ELUTION OF ANTIBIOTICS FROM A NOVEL CROSS-LINKED DEXTRAN GEL: *IN VIVO* QUANTIFICATION

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(ABSTRACT)

Amikacin-, vancomycin- or amikacin/clindamycin-impregnated gel was placed subcutaneously in horses' necks a total of 6 times each. Interstitial fluid was collected at 0, 4, 8, 12 and 24 hours, and days 2 through 10, via capillary ultrafiltration probes placed within the incision (0cm) and 1.5cm laterally. Plasma or serum was collected at days 0, 1 and 7. Biopsy samples were obtained at the completion of the study. A histomorphologic score was assigned to each sample, and the differences in mean scores between treatment (gel) and control incisions were assessed using Wilcoxon signed rank test. Amikacin and vancomycin samples were analyzed via fluorescence polarization immunoassay; clindamycin samples were analyzed via high performance liquid chromatography. Concentrations greater than 1000 times the MIC of amikacin, and greater than 800 times the MIC of amikacin (amikacin/clindamycin gel) were obtained at 0cm. Mean concentrations remained above MIC for vancomycin and clindamycin for 10 days (0cm) and 8 days (1.5cm); for 9 days (0cm) and 7 days (1.5cm) for amikacin gel; and for 9 days (0cm) and 5 days (1.5cm) for amikacin (amikacin/clindamycin gel). Mean plasma amikacin and vancomycin concentrations were negligible; serum clindamycin concentrations were greater than MIC (0.52µg/ml and 0.63µg/ml) at 24 hours and 7 days respectively. There were no significant differences in histomorphologic scores between treatment and control incisions. Cross-linked dextran gel is a safe, effective alternative for local antibiotic delivery in horses, with substantially high local concentrations and minimal systemic absorption for amikacin- and vancomycin-impregnated gels.

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INTRODUCTION

Orthopedic and soft tissue infections are well-recognized problems in equine patients, and may be associated with high morbidity and mortality. The standard approach to these infections often involves aggressive surgical debridement, lavage, and a prolonged course of systemic antibiotics. Systemic delivery of antibiotics to an area of infection is often unpredictable and inefficient due to unreliable penetration into devitalized and/or ischemic tissue. Additionally, presence of such infections is often associated with bacteria growing in biofilms, rendering them up to 1000 times more resistant to antibiotic agents.[1,2] It is widely believed that microbial biofilms are largely responsible for the difficulty encountered in treating many infections with conventional antibiotic therapy. Recently, it has been shown that biofilms may exist in equine wounds, with *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* being the most common microorganisms found.[3]

Use of local antibiotic therapy when combined with aggressive debridement, with or without systemic administration of antibiotic agents, in the management of fractures and orthopedic infections in humans has resulted in significantly improved outcome, reduced hospital stay and decreased medical cost when compared to traditional systemic treatment.[4-7] Additionally, prophylactic use of local antibiotic therapy in soft tissue surgery has been reported,[8-10] with significant reduction in the incidence of infections caused by many major, clinically important microorganisms, and no increase in the occurrence of antibiotic-resistant microorganisms.[8] In horses and other large animals, the use of local antibiotic therapy has provided excellent outcomes when used in the treatment of orthopedic infections.[11-13]

The use of systemic antibiotics is not benign, and may be associated with multiple complications. Amikacin, vancomycin and clindamycin are highly effective antibiotics which are widely used in humans and small animals in the management of moderate to severe

infections; however, they may be associated with adverse effects in horses. Local antibiotic therapy is therefore an attractive choice, as it minimizes systemic antibiotic levels and potentially avoids the risks of adverse effects. Antibiotic-impregnated polymethylmethacrylate (PMMA) is widely used clinically in horses, however it has several disadvantages when compared to biodegradable local antibiotic delivery systems. Construction of the beads is exothermic, antibiotics may be incompletely released, and the precise mechanism of release is still unclear. Bacteria can colonize and grow in biofilms on antibiotic-impregnated PMMA,[14] and bacterial persistence and resistance is recognized in human patients.[15-17] Additionally, PMMA exhibits low biocompatibility due to the induction of a dose-dependent inflammatory and cellular immune response, and apoptosis of lymphoid and osteoblastic cells *in vitro*.[18-20]

Cross-linked dextran[a] is biocompatible, fully biodegradable, and non-immunogenic, and may therefore be a suitable choice for local antibiotic delivery. The gel has a wide range of applications in which it may be utilized, including management of soft tissue and orthopedic infections, and in surgical prophylaxis.

Assessment of local elution of each antibiotic can be achieved *in vivo* by utilizing a capillary ultrafiltration (UF) system.[21] The UF system is a minimally invasive, novel sampling method, allowing withdrawal of extracellular fluid and small molecules at low rates whilst excluding proteins and other cellular matter. Continuous small-volume sampling over prolonged periods of time is possible.[22] The technique was recently reported for evaluating local antibiotic concentrations in the distal limbs of horses after intravenous regional limb perfusion.[23]

The objective of the study was to measure the *in vivo* antibiotic concentrations from vancomycin-, amikacin- and amikacin/clindamycin-impregnated cross-linked dextran gel implanted subcutaneously in horses. Our hypothesis was that use of this gel containing

vancomycin, amikacin or a combination of amikacin and clindamycin would provide effective and predictable elution rates *in vivo*, with concentrations maintained above the MIC of common equine pathogens (4µg/ml for vancomycin; 4 µg/ml for amikacin and 0.5µg/ml for clindamycin) for a minimum of 7 days.

LITERATURE REVIEW

Orthopedic and Soft Tissue Infections in Horses:

Orthopedic and soft tissue infections, whether postoperative, traumatic, hematogenous or idiopathic in nature, are well-recognized problems in equine patients and can be associated with high morbidity and mortality. The reported rate of musculoskeletal infection in humans ranges from 0.8 – 40%, depending on surgical classification,[1,24] and cellulitis and soft tissue infections are estimated to occur in approximately 48 cases per 1000 population.[25] Surgical site infections (SSI) remain a major cause of morbidity and mortality in humans, occurring in up to 38% of surgical patients.[26] Surgical site infections in human patients may significantly increase the risk of readmission, treatment in an ICU, prolonged hospitalization, increased cost of treatment, and death.[26]

In 712 dogs and cats undergoing surgical procedures, surgical sites were classified as inflamed (minor infection) in 5.8% of cases, and infected (major infection) in 3% of cases.[27] This is similar to the overall rate of SSI in human patients[28] In this study, it was found that surgical sites classified as contaminated or dirty were significantly more likely to develop a surgical site infection. Additionally, antibiotic therapy was found to have a protective effect, with patients receiving antibiotics being on average 6 – 7 times less likely to develop SSI.

In horses, the overall postoperative infection rate in equine musculoskeletal surgery is reported to be 10%,[29] with an 8.1% versus 53% infection rate in surgical procedures classified as clean and clean-contaminated respectively. Horses in which surgical procedures were classified as clean-contaminated were 24.3 times more likely to develop a postoperative infection compared to those in which clean surgical procedures were performed. Postoperative abdominal incision infections in horses are often associated with high morbidity, with infection

rates of 10%[30] to 40%[31] reported. Overall outcome is significantly affected by infection, with horses developing incisional drainage being 17.8 times more likely to develop an incisional hernia.[32]

Microbiology of Orthopedic and Soft Tissue Infections:

In horses with musculoskeletal infections, the most common organisms isolated are the Enterobacteriaceae (primarily *Escherichia coli*, *Proteus* species, *Enterobacter* species and *Klebsiella* species), *Enterococcus* species, coagulase-positive staphylococci (*Staphylococcus aureus*), beta-hemolytic streptococci, coagulase-negative staphylococci (*Staphylococcus epidermidis*), *Pseudomonas* species, and anaerobes (*Clostridium* species most common).[33] However, the true incidence of anaerobic infection can often not be determined as anaerobic cultures are not performed in every case. In horses with a septic synovial structure, the etiology of infection (iatrogenic post-injection, penetrating wound, idiopathic or hematogenous in foals) appears to have an effect on the most likely microorganisms associated with the infection.[34] It is important to note that cultures may be negative in approximately 26% of cases,[34] despite other indicators of infection which may make treatment difficult in some cases because of unknown microbial sensitivities. A recent report summarized the most commonly isolated pathogens in horses, confirming that *Streptococcus equi* subspecies *zooepidemicus, S.aureus, E.coli, Corynebacterium pseudotuberculosis, P.aeruginosa,* beta-hemolytic streptococci and *Clostridium* species are most commonly isolated in musculoskeletal infections.[35] In small animals, the most common microorganisms in musculoskeletal infection are *Staphylococcus intermedius, Pasteurella* species, Enterobacteriaceae, *Acinetobacter* species, *Pseudomonas* species, *Enterococcus* species, *Escherichia coli,* and anaerobes such as *Prevotella* species and *Clostridium* species.[36,37]

Microbiology of soft tissue infections, in particular ventral midline celiotomy incisions, in horses is poorly reported. At the Marion duPont Scott Equine Medical Center, the most common organisms isolated from ventral midline celiotomy incisions between 2006 and 2008 were the Enterobacteriaceae (in particular *Enterobacter cloacae* and *E.coli*), *Staphylococcus epidermidis, Enterococcus* species, *Klebsiella* species and *S.aureus* (unpublished data).

Of particular concern is an increasing incidence of methicillin-resistant *Staphylococcus aureus* (MRSA). *S.aureus* is a well-recognized pathogen in both human and veterinary patients, can be associated with a wide range of infections, and is often resistant to multiple antibiotics.[38-41] One of these studies[40] showed that clinical infections attributable to MRSA were present or developed in 11.7% of horses in which MRSA was isolated from nasal swabs, with an incidence rate of nosocomial MRSA infection of 1.8 cases per 1000 cases. An MRSA infection rate of 4.8 cases per 1000 equine cases presented at a veterinary teaching hospital in Austria has also been reported.[41] Nosocomial infections with MRSA are believed to be acquired via transmission from veterinary personnel,[39,42,43] or contamination of the veterinary hospital environment.[44] Additionally, MRSA has been isolated via nasal swabs from horses admitted to a Belgian equine clinic, with 10.9% of horses carrying the microorganism.[45] A retrospective study evaluating MRSA infections in 115 horses,[46] revealed that clinical cases may present with a wide range of clinical signs, including colic, wounds and incision infections. A wide range of body systems may be affected including the skin, bones, joints, tendons, and lungs and the infections appear to be primarily opportunistic. The overall prognosis for survival to discharge appears to be good (83.8%); however, the rates of resistance to commonly used antibiotics can be quite high. Resistance to trimethoprim sulfate, gentamicin and tetracycline was reported in 71.4%, 84.4% and 92% of cases respectively.

Conversely, resistance to chloramphenicol, amikacin and enrofloxacin was reported in 2.4%, 6.2% and 8.1% of cases respectively.

Despite the potential clinical significance of infection, results of anaerobic cultures in soft tissue and orthopedic infections in equine patients are poorly documented. One study [47] revealed that 18% of equine specimens were positive for one or more anaerobes, with gram negative anaerobes such as *Bacteroides* and *Prevotella* predominating. Another study yielded obligate anaerobes in pure culture in 6.1% of cases,[48] with *Bacteroides* and *Fusobacterium* most commonly isolated. Additionally, it was shown that 24.5% of samples were polymicrobial, containing both obligate and facultative anaerobes. In human patients and small animal patients, anaerobic infections are not uncommon, and are a concern due to difficulties in treating these cases. Approximately 26 – 35% of clinical specimens in small animals have yielded obligate anaerobes, with polymicrobial infections of facultative anaerobes and aerobes commonly identified.[49,50] In humans, septic arthritis and osteomyelitis cases may yield obligate anaerobes in up to 77 – 90% of specimens, with 33 – 42% of infections being polymicrobial, with facultative anaerobes and/or aerobes concurrently isolated.[51] Surgical site infections may also be polymicrobial in nature, with obligate anaerobes isolated in up to 55% of cases in one study.[52]

Obligate anaerobes are difficult to isolate from infectious sites, and are often overlooked as a potential source of infection;[51,53,54] however, it is clear from many studies that obligate anaerobes may play an important role in many different types of infections. Treatment of infections in which anaerobes may be found can be complicated by lack of isolation allowing antibiotic susceptibility testing, slow growth of the organisms, the often polymicrobial nature of these infections, and growing resistance of obligate anaerobes to some antibiotic agents.

Biofilm formation is of significant concern due to the problems encountered in managing such cases, and may be largely responsible for the difficulty encountered in treating many infections with conventional antibiotic therapy. A microbial biofilm is composed of adherent microorganisms (sessile) within a polymeric matrix typically comprising exopolysaccharides with interdigitating open water channels.[55,56] The diversity of microorganisms within a biofilm can be quite extensive, and a number of organisms associated with potentially serious infections have been shown to grow in biofilms, including *S.aureus* (including MRSA), *E.coli* and *Pseudomonas aeruginosa.*[57] Additionally, it has recently been shown that biofilms may exist in equine wounds, with *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* being the most common microorganisms isolated.[3] The possibility therefore exists that chronic infections in equine patients may be attributed, at least in part, to biofilm formation. In humans, a major concern regarding biofilm formation has been associated with orthopedic implants and other medical devices such as vascular grafts and intravenous catheters. However, it is now recognized that many chronic infections not related to medical devices may be related to either bacteria not growing and remaining relatively dormant, or growing slowly as biomasses or adherent biofilms on mucosal surfaces.[55] Conditions that elicit a slowing of bacterial growth, such as nutrient limitation or build-up of toxic metabolites, are conducive to the formation of biofilms.[57] Growth of microorganisms within a biofilm (sessile) has been shown to increase the resistance to antibiotics compared to cultures grown in suspension (planktonic) by up to 1000 times.[55,58] However, when sessile bacteria are removed from a biofilm environment, grown *in-vitro* in a conventional planktonic culture, and exposed to antibiotic agents, they become susceptible.[55] A number of mechanisms are proposed for this increased resistance.[55-59] Firstly, lack of antibiotic penetration in to the biofilm may be related to the presence of a glycocalyx (complex hydrophilic polymer) coating,

which is typically anionic. Antibiotics may react with this glycocalyx or become adsorbed, which provides a barrier to antibiotic penetration. Secondly, a relatively large amount of antibiotic-inactivating enzymes may exist within the glycocalyx, providing additional protection to the microbes within the biofilm. Thirdly, it has been shown that oxygen penetrates to only approximately the outer 25% of the depth of a biofilm, resulting in anaerobic areas within the depths of a biofilm and consequently microbes become inherently resistant to aminoglycosides in particular. Lastly, the bactericidal and bacteriostatic mechanisms of many antibiotics are growth-dependant. There is a significant decrease in the growth rate of microorganisms within a biofilm compared to planktonic cells, which results in decreased uptake and decreased function of many antibiotic agents.[57]

The Use of Systemic Versus Local Antibiotic Therapy:

The standard approach to musculoskeletal and soft tissue infections in horses often involves surgical debridement, lavage, and a prolonged course of systemic antibiotics. Systemic delivery of antibiotics to an area of infection (bone, joints, or soft tissue) is often unpredictable and inefficient due to unreliable penetration of antibiotics into devitalized and ischemic tissue. These factors, combined with the potential presence of bacteria growing within a biofilm, may result in an increased minimal inhibitory concentration (MIC) of the infecting microorganisms and therefore inherently increase resistance to systemically administered antibiotic agents. Many of the antibiotics used in hospitals to treat such infections have a narrow therapeutic window – the need for high doses to achieve therapeutic levels in infected tissues may ultimately result in an increased risk of systemic toxicity. Local antibiotic therapy is therefore an attractive alternative, as it is able to provide high local tissue concentrations with minimal systemic absorption.

There is much interest in the use of local antibiotic therapy in the management of human orthopedic and soft tissue infections. Experimental animal models have shown that the use of locally applied antibiotic agents is effective in reducing microbial counts and rate of infection compared to control and systemic antibiotic groups.[9,60] Additionally, in an experimental model evaluating the use of local delivery of enrofloxacin versus intra-muscularly administered enrofloxacin in a swine model,[61] the authors found that locally administered enrofloxacin significantly decreased the risk of resistance of fecal *E.coli* to enrofloxacin, ciprofloxacin, ampicillin and trimethoprim-sulfonamide. The authors therefore concluded that the use of locally administered antibiotic agents represents a therapeutic advantage to the long-term administration of parenterally administered antibiotics.

The use of local antibiotic therapy when combined with aggressive debridement with or without systemic administration of antibiotic agents in the management of fractures and orthopedic infections in humans has resulted in improved outcome, reduced hospital stay and decreased medical cost when compared to traditional systemic treatment.[4-7,62] Additionally, significant decreases in the rates of development of acute wound infections and chronic osteomyelitis postoperatively in humans with compound fractures have been reported.[4,6,7,62,63] In these reports, a positive effect was seen in all fracture types, however the most significant response was seen in those with severe soft tissue damage and gross contamination or impaired vascularity, where it is expected that systemic antibiotic penetration may be inadequate. Prospective and experimental trials evaluating the use of local antibiotic therapy and the rates of development of postoperative incision infections in soft tissue surgery also support the prophylactic use of local antibiotic therapy.[8-10,64-67] All of these studies revealed that the incidence of incision infections was decreased when locally applied antibiotic agents were used alone or in combination with systemic antibiotics, compared to the use of systemic prophylaxis

alone. Local antibiotic application in postoperative incisions not only significantly reduces the incidence of infections caused by many major, clinically important microorganisms, it has also been shown that there is no indication of an increase in the occurrence of antibiotic-resistant microorganisms in patients treated with local antibiotics.[65]

In horses and other large animals, the use of local antibiotic therapy has been reported in the treatment of orthopedic infections[11-13,68-70] and likely improved the outcome of these cases. In equine patients, the most widely used and available substrate for local antibiotic therapy is polymethylmethacrylate (PMMA).

The use of systemic antibiotics can be associated with the risk of organ toxicity. Amikacin, vancomycin and clindamycin may all be associated with adverse effects in horses. Of particular concern in horses is the development of nephrotoxicity associated with administration of aminoglycosides, one of the most commonly administered classes of antibiotic agents, and considered first-choice antibiotics in the treatment of moderate to severe infections.[71] The potential development of nephrotoxicity in equine patients is of particular concern due to the often concurrent administration with other potentially nephrotoxic drugs such as non-steroidal anti-inflammatory drugs (NSAIDs). Additionally, aminoglycosides may result in ototoxicity (which is not of concern in horses, but may be irreversible in humans), and neuromuscular blockade (which is of primary concern due to possible respiratory depression during general anesthesia).[72]

Vancomycin is not commonly administered systemically in horses due to the risks of toxicity In humans, a wide range of adverse reactions have been reported.[73,74] The most common adverse reaction is “red man syndrome” characterized by hyperemia of the upper body and pruritis due to histamine release. Other side effects include ototoxicity and

nephrotoxicity (which are exacerbated when vancomycin is administered concurrently with an aminoglycoside), neutropenia, fever, phlebitis, thrombocytopenia and, rarely, pancytopenia.[74] However, systemic administration of vancomycin in horses has been reported, and no adverse effects were seen.[75]

Clindamycin is widely used in human patients; however systemic administration in adult horses is not recommended due to the risk of bacterial overgrowth, especially *Clostridium difficile*, in the large colon resulting in a potentially fatal pseudomembranous colitis.[72,76] In humans, antibiotic exposure is the greatest risk factor for development of *Clostridium difficile*-associated diarrhea and colitis; the most common antibiotics implicated included clindamycin.[77] Approximately 8 – 10% of human patients administered clindamycin were reported to develop a severe pseudomembranous colitis caused by a toxin secreted by clindamycin-resistant *C.difficile.* Any antibiotic agent may potentially cause enterocolitis, and horses are especially prone to the development of antibiotic-associated enterocolitis because of poor oral absorption resulting in large concentrations of active antibiotic within the gastrointestinal tract.[71] Additionally, for antibiotic agents with good oral bioavailability, there is still a risk associated with biliary excretion and enterohepatic recycling.[71]

Therefore, local delivery of antibiotics that should not be administered systemically in horses allows utilization of highly effective antibiotics without the risk of toxicity. Higher local concentrations may be achieved; therefore, bacteria resistant to the lower serum antibiotic concentrations may be sensitive at the higher local concentrations. This is important when evaluating standard reporting of antibiotic sensitivity and resistance patterns, as these may not be clinically accurate for microorganisms treated via locally administered antibiotics.

Use of Biodegradable Local Antibiotic Delivery Devices:

The polymethylmethacrylate (PMMA)-based bead has been widely used clinically and experimentally in humans and animals. Polymethylmethacrylate is a non-biodegradable, high-density plastic formed by mixing a fluid monomer with a powdered polymer, into which antibiotics may be incorporated.[78] However, PMMA has several disadvantages when compared to biodegradable local antibiotic delivery systems.[11-13,78,79] Often a second surgery is required for removal; this second surgery can result in local soft tissue damage associated with surgical dissection, and may result in the formation of dead space. The beads often become surrounded by a granulation or fibrous tissue capsule,[11-13,80,81] which has been proposed to be due to a non-specific foreign body reaction to the PMMA.[81] In some cases, the PMMA beads may not be able to be removed due to the degree of fibrous tissue capsule formation.[11] All of these factors may predispose to complications associated with the incision or wound.

Construction of the beads is exothermic, and consequently, only antibiotics that are heat-stable can be incorporated into PMMA beads. A proportion of the antibiotics incorporated into PMMA is released to some extent, however the precise mechanism by which the antibiotics are released is still unknown. Studies using methylene blue and gentamicin diffusion through or into PMMA show that the bulk of the beads are essentially impermeable.[82] In vivo studies have shown that as little as 5 – 18% of gentamicin incorporated in to the PMMA may be eluted over a prolonged period of time, which results in uncontrolled release of antibiotics.[78,83]

Importantly, the presence of PMMA itself within an area of infection, and the resultant prolonged release of sub-therapeutic levels of antibiotic from PMMA, may result in increased risk of bacterial persistence and antibiotic resistance. Recent *in vitro* reports have emphasized

that bacteria can colonize, and even grow in biofilms on, antibiotic-impregnated PMMA.[14,83] The slow release of antibiotics from the PMMA, when left *in situ*, is an efficient way to induce and/or select for resistance and the formation of small colony variants.[16] A recent report showed that PMMA may release measurable concentrations of gentamicin for up to 5 years after implantation,[16] and infectious bacteria identified as gentamicin-resistant coagulase-negative staphylococci were recovered from the surfaces of these beads. Antibiotic-impregnated PMMA beads retrieved from surgical patients with orthopedic infections and subjected to in-depth microbiologic analyses have been found to have persistence of bacterial growth and emergence of gentamicin-resistant bacteria (despite preoperative gentamicin susceptibility).[15,17,84] The persistence of these bacteria and growth in biofilms may predispose such patients to recurrent infections.

PMMA exhibits low biocompatibility due to the induction of a dose-dependent inflammatory and cellular immune response,[20,85,86] apoptosis of lymphoid and osteoblastic cells has been demonstrated *in vitro,*[18,87] and inhibition of DNA synthesis and cell growth.[79] Particles of PMMA bone cement have been found in fibrous pseudomembranes surrounding aseptically loose implants, the pathogenesis of which has been linked to the presence of tissue macrophages releasing inflammatory cytokines.[19] PMMA results in early activation of a cytokine cascade, with significantly increased release of TNF-α, IL-6 and IL-1β in vitro, and subsequent lymphocyte proliferation, resulting in an inflammatory response.[19] These inflammatory changes have been associated with stimulation of increased bone resorption (via activation of osteoclasts) and decreased bone formation (via decreased proliferation of osteoblasts).[19,88,89] Compared to other implanted biomaterials (sapphire, titanium, cobalt-chromium-molybdenum alloy), PMMA was shown to result in the greatest tissue reaction, with

dense inflammatory granulation tissue surrounding the implanted PMMA by 7 days, and the greatest capsular thickness.[88]

Experimental evaluation of gentamicin-impregnated PMMA beads in the tarsocrural joints of horses[81] showed that implantation resulted in non weight-bearing lameness in 3/5 treated horses, a significant increase in the joint circumference and synovial fluid total protein in treated horses, and at least mild superficial articular cartilage erosion and thickening of the joint capsule. In clinical cases of orthopedic infection in horses,[11-13] complications associated with PMMA bead implantation have been encountered. These included signs of pain associated with bead implantation (which was confirmed by resolution of lameness when the beads were removed); and breakage of surgical wire or suture during attempts to remove the beads. This was further hampered by the development of thick granulation tissue or fibrous tissue around the beads in multiple cases, and resulted in beads not being able to be removed in one horse.

Cross-linked dextran is biocompatible, fully biodegradable, and non- immunogenic, and may therefore be a suitable choice for local antibiotic delivery. This gel has previously been evaluated experimentally; pathologic changes after implantation in rodent brains were assessed, and it was found to result in no changes compared to saline.[90] Initial evaluation of safety via cytotoxicity studies and skin irritation studies in rabbits revealed no evidence of cell lysis or toxicity, and no evidence of erythema or edema. Initial *in vitro* assessment of elution of antibiotics from the gel suggests they elute for at least 14 days (the end time point of the studies).[91] The cross-linked dextran gel is a novel method of local antibiotic delivery; the product is supplied in a kit that includes two syringes. The first contains oxidized dextran solution; the second syringe contains a cross-linking reagent plus the active ingredient, antibiotic(s) in this case, as solids. The syringes are then connected and the contents are mixed

by reciprocation for approximately 2 – 3 minutes until a viscous gel is formed. This gel is then injected in to the area to be treated, where it sets up within 5 minutes and has a residence time of approximately 3 – 4 weeks. The mechanism of degradation is hydrolysis of the cross-links, followed by enzymatic degradation of the polymer. The gel has a wide range of applications in which it may be utilized, including both soft tissue (such as ventral midline incision infections and other draining tracts) and orthopedic infections. Additionally, there is the potential for prophylactic use as well, especially in ventral midline incisions and fracture repairs.

The perfect local antibiotic delivery system is not available at this time. However, several criteria for local antibiotic delivery agents have been suggested.[56,92,93] It has been proposed that an antibiotic delivery system should be active against the most common bacterial pathogens involved in a particular infection; be locally released at concentrations several times (usually 10 times) the MIC of common pathogens; relatively inert (and not provoke adverse effects); be retained locally; and be stable at body temperature. Biodegradable carriers for local antibiotic delivery offer several advantages over PMMA and systemic administration. Biodegradable delivery vehicles can provide bactericidal concentrations of antibiotics, with potentially altered biodegradability; of the carrier is removed, and sub-therapeutic concentrations of antibiotic cannot be released (which prevents the potential development of resistance). There is no need for a second surgery to remove the carrier, with subsequent requirement for soft tissue reconstruction, and biodegradable carriers are biocompatible, non-immunogenic, and non-toxic.

Antibiotic Choice

Recent studies in humans have evaluated the patterns of antibiotic susceptibility/ resistance in commonly isolated pathogens, revealing that amikacin and vancomycin are among the most effective antibiotics. In horses, the most effective antibiotic against a wide

range of bacteria appears to be amikacin.[33] However, of primary concern in utilizing either of these choices is the development of methicillin-resistant *S. aureus* (MRSA) in human, equine and small animal hospitals, the emergence of vancomycin-resistant enterococci, and an increasing occurrence of multi-resistant organisms.

Vancomycin is a narrow-spectrum bactericidal antibiotic active at clinically achievable levels against most species of Gram-positive cocci and bacilli, including many resistant staphylococci and enterococci.[75] It inhibits synthesis of the linear peptidoglycan in bacterial cell walls during replication, which results in rapid cellular death.[76] It is effective against a wide range of microorganisms, including most strains of MRSA and *Enterococcus.* Resistance has been impeded by high specificity of the drug, as multiple mutations would be required to change the enzymes currently targeted by vancomycin;[94] however, it has been reported. Vancomycin is considered a treatment of choice for methicillin-resistant *S.aureus,*[95] and has been shown to penetrate biofilms formed by *Staphylococcus epidermidis*, where higher concentrations of vancomycin were associated with lower bacterial counts in biofilm.[96] Vancomycin has been evaluated experimentally in horses, where it was administered via intravenous[97] or intraosseous[98] regional limb perfusion; no complications developed during the procedures and the treatment resulted in high local concentrations of vancomycin. Additionally, vancomycin has been used successfully systemically in horses, in the management of severe orthopedic and soft tissue infections.[75] Vancomycin was used either alone or in combination with an aminoglycoside, as dictated by culture and sensitivity results and was considered to be safe and effective for the treatment of resistant staphylococcal or enterococcal infections in horses and foals. Vancomycin-impregnated PMMA has been previously reported in the successful treatment of a methicillin-resistant *Staphylococcus*

epidermidis which was susceptible to vancomycin.[68] Vancomycin-impregnated PMMA beads and cross-linked dextran gel have been used successfully at the authors' hospital.

Amikacin is a broad-spectrum bactericidal aminoglycoside antibiotic, which is composed of an amino sugar linked through glycosidic bonds to an amino cylitol.[94] Aminoglycosides must be actively transported into bacterial cells, which is an oxygen-dependant process. Once in the cells, aminoglycosides bind to the 30S and 50S ribosomal subunits, resulting in impaired protein synthesis. Aminoglycosides have a moderate spectrum of activity, including most aerobic Gram-negative bacteria and select Gram-positive bacteria (especially staphylococcal species). Amikacin may be less susceptible to aminoglycoside-deactivating enzymes when compared to either gentamicin or tobramycin, and is effective against some gram negative bacilli that are resistant to these aminoglycosides.[99] Because of the requirement for oxygen-dependent movement of amikacin in to bacterial cells, anaerobes are inherently resistant.[94] Adverse effects of aminoglycosides primarily include nephrotoxicity, ototoxicity and neuromuscular blockade. Amikacin has previously been reported to be one of the most effective antibiotics available in the treatment of orthopedic infections in horses, and was highly (>90%) effective against coagulase positive staphylococci, Enterobacteriaceae and *Pseudomonas.*[33,34] The use of amikacin-impregnated PMMA beads in the treatment of orthopedic infection in horses has previously been reported, with excellent results.[13,68] Amikacin-impregnated PMMA beads and cross-linked dextran gel have been used in the authors' hospital.

Clindamycin is a semi-synthetic lincosamide antibiotic which inhibits protein synthesis at the 50S ribosomal subunit.[76] Clindamycin exhibits a broad-spectrum of antibiotic activity, including aerobic Gram-positive and anaerobic bacteria, Gram-negative anaerobes, and select mycoplasmas and some protozoa.[100,101] Clindamycin is considered to be bacteristatic, however

in this application (local antibiotic therapy), much higher local tissue concentrations are expected compared to what would be possible with systemic administration, resulting in bactericidal effects.[72] Clindamycin is primarily used in small animal and human medicine in the treatment of skin infections, osteomyelitis and periodontal disease; it is not used systemically in equids because of the risk of bacterial overgrowth and development of a fatal necrotizing enterocolitis. Clindamycin is one of three markedly immune-enhancing antibiotics.[101,102] It results in decreased bacterial adhesion to mucosal surfaces and infected bone; decreases glycocalyx formation by *S.aureus*; and facilitates chemotaxis, opsonization, phagocytosis and bactericidal activity by human and animal polymorphonuclear leukocytes. Clindamycin is rapidly taken up by neutrophils, and peak concentrations in neutrophils is approximately 40 times the extracellular concentrations; additionally, antibiotic activity is maintained within the neutrophils.[103] Exposure to clindamycin inhibits toxins produced by β-hemolytic streptococci, *C.perfringens*, and *S.aureus;,* and also inhibits production of pro-inflammatory cytokines such as TNF-α, IL-1β and inducible nitric oxide synthase by human and animal macrophages. Clindamycin also enhances the production of protective cytokines such as IL-6. Clindamycin has been shown to be effective against common anaerobic pathogens (*Bacteroides* sp, *Fusobacterium* sp, *Clostridium* sp, and *Peptostreptococcus* sp) with susceptibility patterns ranging from 85% to 100% in one study[104] and 78% to 100% in another study.[105] In humans, approximately 96% of anaerobic bacteria isolated in clinical practice are susceptible to easily achieved clindamycin levels.[53]

Clindamycin is frequently used in combination with an aminoglycoside (typically amikacin or gentamicin) for the treatment of mixed aerobic-anaerobic infections.[53,106] Combinations of clindamycin with an aminoglycoside (either gentamicin or amikacin) have been evaluated experimentally, and have shown synergism against resistant anaerobic cocci

and clostridial species.[107,108] When gentamicin and clindamycin were combined in a bone cement, the combination was more effective at preventing biofilm formation compared to gentamicin alone.[109] Importantly, in this study, the presence of clindamycin did not affect the gentamicin release profile. Therefore, in suspected mixed aerobic-anaerobic infections, which are fairly common in veterinary practice, a combination of clindamycin and amikacin may be a highly effective means of managing these infections.

Use of Capillary Ultrafiltration Probes for Collection of Interstitial Fluid:

The capillary ultrafiltration system[b] provides a minimally invasive, novel sampling method, allowing withdrawal of extracellular fluid and small molecules at low rates whilst excluding proteins and other cellular matter. This alternative method can provide continuous small-volume sampling over prolonged periods of time,[22] with collection times of up to 1 to 6 months reported.[110] In ultrafiltration, a negative pressure gradient across a semi-permeable, hollow, hydrophilic membrane with a molecular weight cut-off of 30,000 Daltons causes solvent (water) and small molecules to pass through the membrane.[21,110] The membrane excludes proteins and other cellular matter, while allowing extracellular fluid and small molecules or ions to pass through, creating an ultrafiltrate of tissue fluid. A slow, pulse-free and highly regular movement of the ultrafiltrate is achieved by constant negative pressure (provided via Vacutainers[c]).[111] The rate of fluid collection is typically quite low (1 – 5µL/minute), and the samples are essentially sterile.[21,112] The use of capillary ultrafiltration probes in the measurement of tissue concentrations of systemically administered antibiotics has previously been reported in dogs.[113,114] Recently, successful use of capillary ultrafiltration probes in the distal limbs of horses has been reported, where local concentrations of antibiotics were evaluated after regional limb perfusion.[23] It was concluded that capillary ultrafiltration

probes allowed the authors to successfully evaluate the in vivo pharmacokinetic and pharmacodynamic data of interstitial fluid following intravenous regional limb perfusion.

Measurement of Antibiotic Concentrations:

Vancomycin and amikacin concentrations were analyzed using Fluorescence Polarization Immunoassay (FPIA), and clindamycin concentrations will be analyzed using High Performance Liquid Chromatography (HPLC). FPIA is an accepted analytical method to evaluate concentrations of aminoglycosides, and provides a rapid and reliable method for the therapeutic monitoring of these antibiotics.[115] Automated fluorescence polarization immunoassay (TDx Analyzer)[d] is a competitive binding immunoassay.[116] Briefly, tracer-labeled antigen (fluorescein-labeled antigen) and patient antigen compete for binding sites on antibody molecules. After steady state is reached, the samples are exposed to polarized light (481 to 489 nm) and the net change in fluorescence and light attenuation of the samples is determined. The final fluorescence intensity of the solution is inversely proportional to the amount of chromagen (reagent-analyte complex) formed. Therefore, if a sample contains low concentrations of drug, a small amount of chromagen is produced and polarization is high. Using polarization values generated for each sample in an assay, concentrations of a drug in unknown samples are calculated using calibration curves. Comparison of FPIA with well-established monitoring methods Enzyme Multiplied Immunoassay (EMIT) and Radioimmunoassay (RIA) has indicated an extremely good analytical correlation.[117] Additionally, FPIA offers significant advantages in calibration and reagent stability, and greater sensitivity in the low drug concentration ranges, while maintaining accuracy and precision comparable to those of EMIT and RIA.[117] The Abbott TDx FPIA Analyzer is a totally automated, bench-top fluorescence polarization analyzer, where all processing is carried out in a light-tight, temperature-controlled environment. Together with the great stability of the

FPIA reagents, these factors allow reproducible standard curves to be obtained. FPIA is therefore the analytical method of choice for assessing aminoglycoside concentrations,[118] and is routinely used for therapeutic drug monitoring. The use of this assay for determination of amikacin and vancomycin concentrations in samples from horses has been previously reported.[23,97,98] The standard assay procedure can be modified to increase assay precision to avoid carryover of amikacin and vancomycin from samples of higher concentrations to those of lower concentrations.[119,120]

All samples from the in vivo studies, as well as plasma samples were analyzed by high pressure liquid chromatography (HPLC) using a method developed in the Clinical Pharmacology Laboratory at North Carolina State University.[121] Reference standards for the compound clindamycin hydrochloride were purchased from the United States Pharmacopeia[e]. Clindamycin hydrochloride is 92% clindamycin base and corrections were made when weighing out reference standard to make stock solutions for calibration. Clindamycin HCl was dissolved in distilled water (HPLC grade water) to a concentration of 1 mg/mL clindamycin. From the stock solution, further dilutions were made in distilled water to make up fortifying solutions for serum and to phosphate buffered saline (PBS) solutions to prepare quality control samples, calibration curve samples, and for development of these methods. The stock solution was kept at 4ºC in a tightly sealed dark vial. The stock solutions were added to blank (control) serum or PBS plasma, to make up 9 calibration standards, including zero (range 0.0 µg/mL to 10 µg/mL for serum and up to 20 µg/mL for PBS).

The mobile phase for HPLC analysis consisted of acetonitrile, and 0.05 M potassium phosphate buffer solution, which was modified with 1 N potassium hydroxide to a pH of 5.0. Composition of mobile phase was 30% acetonitrile and 70% buffer (v/v). Fresh mobile phase

was prepared, filtered (0.45 μm), and degassed for each day's run. The laboratory used guidelines and standards published by the United States Pharmacopeia.

The HPLC system consisted of a quaternary solvent delivery system, at a flow rate of 1 mL/min, an autosampler,[f] and UV detector set at a wavelength of 200 nm. The chromatograms were integrated with a computer program.[g] The column was a reverse-phase, 4.6 mm x 15 cm C8 column kept at a constant temperature of 40° C. Retention time of the analyte was 4.9 to 5.0 min.

All incurred serum samples, calibration samples, and blank (control) plasma samples were prepared identically using solid-phase extraction. Solid phase extraction cartridges were conditioned with 1 mL methanol followed by 1 mL distilled water. A serum sample of 500 μL was added to a conditioned cartridge, followed by a wash step of 1 mL potassium phosphate buffer. The drug was eluted with 1 mL 100% methanol and collected in clean glass tubes. The tubes were evaporated at 40° C for 15-20 minutes. Each tube was then reconstituted with 200 μL of mobile phase and vortexed. Fifty μL of each tube was then injected into the HPLC system. A fresh set of calibration and blank samples were prepared for each day's run. All calibration curves were linear with a r^2 value of 0.99 or higher. Limit of quantification for each of the clindamycin in serum samples was 0.05 μg/mL, which was determined from the lowest point on a linear calibration curve that produced an appropriate signal-to-noise ratio. The samples in PBS were diluted appropriately so that the concentrations fell within the upper and lower limits of the calibration curve. Samples were filtered to remove any debris or particles, diluted (if necessary) and injected directly into the HPLC system.

FOOTNOTES

a. Royer Biomedical Inc., Frederick, Maryland, USA.

b. Bioanalytical Systems Inc., Baltimore, Maryland, USA.

c. Vacutainer serum, 4.0mL, BD, Franklin Lakes, New Jersey, USA.

d. Abbott Laboratories, Abbott Park, Illinois, USA.

e. USP, Rockville, Maryland, USA.

f. 1100 Series Autosampler, Agilent Technologies, Wilmington, Delaware, USA.

g. Zorbax Rx-C8, MAC-MOD Analytical, Inc., Chadds Ford, Pennsylvania, USA.

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ELUTION OF ANTIBIOTICS FROM A NOVEL CROSS-LINKED DEXTRAN GEL: *In vivo* QUANTIFICATION

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ABSTRACT

Reasons for performing study: Use of a novel, biodegradable, antibiotic-impregnated gel provides an alternative method of local antibiotic delivery for treatment of musculoskeletal and soft tissue infections in horses.

Objective and Hypothesis: The objective was to measure *in vivo* antibiotic concentrations from vancomycin-, amikacin- and amikacin/clindamycin-impregnated cross-linked dextran gel when implanted subcutaneously in horses. The hypothesis was that use of the antibiotic-impregnated gel placed in an incision would provide effective concentrations above MIC of common equine pathogens *in vivo* for at least 7 days.

Methods: Amikacin-, vancomycin- or amikacin/clindamycin-impregnated gel was placed subcutaneously on either side of horses' necks in 6 sites each. Interstitial fluid was collected at 0, 4, 8, 12 and 24 hours, and days 2 through 10, via capillary ultrafiltration probes placed within the incision (0cm) and 1.5cm laterally. Plasma or serum was collected at days 0, 1 and 7. Biopsy samples were obtained at the completion of the study. A histomorphologic score was assigned to each sample, and the differences in mean scores between treatment (gel) and control incisions were assessed using the Wilcoxon signed rank test. Amikacin and vancomycin samples were analyzed via fluorescence polarization immunoassay; clindamycin samples were analyzed via high performance liquid chromatography.

Results: Concentrations greater than 2000 times the MIC of vancomycin and clindamycin, greater than 1000 times the MIC of amikacin, and greater than 800 times the MIC of amikacin (amikacin/clindamycin gel) were obtained at 0cm. Mean concentrations remained above MIC for vancomycin and clindamycin for 10 days (0cm) and 8 days (1.5cm); for 9 days (0cm) and 7 days (1.5cm) for amikacin alone; and for 9 days (0cm) and 5 days (1.5cm) for amikacin from the amikacin/clindamycin gel. Mean plasma amikacin and vancomycin concentrations were

negligible; mean serum clindamycin concentrations were greater than MIC (0.52µg/ml and 0.63µg/ml) at 24 hours and 7 days respectively. There were no significant differences in histomorphologic scores between treatment and control incisions.

Conclusions and Potential Relevance: Cross-linked dextran gel is a safe, effective alternative for local antibiotic delivery in horses, with substantially high local concentrations and minimal systemic absorption.

INTRODUCTION

Orthopedic and soft tissue infections can be associated with high morbidity and mortality in equine patients. The standard approach to these infections often involves aggressive surgical debridement, lavage and a prolonged course of systemic antibiotics. Systemic delivery of antibiotics to an area of infection is often unpredictable and inefficient due to unreliable penetration into devitalized and/or ischemic tissue. Additionally, such infections are often associated with bacteria growing in biofilms, rendering them up to 1000 times more resistant to antibiotic agents, and potentially resulting in recalcitrance to treatment.[1,2] Recently, it has been shown that biofilms may exist in equine wounds, with *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* being the most common microorganisms found.[3]

Local antibiotic therapy combined with aggressive debridement with or without systemic administration of antibiotic agents in the management of fractures and orthopedic infections in humans has resulted in improved outcome, reduced hospital stay and decreased medical cost when compared to traditional systemic treatment.[4-7] Additionally, prophylactic local antibiotic therapy in soft tissue surgery has been reported to reduce the incidence of infections caused by many major, clinically important microorganisms with no increase in antibiotic-resistant microorganisms.[8-10] In horses and other large animals, the use of local antibiotic therapy has provided excellent outcomes when used in the treatment of orthopedic infections.[11-13]

The use of systemic antibiotics is may be associated with multiple complications. Amikacin, vancomycin and clindamycin are highly effective antibiotics, which are widely used in humans for management of moderate to severe infections, however they may be associated

with adverse effects in horses. Therefore, local delivery allows potential use of effective antibiotics which are otherwise not typically used in horses.

Antibiotic-impregnated polymethylmethacrylate (PMMA) has been widely used in horses, however it has several disadvantages when compared to biodegradable local antibiotic delivery systems. Polymerization of PMMA is exothermic, so only heat-stable antibiotics can be used. Additionally, bacteria can colonize and grow in biofilms on antibiotic-impregnated PMMA.[14] Gentamicin-impregnated PMMA beads retrieved from surgical patients with orthopedic infections have been found to have persistence of bacterial growth and emergence of gentamicin-resistant bacteria.[15-17] Additionally, PMMA exhibits low biocompatibility due to the induction of a dose-dependent inflammatory and cellular immune response, and apoptosis of lymphoid and osteoblastic cells *in vitro*.[18-20]

Cross-linked dextran gel[a] is biocompatible, fully biodegradable, and nonimmunogenic, and may therefore be a suitable choice for local antibiotic delivery. The gel has a wide range of applications in which it may be utilized, including both soft tissue (such as ventral midline incision infections and other draining tracts) and orthopedic infections. There is the potential for prophylactic use as well, especially in ventral midline incisions and fracture repairs. The most commonly used biodegradable antibiotic delivery system used in horses today is calcium sulfate hemihydrate (Plaster of Paris).[21] Other reported biodegradable antibiotic delivery devices in horses are gentamicin-impregnated collagen sponges[22] and an amikacin-impregnated ferric-hyaluronate implant.[23] Both of these biodegradable systems, however, have been shown to have rapid antibiotic elution, with no difference in antibiotic concentrations between these implants and direct intra-articular injection.

Assessment of local elution of each antibiotic was achieved *in vivo* by utilizing a capillary ultrafiltration (UF) system.[24] The UF system provides a minimally invasive, novel

sampling method, allowing withdrawal of extracellular fluid and small molecules at low rates whilst excluding proteins and other cellular matter. This method can provide continuous small-volume sampling over prolonged periods of time,[25] and was recently used in the distal limbs of horses, where local concentrations of antibiotics were evaluated after regional limb perfusion.[26]

The objective of the study was to measure the *in vivo* antibiotic concentrations from vancomycin-, amikacin- and amikacin/clindamycin-impregnated cross-linked dextran gel implanted subcutaneously in horses. Our hypothesis was that use of this gel containing vancomycin, amikacin or a combination of amikacin and clindamycin would provide effective concentrations *in vivo*, with concentrations maintained above the MIC of common equine pathogens (4µg/ml for amikacin,[27,28] 4µg/ml for vancomycin,[29] and 0.5µg/ml clindamycin[30,31]) for a minimum of 7 days.

MATERIALS AND METHODS

Animals:

Eleven healthy adult horses of various breeds were used in the study. The horses weighed 406 to 615 kg, and ages were 5-14 years. Horses were determined to be healthy based on routine physical examination and none had received antibiotic treatment within 2 months of the study. Horses were allocated to receive two of the three antibiotic-impregnated gels placed in incisions on either side of the neck; random allocation was not possible because of the use of amikacin and amikacin-clindamycin gel (placement of these two gels in the same horse would confound results). A washout period of 3 months was used to complete all sample collection. All procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Experimental Methods:

A pilot study using one adult horse was performed to evaluate implantation of the gels (amikacin- and vancomycin-impregnated), placement of capillary ultrafiltration probes, elution rates and systemic absorption. Interstitial fluid samples were collected via capillary ultrafiltration probes placed within the incision (0cm) and 1.5cm lateral to the incision at 0, 4, 8 and 12 hours, and days 1, 2, 4, 8, 10, 14, 18, 22 and 28. Blood was collected on days 0, 1 and 7. Analysis of these interstitial fluid samples revealed that concentrations were below MIC by 14 days for each of the antibiotics.

The second study consisted of placement of two of the three antibiotic-impregnated gels in each horse, such that each gel was placed a total of 6 times. Interstitial fluid samples were collected via capillary ultrafiltration probes placed within the incision (0cm) and 1.5cm lateral to the incision at 0, 4, 8, 12 hours, and days 1 through 10. Blood for serum or plasma was collected via venipuncture of the left or right jugular vein at days 0, 1 and 7.

Antibiotic-impregnated gel and capillary ultrafiltration device implantation:

Horses were placed in stocks and sedated with detomidine hydrochloride (Dormosedan)[b] (0.01 – 0.02mg/kg, IV). Each side of the horses' necks were clipped and prepared for aseptic surgery. The locations of the incisions and placement of probes were marked on the neck using sterile surgical markers[c]. Mepivicaine (Carbocaine-V)[d] was infiltrated subcutaneously in a large, inverted "L".

Capillary ultrafiltration probes were placed 24 hours prior to placement of the gel to allow equilibration within the local tissue environment. In six horses, the capillary ultrafiltration probes were placed 48 hours prior to placement of the gel to allow collection of adequate volumes of blank interstitial fluid. Two capillary ultrafiltration probes[g] were placed

within the incision where the gel was placed (0cm), and two were placed 1.5cm lateral to the incision to measure diffusion of the antibiotics. Duplicate probes were placed to ensure fluid collection at each sample time. To place the probes within the incision, small incisions (less than 1cm in length) through the dermis were created 1.5cm distal and proximal to the end of the incision for the gel. A sterile, hollow introducer needle was then passed subcutaneously from the distal incision to exit the proximal incision. The probes were then inserted in to the hollow introducer needle, and the introducer needle was withdrawn out the proximal incision to leave the probes lying directly underneath the gel. The same procedure was performed to place the probes 1.5cm lateral to the incision, again leaving the probes lying subcutaneously.

For placement of the antibiotic-impregnated gel, two full-thickness vertical incisions were created, 7cm in length, on either side of each horse's neck. The cross-linked dextran gel was provided in two syringes; one syringe containing dextran liquid, and the other a cross-linking polymer and active agent (antibiotic) as a powder. The two syringes were conjoined, and reciprocation for approximately 2 minutes resulted in a viscous gel being formed. The entire amount of gel (2ml – 125mg vancomycin, 125mg amikacin or 100mg amikacin/50mg clindamycin) was injected in to the incision. The incisions were closed using a non-absorbable, monofilament nylon (Ethilon)[e]. A control incision was created on each side of the neck, also 7cm in length. These incisions were injected with a similar volume of sterile 0.9% saline[f].

The incisions were bandaged, and the capillary tubing was connected to plain (serum) vacutainers[g] as a negative pressure source. These were sutured to the horses' necks either side of the incision (Figure 1). To protect the sites, the horses were maintained in slicker hoods[h] throughout the duration of the study.

Sample collection:

Samples of interstitial fluid were collected by replacing the Vacutainers at each time point during the study. The volume of interstitial fluid obtained was measured prior to storage. To obtain serum or plasma samples, blood was collected via venipuncture from either the left or right jugular vein. Blood was allowed to sit for 30 minutes to clot, prior to centrifugation[i] (1,900 X g for 10 minutes at 4 C) and collection of serum. Samples were stored at -80°C until assayed.

At the completion of the study, biopsy specimens of the treatment incisions (antibiotic-impregnated gel), control incisions, and skin overlying the capillary UF probes were collected and placed into 10% formalin solution to be analyzed histologically. A histomorphologic scale was used, which was modified from a previously reported scale,[32] to evaluate the different biopsy specimens (Table 1). The slides were evaluated blindly, and the variables listed were assigned a score out of 3 (0 for absent, 1 for mild, 2 for moderate and 3 for severe), for a maximum score of 18. The slides were evaluated in triplicate, with the mean score being used.

Sample analysis:

Vancomycin and amikacin concentrations were detected by use of an automated fluorescence polarization immunoassay (TDx Analyzer).[j] The use of this assay for determination of amikacin and vancomycin concentrations in samples from horses has been previously reported.[26,29,33] The standard assay procedure was modified to avoid carryover of amikacin and vancomycin from samples of higher concentrations to those of lower concentrations.[34,35] The upper limits of detection are 50µg/ml and 100µg/ml for amikacin, and vancomycin respectively. Concentrations presented as "low" on the TDx analyzer were recorded as zero for analytical purposes. Accuracy of this assay for determining amikacin and

vancomycin concentrations in interstitial fluid samples via the modified procedure was evaluated by analysis of interstitial fluid samples containing known concentrations of amikacin[i] and vancomycin.[k] Serial dilutions of these samples were also performed, and the analyzer was then calibrated to run these serial dilutions. Serial dilutions of some of the interstitial fluid samples obtained during the experiment were performed, because the concentrations obtained were greater than the upper limits of detection. Samples of known amikacin and vancomycin concentrations in interstitial fluid and test control samples provided by the manufacturer[j] were assayed in tandem with each batch of interstitial fluid or serum samples. All samples were run in triplicate.

Clindamycin concentrations were analyzed by reverse phase high pressure liquid chromatography (HPLC) using a method developed in the Clinical Pharmacology Laboratory at North Carolina State University.[36] The mobile phase for HPLC analysis consisted of acetonitrile, and 0.05 M potassium phosphate buffer solution, which was modified with 1 N potassium hydroxide to a pH of 5.0. The HPLC system consisted of a quaternary solvent delivery system[l] at a flow rate of 1 mL/min, an autosampler[m], and UV detector set at a wavelength of 200.[n] The chromatograms were integrated with a computer program[m]. The column was a reverse-phase, 4.6 mm x 15 cm C8 column[o] kept at a constant temperature of 40° C. All calibration curves were linear with a r^2 value of 0.99 or higher. Limit of quantification for each of the clindamycin in serum samples was 0.05 µg/mL. Samples were diluted appropriately so that the concentrations fell within the upper and lower limits of the calibration curve. Samples were filtered to remove any debris or particles, diluted (if necessary) and injected directly into the HPLC system.

Data analysis:

The volume of interstitial fluid within the length of tubing connecting the capillary ultrafiltration probes to the vacutainers (40cm) was determined to be 140µL. The duration of time required for a specific volume of interstitial fluid to travel the length of the tubing was calculated for each time point within the first 24 hours. This was designated the mean lag time. The mean lag time was then subtracted from the actual time at which the Vacutainers were changed (4, 8, 12 and 24 hours), to account for the volume of interstitial fluid within the tubing at these time points. These times were determined to be 2 hours 48 minutes, 6.5 hours, 10 hours 48 minutes and 21 hours 54 minutes respectively. These time points were then used in the graphical representation of the data.

Statistical analysis of the mean histomorphologic scores was performed using Wilcoxon signed rank test. Significance was set at a P-value of <0.05.

RESULTS

All horses tolerated placement of the capillary ultrafiltration tubes and antibiotic-impregnated gel. Throughout the experiments, most horses tolerated the presence of the capillary ultrafiltration tubes well. One horse rubbed out two probes, and required a second procedure to replace the probes.

Collection of interstitial fluid with the capillary ultrafiltration probes was successful at all time points because of placement of two probes at each site. Reasons for unsuccessful sample collection included kinking or puncture of the tubing connected to the vacutainer, or slippage of the vacutainer from its holder; in many instances, the precise reason for lack of interstitial fluid was not evident.

Antibiotic concentrations were not detected in serum or plasma at time 0 hours (prior to placement of the antibiotic-impregnated gel). Mean (±SD) concentrations of vancomycin, amikacin, and amikacin and clindamycin (from amikacin/clindamycin gel) at 24 hours and 7 days post-implantation are presented in Table 2. Antibiotic concentrations were not detected in interstitial fluid at time 0 hours (prior to placement of the antibiotic-impregnated gel). Mean (±SD) concentrations of vancomycin, amikacin, and amikacin and clindamycin from amikacin/clindamycin-impregnated gel obtained at 0cm and 1.5cm are presented in Figures 1a and 1b, and 2a and 2b respectively.

The C_{max} (µg/ml) and T_{max} (hours) of the antibiotics in interstitial fluid, as well as the C_{max}:MIC for amikacin and amikacin from amikacin/clindamycin-impregnated gel, and T>MIC (days) for vancomycin and clindamycin from amikacin/clindamycin-impregnated gel, are presented in Table 3.

Surgical incisions healed well in all but two horses, which developed seromas associated with the gel incision on one side of each of their necks (amikacin, vancomycin). Mean histomorphologic scores for amikacin-, vancomycin- and amikacin/clindamycin-impregnated R Gel incisions, control incisions and skin obtained from over the capillary ultrafiltration probes are presented in Figure 3. There was no significant difference between in the mean histomorphologic scores between each of the antibiotic-impregnated gel treatment incisions and their corresponding control incisions. All incisions resulted in some degree of inflammation within the dermis, which varied between horses (Figures 4 and 5). The inflammatory component consisted of a mixed population of inflammatory cells which were primarily lymphocytic-plasmacytic, with mild to moderate eosinophils (this varied between horses) and macrophages, and occasional neutrophils. Distribution of inflammatory cells was variable and ranged from scattered lymphocytes and plasma cells within the dermis, to

extensive accumulation of inflammatory cells. Extent of fibroplasia also appeared to vary between horses, with some horses having extensive fibroplasia extending from the superficial dermis to deeper within the dermis, whereas other horses appeared to have only mild fibroplasia. Placement of the capillary ultrafiltration probes did not result in any clinically significant inflammation as evident by the lower histomorphologic scores.

DISCUSSION:

Subcutaneous placement of a cross-linked dextran gel containing vancomycin, amikacin or a combination of amikacin and clindamycin in incisions provided effective and predictable elution rates *in vivo*, with concentrations maintained above the MIC of common equine pathogens for greater than 7 days for all antibiotic-impregnated gels at 0cm, and all except amikacin (amikacin/clindamycin-impregnated gel) at 1.5cm. Use of a cross-linked dextran gel containing vancomycin, amikacin or a combination of amikacin and clindamycin placed in an incision provided effective and predictable elution rates *in vivo*, with concentrations maintained above the MIC of common equine pathogens for greater than 7 days for all antibiotic-impregnated gels at 0cm, and all except amikacin (amikacin/clindamycin-impregnated gel) at 1.5cm. The time-dependent nature of clindamycin and vancomycin means that tissue concentrations must be maintained above MIC in order for the antibiotics to have effect. This was achieved out to 10 days at the site of implantation for both of these antibiotics. Amikacin, being a concentration-dependent antibiotic, relies more on high peak concentrations above MIC in order to be effective, with a recommended C_{max}:MIC ratio of ≥10.[26] This was also achieved, with mean ratios of 1531.58 and 986.92 at 0cm and 324.67 and 288.29 at 1.5cm distant to gel implantation for amikacin and amikacin from amikacin/clindamycin-impregnated gel respectively. Additionally, concentrations greater than 800 times the MIC of amikacin were achieved for both amikacin- and amikacin/clindamycin-impregnated gels at the site of

implantation. The post-antibiotic effect associated with concentration-dependant antibiotics mean that the efficacy of amikcin-impregnated gels may be prolonged beyond 10 days.

Comparison of concentrations obtained from the different antibiotics was not performed in this study, as we were primarily interested in assessing whether or not use of the gel resulted in clinically significant concentrations within the local tissue environment. When examining Figures 1 and 2, it is clear that concentrations obtained from the amikacin- and vancomycin-impregnated gels are higher than those from the combination (amikacin/clindamycin) gel. The likely reason for this is differences in concentrations of the antibiotics in the different gels supplied by the manufacturer;[a] the amikacin- and vancomycin-impregnated gels contain 125mg of antibiotic each, whereas the combination gel contains 100mg amikacin and 50mg clindamycin. Another possible reason is antagonism between amikacin and clindamycin in the combination gel, which has been previously reported.[37] Other studies, however, have indicated no significant antagonism and potential synergism against resistant anaerobic cocci and clostridial species.[38-40] The reason for this synergism is unknown, as anaerobes are inherently resistant to aminoglycosides. It has been proposed that combination therapy consisting of amikacin and clindamycin may delay emergence of antibiotic resistance, provide broad-spectrum coverage for infections of unknown or mixed etiology or generate greater antibiotic effect against specific pathogens that is achievable with a single drug.[41] It would have been ideal to evaluate gels with the same concentrations in them, as well as assessing a gel with just clindamycin alone to assess whether or not there is an interaction between the amikacin and clindamycin. This was not possible, as the gels are provided as is by the manufacturer, and are marketed as such.

Minimal systemic absorption was evident for amikacin-, vancomycin- and amikacin (amikacin/clindamycin)-impregnated gels, with concentrations below MIC at 24 hours and 7

days. The serum clindamycin concentrations were higher than expected, being greater than MIC (>0.5µg/ml) at both 24 hours and 7 days; and the reason for this is unclear. One possible reason for this may be differences in protein-binding between the three antibiotics. Capillary ultrafiltration probes act by excluding all molecules which are greater than 30,000 daltons, which includes all proteins. As such, the interstitial fluid collected via ultrafiltration contains only free (un-bound), or pharmacologically active, antibiotic. This is in contrast to serum or plasma, which will contain both protein-bound and un-bound antibiotic. Amikacin has been shown to be minimally protein-bound in plasma, with only around 6% to 7% of the total amount of amikacin being protein-bound.[42] Vancomycin is variably protein-bound in plasma, with 28% to 56% of the total amount of drug being protein-bound.[43] Interestingly, it has been shown that when vancomycin concentrations increase, the amount of protein-binding decreases.[44] Clindamycin, on the other hand, is approximately 94% protein-bound in serum.[45] This may therefore account for both the lower clindamycin concentrations experienced locally (because the protein-bound clindamycin is excluded from the interstitial fluid), as well as the higher systemic concentrations. Despite the relatively high systemic concentrations, none of the horses in the study developed any clinical signs, however this should be considered when using the amikacin/clindamyin-impregnated gel in systemically compromised horses. Pharmacokinetic studies evaluating clindamycin in horses are lacking because of the obvious risks associated with systemic administration. Because of the limited number of serum samples in our study, it is difficult to determine whether higher concentrations were reached, and for how long these systemic concentrations persisted. Pharmacokinetic studies in dogs and cats indicate that mean serum concentrations after systemic administration may reach up to 9.4µg/ml[31] and 7.4µg/ml[46] respectively, without adverse effects. Some degree of systemic clindamycin absorption has been shown in the use of topical vaginal creams in humans,[47]

however the concentrations obtained are quite low. There does appear to be a difference in the amount of systemic absorption between different formulation, which has been proposed to be associated with both the formulation used and the physiology of the patient.

Use of an antibiotic-impregnated gel is a viable alternative to the use of Plaster of Paris, and potentially allows greater clinical application because it is injectable. The gel is completely broken down and removed via hydrolysis and enzymatic degradation within 3 – 4 weeks, meaning that all antibiotics are gone within this time frame, and there is no prolonged residence and possible selection for resistant bacteria, as has occurred with antibiotic-impregnated PMMA.

In conclusion, use of a novel cross-linked dextran gel impregnated with either amikacin, vancomycin or a combination of amikacin and clindamycin results in excellent local soft tissue concentrations with minimal systemic absorption. Further, the gel does not appear to result in any clinically significant inflammation in the soft tissue of normal horses.

FOOTNOTES:

a. Royer Biomedical Inc., Frederick, Maryland, USA

b. Pfizer Animal Health, Exton, Pennsylvania, USA.

c. Medline Industries Inc., Mundelein, Illinois, USA.

d. Ethicon, Somerville, New Jersey, USA.

e. Baxter Healthcare Corporation, Deerfield, Illinois, USA.

f. Bioanalytical Systems Inc., Baltimore, Maryland, USA.

g. Vacutainer serum, 4.0mL, BD, Franklin Lakes, New Jersey, USA.

h. Ultraflex Stretchy Mesh Slicker Hoods, Schneider Saddlery, Chagrin Falls, Ohio, USA.

i. Fisher Scientific, Fair Lawn, New Jersey, USA.

j. Abbott Laboratories, Abbott Park, Illinois, USA.

k. Sigma-Aldrich, St Louis, Missouri, USA.

l. USP, Rockville, Maryland, USA.

m. 1100 Series Autosampler, Agilent Technologies, Wilmington, Delaware, USA.

n. Zorbax Rx-C8, MAC-MOD Analytical, Inc., Chadds Ford, Pennsylvania, USA.

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FIGURES:

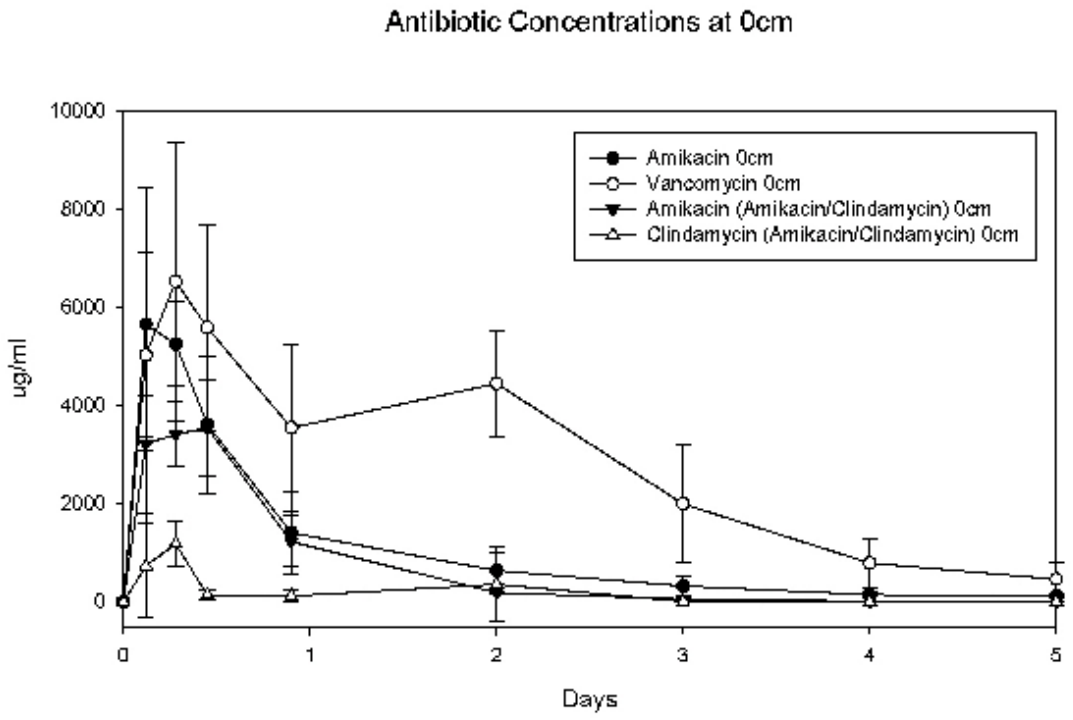

Figure 1a: Mean (±SD) concentrations (µg/ml) of amikacin, vancomycin and amikacin and clindamycin from amikacin/clindamycin-impregnated gel present within the incision (at 0cm).

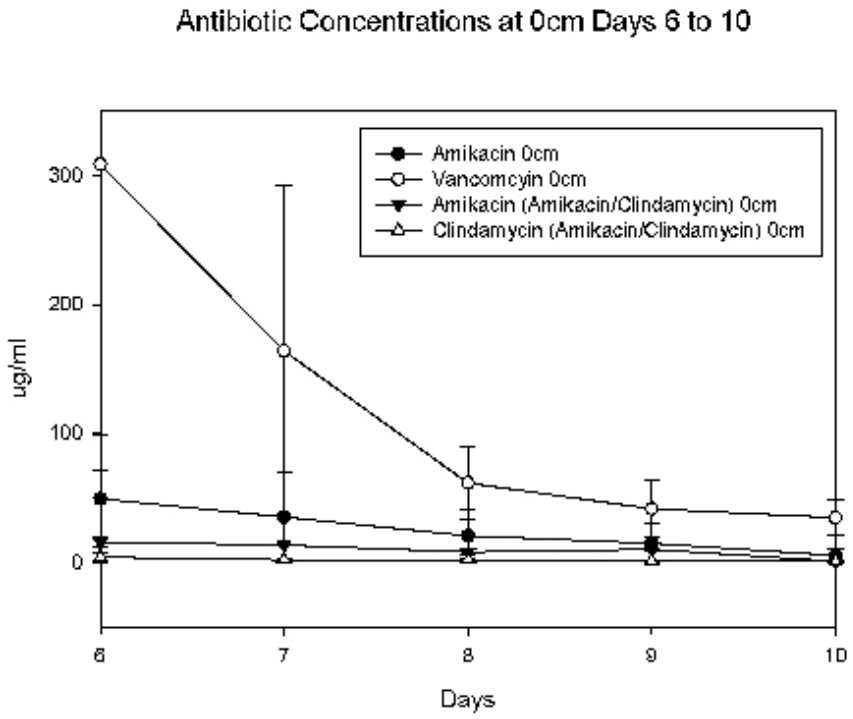

Figure 1b: Mean (±SD) concentrations (µg/ml) of amikacin, vancomycin and amikacin and clindamycin from amikacin/clindamycin-impregnated gel present within the incision (at 0cm) from days 6 through 10.

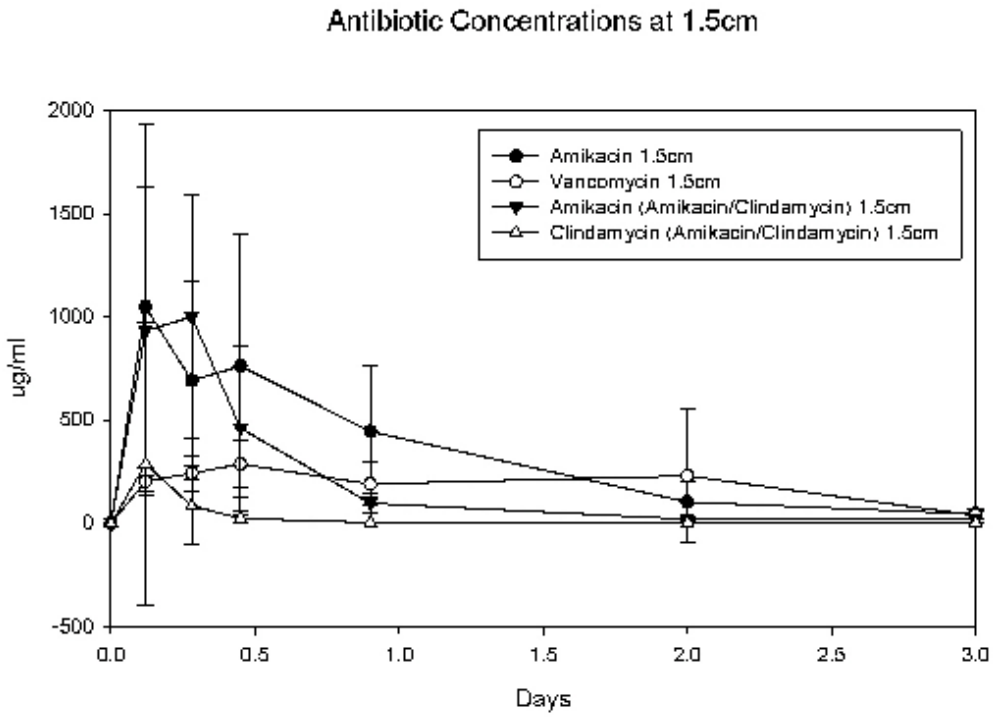

Figure 2a: Mean (±SD) of amikacin, vancomycin and amikacin and clindamycin from amikacin/clindamycin-impregnated gel present within the incision (at 0cm) from days 0 through 3.

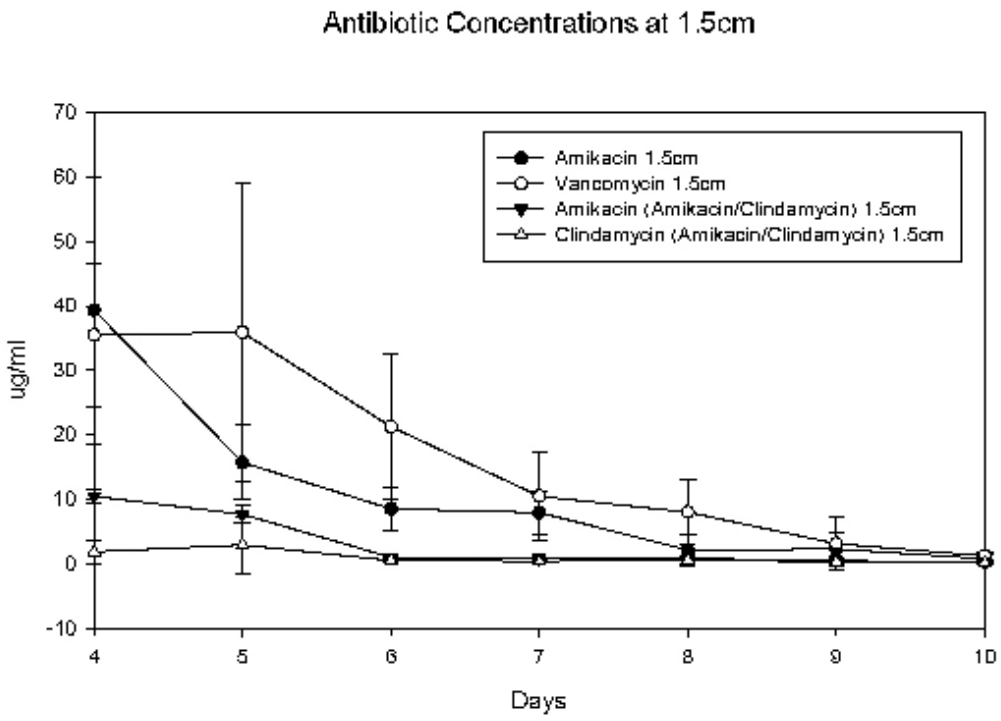

Figure 2b: Mean (±SD) of amikacin, vancomycin and amikacin and clindamycin from amikacin/clindamycin-impregnated gel present within the incision (at 0cm) from days 4 through 10.

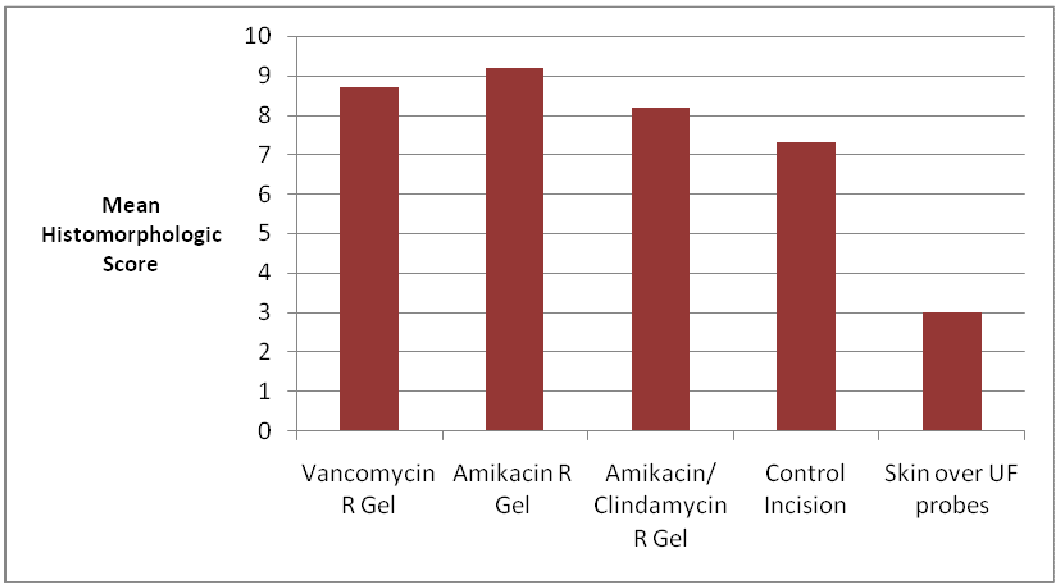

Figure 3: Mean histomorphologic scores for vancomycin-, amikacin-, and amikacin/clindamycin-impregnated R Gel incisions, control incisions, and skin overlying capillary ultrafiltration probes.

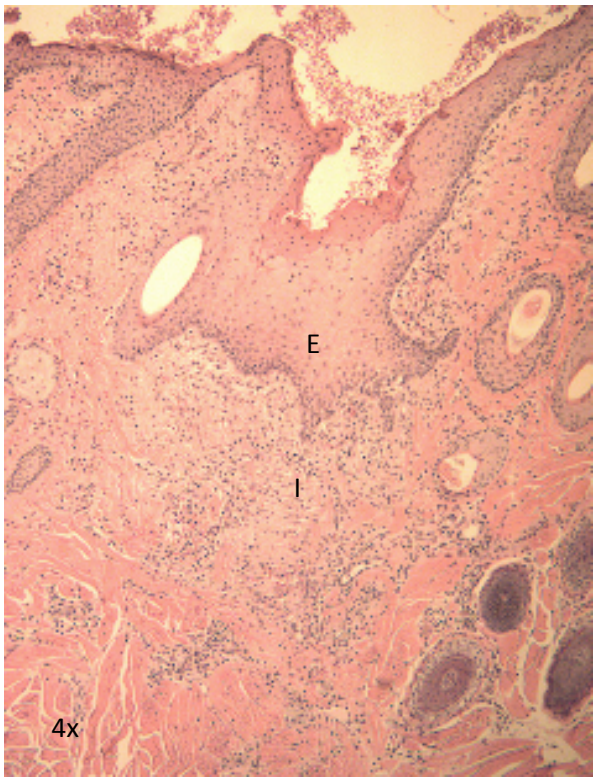

Figure 4: H&E stained slide from an incision containing vancomycin-impregnated gel, with epithelial hyperplasia (E) evident, and a moderate, mixed inflammatory cell infiltrate within the dermis (I).

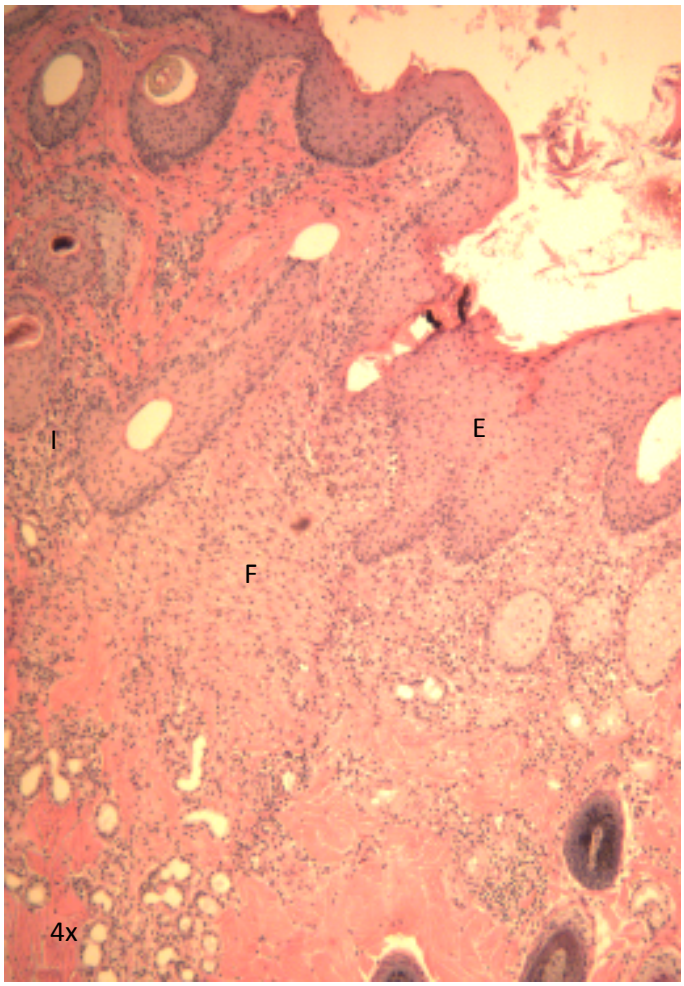

Figure 5: H&E stained slide from an incision containing amikacin-impregnated gel, with epithelial hyperplasia (E) evident, moderate fibroplasia within the dermis (F), and a mixed inflammatory cell infiltrate within the dermis (I).

TABLES:

Variable	Maximum Score
Epidermal Hyperkeratosis	3
Epithelial Hyperplasia	3
Vascular Proliferation	3
Fibroplasia	3
Inflammatory Cell Infiltrate	3
Hemorrhage	3
TOTAL	**18**

Table 1: Modified histomorphologic scoring system applied to blindly viewed slides.

	24 hours	**7 days**
Amikacin	0.06 (±0.073)	0.028 (±0.06)
Vancomycin	0.06 (±0.14)	0 (±0)
Amikacin (Amikacin/Clindamycin)	0.017 (±0.019)	0 (±0)
Clindamycin (Amikacin/Clindamycin)	0.52 (±0.19)	0.63 (±0.4)

Table 2: Mean (±SD) serum (clindamycin) and plasma (amikacin, vancomycin) concentrations in µg/ml at 24 hours and 7 days after implantation of the corresponding antibiotic-impregnated gels.

Variable	Treatment	Interstitial Fluid 0cm	Interstitial Fluid 1.5cm
C_{max} (µg/ml)	Amikacin Vancomycin Amikacin (Amikacin/Clindamycin) Clindamycin (Amikacin/Clindamycin)	5660.25 (±1472.34) 6533.3 (±3409.04) 3420.2 (±649.9) 1186.3 (±445.6)	1048 (±890.64) 286.8 (±111.19) 1000.67 (±561) 286.71 (±684.06)
T_{max} (hours)	Amikacin Vancomycin Amikacin (Amikacin/Clindamycin) Clindamycin (Amikacin/Clindamycin)	4 8 8 8	4 12 8 4
C_{max}:MIC	Amikacin Amikacin (Amikacin/Clindamycin	1531.58 (±368.09) 986.92 (±79.3)	324.67 (±218.56) 288.29 (±170.1)
T>MIC (days)	Vancomycin Clindamycin (Amikacin/Clindamycin)	10 10	8 8

Table 3: Pharmacokinetic values presented as the mean (±SD) of amikacin, vancomycin, amikacin (amikacin/clindamycin) and clindamycin (amikacin/clindamycin) in samples of interstitial fluid and serum or plasma after subcutaneous implantation of the respective antibiotic-impregnated gels.