any approach used to collect interstitial fluid (tissue biopsies, tissue cages) may affect the concentration of the drug and the estimates of pharmacokinetic parameters.[22] Homogenization of tissue biopsy specimens can overestimate lipophilic drug concentrations and underestimate hydrophilic drug concentrations due to combination of interstitial fluid, intracellular fluid, and blood through the homogenization process. The ultrafiltration probes allow collection of the protein-unbound fluid from the interstitial fluid and provide a sample ready for analysis. The antibiotic concentration measured is the drug concentration available compared with tissue samples that may have both protein bound and un-bound antibiotic. To our knowledge no studies have been performed in horses to determine pharmacokinetic and pharmacodynamics of antimicrobial drugs using ultrafiltration proves. We believe that the use of ultrafiltration probes offers a valuable tool of *in vivo* sampling.

OBJECTIVES

The purpose of this study was to investigate regional limb perfusion for administration of enrofloxacin and a low dose of amikacin in standing horses. The specific objectives were:

1) to determine the pharmacokinetics of intravenous limb regional perfusion of enrofloxacin and amikacin on drug concentrations in systemic blood, synovial fluid, interstitial fluid of subcutaneous tissue and bone marrow in standing horses,

2) to determine pharmacokinetics/pharmacodynamic indices for either amikacin or enrofloxacin in these tissues using a minimum inhibitory concentration (MIC) that would cover the most common equine pathogens (MIC 16 μg/ml for amikacin and 0.5 μg/ml for enrofloxacin),

3) to evaluate an ultrafiltration system for collection of interstitial tissue and bone marrow fluid for *in vivo* sampling of drugs in horses.

MATERIALS AND METHODS

Animals

Fourteen adult, healthy horses of various breeds were studied using a parallel design: group 1 (n=7) received enrofloxacin and group 2 (n=7) received amikacin. Drugs and perfused front limbs were randomly assigned. Weights ranged from 375 to 513 kg (mean ± SD 451.7 ± 44.9 kg) and age ranged from 2 to18 years (mean ± SD 10.6 ± 5.0 years). Horses were free of radiocarpal disease based on history, clinical, and previous radiographical examination. Horses had not received antimicrobial therapy within 3 months of the current study. All procedures were approved by the Auburn University Institutional Animal Care and Use Committee.

Experimental Methods

Twenty-four hours prior to perfusion an IV catheter was placed at a jugular vein for drug administration. Two capillary ultrafiltration probes were positioned in one of the fore limbs (see below) for *in-vivo* collection of interstitial fluid from the subcutaneous tissue and bone marrow of the third metacarpal bone (MCIII). Serum samples were obtained via venipuncture from either the right or left jugular vein, contralateral to the site of jugular catheter placement. Interstitial fluid samples were collected from the ultrafiltration probes and synovial fluid from the radiocarpal joint. The first collection occurred prior to tourniquet release (time 0) (approximately 25-30 minutes after perfusion) and at 0.5, 1, 4, 8, 12, 24, and 36 hours after the tourniquet was released

Horses were hospitalized and treated with phenylbutazone (4.4 mg/kg PO SID) for 4 days, which started after the end of the experimental protocol (after last collection). Horses were stall rested for 4 weeks and closely monitored for any evidence of lameness, swelling, or infection. Limbs were bandaged and changed on alternate days until the surgical staples were removed 14 days after surgery.

Placement of Capillary Ultrafiltration Device

Horses were placed in stocks and sedated with detomidine hydrochloride (0.02 mg/kg IV). A high four point nerve block and local infusion of 2% mepivicaine hydrochloride around the dorsal aspect of MCIII provided regional anesthesia at probe placement sites. Capillary ultrafiltration probes (**Figure 1**) were placed in the medullary cavity and subcutaneous tissue over the third metacarpal bone (MCIII) (**Figure 1**). A 2 cm stab incision was made through the skin, subcutaneous tissue, and periosteum on the proximo-lateral aspect of MCIII, approximately 4 to 5 cm below the carpometacarpal joint. Access to the medullary cavity was achieved by drilling a 4.5mm bicortical hole of approximately 70mm in length in a proximo-lateral to distal-medial direction at a 60-70 degree angle to MCIII. A second stab incision was made at the point of exit of the drill bit at the distal-medial aspect of the limb. A 70mm, hollow, stainless steel cannula containing the ultrafiltration probe was introduced into the medullary cavity from the proximal to the distal cortical hole. Once the ultrafiltration probe was inserted into the medullary cavity, the hollow cannula covering was removed through the distal hole, leaving the ultrafiltration probe in place. A second ultrafiltration probe was placed dorsolaterally or dorsomedially in the subcutaneous tissue, approximately 3cm distal to the first ultrafiltration probe and parallel to MCIII. A 2 cm stab incision was made

through the skin and subcutaneous tissue and the hollow, stainless steel cannula was introduced from proximal to distal into the subcutaneous space. A second incision was made over the distal tip of the cannula and the ultrafiltration probe was inserted using the same technique as described for the medullary cavity probe. The probes were secured with skin staples and a sterile bandage was applied from the distal antebrachium to proximal metacarpophalangeal joint.

Regional Intravenous Limb Perfusion

Horses were restrained in the stock and sedated with detomidine hydrochloride (0.02 mg/kg IV). An Esmarch bandage tourniquet 5" wide was placed 25-30 cm above the accessory carpal bone and a second tourniquet was placed at the level of the metacarpophalangeal joint. Roll gauze was placed over the cephalic vein before application of the tourniquet to attain better compression of the vascular structures. After aseptic preparation, a 20-gauge, 2" catheter was introduced into the cephalic vein and a 7" IV extension set was attached to the catheter to assist during perfusion. Perfusion was performed with either 250 mg of amikacin or 1.5 mg/kg of enrofloxacin diluted with Lactated Ringers Solution[j] to a total of 60 ml. The infusate was slowly administered manually over 5 minutes and tourniquets remained in place for 30 minutes after perfusion. To limit variability of tourniquet application, the same clinician (AP) placed the tourniquets and perfused all horses in the study. After perfusion of the antibiotic but before removing the tourniquet, the 20-gauge catheter was removed and a pressure bandage was applied to the venipuncture site.

Sample Collection

At each collection period, samples for analysis were collected from the interstitial fluid of the subcutaneous tissue and bone marrow of MCIII, synovial fluid of the radiocarpal joint, and serum. Samples from the interstitial fluid were collected by replacing the vacutainers (**Figure 2),** which provided a continuous suction on the ultrafiltration probes. Two milliliters of synovial fluid were collected by aseptic arthrocentesis of the radiocarpal joint. A 20-gauge, 1" needle was introduced into the palmar pouch of the radiocarpal joint, 1cm proximal to the accessory carpal bone and 1cm caudal to the radial epiphysis. Synovial fluid was aspirated with a 3ml sterile syringe. Whole blood samples were obtained by venipuncture of a jugular vein. Blood samples were centrifuged and serum was separated and stored at -80º C until assessed. Limbs were re-bandaged after each collection. After the final collection, the probes were removed and probe placement incisions were closed using surgical staples.

Sample Analysis

All samples were stored at -80º C pending assay. Both enrofloxacin and amikacin assays were validated for each tissue studied. Amikacin was detected using fluorescence polarization immunoassays (FPIA). Enrofloxacin was detected by high pressure liquid chromatography (HPLC) using previously described methods.[84] Plasma samples were defrosted and centrifuged through a 10-kD membrane at 3,000 rpm for 2 hours to remove cellular debris and proteins. The resulting filtrates were analyzed using reverse-phase chromatography (Luna C-8 Column), a mobile phase comprised of phosphate buffer, methanol, and acetonitrile and fluorescence detection with excitation at 280nm and emission at 450nm. The upper and lower limits of detection for enrofloxacin were 30 and 5000 ng/ml

respectively. Controls spiked with antibiotics to spanned concentrations detected in samples predicted within 15% of the known concentration. For amikacin, the upper and lower limits of detection were 0.8 and 50 ng/ml respectively. Controls spiked with antibiotics to spanned concentrations detected in samples predicted within 15% of the known concentration.

Data Analysis

Tissue drug concentrations (log) versus time curves were subjected to noncompartmental linear regression analysis WINONlin using the trapezoidal method to determine area under the curve to infinity. Additional parameters included peak concentration (Cmax, reported as actual value), the time to reach peak concentration (Tmax), area under the curve (AUC), and disappearance half-life (T½). Two pharmacokinetic/pharmacodynamic indices were calculated for each tissue: Cmax/MIC and AUC/MIC. An MIC of 16µg/ml was used for amikacin and 0.5µg/ml for enrofloxacin.[17] Descriptive statistics was determined for pharmacokinetic parameters and pharmacodynamic/pharmacokinetic indices for each tissue and drug. Data were expressed by mean ± standard deviation (when normally distributed data) or by median and range (when not normally distributed). Comparisons were not made in indices between drugs because doses were not comparable. Thus comparisons were limited within treatment groups among tissues using non-parametric or parametric ANOVA test and suitable multiple comparison test to identify where significant differences occurred. Significant differences were set at $p \leq 0.05$. Computer software was used for data analysis

RESULTS

Lameness was not detectable at the walk in any of the horses following the drilling of MCIII and implantation of the ultrafiltration device. No complications occurred from placement and maintenance of the ultrafiltration probes or during regional limb perfusion with amikacin. Signs of inflammation (transient perivascular edema and mild cellulites) occurred at the site of infusion in three horses perfused with enrofloxacin. Signs were noticed approximately 6-8 hours after perfusion and resolved without treatment within 48 hours. Collection of interstitial fluid with the ultrafiltration probe was successfully obtained except in two horses. In one horse the permeable area of the ultrafiltration probe shrunk, and in another horse constant slippage of the leg bandage obstructed both probes at the level of the non-permeable portion. As a result, insufficient fluid was collected for drug detection at some of the collected time points in these horses. These samples were considered missing samples for the analysis. The volume of interstitial fluid sample collected with the ultrafiltration probes at each time period did not differ between the probes on horses perfused with amikacin **(Table 1).**

Pharmacokinetics

The highest tissue concentration after regional limb perfusion of enrofloxacin was found in synovial fluid (Median (Range) - 13.22 (0.254 - 167.9) µg/ml), followed by interstitial fluid of the subcutaneous space (3.99, (0.44 - 197.6) µg/ml), and then the interstitial fluid of the bone marrow (1.09, (0.27 - 67.9) µg/ml). The Cmax for the serum

was 0.70, (0.58 - 8.7) µg/ml. **(Table 2)**. For amikacin, the highest Cmax again occurred in synovial fluid (26.2, (5.78 - 50.0) µg/ml), followed by interstitial fluid of the subcutaneous space (19.4, (4.1 - 29.4) µg/ml), then the interstitial fluid of the bone marrow (6.6, (2.8 - 11.1) µg/ml). Cmax for amikacin in the serum was 1.9, (1.1 – 3.5) µg/ml. The Tmax were high variable among tissues **(Table 2).** See Table 2 for pharmacokinetic data.

Pharmacokinetic / Pharmacodynamic indices

In vitro studies for concentration-dependent antimicrobials suggest that antibacterial efficacy is more likely if the Cmax/MIC is ≥ 10, or the AUC/MIC is ≥ 125. Based on those targeted indices, enrofloxacin Cmax/MIC was achieved in all tissues in 3 of 6 horses, whereas AUC/MIC was achieved in all horses for the synovial fluid and interstitial fluid of the subcutaneous tissue, 4 of 6 horses in the bone marrow interstitial fluid, and none in the serum**.** For amikacin, target Cmax/MIC nor AUC/MIC was not achieved in any of the horses or tissues. **(See Table 3 and Figure 3**). No significant differences were observed between tissues in Cmax/MIC or AUC/MIC in horses perfused with either enrofloxacin or amikacin.

DISCUSSION

The activity of both fluorquinolones and aminoglycosides against microorganisms is regarded as concentration-dependent.[10] Thus the greater the antibiotic concentration reached in the tissue above the MIC, the greater the rate of bacterial killing.[85] Therefore intravenous regional limb perfusion (RILP) is an excellent method of administering these concentration-dependent antibiotics due to the high concentrations that can be reached in the distal limb of horses.[7] Recent scientific investigations have reported high antibiotic concentrations in bone and synovial fluid of horses for up to 24 hours after RILP with gentamicin and amikacin in anesthetized horses.[5,7] Others have also described the pharmacokinetics of time-dependent antibiotics such as vancomycin and ceftiofur after RILP, also in anesthetized horses.[9,75] In field and hospital conditions, equine practitioners routinely perform RILP with horse standing using chemical restraint. The standing procedure appears to be well tolerated by horses and is easy to perform as no specialized facilities or equipment is required. In this study, administration of 1.5 mg/kg of enrofloxacin by RILP in standing horses resulted in therapeutic antibiotic concentrations for approximately 24 hours in interstitial tissue and synovial fluid, and 36 hours in bone marrow. In marked contradiction, a low dose of amikacin (250 mg) did not result in efficient therapeutic antibiotic concentrations in any of the tissues.

The pharmacokinetic parameters (Cmax and AUC) provide guidance concerning achievable concentrations in living tissues as high values for Cmax and AUC are

important for the efficacy of concentration-dependent antimicrobials. Conversely, pharmacodynamic aspects of antimicrobial drugs describe the interaction between the drug and the microorganisms. (i.e. *in-vitro* activity with regard to MIC). Pharmacodynamic predictors that evaluate the efficacy of concentration-dependent antibiotics include the ratio between Cmax and MIC (Cmax/MIC) and the ratio of area under the plasma drug concentration versus time curve to the MIC (AUC/MIC). The maximum efficacy of these concentration-dependent antibiotics appears to occur when Cmax/MIC $\geq$ 10 or the AUC/MIC is $\geq$ 125. The AUC/MIC has been shown to be the most accurate predictor of bacteriological and clinical response to fluoroquinolone.[86] Exceeding these targeted indices not only enhances bacterial killing, it also reduces the risk of microbial resistance. In this study potential efficacy as indicated by Cmax / MIC and AUC/MIC varied among antimicrobial agents. The results presented here demonstrate that a dose of 1.5 mg/kg of enrofloxacin produced sufficient tissue concentrations for Cmax/ MIC and AUC/MIC ratios high enough for most susceptible bacteria causing orthopedic infections in horses.[87] Using the MIC value of 0.5μg/ml, enrofloxacin (1.5 mg/kg) by RILP resulted in pharmacodynamic values well above the cited surrogate markers for successful therapy in 3 of 6 horses for Cmax / MIC. This dose also exceeded AUC/MIC targeted ratios for all horses for the synovial fluid and interstitial fluid of the subcutaneous tissue, and in 4 of 6 horses for the bone marrow interstitial fluid.[88] In contrast, 250 mg of amikacin did not result in Cmax/ MIC nor AUC / MIC ratios effective for treatment of organisms with an MIC $\geq$ 16 μg/ml for any tissue.

Although enrofloxacin was more predictable than amikacin in achieving therapeutic concentrations in the different tissues, concentrations achieved in the different

tissues were highly variable for both antibiotics used in this study. To our knowledge, no published population statistics for equine pathogen causing osteomyelitis for pharmacokinetic / pharmacodynamic indices are available. Therefore, we selected targets based on (CLASI) susceptible MIC due to the high possibility that these results would be relevant for any isolate considered susceptible to amikacin or enrofloxacin.[89] Although an MIC of 16µg/ml is known to be satisfactory for amikacin, there are no official MIC standards for enrofloxacin to apply to equine pathogens. An MIC of 0.5µg/ml covers the most important pathogens, including coliforms, enterobacters, and Staphylococcus spp.[89] Based on the pharmacodynamic findings in this study, enrofloxacin at a dose of 1.5 mg/kg by RILP in standing horses will be a good alternative. In contrast, a higher dose of amikacin (500 mg to 2g) for RILP administration in standing horses is recommended.

Enrofloxacin was selected for this study because the antibacterial properties are ideal against most common pathogens involved in orthopedic infections in horses.[23] However, high concentrations of enrofloxacin, as might be obtained with local administration (intra-articular and/or by RILP) have been questioned because of the adverse effects to chondrocytes and tendon metabolism *in-vitro*.[14,15] The chondrotoxic effects are characterized by suppression of proteoglycan synthesis, proteoglycan degradation, and chondrocyte necrosis.[15] In this study the maximum concentration (Cmax) of antibiotic reached in the synovial fluid was [mean ± SD 54.37 ± 69.07, median (minimum-maximum) 13.22 (0.254 - 167.9 µg/ml)]. Based on *in-vitro* studies, this concentration would not be expected to reach concentrations high enough to cause chondrocyte damage as previously reported (>1000 mcg/ml).[14] The effects of sustained concentrations of antibiotic, as can be achieved with multiple treatments with RILP, still requires further

investigations. Until proven otherwise, we do not recommend using enrofloxacin by RILP in young horses (≤ 2 years of age) because these horses appear to be more susceptible to chondrotoxic effects of quinolones.[11]

The enrofloxacin concentration achieved in the synovial fluid following RILP was approximately 5-10 times greater than reported after systemic administration of 5.0 mg/ kg.[90] Interstitial fluid antibiotic concentrations of the bone marrow and subcutaneous tissue also reached high concentrations and remained higher for a longer period (36 hours) than for synovial fluid (24 hours). The high lipid solubility of the drug most likely resulted in an excellent distribution and preferential accumulation in these tissues. In horses with orthopedic infections these properties are crucial to maintain high concentrations of antibiotics for extended times, while minimizing dosing intervals during the treatment of infected soft tissues or osseous structures. In addition to increased bacterial killing and prolonged post-antibiotic effect, longer dosing intervals can reduce the chances for development of adaptive resistance by microorganisms.[91] Considering the strong post-antibiotic effect of enrofloxacin and the results of this study in which antibiotic concentrations remained above the MIC for 36 hours in the bone marrow and interstitial fluid and 24 hours for the synovial fluid, we recommend performing RILP with enrofloxacin no less than every 36 hours. However, because antimicrobial activity at infected sites can be diminished, pharmacokinetics/dynamic studies under septic conditions require further investigations.[92]

The perivascular edema and vasculitis of the distal limb that occurred in three horses perfused with enrofloxacin was most likely due to extravasation of the drug solution during RILP, causing an inflammatory response in the surrounding tissue. Irritation is most likely

due to the vehicle used to suspend the drug or the pH adjustment for stability of the product. In a pilot study looking at different doses and formulations for use in the current study, the small animal preparation (50mg/dl) or doses >2.0 mg/kg caused severe cellulitis in the perfused limb of three horses. In our clinical experience there is always some degree of solution extravasation after RILP in standing horses because high hydrostatic pressure forces solution leakage at the venipuncture site. To limit extravasation, we recommend: (1) using an intravenous catheter for perfusion; (2) not removing catheters before tourniquets are released; (3) locating the infusion site 6-10 cm distal to the proximal tourniquet; (4) diluting the perfusate with saline to make a total volume of 60 cc; and (5) slowly administering the perfusate over 5 minutes. By following these steps, in the last year we have administered enrofloxacin by RILP to 12 horses with distal limb infections (i.e. septic arthritis) with no adverse effect.

Amikacin has been considered the antibiotic of choice for local and regional administration because it is effective against a wide range of microorganisms implicated in septic conditions. The administration of 150 mg to 1 gram of amikacin by intravenous and intraosseous regional limb perfusion has been reported to result in high antibiotic concentration in synovial fluid and bone in anesthetized horses.[7, 67] In the present study, a smaller dose of amikacin (250 mg) was utilized to determine if a lower dose than usually used by practitioners (500mg -2 grams) would deliver effective antibiotic concentration. Amikacin tissue concentrations in the horses of this study were significantly (2-5 times) lower than previously reported.[7,5171] In addition, pharmacodynamic data (AUC/MIC and Cmax/MIC) demonstrate that the 250 mg dose did not reach adequate concentrations for effective therapy. The most likely reason for

the difference in our findings (inadequate amikacin concentration) with RILP compared to other reports is the difference in methodology. We performed RILP with horses standing instead of under general anesthesia and lateral recumbency. Systemic blood pressure in anesthetized horses is decreased as a result of decreased cardiac output and peripheral vascular resistance due to the influence of anesthetic agents and position.[93,94] In addition, systemic pressures in the distal limb of standing horses are likely greater because of the weight of the blood in the vein and changes in peripheral vascular resistances due to motion.[95] Perhaps the high pressure found in standing horses compared to horses under general anesthesia influences leakage of the perfusate to the systemic circulation and this leads to lower antibiotic tissue concentrations in the perfused region. Furthermore, in most of the other studies a higher dose was used.

The high variability of tissue concentrations identified for both antibiotics in this study was comparable to previous reports after RILP with amikacin and other antimicrobials[5,7,51,50] This variability may be due to differences in placement of the tourniquet, differences in location (distal limb versus antebrachium), and pressure application of the tourniquet (i.e. individual variances between antebrachial muscle), differences in medullary volume of involved bones, and leakage of the perfusate during perfusion.[51] Other investigators have blamed discrepancies in body weight and antimicrobial dose as causes of high Cmax variability.[8] However, the results of this investigation demonstrate the same variability with both antimicrobials, in which horses perfused with amikacin received the same antibiotic dose of 250 mg and horses perfused with enrofloxacin received a dose based on body weight. Even with tourniquet placement

and RILP procedures performed by the same investigator (AP), discrepancies likely occurred between horses on position and pressure applied to the tourniquet.

Absorption of the perfusate into the systemic circulation during the RILP procedure occurred in all horses in this study, with serum antibiotic concentrations being detected prior to releasing the tourniquet in eleven out of twelve horses. This apparent failure of the tourniquet may be due to (1) inadequate pressure applied to the tourniquet. In this study a pneumatic tourniquet was not used in order to imitate clinical situations where an elastic tourniquet is likely used. Thus, application of pressure of each tourniquet was not monitored; (2) no exsanguination of the perfused region. The increased venous blood volume in the region due to the combination of the perfusate and the blood may cause reduction in the efficacy of the tourniquet;[96] (3) the difference between performing the procedure standing versus under general anesthesia may also affect tourniquet physiology. Ideally, vascular occlusion occurs when external pressure is higher than arterial systolic pressure. However, tourniquet application to the distal limb of anesthetized horses at a pressure of 600 mmHg did not completely occlude blood flow to the limb.[92] This might be of greater influence in standing horses. Continued arterial blood flow with a greater degree of venous obstruction can cause engorgement of the vessels distal to the tourniquet and contribute to pain, which can induce restlessness and motion of the limb. This might then result in variations of tourniquet pressure compared to total immobilization of the limb during general anesthesia and lateral recumbency. Another important factor to consider is the difference in cardiovascular variables between the anesthetized horses compared to awake, standing horses as described in the previous paragraph. These findings have not been previously reported, and are very important

concepts, because RILP is more often performed by equine practitioners with the horse standing using similar techniques as used in our study.

In the present study we successfully utilized capillary ultrafiltration probes for *in-vivo* collection of fluid samples in standing horses. This device allowed us to successfully evaluate the pharmacokinetic and pharmacodynamic data of the two antimicrobials in two different tissue compartments in vivo after RILP. The collection device was well tolerated, easy to place and maintain, and effective for the length of the collection period. The volume of fluid samples between probes did not significantly differ and the volume was adequate for direct analysis in all collections. The volume of sample collected represented the time elapsed between collections rather than a specific measurement time point. Therefore higher volumes were collected when more time elapsed between collections. Ultrafiltration probes have been used to collect interstitial fluid in different species including rats, dogs, mice, cats, and humans for analysis of drugs and interstitial tissue electrolytes, proteins, and metabolites for up to 30 days.[20,83] The main advantages of using this ultrafiltration probe in horses include (1) continuous fluid collection, (2) little or no manipulation of the sample prior to analysis, (3) the ability to use the catheter 24 hours after implantation, and (4) allowance of free movement of animals in a stall. The only disadvantage of these devices is that they are friable and care must be taken when using them. In two horses, shrinkage of the capillary portion of the probe and obstruction of the non-permeable portion of the probe resulted in insufficient volume of sample for analysis. This technical error can be avoided by correct placement of the vacutainer and using caution with bandage application over the device.

In conclusion, 1.5 mg/kg of enrofloxacin is an alternative for RILP administration in horses with orthopedic infections. However, care must be taken to avoid extravasation of the drug, which can develop serious complications. Further studies are required to determine the efficacy of enrofloxacin at 1.5 mg/kg by RILP to treat septic condition of the distal limb of horses. Regarding to administration of amikacin by RILP in standing horses, a dose greater than 250 mg is recommended when using the cephalic vein for perfusion to achieve effective therapeutic levels at the carpus and MCIII. The findings of this study also emphasize the importance of careful placement of tourniquets for performing intravenous regional limb perfusion in standing horses. Finally, this is the first study to demonstrate the efficacy of capillary ultrafiltration probes for *in-vivo* collection of interstitial fluid in standing horses.

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FIGURES

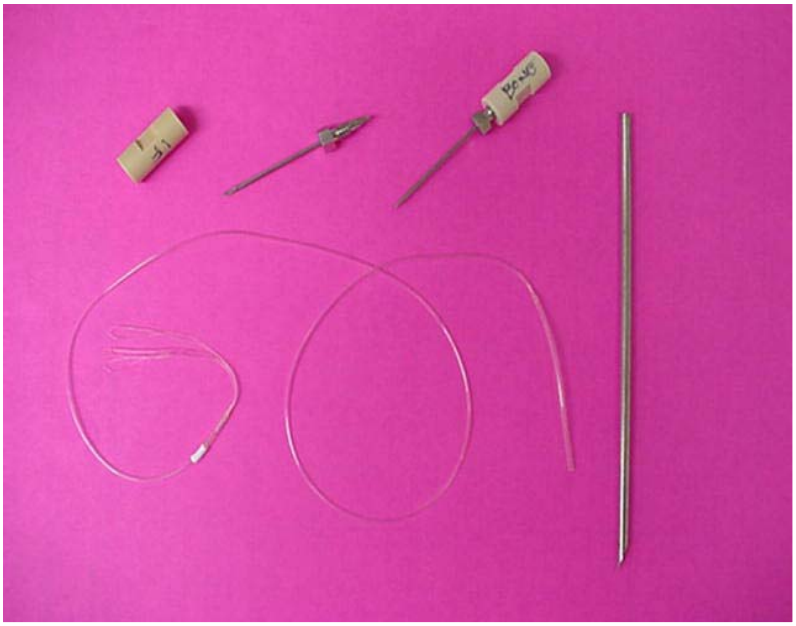

Fig. 1: *In –vivo* capillary ultrafiltration probes for collection of interstitial fluid samples. Capillary ultrafiltration probe, needle introducer, and connecting barrel.

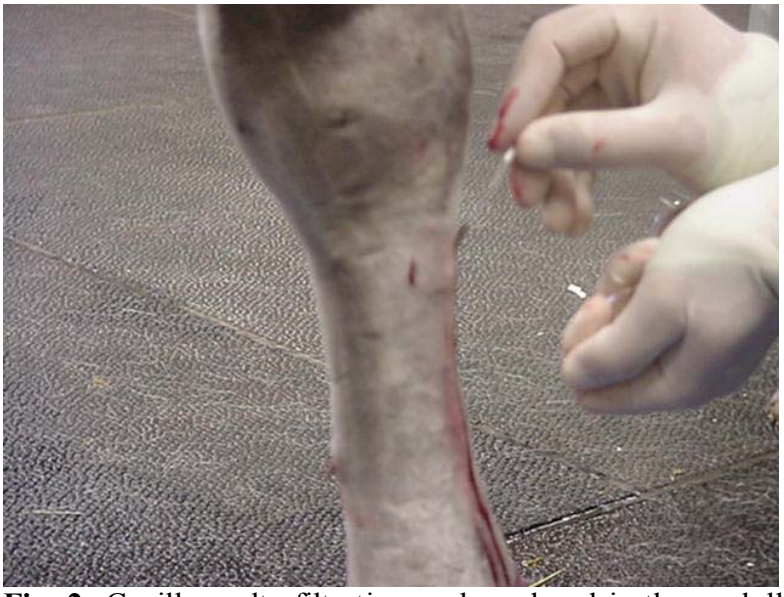

Fig. 2: Capillary ultrafiltration probes placed in the medullary cavity and subcutaneous tissue into and over the third metacarpal bone, respectively.

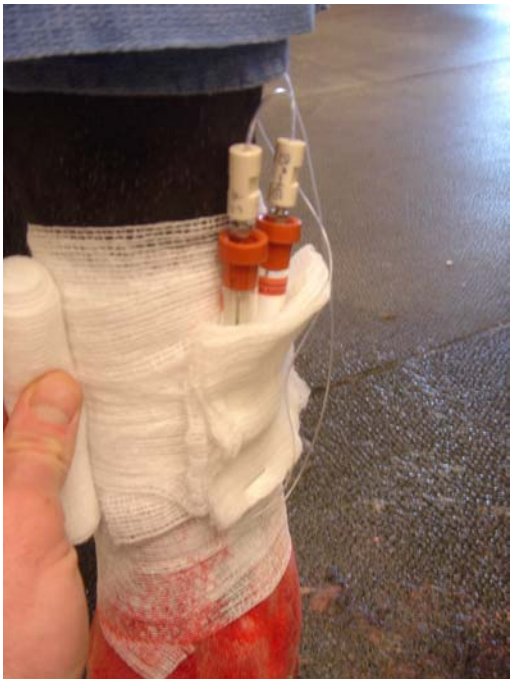

Fig. 3: A vacutainer attached to the ultrafiltration probe was used to create negative pressure necessary to collect fluid from the bone marrow cavity and subcutaneous space.

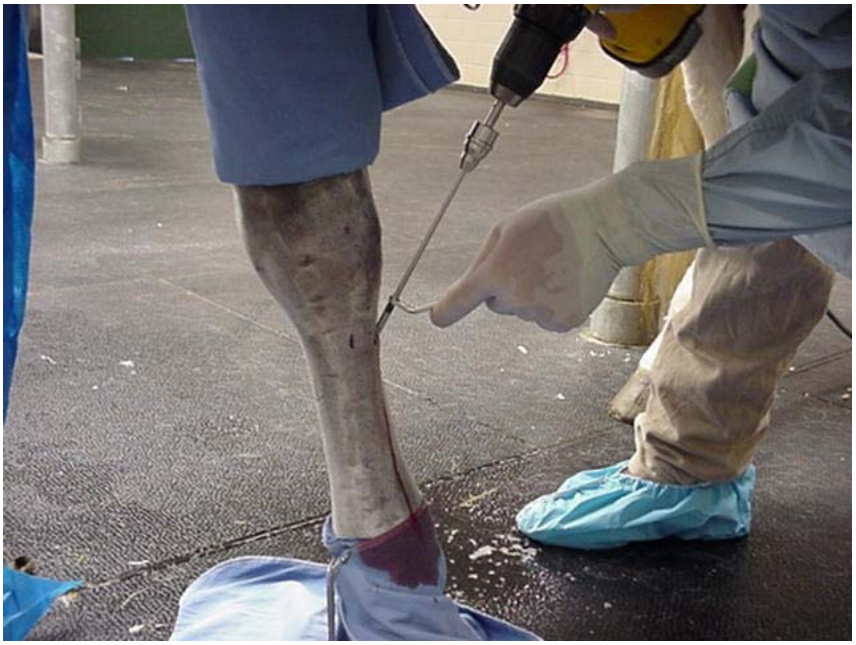

Fig. 4: Access to the medullary cavity was achieved by drilling a 4.5mm bicortical hole of approximately 70mm in length in a proximo-lateral to distal-medial direction at a 60-70 degree angle to MCIII

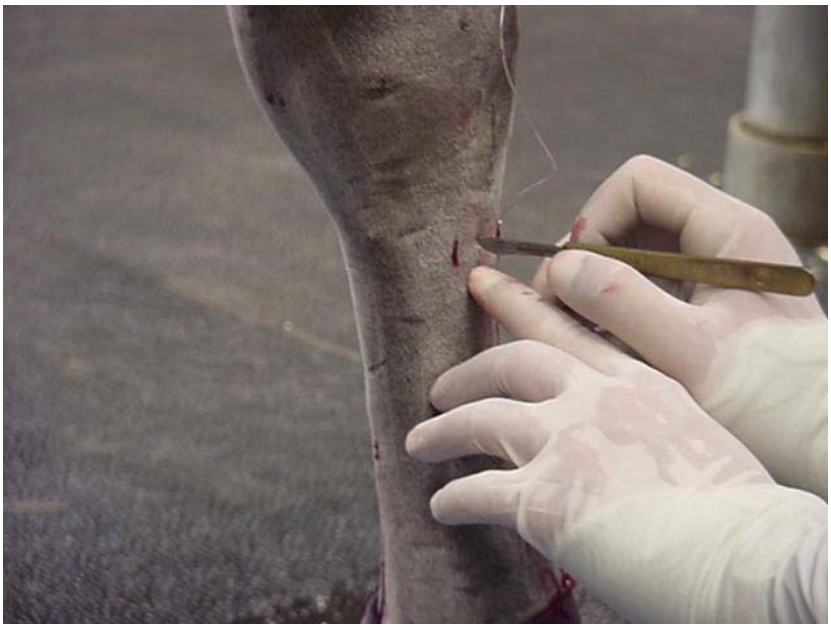

Fig. 5: A second ultrafiltration probe was placed dorsolaterally or dorsomedially in the subcutaneous tissue, approximately 3cm distal to the first ultrafiltration probe and parallel to MCIII. A 2 cm stab incision was made through the skin and subcutaneous tissue and the hollow, stainless steel cannula was introduced from proximal to distal into the subcutaneous space

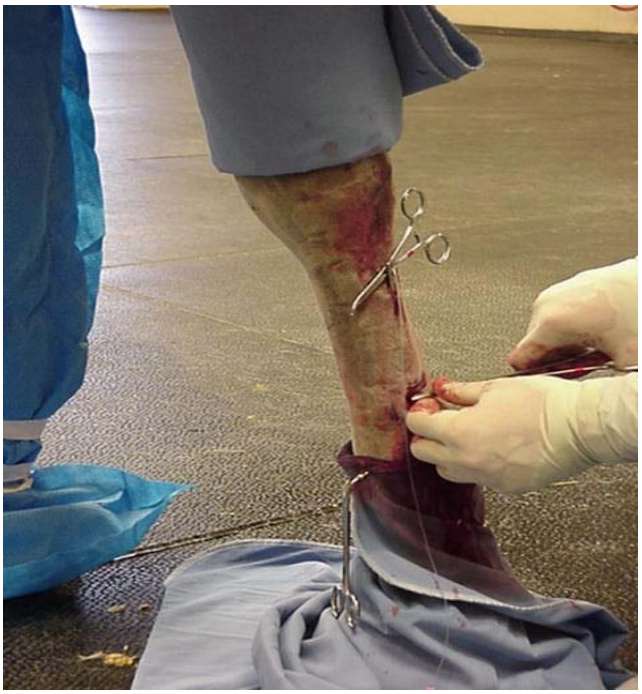

Fig. 6: A second incision was made over the distal tip of the cannula and the ultrafiltration probe was inserted using the same technique as described for the medullary cavity probe

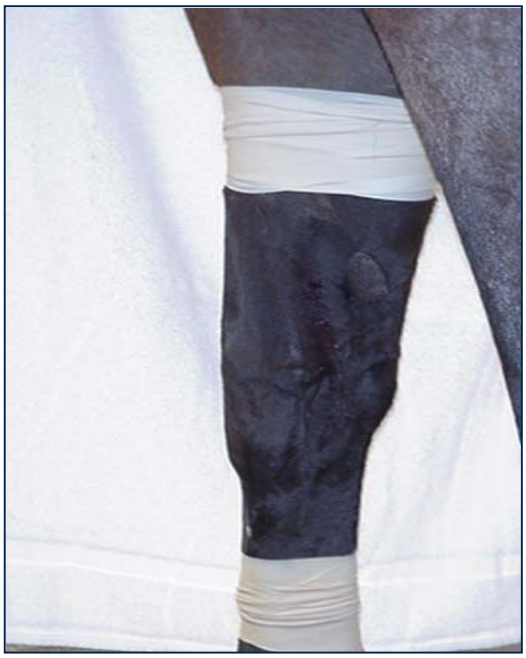

Fig. 7: An Esmarch bandage tourniquet five inches wide was placed 25-30 cm above the accessory carpal bone and a second tourniquet was placed at the level of the metacarpophalangeal joint

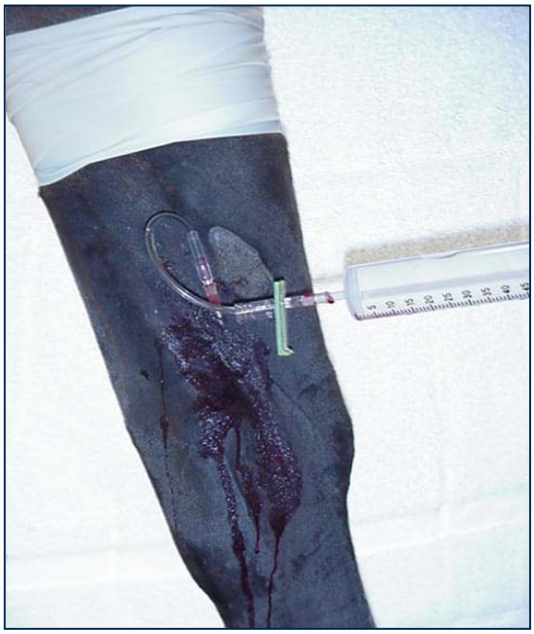

Fig. 8: After aseptic preparation, a 20-gauge, 2" catheter was introduced into the cephalic vein and a 7" IV extension set was attached to the catheter to assist during perfusion

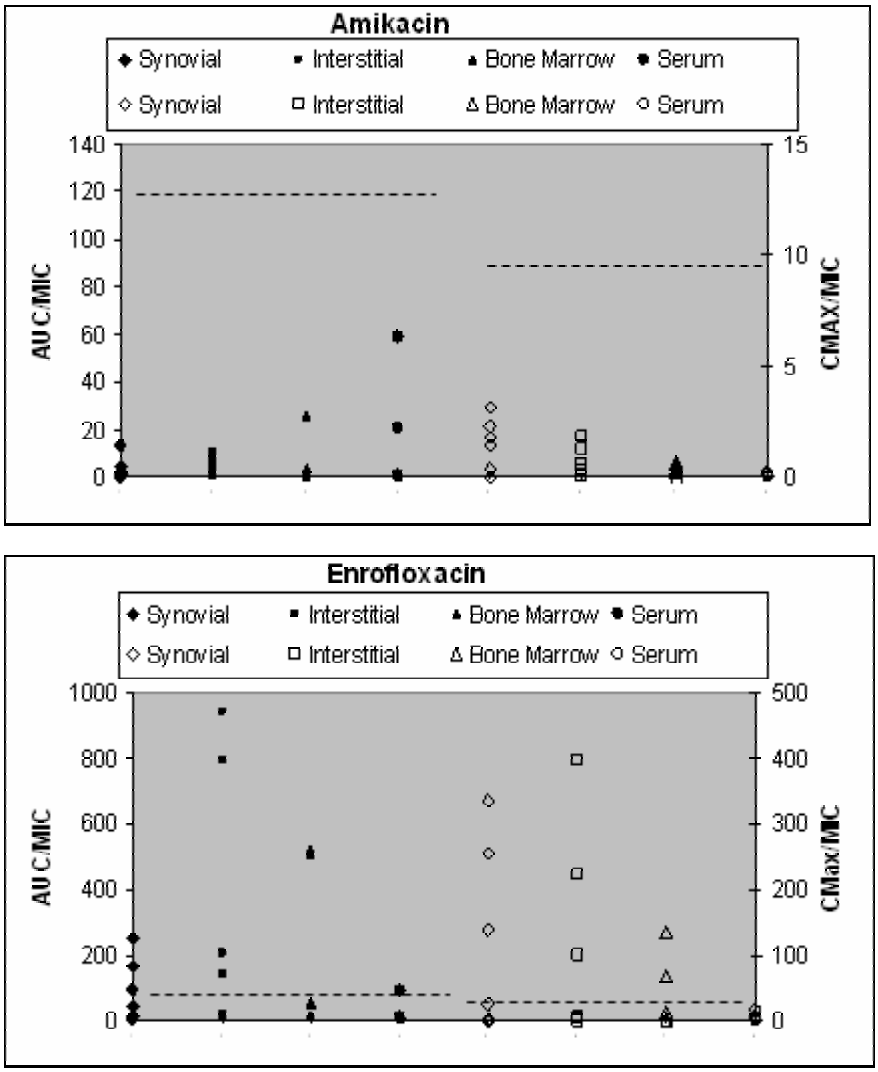

Fig. 9: AUC/MIC (left) and Cmax/MIC (right) achieved for each body tissue (n=4) in each horse (n=6) following administration of either amikacin (top; 250 mg) or enrofloxacin (bottom; 1.5 mg/kg) using standing regional limb perfusion. Hatched line indicates the target ratio for each indice (125 for AUC/MIC and 10 for Cmax/MIC). Susceptible breakpoints for each drug as established by CLASI ($\leq$ 16 mcg/ml for amikacin and 0.5 $\leq$ mcg/ml for enrofloxacin) were used for population pharmacodynamic (MIC) data. Notice the difference in scale values for each antibiotic.

TABLES

Time (hrs)	Subcutaneous tissue (ul)	Bone Marrow(ul)
0	61.4 ± 20.3	114.2 ± 62.6
0.5	87.1± 93.9	57.1 ± 18.8
1	178.5 ± 99.4	257 ± 123.9
4	314.2±157.3	335.7 ± 165.1
8	314.2 ± 121.2	335.6 ± 165.1
12	928.3±345.3	814.2± 409.9
24	800 ± 288.6	635.7 ± 430.8
36	928.5 ± 411.1	771.4 ± 394.6

Table 1 Volume of interstitial fluid collected at each measured time point for the ultrafiltration probes placed in the subcutaneous tissue and bone marrow of the third metacarpal bone of horses perfused with 250 mg of Amikacin.
Data is presented by Mean ± Standard deviation

		AMIKACIN			ENROFLOXACIN		
TISSUE	**Units**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**
Bone Marrow	**AUC (µg*hr/ml)**	62.96	28.30	419.50	25.37	5.43	262.81
Subcutaneous Tissue		108.74	28.36	176.99	70.76	5.26	469.50
Synovial Fluid		66.01	12.77	214.82	71.49	7.60	126.70
Serum		10.70	1.70	943.85	6.63	3.14	45.80
Bone Marrow	**Cmax (µg/ml)**	6.63	2.81	11.18	1.09	0.27	67.9
Subcutaneous Tissue		19.41	4.1	29.4	3.99	0.44	198.0
Synovial Fluid		26.27	5.7	50.0	13.22	0.25	167.9
Serum		1.95	1.19	3.57	0.70	0.58	8.7
Bone Marrow	**T1/2 (hrs)**	5.41	2.15	67.07	27.76	9.80	37.17
Subcutaneous Tissue		2.01	1.64	15.40	12.04	4.22	463.45
Synovial Fluid		2.04	1.06	10.59	13.24	7.08	90.0
Serum		4.47	0.77	269.64	6.76	5.12	16.99
Bone Marrow	**Tmax (hrs)**	4	1	4	1	0	4
Subcutaneous Tissue		4	1	4	1	1	4
Synovial Fluid		.25	0	1	0	0	4
Serum		.25	0	4	0	0	.5

Table 2 Pharmacokinetic values determined for 7 horses perfused with amikacin and 7 with enrofloxacin: Area under the curve (AUC), maximum concentration (C_{max}), half life of the drug (T1/2) and the time to reach peak concentration (T_{max}).

	AUC: MIC	Cmax : MIC
AMIKACIN	**Median (Min-Max)**	**Median (Min-Max)**
Bone Marrow	3.94 (1.77 - 26.22)	0.41 (0.17 - 0.70)
Subcutaneous Tissue	6.80(1.77 - 11.06)	1.23 (0.26 - 1.84)
Synovial Fluid	4.13(0.79 -13.43)	1.64 (0.36 - 3.10)
Serum	0.67(0.11 -58.99)	0.12 (0.07 - 0.22)
ENROFLOXACIN		
Bone Marrow	50.7 (10.8 – 525.5)	2.19 (0.54 -135.8)
Subcutaneous Tissue	141.5 (10.5. – 939.0)	7.96 (0.98 - 396.13)
Synovial Fluid	142.9 (15.21 – 253.4)	26.5 (0.5 - 335.95)
Serum	13.2. (6.2 – 91.5)	1.40 (1.16 - 17.4)

Table 3 Pharmacodynamic values for tissue concentrations after Regional Limb perfusion with amikacin and enrofloxacin. Cmax/MIC and the area under the inhibitory curve AUC/MIC were determined for all the tissue fluid samples. An MIC of 16µg/ml was used for amikacin and 0.5 µg/ml for enrofloxacin.