The development of a biodegradable drug delivery system for orthopedic infection in the horse: in vitro and in vivo biocompatibility and gentamicin elution characteristics

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The development of a biodegradable drug delivery system for orthopedic infection in the horse: *In vitro* and *in vivo* biocompatibility and gentamicin elution characteristics

by

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This is to certify that the Master’s thesis of

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has met the thesis requirements of Iowa State University

Signatures have been redacted for privacy
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CHAPTER 1. GENERAL INTRODUCTION

Thesis Organization

This thesis is organized in alternate format and contains two studies which are presented as journal papers. The first paper is titled "Biocompatibility and gentamicin elution characteristics of a biodegradable drug delivery system for the treatment of orthopedic infection in the horse". The second paper is titled "Biodegradable injectable polymers for sustained antimicrobial drug release in the equine joint: An in vivo pilot study". Each paper contains its own summary, introduction, materials and methods, results, discussion and references. The literature review is designed to give a comprehensive overview and background of current knowledge that created the ideas to pursue these projects. The general conclusion summarizes the results of this project and reflects on problems, which had occurred during the study period. It also states the overall value and impact of this project.

Introduction

Septic arthritis and septic tenosynovitis are the terms used to describe a bacterial infection of a closed synovial cavity, either in the joint or in the tendon sheath respectively. Joint infections are devastating and performance limiting problems in horses that can potentially result in irreversible cartilage damage and joint capsule fibrosis resulting in chronic painful arthritis(1,2). In addition, chronic scarring and adhesions of tendons within their synovial sheaths as a result of infection may cause loss of gliding function and persistent lameness.

To prevent chronic tissue damage, high local antibiotic concentrations are necessary to quickly eliminate the bacterial infection. Systemic administered antibiotics are not as effective in eliminating infection due to lower tissue concentrations than can be achieved by intra-articular injection, use of antimicrobial impregnated non-biodegradable polymethylmethacrylate (PMMA), or regional perfusion (3,4).

The objective of our research was the development of a local drug delivery system with unique characteristics and benefits for an entirely new clinical approach to the treatment of musculoskeletal infection in the horse. Biodegradable polymers can be designed to release antimicrobial drugs at a rate and concentration to the target site that permits optimal therapeutic activity while reducing undesirable side effects to a minimum. The use of a biodegradable material for local delivery has both logistical and economic advantages. These materials provide a sustained high concentration of antibiotics within a synovial cavity over a period of days without the need for repeated intra-articular injections, and they do not require retrieval at a later date. Because horses have large joints, and a relatively high incidence of joint infection, they are a good model for the development of a local drug delivery system. Biocompatibility was an important issue in this project.

Although a variety of biodegradable polymers have been developed, very few were designed for biomedical use and even fewer have been evaluated under clinically controlled conditions. Because synovial
structures such as joints and tendon sheaths are sensitive to exposure to foreign material, it was important to test the polymer for tissue reactivity.

**Literature Review**

**Orthopedic Infection in the Horse**

Retrospective studies of horses with septic arthritis/tenosynovitis receiving multiple treatments revealed survival rates of 85% in adult horses. However, follow-up racing data show that only 56.5% of all horses of racing age return to racing and only 45% of these horses are able to make more than five starts. Survival rates in foals are much lower. Only 45% survive to be released from the hospital(1). The bacterial families most commonly isolated from synovial infections include *Staphylococcus, Enterobacteriaceae* and anaerobes (1). The most common organisms isolated from foals are *Enterobacteriaceae*(1). Culture and sensitivity results of previous synovial fluid samples from horses with septic arthritis, tenosynovitis and osteomyelitis showed good susceptibility to aminoglycosides(1,2).

Treatment protocols include: lavage of the synovial structure with or without arthroscopic visualization, systemic and intra-synovial antibiotic infusion, surgical drainage with or without arthrotomy, partial synovectomy and non-steroidal anti-inflammatory medication(1,2).

The benefits of anti-inflammatory medication in conjunction with antibiotics in the treatment of synovial infection are now better understood with the discovery of cytokine and eicosanoid pathways. Cytokines (eg. IL-1alpha, TNF alpha and IGF-1) and eicosanoids (eg. PGE2, TXB2 and LTB4) are very sensitive indicators of inflammation and they play an important role in the occurrence of damage to chondrocytes and synovium associated with bacterial infection(5).

The release of lipopolysaccharide (LPS) into synovial structures from gram-negative bacteria induces polymorphonuclear leukocytes, chondrocytes and synovial cells to produce proteolytic enzymes such as serine proteinases, matrix metalloproteinases and cytokines. All of these enzymes can destroy cartilage.

Levels of inflammatory mediators are elevated in synovial fluid of equine arthritis patients. Joint infection usually results in elevated concentrations of IL-1, IL-6, TNF-alpha and prostaglandin E2 (PGE2). Elevation of these same mediators occurs in *in vitro* models using *Staph. aureus* infected synoviocytes(5).

Aggressive antibiotic treatment of a synovial infection along with the concurrent use of anti-inflammatory medication and establishing drainage are important principles of a successful outcome. Local intra-synovial antibiotic injections and non-degradable antibiotic impregnated polymethylmethacrylate (PMMA) beads provide 10-100 fold higher antibiotic levels than can be achieved via systemic antibiotics; and they can significantly improve the clinical outcome (11,12,13). High local antimicrobial drug concentrations are achieved via intra-articular (IA) injection of gentamicin. In one study, the injection of 150 mg of gentamicin sulfate into the radio-carpal joint resulted in a peak mean synovial fluid concentration of 1,828 µg/ml. This drug level was significantly greater than that after intravenous (IV) administration alone (2.53 µg/ml), but less
than simultaneous IV and IA administration (5,720 µg/ml). Pharmacokinetic studies involving the release of gentamicin from PMMA beads produce a drug concentration of 250-600 µg/ml and higher for 30-60 days at the site of the infection (16). In fact, previous studies revealed that local delivery of an antibiotic can lower the Minimal Inhibitory Concentration (MIC) for a non-susceptible organism, thereby making the bacteria susceptible to the applied antibiotic (16). Multiple studies in humans and dogs confirmed minimal systemic absorption and no nephrotoxicity (16,17,18). Local delivery systems have been used successfully in human and veterinary surgery in the treatment of chronic osteomyelitis, infectious arthritis and osteomyelitis associated with fracture repair (10,13). The impregnation of PMMA bone cement with antibiotics is routinely used in human and equine orthopedic surgery, and it has made a significant difference in the clinical management and prognosis of these patients (31,19).

**Biodegradable Controlled Drug Delivery Systems**

**General considerations**

Currently, biodegradable polymers are being developed to carry and deliver a drug to a specific tissue site at therapeutic concentrations while disintegrating over a pre-determined time period. This principle of drug delivery is designed to avoid systemic side effects including nephro-and hepatotoxicity, which are commonly observed with some drugs at therapeutic concentrations.

Although most bioerodible polymers developed for biomedical use were designed for use as suture materials rather than for controlled release application, these materials are currently being investigated for drug delivery. These polymers can be prepared as various carrier systems including: macromolecular drug carriers, micellar systems (block copolymers composed of hydrophilic and hydrophobic segments), liposomes, nanoparticles and microspheres(20). Once the carrier is implanted at the tissue site for sustained drug release, biological mechanisms within the tissue environment, such as phagocytosis and opsonization, are triggered to help eliminate the carrier from the body as a natural response to foreign bodies. The character and severity of this response depends on the site of implantation and the nature of the polymer. The study of this response has developed into a separate area of material science research called biocompatibility.

A variety of characteristics of an ideal targeting system have been established, including:

- compatibility with the body in terms of toxicity, biodegradability, and antigenicity
- protection of the drug until it reaches its site of action
- maintenance of the drug-carrier integrity until the target is reached
- avoidance of interaction with normal cells
- an ability to traverse intervening membranes
- target recognition and association
- controlled drug release to achieve the desired therapeutic effect
- carrier elimination from the body following drug release.
Biodegradable polymers used in drug delivery research may be classified as of natural or synthetic origin. Natural polymers such as collagen, gelatin, starch, cellulose and hyaluronic acid can be formulated into microparticles and are relatively inexpensive; however, they may cause a significant immune response due to their foreign protein nature. Synthetic polymers have been developed over the past 5-10 years and include a variety of materials including: polyglycolic acid/polyactic acid copolymers (PLGA), polycaprolactones (PCL), polyhydroxy-butyrate/-valerate copolymers, polyanhydrides, polyethyleneoxide/polybutylene terephthalate copolymers, and poly(ortho esters) (POE).

Some of these materials degrade via bulk erosion. Bulk erosion occurs when water imbibes into the center of the matrix, resulting in homogeneous breakdown of the polymer. This makes it difficult to achieve zero-order release of drugs and to control release kinetics. For instance, polyesters such as lactic acid-glycolic acid copolymers display bulk erosion, resulting in significant degradation in the matrix interior causing "dumping" of the incorporated drug into the environment(21). The drug release rate in surface-eroding systems is proportional to the polymer erosion rate. For surface erosion to occur, the degradation rate on the polymer matrix surface has to be much faster than the rate of water penetration into the matrix bulk. Ideally, the polymer should be hydrophobic but should also have water-labile linkages connecting monomers. Currently, only two polymer materials exhibit these characteristics, the polyanhydrides and their copolymers and poly(ortho esters). Unfortunately, two of the most widely investigated and advanced synthetic polymers in terms of the available toxicological and clinical data, poly lactic acid (PLA) and poly glycolic acid (PLGA), biodegrade homogeneously (bulk degradation) due to hydrolytic desterification of their constituent monomers. Although PLA and PLGA possess excellent biocompatibility and their rate of biodegradation can be modulated via copolymerization, they are not feasible for drug delivery systems where a precise and controlled mechanism of drug release and kinetics is required.

**Pharmacological consideration**

In addition to the polymer, the nature of the drug being incorporated into the matrix of the polymer also requires consideration in the early stage of the design process of a targeted delivery system. The physicochemical characteristics, dose, and required release pattern of a drug dictate design feasibility(22). For example, insulin should be released at a constant rate most of the time, but supplemented by increases near mealtime to control higher glucose levels. Another example is the dosing of aminoglycosides such as gentamicin. Once-a-day dosing has been shown to be more efficacious and least nephrotoxic. This dosing regimen is based on a number of pharmacodynamic properties including: (1) mechanism of bactericidal activity; (2) first-exposure adaptive resistance; (3) post-antibiotic effect (PAE), and (4) mechanism of aminoglycoside-induced nephrotoxicity(23). The post antibiotic effect (persistent suppression of bacterial growth following removal of the antimicrobial agent) is significantly prolonged when gentamicin is given once daily (6.6 mg/kg) compared with three times dosing intervals(24). In general, the higher the concentration of
gentamicin achieved at an infection site, the greater the bacterial killing and the greater the duration of the PAE. Therefore, it would be clinically desirable to have a high burst release of gentamicin once daily from the polymer matrix versus constant high drug concentrations. A variety of solutions are currently being investigated to achieve these kinetics. They include the incorporation of small magnetic beads into the polymer, which, when exposed to a magnetic field, increase drug release by compressing and expanding the matrix channels, thereby "squeezing" out more drug(25). Other approaches for achieving modulated sustained release involve ultrasound or enzymatic processes, and pulsatile delivery systems are being developed using thermo, electrically, light, pH and ionic responsive systems.

The concentration of the antibiotic released from the polymer at the site of implantation which will effectively kill the microorganism, must be considered in order to be able to determine the amount of drug that is incorporated into the polymer. A rough estimate of the desired delivery rate (release rate \( \frac{dm}{dt} \) as a function of time \( [dt] \)) may be obtained by its equivalence with the rate of drug elimination at steady state:

\[
\frac{dm}{dt} = C_{ther} \times CL
\]

where \( C_{ther} \) and CL are the therapeutic drug concentrations in, and drug clearance from the blood circulation (eg. systemic treatment) or local tissue (local treatment), respectively(26). The therapeutic drug concentration for gentamicin is extrapolated from the Minimal Inhibitory Concentration (MIC) (see below) which is determined in vitro. Previous studies, which investigated the plasma and synovial fluid concentrations of gentamicin after the administration of 150 mg into the radio-carpal joint, revealed that mean synovial fluid clearance \( (C_{lb}) \) is 1.22 \( \pm \) 2.54ml/min and the apparent half-life \( (t_{1/2}) \) is 259.2 \( \pm \) 123 min. For local therapy, the decline in drug concentration as a function of position away from the implant site may also need to be considered. The total drug mass required in the polymer may then be estimated by the product of the release time and \( dm/dt \), or by the integral of the release profile.

Sensitivity of a microorganism to an antibiotic is routinely determined via antimicrobial susceptibility testing using the dilution or the agar disk-diffusion (Kirby Bauer) test and reveals the MIC in \( \mu g/ml \) in relation to breakpoints. Although the length of time an effective concentration of an antimicrobial must be maintained at the site of infection for treatment to be effective has not been determined, it has been suggested that the concentration in the adjacent tissues should be maintained above the MIC for 3-4 weeks(27). This would mean in the case of Staph. aureus, that gentamicin release from the polymer would need to maintain a concentration higher than 2 \( \mu g/ml \), the concentration of gentamicin effective in inhibiting the growth of Staph. aureus.

Prolonged high drug concentration and sub-inhibitory concentrations of antibiotics have also raised concerns regarding the development of bacterial resistance and hypersensitivity reactions. However, bacteriological investigations over a three year period on 544 patients with bone infection treated with
gentamicin-PMMA beads did not reveal an increase in the frequency of resistant strains during this period. On the contrary, in several patients, a decrease in the percentage of resistance was observed(28).

**Antibiotic delivery systems for orthopedic infection**

Several biodegradable polymers including plaster of paris, a combination of fibrin and cancellous bone, microspheres or sponges of poly(D,L-lactic-co-glycolic acid) (PLGA), the bone cement poly(propylene fumarate), polyanhydride, and the natural materials collagen, gelatin, and inorganic phosphates have been investigated recently and found effective for the delivery of antibiotics to infected bone(10,15,28). A copolymer mixture of poly(lactide/glycolide) (PLG:PGA, 50:50) was used for the controlled delivery of gentamicin and compared with PMMA beads in a canine osteoarthritis model. A two-week gentamicin release was obtained, and soft tissue and bone gentamicin levels as well as microbial data indicated that the PLG-GA copolymer was as effective as the PMMA implants(29). Poly(L-lactic acid) based implants with 10% gentamicin sulfate content, which were placed subcutaneously into the back of rats just lateral to the midline, have been shown to release the incorporated antibiotic throughout 30 days with a decreasing delivery rate. During the first 24 hours, the implants released a total amount of 3600 µg/ml gentamicin. Very good tissue compatibility was indicated by the absence of inflammatory cells throughout the entire time of implantation as shown by microscopic examination(30). As a result of their excellent biocompatibility the FDA has approved PLA and PLGA polymers. However, the control rate of these materials for the drug release is difficult to adjust and the concentrations achieved in vitro may not necessarily be the same as they are in vivo(30,37).

Gentamicin impregnated collagen sponges (Gentacoll®, Schering-Plough, Denmark) and polyanhydride beads (Septacin®, Nova Pharmaceuticals Corp. Abbott-Laboratories, Chicago) have been commercially developed and are currently being evaluated in human clinical trials. The polyanhydride beads consist of a copolymer of sebamic acid and a fatty acid dimer, both naturally occurring substances. In several well-designed studies of experimentally induced osteomyelitis in dogs and rabbits, the efficacy of the implants was compared to surgical debridement alone, debridement plus placebo beads, and long-term systemic antibiotic therapy(32). The results of these studies revealed that the polyanhydride beads were either as effective or better than long-term systemic antibiotics. Microbiological examination of the implant site and surrounding tissue showed a significantly reduced bacterial count when compared with infection sites from animals, which had only received debridement as the treatment. Analysis of serum for gentamicin concentrations revealed that levels never failed to fall below trough concentrations throughout the study period. Polyanhydrides have received a considerable amount of attention because of their degradation via surface erosion and excellent biocompatibility. In addition, erosion rates can be programmed to last from one day to several years, depending on the copolymer composition.
Recently, a copolymer of sebacic acid (20% gentamicin) was investigated in horses for the treatment of septic arthritis using an *in vivo* model. Infection was successfully eliminated from the joint and it was concluded, that this copolymer may assist in the treatment of joint infection in horses(33).

**The family of poly(ortho esters)**

The polymer investigated in the first paper is a recently synthesized polymer of the family of poly(ortho esters) (POE) (25,26). Poly(ortho esters) are bioerodible hydrophobic polymers that can undergo an erosion process confined to the polymer-water interface known as surface erosion. A major advantage is that they contain acid-labile linkages in the polymer backbone so that their hydrolysis rate can be adjusted within wide limits by the incorporation of acidic or basic excipients(38,39,40). As water penetrates the device, the acidic excipient ionizes and the pH in the outer layers is lowered with consequent acceleration of hydrolysis rate of the acid-sensitive linkages. As a result of this process, an eroding front develops which moves into the device as surface erosion takes place. Biocompatibility data concerning the ultimate hydrolysis products are available for some of the older and newer generation POEs. An investigation of the two hydrolysis products of older generation POEs including 1,2,6-hexanetriol showed that they possess no significant toxicological hazards and that the triol is excreted without metabolism through the kidneys(49). Hydrolysis products of newer generation POEs include triethylene glycol, glycolic acid, pentaerythritol, and propionic acid ®. Limited biocompatibility data is available for these products and are discussed below.

The POE is synthesized under anhydrous conditions by the condensation of alkyl orthoacetate and 1,2,6-hexanetriol(6). The use of certain triols can produce polymers that have very flexible chains, and are viscous pastes at room temperature even though molecular weights are in excess of 50,000 Da. These materials represent a unique type of bioerodible material because therapeutic agents can be incorporated without the use of solvents or elevated temperatures. They are particularly well suited for the incorporation of sensitive molecules, which require release by zero order kinetics.

Investigation of the POE in rats (with a gel of sodium hyaluronate (SH) used as a control because of its well-known biocompatibility) utilized the subcutaneous cage-implant system(42). A volume of 0.5 ml POE was injected in a stainless steel wire cage implanted in the back of a rat at the level of the panniculus carnosus, and the exudate surrounding the implanted material within the cage system was collected for the quantification of the inflammatory components. Results revealed an initial mild inflammatory response after injection, which was very minimal after 14 days. Concentrations of PMNs and lymphocytes, as well as the extra- and intracellular activities of acid and alkaline phosphatase were not different from the controls (empty cage) and the SH. The total leukocyte and macrophage concentrations were higher only at seven days for both SH and POE compared with the control. This indicated a response in the acute phase comparable to the response obtained with SH.
The biocompatibility of the POE was investigated for the ocular delivery of 5-fluorouracil after glaucoma filtration surgery in people(6,43). Formation of granulation tissue is a common sequel of glaucoma filtration surgery. The current therapy consists of daily subconjunctival injections for up to seven days, which is very painful for the patient and increases the risk of side effects and infection. Sustained delivery of 5-fluorouracil from the POE, implanted at the time of surgery, would eliminate the need for repeated injections.

The study quantified the reaction of the anterior segment of the eye to the POE after subconjunctival injection in the rabbit. Sodium hyaluronate and medical grade silicone oil were used as controls. Histologic evaluation identified a mild eosinophilic reaction at three days after injection of the POE. The focal inflammation consisted of 50% eosinophils and 50% lymphocytes, densely packed with extracellular material. At ten days, the tissue returned to normal. Overall, there were more inflammatory cells observed than with the SH and less than with the silicone oil. The POE did not become encapsulated, and it degraded in a short period of time in vivo. No irreversible changes occurred in the tissues, indicating excellent biocompatibility. It was concluded that the POE showed interesting properties for use after glaucoma filtration surgery; and it deserved further investigation related to its in vivo release characteristics.

Recently, a POE has been used for the development of a drug delivery system for the treatment of periodontal disease(44). Periodontitis is a group of dentoalveolar infections that remains one of the major causes of adult tooth loss. These infections are caused by a pathogenic flora established within the gingival sulcus. The bacterial microflora differs significantly from that of the supragingival environment in that it contains more anaerobes, more gram-negative organisms, and a greater portion of motile species. Current treatment consists of mechanically removing supra- and subgingival plaque and calculus, followed by systemic or local antimicrobial agents. Tetracycline was incorporated into the POE, which released therapeutically effective concentrations locally within the periodontal pocket for about ten days. It was concluded that because polymer erosion time and rate of drug release could be readily varied, residence times and rates of tetracycline release could be adjusted to the requirements of a therapeutically useful system.

The POE has also been evaluated as a biodegradable carrier of demineralized bone in the reconstruction of large diaphyseal defects in rats(40). Diaphyseal defects, resulting from trauma, infection, or resection of a tumor, remain a challenging problem in people. While autogenous bone is considered the best material for bone grafting, it may be difficult to obtain sufficient amounts of this substance, and its use requires an additional operation. A radial defect in rats was used as a diaphyseal segmental-defect model for the evaluation of material for repair. Radiographic evaluation revealed that areas of new bone were significantly greater in defects that had been filled with the composite of demineralized bone and POE or demineralized bone alone compared with those that had been filled with the POE alone. Histology confirmed these results. The study concluded that the POE may prove to be valuable for sustained release, because it does not inhibit osteogenesis, induces little inflammation, provides local homeostasis, permits molding and contouring, and is resorbed by four weeks.
In another study, the POE showed its usefulness in the delivery of the anti-inflammatory drug, indomethacin for the inhibition of heterotopic osteogenesis in rats. The POE/indomethacin system successfully prevented abnormal osteogenesis and was recommended for further investigation(51).

Biocompatibility evaluation

The concept of biocompatibility is based on the interactions between a material and its biological environment. The failure of a biomaterial to display good biocompatibility in a clinical situation is often revealed by a breakdown in the desired material properties and/or an unsatisfactory clinical response(35). One of the most important factors in determining the biocompatibility of an implanted material is the cellular response to the material at the tissue-material interface. Cell-polymer interaction has mainly been investigated in vitro using a large variety of techniques including cell adhesion counting, cell morphology observation (histopathology), proliferation quantification and evaluation of cell function.

Traditional staining techniques (H&E, van Giesen, Periodic Acid-Schiff, and avidin-biotin-horseradish peroxidase) may be used in the classification and identification of inflammatory cells such as macrophages, polymorphonuclear leukocytes (PMN), and T- and B-lymphocytes. These cell types reflect the inflammatory response in the presence of an implanted biomaterial within a body adjacent to the site of implantation. As a result of tissue damage, platelet and endothelial cell activation stimulates neutrophil and macrophage migration from blood vessels into damaged tissue areas. These cells may trigger the influx of other cells or may produce factors, which stimulate proliferation of fibroblasts, collagen synthesis and extracellular matrix formation(36).

Processes leading to the activation and production of inflammatory cells are regulated at the molecular level by soluble mediators (cytokines, chemokines, and growth factors) which act through different cell surface specific receptors. Cytokines are a heterogeneous group of low molecular weight proteins that act locally to modulate inflammation and immunity. They include Interleukins 1-13 (IL-1 to IL-13), granulocyte- and macrophage colony stimulating factors (GM and GM-CSF), and Tumor Necrosis Factor alpha and beta (TNF-alpha and TNF-beta). Growth factors such as Transforming Growth Factor-beta (TGFbeta) and Platelet Derived Growth Factor (PDGF) are a group of potent hormone-like polypeptides that act as intercellular mediators(37).

One approach to investigating the host response to a material is the use of isolated or cultured cell lines in vitro. Measurements of enzyme production, chemokinesis, complement and metabolic responses are useful for defining specific responses of tissue; however, they may have limited value in predicting the response to the same material in an in vivo environment.

Immunohistochemistry has been used extensively to analyze the kinetics of the inflammatory response to identify the type, number and spatial distribution of cells with respect to the implant in tissue section or fluid (exudate). Enzyme-linked immunosorbent assays (ELISA) and radioimmunoassay (RIA) using polyclonal or monoclonal antibodies towards specific cell types and cytokines have become routine screening methods in biocompatibility assessment.
Translation or synthesis of any protein or glycoprotein requires the presence of corresponding messenger RNA (mRNA) molecules within the cell. The mRNA molecules are formed during transcription of the genetic information within the cell nucleus and represent the genetic information being used to make protein. Consequently, the analysis of mRNA via Northern blot to determine which genes are being expressed, implies that the corresponding protein is being actively synthesized(38). In situ hybridization has been developed in order to gain more information about the cell type and location of the cells expressing specific genes. In situ hybridization of a labeled nucleic acid probe can be used to detect specific RNA expression within whole cells, and it may be applied to cytopsins, frozen or fixed tissue sections(39).

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CHAPTER 2. BIOCOMPATIBILITY AND GENTAMICIN ELUTION CHARACTERISTICS OF A
BIODEGRADABLE DRUG DELIVERY SYSTEM FOR THE TREATMENT OF ORTHOPEDIC
INFECTION IN THE HORSE

A paper to be submitted for publication to Veterinary and Comparative Orthopaedics and Traumatology

Summary:

In a preliminary study, the in vitro release kinetics of poly(ortho ester) (POE) impregnated with 5% gentamicin sulfate were determined using a double diffusion chamber with synovial fluid as the medium. Gentamicin concentrations were measured via fluorescent polarization immunoassay (TDx) over a 14 day period. The POE released gentamicin concentrations in therapeutic concentrations, however, assay- and sampling procedures require evaluation in further studies.

In two separate cell culture studies the efficacy of the POE to successfully eliminate infection and the biocompatibility (toxicity) of the POE was evaluated using synovial and cartilage explants. Synovial membrane was harvested from the dorsal aspect of the radiocarpal joint of two horses and maintained in explant culture. Staphylococcus aureus was used to create infection in the synovial explant culture, which was then treated with the gentamicin-impregnated POE. Infection was eliminated after 24 hours as determined by negative culture of the medium. Cell function (HA) and the expression of IL-1 and IL-6 remained at control levels after infection was eliminated, however, hyaluronic acid (HA) concentration gradually decreased during the following days. Infection was detrimental to the untreated explants.

Poly(ortho ester) was added to synovial-and cartilage explants for five days and cell viability determined via the Trypan Blue method and immuno fluorescence technique (Propidium Iodide). Cell viability in the synovial explants was not significantly compromised by the POE; however, there was a decrease in viability in the cartilage explants. No detrimental effects of the POE on joint tissue were found that would prevent its testing in vivo. This local biodegradable drug delivery system may be a valuable aid in the treatment of septic musculoskeletal conditions.

Introduction:

Orthopedic infections in the horse, including septic arthritis, septic tenosynovitis and osteomyelitis, remain a serious problem that may result in irreversible cartilage damage, capsular fibrosis, intrathecal adhesions, pathologic fractures, and chronic lameness when infection is not rapidly eliminated from the synovial space(1). Retrospective studies of horses with septic arthritis or tenosynovitis receiving several different treatment approaches, report survival rates of 85% in adult horses(2). However, follow-up racing data
show that only 56.5% of all affected horses of racing age return to racing, and only 45% of these horses are able
to start more than five times. Survival rates in foals are much lower with only 45% surviving to be released
following hospitalization.

Treatment usually includes aggressive long-term antibiotic administration. Providing high drug
concentrations at the site of infection is an essential part of an effective treatment protocol. Currently,
antibiotic-impregnated polymethylmethacrylate (PMMA) beads or rods are the treatment of choice in horses
and humans with established musculoskeletal infection and may significantly contribute to a successful
outcome(3). Controlled release technology offers a novel approach to antibiotic delivery by targeting specific
tissues with optimal therapeutic concentrations while minimizing undesirable systemic side effects.

Biodegradable polymers are of increasing interest for a variety of therapeutic applications including their use
for sustained drug delivery in the treatment of orthopedic infection. The use of biodegradable polymers has the
inherent advantage over non-biodegradable alternatives such as PMMA of alleviating the need for surgical
removal of the delivery system at a later date(3).

The development of controlled release systems using bioerodible polymers has been significantly
advanced over the past five years. However, biocompatibility has become an important issue with the
introduction of these novel biomedical materials for drug delivery. Biocompatibility has been defined as the
ability of a material to perform with an appropriate host response in a specific application, and has been
classically described using four criteria: (1) the initial interfacial reactions (dominated by macromolecular
absorption onto the material surface), (2) the effect of the implant on the surrounding tissue, (3) the effect of the
tissue on the material, and (4) the systemic response to the implanted material(4). Using these criteria, an array
of both biodegradable and nonbiodegradable polymers has been demonstrated to induce a marked inflammatory
reaction(5). Therefore, careful selection and evaluation of the carrier material, including toxicity screening, is
needed in the development of a polymeric material suitable for use as part of a controlled drug delivery system.
Furthermore, a detailed understanding of the characteristics of the target tissue, the drug structure and action,
the biologic properties of the implanted polymer, and the elimination of the carrier from the body following
drug release is essential for achieving maximum therapeutic benefit(6). Failure of a biomaterial to display good
biocompatibility, particularly in a clinical situation, may result in a premature or delayed breakdown of the
desired material properties, or an unsatisfactory biological response.

Poly(ortho ester)s (POE) is a family of synthetic biodegradable polymers that consist of three distinct
generations(7). They are hydrophobic polymers, which undergo an erosion process confined to the polymer-
water interface known as surface erosion. Recently, a new variation of the POE-polymers was developed,
which is synthesized by the addition of diols to 3,9,-diethylidene-2,4,8,10-tetraoxaspiro[5.5]undecane, where a
glycolic acid dimer segment is incorporated into the polymer to adjust polymer erosion.

The POE consists of a semisolid, hydrophobic and injectible form of poly(ortho esters) and degrades
by surface hydrolysis. The hydrolysis process of the POE can be modulated by the addition of glycolic acid
dimer segments without the use of solvents, so that the residence time of the device and the rate of drug delivery can be controlled(7). These differences may be even more important in septic and acidic environments as they occur in infectious arthritis. In addition, sterilization techniques can significantly affect the mechanical and physical properties of PLA-PGA devices. All current standard sterilization techniques cause deformation, degradation, melting and softening of the polymer and result in toxic residues. In contrast, the POE can safely be sterilized by gamma-irradiation without toxic residues and deterioration and can be stored long-term under unhydrous conditions(7).

A variety of drugs have been incorporated into members of the POE-family and therapeutically useful delivery systems developed including 5-fluoruracil, the narcotic antagonist naltrexone, contraceptive steroids such as norethindrone and levonorgestrel, and indomethacine(8,9,10). The only antimicrobial agents that have been investigated therapeutically so far using the POE as a carrier in a sustained release system include tetracycline and mitomycin C(11,12). In order to determine the response of synovial and articular tissue to the polymer we applied a previously established in vitro model which uses changes in cell parameters including hyaluronic acid (HA) synthesis and cytokine production as sensitive indicators of tissue health. We hypothesized that, should the polymer have an undesired effect on normal cells, HA concentrations, histopathology scores and/or IL-1 and IL-6 levels will be altered when compared with a control group. The objectives of the study reported here were to evaluate the biocompatibility of the POE impregnated with gentamicin in vitro, and to determine whether this drug delivery system could rapidly eliminate infection.

**Materials and Methods:**

**Experimental design**

In a preliminary study, the elution characteristics of gentamicin sulfate from POE were studied by measuring the concentration of the antibiotic released from the polymer into synovial fluid into two double diffusion chambers over a 14-day period using an immunofluorescence (TDx) assay. Then two in vitro explant culture studies were conducted to evaluate the efficacy and toxic effects of the POE-gentamicin drug delivery system. A controlled randomized block design was used to evaluate the in vitro response of synovial and articular cartilage explant cultures to POE. This study was approved by the Iowa State University Animal Care Committee.

**Synthesis of POE**

Poly(ortho ester) was synthesized using a previously established protocol(13). Briefly, diols were added to 3,9-diethylidine-2,4,8,10-tetraoxaspiro[5,5]undecane, where a glycolic acid dimer segment is incorporated into the polymer. Polymerization was done under anhydrous conditions and the solution concentrated on a rotovaporator with the remaining solvent removed in a vacuum oven at 40°C. The polymer was mixed with 5% gentamicin sulfate powder\(^a\) (1 gr equivalent to 333.33 mg of gentamicin base) and had a

\(^a\) Garacin; Schering-Plough Animal Health Corp., Kenilworth, NJ
Double diffusion chamber for *in vitro* release kinetics from POE made from two 20ml syringes. Synovial fluid was harvested through the openings on the chamber surface. Note the Micropore membrane used to separate both chambers.

viscosity which made it possible to inject it through a 14 gauge needle using light pressure. The material was then sterilized in a 12 ml syringe using gamma-irradiation (2.5 mrad).

**Efficacy study - Synovial tissue explant cultures**

A modification of a previously established explant culture model was used (14). Synovial membrane tissue was aseptically harvested from the dorsal aspect of the midcarpal joint of one musculo-skeletal disease-free horse (one year of age, 345 kg body weight) within ten minutes of death, and placed in serum-free Dulbecco's modified Eagle's minimum essential medium* (DMEM) supplemented with 100 IU of sodium penicillin/ml and 100 mg of gentamicin sulfate* /ml on ice for transportation to the laboratory. There were no abnormalities on clinical examination of the joint and histologic examination of a synovial tissue sample.

Synovium was dissected from subsynovial connective tissue and fibrous capsule and sliced into 2-4 mm² explants (approximately 100 mg wet weight per culture well) for tissue culture. Explants were incubated for a total of four days in DMEM supplemented with 10% fetal calf serum without antibiotics (3 ml volume per well) at 37°C and 100% relative humidity in 5% CO₂ with daily harvesting, culture and exchange of media. Explants were randomly assigned to one of three groups (n=12 per group) (see Table 1).

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a Sigma Chemical Co., St. Louis, MO  
b Gentocin, Schering, Kenilworth, NJ
Group 1 served as a sterile, POE-free control group throughout the experiment (SC). Group 2 and 3 explants were stabilized in culture for 24 h, and then both infected with 405 CFU/mL of a *Staphylococcus aureus*, which had been previously cultured from an infected carpal joint and frozen down at -70°C. Group 2 served as the infected control group (SI) and group 3 (SIP) had 250 mg of POE (containing 12.5 mg gentamicin sulfate) added to each explant culture 18 hours post-infection. At the end of the study, all tissue explants were placed in 10% formalin for histologic examination. Two drops of the pooled media from each group were submitted for bacterial culture and the remainder stored frozen at -60°C for cytokine and hyaluronic acid analysis.

Table 1. Treatment protocol for the synovial explant groups at different times after establishment of cultures.

<table>
<thead>
<tr>
<th>Group/hours</th>
<th>24</th>
<th>42</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTR (SC)</td>
<td>Mc\textsuperscript{a}</td>
<td>Mc</td>
<td>Mc</td>
<td>Mc</td>
</tr>
<tr>
<td>INF (SI) + <em>S. aureus</em> \textsuperscript{b}</td>
<td>Mc</td>
<td>Mc</td>
<td>Mc</td>
<td>Mc</td>
</tr>
<tr>
<td>INF/POLY (SIP)+ <em>S. aureus</em></td>
<td>Mc +POE\textsuperscript{c}</td>
<td>Mc</td>
<td>Mc</td>
<td>Mc</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Media change; \textsuperscript{b}Staphylococcus aureus ; \textsuperscript{c}250 mg/culture

*Toxicity study - Synovial & Cartilage explant cultures*

Synovial membrane tissue was harvested from one horse (six months of age) using the same protocol as above. Explants were randomly assigned to one of two groups (n=12/group): Group 1 served as a sterile, POE-free control group (SC\textsubscript{tox}). Group 2 had 250 mg of POE added to the cultures after an initial stabilization period of 24 h (SP\textsubscript{tox}).

Explants were incubated for five days in DMEM supplemented with 10% fetal calf serum without antibiotics (3 ml volume per well) at 37°C and 100% relative humidity in 5% CO\textsubscript{2} with daily harvesting and exchange of media.

Full thickness articular cartilage was excised aseptically from the metacarpo- and metatarsophalangeal joints of one horse (2.5 years of age) within ten minutes of death. Cartilage sections were pooled and maintained in sterile tubes in 30 ml serum-free maintenance medium (Dulbecco's modified Eagle medium/F 12 DME/F12, suppl. with ITS\textsuperscript{c} in a 5% CO\textsubscript{2} at 37°C. After 24 h, the cartilage slices were cut into 2-3 mm\textsuperscript{3} explant fragments, pooled, and mixed thoroughly. The cartilage was placed into preweighed, sterile, 12 x 75 mm\textsuperscript{3} polystyrene tubes\textsuperscript{d} with caps containing 2 ml of the maintenance medium. The tubes were reweighed to

\textsuperscript{c} Sigma Chemical Co., St. Louis, MO
\textsuperscript{d} Amersham Life Sciences, Buckinghamshire, UK
determine the wet weight of the cartilage and the weight adjusted to 100 ± 5 mg of tissue. The explants were allowed to stabilize in DMEM for an additional 24 h at 37°C. The chondrocyte explant cultures were then randomly assigned to one of two groups (n=12) as follows: Control, polymer free group (CCtox), and a second group to which POE polymer was added at 24 hours (CPtox). Medium was harvested and replaced every 24 h for five days. Harvested media was frozen at -70°C until assayed.

**Bacterial culture of media**

Two drops of media harvested from the infected culture groups at 42 h and 72 h and from the infected groups which had received the polymer at 72 h and 96 h were cultured for bacterial growth on Blood agar, McConkey, and anaerobically.

**Hyaluronic acid concentration**

Hyaluronate (HA) in medium from synovial cultures of the efficacy study was quantified at 24 h (SCeff and SPeff groups), 42, 72 and 96 h (all groups) using a radiometric assay in triplicate. The test utilizes 125I-labeled HABP (hyaluronic acid binding protein) from bovine cartilage. A 100 µl sample of medium from each culture was pipetted into the tubes. The tubes for the standards consisted of HA concentrations at 5, 12.5, 25, 50, 75, 100, 500, and 1000 µg/l. To each sample, 100 µl of HABP-125I-solution was added, and the mix incubated for 60 minutes at 20°C. Then 100 µl of HA-Sapharose were added to each tube and the mixture incubated for 45 minutes at 20°C. Decanting suspension (2 ml) was then added and the tubes centrifuged for ten minutes at 1500 x g. Following centrifugation the tubes were decanted, the remaining moisture absorbed, and radioactivity measured by scintigraphy. Concentrations were determined using the standard curve.

HA concentrations were also quantified in the synovial cultures of the toxicity study (SCtox, SPtox) using the same protocol.

**Histology**

At the end of the culture period in the efficacy study, synovial explants were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned, and examined histologically using routine hematoxylin and eosin (H&E) staining techniques.

Qualitative morphologic evaluation was performed using a previously established scoring system and evaluated by two pathologists that were masked to treatment(15). Scores were assigned to (1) presence of bacteria, (2) loss of differential staining, and (3) cellular debris.

Synovial membrane explants in the toxicity study were graded for (1) synovial hyperplasia, (2) subintimal edema and (3) subintimal fibrosis, (4) loss of staining and (5) debris. These variables were scored from 0-4 (0, normal; 1, occasional changes noted in section; 2, changes noted in approximately 50% of section; 3, changes noted in 75% of section; 4, changes noted in 100% of section).

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*Pharmacia AB, HA Test, Uppsala, Sweden*
3, changes noted in >50% of section; 4, changes throughout section) and the median grade for each variable was calculated for each of the two explant groups (SC\textsubscript{tox} and SP\textsubscript{tox}).

Full thickness cartilage explants from the toxicity study were evaluated using H&E, Alcian Blue and Safranin O stains. Cartilage explants were similarly evaluated for (1) chondrocyte necrosis, (2) chondrone formation, and (3) intercellular matrix staining. The median grade for these variables were added to give an articular cartilage score for each section with a range from 0 (normal) to 12 (very abnormal in all characteristics). A cartilage explant score was then calculated for each specimen group (CC\textsubscript{tox} and CP\textsubscript{tox}).

\textbf{Viability staining of toxicity explant groups}

Viability staining of synovial-and cartilage explants was performed in the toxicity study (n=12/group) at the end of the experiment (day 5). Synovial explant viability was assessed using the Trypan Blue Exclusion method as previously described\cite{16}. Briefly, each explant was rinsed in fresh DMEM without FBS before being placed in digestion vials and incubated with 3 ml of 0.25% trypsin-EDTA. The vials were stirred for 30 minutes at 37°C and the explants then again rinsed in fresh culture medium three times before they were incubated further in 3 ml DMEM with collagenase type IV\textsuperscript{f} (200 IU/ml of DMEM media) for 20 minutes at 37°C. The filtrate from the base of the digestion vial was collected and centrifuged for two minutes at 6500 g. The centrifugation pellet was placed on a glass slide and 30 µl of 0.1% trypan blue\textsuperscript{g} in PBS solution was added. Two hundred cells were immediately counted and the viability calculated as a percentage of unstained cells (stained cells and unstained cells) x 100.

Cartilage explant viability was performed using fluorescence microscopy. Explants were rinsed three times in fresh DMEM and a 2-3 mm\textsuperscript{2} piece of cartilage from each explant group was mounted on the tissue stage of a Microslicer\textsuperscript{h} with 0.1 mol PBS as the buffer solution. The articular surface was sectioned into 70-100 µm slices and immediately placed on a glass slide. Two drops of Mounting Medium with propidium iodide\textsuperscript{i} were added to the tissue slice and examined at 10X magnification under a fluorescence microscope\textsuperscript{j}. Photomicrographs were taken from each section with and without fluorescence light. Because the cartilage thickness and cell density varied, cell viability was determined via image analysis counting the number of fluorescing dead (yellow) cells per unit area (mm\textsuperscript{2}) on a photomicrograph compared with the same unit area of a photomicrograph without a fluorescence filter (all cells).

\textsuperscript{f} Sigma Chemical Co., St. Louis, MO
\textsuperscript{g} Sigma Chemical Co., St. Louis, MO
\textsuperscript{h} DTK 1000, Pelco, Tustin, CA
\textsuperscript{i} Vectashield® H-100, Vector Laboratories, Burlingame, CA
\textsuperscript{j} Olympus BX 60/BX-FLA, Sun Valley, CA
Cytokine expression

Samples of previously harvested culture media in the efficacy study were thawed and assayed for IL-1β and IL-6 using commercially available ELISA kits\(^k\). These assays employ the quantitative sandwich enzyme immunoassay technique with monoclonal antibodies specific for recombinant human cytokines pre-coated onto a microtiter plate. The sensitivity for these assays has been determined to be less than 1 pg/mL and less than 0.7 pg/mL for IL-1 and IL-6, respectively.

Media from all groups of the toxicity study (SC\(_{tox}\), SP\(_{tox}\), CC\(_{tox}\), CP\(_{tox}\)) was also assayed for IL-1β and IL-6.

Cytokine concentrations were expressed as a ratio to the baseline value for each synovial explant treatment group.

Data Analysis

Data for all variables was averaged and expressed as a mean ±SEM. For synovial explants, the toxicity portion of the experiment (SC vs SP) was evaluated separately from the efficacy portion of the experiment (SC vs SI and SIP). Values were compared by 2 way-ANOVA with repeated measures. Where significant differences were identified, post-hoc comparisons were made using Sheffe's test and significance recognized for values of P<0.05.

Results:

In vitro gentamicin release (chamber & cultures)

The release of gentamicin from the polymer (500 mg) into the two diffusion chambers at 12 and 24 hours averaged 105.5 µg/ml and 202 µg/ml, respectively. During the following 24 h a high release of gentamicin measured 3830 µg/mL from the POE, which was followed by a plateau of sustained release for the following 13 days averaging 4269.23 µg/mL per day. The highest drug concentration was measured at 96 h with 6500 µg/mL. The polymer visibly degraded and dissolved within the synovial fluid over a 4-5 day period; however, high gentamicin levels were maintained throughout the study period. The release of gentamicin in the synoviocyte and cartilage cultures was fairly similar during the study period with 2475 µg/mL and 1581 µg/mL at six hours and 3420 µg/mL and 3360 µg/mL at 24 hours, respectively. No gentamicin levels were found in the media prior to adding the polymer at 18 hours.

Subjectively, the polymer material appeared to disintegrate and dissolve slightly faster in the cultures compared with the degradation within the synovial fluid chamber. This was particularly distinct in the synovial explants when compared with the cartilage explants.

\(^k\) Quantikine, R&D Systems, Minneapolis, MN
**Hyaluronan synthesis**

The mean hyaluronan concentrations from the SC group did not differ significantly from that of the SP group over a 72 h time period. However, there was a time effect with HA values found to be significantly higher at 24 h than 42 h or 72 h ($P<0.05$). For subsequent comparisons among SI and SIP groups, SC and SP values were pooled as controls (SCP). The mean HA values of the control group was significantly greater than values for the SI and SIP groups ($P<0.001$), and mean SIP values were significantly greater than mean SI values ($P<0.001$). Significant differences in values between 42 h and 96 h were also found ($P<0.01$). This decline in HA concentration was most apparent in the SI group after 18 h of exposure to *S. aureus*. The concentration of HA in the SIP group was similar to the levels in the control group at 42 hours, but significantly declined during the remainder of the culture period.

Hyaluronan concentrations in the toxicity study were not significantly different between SC$_{tox}$ and SP$_{tox}$, however, there was a statistically significant difference present between the two cartilage groups (CC$_{tox}$ and CP$_{tox}$). HA concentration had significantly declined in the CP$_{tox}$ group after 48 hours of incubation with the POE.

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**Figure 1.** HA concentrations in the synovial explant efficacy study. Note the very low HA concentration in the infected and untreated group at 42 hours. HA concentration in the infected and POE treated group reached almost control levels.
IL-1\(\beta\) and IL-6

Apparent differences in IL-1\(\beta\) and IL-6 levels among synovial explant groups in the efficacy study were not statistically significant. There was a trend for increased expression of IL-1 \(\beta\) in the SI group at 42 hours and an increase of IL-1 \(\beta\) in the SIP group. However, cytokine levels declined significantly over time (\(P<0.05\)). Interleukin-6 showed an increased expression from the baseline in the SI group compared with the SP and SIP groups.

No difference between IL-1\(\beta\) and IL-6 expression was found among the synovial explants in the toxicity study, but we observed an increased expression of IL-1\(\beta\) and IL-6 in the cartilage explant group (\(\text{CP}_{\text{TOX}}\)) after the POE had been added for 24 hours.

Histology and Viability staining

All synovial and full thickness cartilage samples harvested were histologically normal prior to tissue culture.

Histopathology scores for the SC group in the efficacy study remained low except that the score for bacterial presence was almost as high as in the infected group. Scores for the SI group were significantly higher compared with SC and SIP. Scores between SC and SIP were not significantly different (\(p < 0.05\)), but SIP had a lower score for bacterial presence.

Synovial explant viability staining was \(>80\%\) in the control groups of the toxicity study. Groups which had the polymer added showed a viability of \(>90\%\). Histopathology scores of the control group and the group, which had POE added, were not significantly different (\(p < 0.05\)). However, there was a significant difference among the cartilage groups concerning cell viability and histopathology scores. Viability in the control group (\(\text{CC}_{\text{TOX}}\)) reached 245 cells/unit area compared with 98 cells/unit area in the \(\text{CP}_{\text{TOX}}\) group.

Histopathology scores were higher in the \(\text{CP}_{\text{TOX}}\) group. Chondrocyte necrosis was present in 50\% of the explants with fibrillar matrices and patchy eosinophilic stains.

Microbiology (Culture of Media)

All groups, which were intentionally infected with a \(S.\,\text{aureus}\) cultured positive at all times. The SIP group was negative for \(S.\,\text{aureus}\) 24 h after addition of the polymer. All other groups including the explants from the toxicity study cultured negative for bacteria at all times.

Discussion:

We evaluated the material under \textit{in vitro} conditions to gain basic data concerning the effects of the material on joint tissue. This allows estimation of the response that can be expected when the polymer is injected into a synovial cavity \textit{in vivo} and ultimately determines whether this polymer is suitable for controlled
drug release in the joint. Also, we used fairly young animals for our explants because we considered juvenile tissue to be more sensitive and amplify any cellular insult that may result from the presence of the polymer.

The POE was chosen for the development of a controlled drug release system because previous studies have indicated excellent biocompatibility. In recent studies involving second generation poly(ortho esters), it was found that the two ultimate hydrolysis products possess no significant toxicological hazards and are excreted without metabolism through the kidneys(17). The addition of polymer to synovial membrane explants did not result in significant changes of cell parameters including HA-synthesis and cytokine production. Hyaluronan concentration remained at high levels at all times in the SC and SP groups. Histopathology scores were not significantly different from scores from the control groups, which may indicate that the breakdown products during polymer degradation do not cause harmful or compromising effects on synovium.

The gentamicin release from the POE eliminated infection from the synovial explants and significantly reduced bacteria counts compared with the SI group. However, the relatively high bacteria score in the control group precluded distinguishing more between the SIP group and the control group. Possible reasons for this may be contamination at the time of harvesting the synovial membrane or during and after the culture process. The most likely reason for the bacterial presence in the culture may be that bacteria were introduced during processing, because no significant morphologic cell changes were observed at the end of the culture period and HA concentration remained at high levels at all times. The increased expression of IL-1 at 96 hours in the control group may further support a late introduction of the bacteria.

Histopathology scores in the SIP group almost reached levels of the SC group after POE was added to the previously infected synovial explants. Although HA-concentration reached control levels after infection had been eliminated, we observed significant deterioration of HA-synthesis at 72 hours in the SIP group. This may be either the result of decreased synthesis or increased degradation of HA by bacterial hyaluronidase. Because we did not evaluate cell viability in this group, it is possible that cell death may have been the cause for the decreased HA concentration. However, HA concentration remained at normal levels after elimination of infection, which makes acute cell death as a cause more unlikely. Lower HA concentrations have been found in septic joints and have also previously been reported in an in vitro synovial explant infection model(18,19). Hardy et al. (1998) reported that filtered media from previously infected synovial cell cultures added to synovial explants caused inflammatory mediators to raise and HA concentrations to fall. When filtered media was added to cartilage explants, matrix degradation occurred.

Cook et al.(14) found that HA concentrations in infected synovial explants were dramatically decreased after infection had been eliminated with a biodegradable drug delivery system. Even though infection is rapidly eliminated from joint tissue, ongoing deterioration of cells may account for the low HA levels. It has been suggested that decreased HA synthetic rate, rather than increased degradation may be the mechanism for decreased HA concentration in culture medium(18). Our results confirm these findings, although HA concentrations in our explants temporarily reached control levels.
Cartilage explants responded very differently to the POE. Histopathology scores in the CP10X group were significantly higher compared with the control group. Saffranin O staining indicated that most of the proteoglycan had been washed out from the extracellular matrix. Why the scores are so much different from the synovial explant can only be speculated. Although one may expect a more dramatic response by the synovium as synoviocytes commonly are the first line of defense in injury, chondrocytes appear to be more sensitive in this case. This, however, was not reflected by an increase in cytokine levels in the cartilage explants, which may be a result of matrix damage, but not damage to the nucleus. By using smaller amounts of the POE, this response may be alleviated. However, only in vivo testing may reveal whether or not this is a significant finding. Future in vivo studies with the POE need to include the evaluation of glycosaminoglycan, chondroitin sulfate, or keratan sulfate concentrations of cartilage specimens.

Immunohistochemistry is being used extensively in the assessment of biocompatibility of biomedical implants. Immunohistochemistry has also been a valuable tool in the investigation of joint disease and serves as a sensitive indicator for saddle changes in the normal environment of synovium and cartilage in the joint. We assumed that any detrimental effects of the polymer on synoviocytes and cartilage would be expressed as an increase in certain inflammatory mediators including cytokines. This model focused on the expression of IL-1 and IL-6 because they have been consistently found to be released in medium and synovial fluid by synovial cells as a response to injury. IL-1 is a key mediator of the inflammatory host response to microbial invasion and contributes to the destruction of cartilage(20,21). Both equine mononuclear cell factor (MCF) and recombinant huIL-1, alpha and beta, have been shown to stimulate the production of PGE_2 by equine chondrocytes and synovial cells in vitro and inhibit proteoglycan synthesis in equine articular cartilage(22). We used human ELISA test kits because hybridization studies of human, murine and equine IL-1 revealed high homology among them(23,24).

At no time did the presence of the polymer in the synovial explants cause an increase in cytokine levels compared with the control groups. Our other results of this study concerning IL-1 and IL-6 production differ in some aspects from previous studies. There was an increase in IL-1 expression in the infected group, which had been treated with the POE. This may reflect a post-infectious rebound-response of the synoviocytes after the invading pathogen has been eradicated from the system. It has been documented that synovium and cartilage are undergoing continuous degenerative matrix changes even though the infection has been eliminated. These ongoing changes have been associated with the sustained synthesis of inflammatory mediators such as IL-1 by synoviocytes and have been referred to as "post-infectious arthritis"(25). In a previous synovial explant study using S. aureus to infect the synoviocytes, it was found that IL-1beta concentration ratio remained significantly high in groups which had received bacteria-free (filtered) media from previously infected synovial explants, even after culture was continued with standard medium(18). We did not observe the early increase in IL-1 after S. aureus infection that has been seen in previous studies, which may be related to the dose of antimicrobial exposure. Although the amount of bacteria (405 CFU/mL) that was added
to the explants for infection has been shown to result in sufficient cellular damage without causing cell death, our explants may have become more compromised by the amount of bacteria making them unable to respond. The fact that HA-concentrations were significantly decreased in this group may indicate a detrimental insult to the cells that might have lead to cell death. On the contrary, when *S. aureus* was added to the SIP group and infection was eliminated with the POE, cells retained the ability to synthesize HA, which indicates normal cell function.

Interleukin-1 is a potent inducer of IL-6, which is also produced by synovial cells(26). Although there was a trend in the SI group for increased expression of IL-1, the expression of IL-6 was more significant in the same group at the same time. Assuming, that IL-6 typically shows a delayed appearance, which is stimulated by IL-1 or TNF, then our data would suggest that IL-1 beta may have become elevated shortly after infection to cause IL-6 to raise later on.

The overall good biocompatibility of the POE in this culture model is consistent with data from other groups(27). Bernatchez et al. found that subconjunctival injection of the POE caused a mild eosinophilic inflammatory response at injection sites in laboratory animals when compared with sodium hyaluronate and silicone oil. The acute inflammation resolved completely by day ten and no encapsulation with fibrous tissue occurred. However, these *in vitro* results concerning the tissue response towards the POE may not necessarily correlate with the response in an *in vivo* environment and therefore need to be interpreted carefully when extrapolated to injection of a joint. Although the hydrolysis products of the POE including triethylene glycol, glycolic acid, pentaerythritol and propionic acid have not been associated with toxicological effects in laboratory animals, injection of the POE into a synovial structure in the horse may result in undesired site effects.

The release of gentamicin from the POE in our preliminary kinetic study occurred in a burst during the first 24 hours; however, the drug release during the next 13 days was more characterized by a high sustained release of gentamicin. This phenomena has been well recognized in the synthesis of polymers and constitutes one of the greatest problems in the design of controlled drug delivery systems. "Dumping" of the incorporated drug may lead to undesired toxicity of the drug and makes the release kinetics much more random. Ideally, the main mechanism responsible for the release of drug from a biodegradable polymer is surface erosion while the drug is uniformly distributed through the polymer facilitating zero-kinetic release(28). In order to obtain zero-order release, a geometry may be necessary that does not change its surface area as a function of time(29). Bulk erosion in addition to surface erosion, as seen in both the kinetic- and the culture study, is a result of the polymer imbibing water into the center of the matrix and has been observed with other polymers as well. Polylactic acid (PLG) and polyglycolic acid (PLGA) polymers used for biomedical application are well known for this problem which makes the controlled release in these materials particularly difficult(30,31). We also accounted some problems with the diffusion chamber set up. While we were using a 500 mg POE sample which had a total amount of 25 mg gentamicin incorporated for release, the total amount
of gentamicin that was found in the synovial fluid over the entire time period added up to approximately 80 mg. This discrepancy may be the result of an invalid assay procedure. While the immunofluorescence assay (TDx) is routinely being used to determine gentamicin levels in serum this assay is not validated for the measurement of drug levels in synovial fluid. For that reason, a different technique may need to be applied such as High Performance Liquid Chromatography (HPLC) in order to get accurate drug concentrations.

One of the significant advantages of the POE over other polymers is that the release of drugs from this polymer matrix can be predictably controlled and adjusted by varying the concentration of glycolic acid dimers. For future studies with the POE, particularly in vivo, the release of drug during the first 24 hours needs to be adjusted to a more homogenous system in which high drug concentrations are reached fast and then maintained at that level for the desired period of time. Gentamicin was chosen to incorporate into the polymer, because of its broad gram-negative spectrum and efficacy for the most commonly cultured organisms found in infected joints(2). Previously reported susceptibility patterns for these organisms have not differed from findings in our hospital. However, from a pharmacokinetic point of view, aminoglycosides may not be the drug of choice for sustained release because of their significantly increased efficacy when administered once daily at a high dose. Once-a-day dosing prolongs the "post-antibiotic effect" and has been associated with the least potential for nephrotoxicity. Therefore, it may be clinically more beneficial to release gentamicin in a "pulsatile" fashion, which is currently being investigated in stimuli-responsive ("intelligent") delivery systems(32,33).

Pharmacokinetic studies commonly use buffer systems such as PBS (pH 7.3, 154 mmol/L) to determine the concentrations of drugs released into a closed system(34). We used synovial fluid because we assumed that the release into an environment such as the joint may be different from the release into a phosphate buffered saline environment. Several factors may significantly influence the release of drug as a function of time, particularly when considering microparticles (microspheres) and ointments as the drug carrier matrix. These may include differences in viscosity of the fluid surrounding the polymer and the difference in the composition of the fluid. Synovial fluid is an ultrafiltrate of plasma and contains ions and molecules such as hyaluronan in high concentrations (0.5 mg/ml)(35). It is unknown at this time how these factors influence the release of drug into the environment and which impact they have on the polymer matrix itself. By using previously harvested normal synovial fluid as the medium from horses unaffected by joint disease, we attempted to simulate in vivo conditions, which would give us a more realistic idea about the release from inside a closed synovial cavity.

In summary, the POE was able to effectively eliminate infection from a synovial explant culture. Although HA-synthesis was maintained during and after the infection, HA-concentrations significantly declined shortly post-infection. POE did not cause an increase in cytokine levels in the synovial explant groups and remained within control-levels or below. The gentamicin-impregnated polymer did not decrease synovial explant viability, however, cartilage explants showed a moderate decline in viability and some significant histopathologic changes after five days of exposure to gentamicin-impregnated POE.
Picture 2. Photomicrograph (10x) of cartilage explants. Left: Explant with POE added for five days. Right: Control explant. Note the pyknotic and karyorrhectic chondrocytes.

Our data did not reveal any detrimental effects of the POE on joint tissue that would prevent its testing in vivo. Because polymer erosion time and rate of drug release can be readily varied for this material, residence times and rates of gentamicin release may be adjusted to the requirements of a therapeutically useful system. The material may be a valuable adjunct therapy in the treatment and prophylaxis of septic musculoskeletal conditions in the horse.

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CHAPTER 3. BIODEGRADABLE INJECTABLE POLYMERS FOR SUSTAINED ANTIMICROBIAL DRUG RELEASE IN THE EQUINE JOINT:

AN IN VIVO PILOT STUDY

A paper to be submitted to Veterinary and Comparative Orthopaedics and Traumatology


Summary:

Microspheres consisting of poly(DL-lactide-co-glycolide) (PLGA) and poly(e-caprolactone) (PCL) were prepared via double emulsion technique and impregnated with 15% gentamicin. The gentamicin release from both polymers into double diffusion chambers filled with synovial fluid were evaluated over an eight day period via a radioimmunoassay. Release of gentamicin from PLGA and PCL measured 2.05 µg/ml and 40.4 µg/ml at 12 hours, respectively. Synovial fluid samples from both polymers inhibited the growth of a S. aureus in vitro.

The articular response to PLGA microspheres injected into the radio-carpal joint of three horses was compared with the injection of another polymer, Poly (ortho ester) (POE) ointment which was injected into the tibio-tarsal joint of three horses over a 4 day period. Biocompatibility data revealed a mean WBC of 28,945 cells/µL and 91% mildly degenerated neutrophils in the PLGA-injected joints versus 3,528 cells/µL and 90% neutrophils in the POE injected joint.

Protein levels, lymphocyte and macrophage counts were also elevated. Total cell count in the injected joints reached control levels after 96 hours. None of the horses showed significant clinical signs from the PLGA or POE injection. Although both polymers POE and PLGA cause a short-term moderate synovitis and may be suitable as a biodegradable drug delivery carrier in the equine joint, the POE seems to cause less irritation of the synovial membrane. Because in vitro drug release from the PCL polymer was promising based on the levels of drug eluting, PCL deserves further evaluation for drug delivery in the equine joint.

Introduction:

Septic arthritis is a disorder that results from sequestration of bacteria in a joint. The cause of septic arthritis can be (1) hematogenous in origin which is common in foals but rare in adults; (2) spread from an adjacent infected soft-tissue focus; (3) occur as the result from a penetrating wound; or (4) occur secondary to surgery (eg. arthroscopy). Other sources for joint infection in the adult horse are inoculation during intra-articular injection and open fractures, which are extending into joint cavities. The incidence in foals on breeding farms has been reported to be 1%, but causes mortality in 0.25%(1). The risk of infection was the highest in the first 30 days. Polyarthritis is common in foals (50% of those with infectious arthritis) and uncommon in adults (1.5%) (2).
Although therapy selection including a combination of antimicrobial and joint drainage protocols has significantly improved the prognosis and mortality rate, septic arthritis remains one of the most detrimental and performance limiting injuries in the horse industry. This is in part due to severe and chronic damage to the synovial structures which is triggered by the induction of an inflammatory process by the invading microorganism.

A significant contributing factor to the development of traumatic infection is extensive soft-tissue damage that causes disruption of blood supply and lymphatic drainage as well as compromising local immune response against initial contamination (3). This results in low antimicrobial tissue levels at the site of infection when antibiotics are administered systemically. To provide therapeutic antimicrobial drug concentrations, local delivery has become an important adjunct therapy in the treatment and prophylaxis of musculoskeletal infection. Implantation of antimicrobial-impregnated polymethylmethacrylate (PMMA) beads into a site of infection has been an effective method for providing sustained high concentrations of antibiotics locally. PMMA beads are currently recommended for use in the treatment of open fractures and acute and chronic bone and joint infections. However, removal of the beads from immobilized joints after elimination of the infection can be difficult due to granulation tissue or fibrous scar tissue formed around the beads. To overcome this disadvantage, biodegradable materials have been used to carry and release antimicrobial drugs over a sustained period of time. Some of the first materials investigated for this purpose were poly(lactic acid) (PLG) and poly(glycolic acid) (PLGA) polymers and their copolymers, because they display important advantages of biocompatibility, predictability of biodegradation kinetics, ease of fabrication, and regulatory approval by the FDA. Importantly, these polymers can be purchased commercially. Biocompatibility as well as biodegradation rate of PLG and PLGA polymers depends on their components and molecular weight, respectively. Other influential factors include the repeat structure of the copolymer, the pH and ionic strength of the external medium, and the nature of the drug incorporated into the delivery device.

However, biocompatibility of PLA and PGA is still somewhat controversial and the majority of available data is from laboratory animals or has been established using cell culture systems. Extensive experimental and clinical data support good tissue receptivity by implantation in bone. Cutright et al. (19) investigated different polymers and copolymers of PLA and PLG in rat femurs and in the mandible of rhesus monkeys. They found that the polymers were replaced by connective tissue, bone and marrow tissue, thus, suggesting good biocompatibility. In contrast, non-specific foreign-body reactions with osteolysis has been reported once degradation of the PLA began(18). To our knowledge, there is currently no data available on the use of PLG microspheres within synovial membrane cavities in companion animals.

Poly(ortho ester) consists of a semisolid, hydrophobic and injectible form and degrades by surface hydrolysis. The hydrolysis process of the POE can be modulated by the addition of glycolic acid dimer segments without the use of solvents, so that the residence time of the device and the rate of drug delivery can be controlled(7). These differences may be even more important in septic and acidic environments as they
occur in infectious arthritis. The POE can safely be sterilized by gamma-irradiation without toxic residues and deterioration and can be stored long-term under unhydrous conditions(7). In contrast, sterilization techniques can significantly affect the mechanical and physical properties of PLA-PGA devices due to deformation, degradation, melting and softening of the polymer and may result in toxic residues.

To our knowledge, Poly(ortho ester) has not been investigated for the use of controlled drug delivery in companion animals. Only limited data concerning the biocompatibility have been reported including rat studies investigating POEs for ocular delivery of 5-fluorouracil after glaucoma filtration surgery in people. The POE was compared with HA and medical grade silicone oil. POE has recently been investigated for the use in the treatment of orthopedic infection in the horse. Cell culture studies have shown that gentamicin impregnated POE can successfully resolve a Staph. aureus infection of synovial membrane (20). However, a 250 mg sample of the POE, which was added to synovial, explants and cartilage explants, revealed that cartilage may be more sensitive to the material than synovium as indicated by viability staining. It was concluded that POE may be a valuable aid in the treatment of orthopedic infection. In vivo data may be needed to ultimately determine the biocompatibility.

The purpose of our study was (1) to determine the in vitro elution kinetics of gentamicin in synovial fluid from 50/50 drug loaded poly(DL-lactide-co-glycolide) (PLGA) microspheres which was compared with the gentamicin elution characteristics of poly(e-caprolactone) (PCL) which is another commonly used polymer for drug delivery, and (2) to establish biocompatibility data of two polymers (PLGA and POE) after injection into joints of horses.

Materials and Methods:

Preparation of polymers

50/50 poly(DL-lactide-co-glycolide)a and poly(e-caprolactone)b microspheres of 80-120 µm diameter in size impregnated with 15% of gentamicin sulfatec were prepared via the double-emulsion-evaporation technique as previously described(12). The size of the microspheres were determined via a microscope and sterilized using ethylene-oxide.

Poly(ortho ester) was synthesized using a previously established protocol(13). Briefly, diols were added to 3,9-diethylidine-2,4,8,10-tetraoxaspiro[5.5]undecane, where a glycolic acid dimer segment is incorporated into the polymer. Polymerization was done under anhydrous conditions and the solution concentrated on a rotovaporator with the remaining solvent removed in a vacuum oven at 40°C. The polymer was mixed with 5% gentamicin sulfate powderd (1 gr equivalent to 333.33 mg of gentamicin base) and had a

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a Birmingham Polymers, Inc., Birmingham, AL
b Birmingham Polymers, Inc., Birmingham, AL
c Gentocin, Schering Corp, Kenilworth, NJ
d Garacin, Schering-Plough Animal Health Corp., Kenilworth, NJ
viscosity which made it possible to inject it through a 14 gauge needle using light pressure. The material was then sterilized in a 12 ml syringe using gamma-irradiation (2.5 mrad).

**In vitro release kinetics**

The *in vitro* release was assessed with the use of a double diffusion chamber separated by a Millipore membrane. The donor side contained 50 mg microspheres diluted in a 5 mL saline solution and the receiver side only synovial fluid (15 mL volume). The chamber was continuously agitated and samples of synovial fluid (1 ml) from the receiver side collected and replaced with fresh synovial fluid over a ten day period. Synovial fluid was assayed in duplicates using a radioimmunoassay.

**Documentation of Gentamicin Bioactivity**

Aliquots of the harvested synovial fluid samples from the above study were also tested for bioactivity against a lawn of *S. aureus*. A virulent *S. aureus* isolate from an infected equine joint was kept frozen at -70°C for use in this study. A suspension matching a 0.5 McFarland turbidity standard was prepared and used to create lawns of *S. aureus* on Mueller Hinton agar plates. Six mm holes were punched into the agar using a disposable biopsy punch and filled with 100 µL synovial fluid collected at days 1, 3, 5, 8 and 10. There were three holes/plate which were incubated for 24 hours at 37°C and then the zone of inhibition was measured.

**In vivo preparation**

Six horses (two 18 years of age) were divided into two groups, a PLGA and a POE group, of three horses each. The study involving the PLGA group was performed first to gain basic knowledge about catheter placement and joint response towards the polymer. Because problems with the maintenance of the catheter were experienced, a larger joint—the tibio tarsal joint—was chosen for the POE group. This facilitated arthrocentesis and consistently provided adequate amounts of synovial fluid.

Horses of both sexes and various breeds with no prior history of joint disease were used. Each horse was judged to have clinically normal antebrachiocarpal- and tibio tarsal joints if effusion, heat, or signs of pain on palpation were lacking. In addition, all horses were considered sound if no lameness was observed when trotted in a straight line. The Iowa State University Animal Care Committee approved this study.

In the PLGA group, the left and right radio-carpal joint of three normal horses were aseptically prepared. One antebrachiocarpal joint (left) was chosen for treatment in each horse before the experiment. A catheter (2.5 cm long, 0.8 mm in diameter) was aseptically introduced into each joint at the palmarolateral

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*e* Type NY60, Millipore Corporation, Bedford, MA  
*f* GammaCoat™, Incstar Corp., Stillwater, MN.  
*g* Disposable Biopsy Punch, 6 mm, Premier.  
*h* Angiocath, The Deseret Co., Sandy, UT
A 200 mg polymer sample was dissolved in 4.85 mL H2O, 0.05 mL Tween 80, and 0.1 mL 2% Carboxymethylcellulose and injected under aseptic conditions into one antebrachio-carpal joints of each horse.

In the POE group, a 4 gram sample of self-catalyzed ointment was injected under sterile conditions using a 10 ga 1½ inch needle into the right tibio-tarsal joint in each horse. The opposite joint served as the control and was sham-injected with 5 ml of sterile saline. Synovial fluid collection was performed via arthrocentesis.

A synovial fluid sample was collected from all joints prior to polymer injection for cytology.

**Synovial fluid cytologic examination**

Synovial fluid specimens for cytologic examination were obtained in both groups using a 1 ml tuberculin syringe and a 2.5 cm long, 0.8 mm in diameter hypodermic needle, at post-injection hours (PIH) 24, 48, 72, and 96. To prevent clot formation, the specimens were transferred immediately to tubes containing the disodium salt of edetate. Smears for the differential leukocyte count were made immediately.

Specimens were refrigerated at 4°C and were evaluated within 24 hours of collection for color and transparency, and pH. Refractive index was measured principally to estimate fluid total protein concentration. White blood cell counts (WBC) and neutrophils, large mononuclear, as well as small mononuclear cells were quantitated by examination of Wright-stained smears.

**Synovial fluid gentamicin concentration**

Gentamicin levels in the collected samples from the POE- and the PLGA group were quantified using an automated fluorescence polarization Immunoassay (FPIA) analyzer. Hyaluronidase lyophilized desiccated was added to each sample to decrease the viscosity of the synovial fluid for analysis by the TDx-analyzer.

**Clinical evaluation**

All parameters were assessed prior to polymer injection and daily prior to synovial fluid collection throughout the study period and until three days after the last collection. Parameters included joint circumference, carpal flexion, and degree of lameness. Lameness was graded using a scale from 0 to 5. (0 = no lameness at a walk or trot; 1 = no lameness at a walk, alterations of gait at a trot; 2 = alteration of gait at a walk, head bob at a trot; 3 = obvious head bob at a walk or trot; 4 = intermittent non-weight bearing; 5 = persistent

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i Sterile blood collection tubes, Monoject, St. Louis, MO
j Total solids meter, American Optical Corp., Buffalo, NY
k Improved Neubauer Ruling Hemacytometer, American Optical Corp, Buffalo, NJ
l TDx Gentamicin Assay, Abbott Laboratories, Chicago, IL
m Hyaluronidase Type I-S; Sigma Chemicals, St. Louis, MO
non-weight bearing). Carpal flexion was subjectively assessed by slowly flexing the polymer-treated carpus until the horse resisted. When significant resistance in carpal flexion was noted, the lameness score was upgraded for 1 grade at the scale from 0-5. Carpal circumference was measured in centimeters at the level of the mid-accessory carpal bone using a flexible tailor's tape. Tarsal joint circumference was measured at the level of the polymer-injection site, which was marked by a simple interrupted suture using 2-0 nylon ®.

Statistical analysis

Paired t test, with a Bonferroni adjustment for the number of comparisons made, was used to determine whether statistically differences existed between results from specimens obtained at PIH 0 and from all other times within each group. This analysis was used as a variance-stabilizing transformation and was performed on the logarithm of the observations. The Wilcoxon signed rank test was performed on the same data to verify that results obtained were not reliant on the t test's assumption of an underlying normal distribution. The Tukey w procedure, which corrected for subsequent multiple comparisons, was used to determine statistically significant differences in gentamicin concentrations between the two polymers. Statistical significance was established at p <0.05.

Results:

Gentamicin concentrations from PLGA and PCL

Concentrations resulting from the release of gentamicin from PLGA and POE into synovial fluid over the study period reached levels of 2.2 µg/mL and 15.6 µg/mL, respectively, at 24 hours (Table I). The release concentrations peaked at 96 hours for the PCL at 59.3 µg/mL and at 2.6 µg/mL at 168 hours for the PLGA.

Although higher gentamicin release was found with the PCL there was a marked fluctuation in the drug concentrations and the release occurred very non-linear. The drug concentrations in the synovial fluid from the PLGA microspheres were fairly constant. The nearly constant gentamicin concentration of approximately 2 µg/mL was at the MIC (Minimal Inhibitory Concentration) which has been reported for most clinical organisms. Bioactivity assays confirmed that the concentration of gentamicin within the synovial fluid samples were high enough to inhibit growth of S. aureus. No significant antimicrobial activity was detected in the samples from joints without the PLGA. The zones of inhibition for S. aureus growth for PLC and PLGA were proportional to the gentamicin concentration measured in the same sample (PCL/PLGA, day 1, 28 mm, 17 mm; day 3, 32 mm, 19 mm; day 5, 25 mm, 15 mm; day 8, 20 mm, 17 mm; day 10, 9 mm, 10 mm, respectively).
Table 1. Gentamicin elution concentrations from 50/50 PLGA and PCL in synovial fluid in vitro.

<table>
<thead>
<tr>
<th>Times (hours)</th>
<th>50/50 PLGA µg/mL</th>
<th>PCL µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.298</td>
<td>39.82</td>
</tr>
<tr>
<td>1</td>
<td>0.863</td>
<td>16.88</td>
</tr>
<tr>
<td>3</td>
<td>1.456</td>
<td>24.98</td>
</tr>
<tr>
<td>6</td>
<td>1.574</td>
<td>20.01</td>
</tr>
<tr>
<td>12</td>
<td>1.62</td>
<td>25.66</td>
</tr>
<tr>
<td>24</td>
<td>2.16</td>
<td>15.61</td>
</tr>
<tr>
<td>36</td>
<td>1.885</td>
<td>45.6</td>
</tr>
<tr>
<td>48</td>
<td>1.882</td>
<td>27.02</td>
</tr>
<tr>
<td>72</td>
<td>1.612</td>
<td>42.6</td>
</tr>
<tr>
<td>96</td>
<td>2.304</td>
<td>59.32</td>
</tr>
<tr>
<td>120</td>
<td>1.688</td>
<td>54.16</td>
</tr>
<tr>
<td>144</td>
<td>2.287</td>
<td>43.26</td>
</tr>
<tr>
<td>168</td>
<td>2.56</td>
<td>35.61</td>
</tr>
<tr>
<td>192</td>
<td>NA</td>
<td>36.23</td>
</tr>
</tbody>
</table>

Synovial fluid cytological findings

Cytological parameters such as total cell count and protein differed significantly between the PLGA and the POE injected joints.

At PIH 0, synovial fluid specimens from all horses from both groups, POE and PLGA, prior to catheter placement and polymer injection had pale yellow to almost white color and ranged from clear to cloudy transparency. This changed in the polymer treated joints after 24 hours to a more turbid appearance and the control joints remained cloudy throughout the study period. There was no significant change in pH levels (pH 7.0) between polymer-treated and control joints at anytime.

Injection of the PLGA polymer into the joint resulted in a mean WBC count of 28,945 cells/µL with 91% mildly degenerated neutrophils versus 8,230 cells/µL and generally well preserved neutrophils in the control joints. The greatest increase was observed at PIH 48. This count, however, decreased significantly at PIH 72 hours and reached almost levels of the control (catheterized) joints. The mean small mononuclear (SM) cell count was high at PIH 24 for both joints; the greatest increase was observed for the catheter-control group (13.33%, PIH 24), but was statistically not significantly different. By PIH 48, the mean SM counts for both joints in all horses were similar and reached 2.66% and 4.33% in the PLGA- and control joint by PIH 72, respectively. The mean SM counts for the entire study period were 3.92% in the PLGA joint and 6.92% for the control joint. There was a significant difference in the large mononuclear (LM) cell counts between the PLGA joint and the control joints. LM counts increased in the control joints (81%, PIH 24) and maintained elevated compared with the PLGA joint (62%, PIH 24). This difference was particularly significant during PIH 24 through 72.
Figure 1. Mean Total Leucocyte Count after PLGA injection

At PIH 96 the difference was not significant anymore and the LM counts for both joints were decreased. The mean LM counts for the entire study period for the PLGA joints and the control joints were 28.75% and 48.66%, respectively. Few macrophages appeared activated, which was most prominent at 48 hours.

The mean refractive index of all synovial fluid specimens for the control joints in the PLGA group (catheterized, but not polymer-treated) was 1.54 g/dl (range 1.0 g/dl to 2.46 g/dl). One horse (horse 1) out of this group had a significantly higher mean index than the two other horses (2.46 g/dl). However, the same horse had an overall slightly higher index in the catheterized joint compared with the catheterized and polymer treated joint and also had a significantly higher pre-catheter index (1.1 g/dl) than the two other horses. The mean refractive index in the polymer-treated joint reached 3.48 g/dl. There was a statistically significant difference in the index from horse one and the two other horses. The index in horse one (1.96) was much lower than the average of the two other horses (4.25).

Figure 2. Mean Total Leucocyte Count after POE injection.
Injection of the POE polymer resulted in a mean WBC count of 6,091 cells/µl with mostly well preserved neutrophils. The mean WBC counts in the control joint was 2,588 cells/µl with well preserved neutrophils. The largest increase in total white cells occurred at PIH 24 as seen in the PLGA group, however, this increase was not as significant. Total cell counts remained closer at control levels over the rest of the study period. The mean refractive index in the control group (sham injected) was 1.23 g/dl versus 1.98 g/dl in the POE group. Although the most significant increase occurred again at PIH 24 and remained elevated in both groups, this increase was not as significant in the POE group when compared with the PLGA group.

Table 1. Mean Differential Cell Count of PLG and POE polymer injected joints in comparison. NA=Not available

<table>
<thead>
<tr>
<th>Cell type/polymer</th>
<th>Pre-Injection</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>77.6 PLG 48.3 POE</td>
<td>81.3 PLG 46.3 POE</td>
<td>83.3 PLG 43.3 POE</td>
<td>69 PLG 39 POE</td>
<td>76 PLG 20 POE</td>
<td>NA PLG 14 POE</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>10.6 PLG 11.6 POE</td>
<td>0 PLG 33.3 POE</td>
<td>2.3 PLG 9.3 POE</td>
<td>2.6 PLG 3.5 POE</td>
<td>3.8 PLG 9.3 POE</td>
<td>NA PLG 21 POE</td>
</tr>
<tr>
<td>Mononuclear</td>
<td>62 PLG 40 POE</td>
<td>15 PLG 35.3 POE</td>
<td>14 PLG 41.3 POE</td>
<td>24 PLG 57.5 POE</td>
<td>28.7 PLG 67.6 POE</td>
<td>NA PLG 59 POE</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.6 PLG 40 POE</td>
<td>15 PLG 35.3 POE</td>
<td>14 PLG 41.3 POE</td>
<td>24 PLG 57.5 POE</td>
<td>28.7 PLG 67.6 POE</td>
<td>NA PLG 59 POE</td>
</tr>
<tr>
<td>Macrophages</td>
<td>15 PLG 40 POE</td>
<td>15 PLG 35.3 POE</td>
<td>14 PLG 41.3 POE</td>
<td>24 PLG 57.5 POE</td>
<td>28.7 PLG 67.6 POE</td>
<td>NA PLG 59 POE</td>
</tr>
</tbody>
</table>

Picture 1. Control: Mononuclear cell prior to PLGA injection.
Clinical evaluation

None of the horses in both groups became lame. There were no significant differences among the horses and joints concerning lameness grades (p < 0.05). However, there was a trend for increased joint circumference in the POE injected joints. Increase in joint circumference could be palpated mainly over the dorsal aspect of the carpal joint or as generalized effusion of the tibio-tarsal joint. This joint distension, however, did not cause resistance when the joint was flexed.

There was minimal swelling present at the catheter site while the catheter was in place and after it had been pulled. Carpal flexion was not significantly decreased, however, one horse showed some resistance to flexion at PIH 24. This response subsided at PIH 36 after the catheter had been pulled and completely resolved at PIH 48. Although there was a significant difference in the total cell count and the protein levels between both polymers, this difference was clinically not appreciable.

Discussion:

Our findings concerning the gentamicin release concentration from PLGA polymers differ from other studies. Although previous studies used phosphate buffered saline as the medium the drug eluted into, the concentrations in this study were significantly lower from results in other studies (11, 12). This may have been caused by the relatively high viscosity and high concentration of hyaluronan of synovial fluid, which may interfere with the erosion process from PLGA polymers. Low molecular weight copolymers such as the 50/50 DL-PLG can be expected to degrade very rapidly, which is one of the reasons this polymer was chosen for
evaluation. The degradation profile will typically show an initial lag time during which diffusion of water occurs. We did not observe this characteristic lag time during the study period; however, it might be possible that hydrolysis was delayed by the different pH and environment of the synovial fluid. Hydrolysis is also affected by the size, hydrophilicity, and crystallinity of the particular polymer and requires consideration when designing a drug delivery system.

Another reason for the low concentrations may have been the gentamicin loading (15%), which may not have been high enough to release the desired concentrations. However, higher release concentrations of gentamicin with a similar loading have been reported(13). Clinically, it would be desirable to have higher concentrations of drug in the synovial fluid because concentrations 100 times greater than the MIC for an organism may aid in the elimination of infection.

PCL is an aliphatic high molecular weight homopolymer with a slow degradation rate, which may be increased by copolymerization. PCL has a high permeability to many drugs, which provides a rapid release and is characteristic for PCL devices(14). The principle mode of degradation, hydrolysis, does not differ from lactide and glycolide polymers. Degradation proceeds first by diffusion of water into the material (initially into the more amorphous zones) followed by random hydrolysis, fragmentation of the material, and finally a more extensive hydrolysis possibly accompanied by phagocytosis, diffusion, and metabolism. Although the PCL exhibited a very random release of gentamicin, concentrations were more desirable clinically. Considering the clinical use of aminoglycosides, the release behavior of the PCL may be more advantageous than the constant low levels observed with the PLGA. Aminoglycosides have a prolonged "post-antibiotic" effect and are less nephrotoxic when administered at a high dose in single daily injections(15). Therefore, the more pulsatile release of gentamicin from the PCL may be clinically beneficial.

Synovial fluid cell parameters were used to establish preliminary clinical data concerning the injection of these polymers into synovial cavities. Synovial cell parameters including the WBC are helpful parameters in the diagnosis of joint disease (21). Although there is no clear definition what degree of elevation in the WBC may indicate traumatic versus infectious arthritis, WBC counts are valuable diagnostic and prognostic tools concerning the health of the synovial environment. The WBC of normal equine synovial fluid has been reported by different authors as 167 ± 21 and 87 cells/mm³. Neutrophils, lymphocytes and large mononuclear cells are observed, but the percentage of the neutrophils is generally less than 10% in normal fluids. Quantitative and qualitative changes in the leukocytes can provide an indication of the magnitude of synovial membrane inflammation.

PLGA and POE cause a short-term moderate synovitis in the equine joint without significant clinical signs. There was a sharp rise in the mean WBC 24 hours after PLGA injection, which reached control levels at 72 hours. This raise was significantly lower in the POE group compared with the PLGA and the control joint. Although no clinical data are available concerning the response of synovial structures to POE or PLGA, clinical signs including formation of fibrous tissue, osteolytic reactions and bone resorption have been well documented.
for PLG and PLGA implants in human medicine. These findings have been more frequently observed at around 12 weeks after implantation(18). A non-specific inflammatory response in these studies was connected with the breakdown of the polymer and the accumulation of degradation products.

Biocompatibility of PLGA polymers has been evaluated for their use as suture material, bone plates, implant materials, bone graft substitutes, and nerve graft substitutes(4,5,6,7). Intramuscular administration of PLGA microcapsules of various particle sizes in rats evoked a sharply localized acute inflammatory reaction followed by minimal connective tissue, macrophages, and foreign body cell response(8). These responses declined in parallel with microsphere degradation. A dose dependent effect was observed by Julienne et al. following IV injection of PLGA particles in mice(9). At a level of 665 mg/kg, microspheres were well tolerated, whereas at 1000 mg/kg, 20% of the mice died from cyanosis. Although tissue responses from PLGA and PGA implants are generally considered mild without adverse immunological and toxicological effects, significant inflammatory responses have been observed. PGLA polymers were tested as strips deployed longitudinally across 90° of the circumferential surface of coil wire stents and implanted in porcine coronary arteries of 2.5- to 3.0 mm diameter(10). Stent patency was assessed by angiography followed by microscopic examination of the coronary arteries. Multinucleated giant cells and macrophages surrounded by proteinaceous debris were observed at the interface between polymer and tissue. Signs of acute inflammation included the presence of granulocytes (mainly eosinophils), lymphocytes, and occasional plasma cells at the site of the polymer strips. It was concluded that the marked inflammatory reaction within the coronary artery had not been expected on the basis of in vitro tests and may be attributable to a combination of parent polymer compounds, biodegradation products, and possible implant geometry.

In contrast, poly(lactic acid) polymers have been evaluated by several investigators as carriers of antibiotics for the treatment of osteomyelitis. In one study, implants consisting of pure poly(L-lactic acid) (L-PLA), copoly(DL-lactic-co-glycolic acid) and 20% poly(DL-lactic acid) (DL-PLA) impregnated with gentamicin were placed subcutaneously into the back of rats just lateral to the midline(11). During the first 24 hours L-PLA and DL-lactic acid-glycolic acid copolymer implants released a total amount of 3600 µg/ml and 1900 µg/ml gentamicin, respectively. Very good tissue compatibility was found based on the absence of an inflammatory reaction throughout the entire time of implantation as shown by microscopic examination.

Histologically, a typical foreign-body reaction was seen with abundant macrophages and multinucleated foreign-body giant cells after polymer injection. We observed few macrophages, but our study period covered only an initial time period. Long term evaluation may reveal a different cytologic picture. Also, the amount of polymer injected into the joint may play a significant role concerning the magnitude of the cytologic response.

The lymphocyte count was consistently higher in the control joints of two horses and was higher in the PLGA joint of the third horse. The greatest difference occurred at PIH 24 in two horses of the PLGA group and then subsided to non-significant levels. The mononuclear cell count was elevated only in the control joint
of one horse in this group, which had also a significantly elevated lymphocyte count in the control joint. There was a profound mononuclear cell response in the POE injected joints compared with the PLGA group. Also, the lymphocyte count was consistently higher in the POE group than in the PLGA group. Because the POE has never been evaluated for the use in synovial structures such as the joint only limited biocompatibility data is available for older, second generation, POEs. Previous studies in rabbits revealed a strong eosinophilic reaction with densely packed lymphocytes after subconjunctival injection. A 200 µl sample of POE was injected into the subconjunctival nasally to the superior rectus muscle. Daily clinical observations were performed and tissue blebs were identified. On histologic evaluation eosinophils were the most frequent type of inflammatory cells at three days. At ten days, fibroblasts were the predominant cell type at the site of injection and the POE had degraded. This significantly differs from our study, which did not show a significant contribution of eosinophils to the tissue response. The study further concluded that purification technique improves the biocompatibility. Therefore, it may be the amount of polymer injected, the degree of POE purification and the site of injection that ultimately may dictate the type of cell response to the POE.

Catheterization caused a mild inflammatory response of the synovium in the control joint as seen in previous studies (16). The swelling over the dorsal aspect of the carpal joints and increase in joint circumference can at least in part be attributed to the repeated intra-articular injections after the catheter was pulled. Harvesting of synovial fluid via repeated arthrocentesis does by itself cause some degree of inflammation and may have attributed to the elevation in cell counts. We experienced difficulties in maintaining catheter patency and decided to remove the catheter at PIH 36. Because arthrocentesis was performed using the dorsal joint pouch, the swelling was localized to that site of the joint. A similar observation was made in the POE injected joints. Swelling mainly occurred at the site of arthrocentesis, which was in the medial and lateral pouch of the tibio-tarsal joint.

Although there was a significant raise in the WBC in the PLGA injected joints, none of the horses became significantly lame. The mild joint effusion and reduced carpal flexion resolved clinically in all joints after 8-10 days.

In summary, the PLGA showed acceptable biocompatibility in vivo, but did not release gentamicin in clinically relevant concentrations in vivo and showed poor elution characteristics compared with the PCL. Polycaprolactones deserve further evaluation in vivo, because they released high gentamicin concentrations in vitro and exhibited random release kinetics. The POE on the other hand proved to be less irritating to the synovial membrane compared with the PLGA but did also not provide enough drug within the joint to be of clinical significance. Because the preliminary results of this study may only be applicable to the specific polymers investigated, biocompatibility and drug elution from the polymers may change with altering molecular weight, polymerization catalysts, plasticizers, and glycolic unit content. Any future studies developing an effective drug delivery system will have to take into account drug elimination rate of the synovial membrane.
References:


CHAPTER 4. GENERAL CONCLUSIONS

The research presented in this thesis establishes the groundwork for further investigation into the use of biodegradable materials as drug carriers for the treatment of infectious musculoskeletal conditions in the horse. Sustained local drug delivery is becoming the route of choice for a large number of therapeutic agents and will significantly re-shape drug administration in companion animals and humans in the future.

We were able to show that the antibiotic-impregnated poly(ortho ester) can rapidly eliminate infection from a cell culture system and maintain cell function as indicated by normal HA-concentrations. However, one of the most important findings in our study was that although infection is eliminated, ongoing detrimental changes occur. This was indicated by the rapid decline of HA-concentration in our cell cultures post-infection and emphasizes that (1) any infection may potentially cause chronic damage and (2) antimicrobial therapy as the only treatment will not prevent or decrease these ongoing changes. One surprising finding of our in vitro study was the fact that the POE appeared to cause more changes in the cartilage cultures versus the synovial cell cultures. This will require more investigation particularly concerning the long-term outcome of joint infections. Any local drug delivery will need to also address the inflammatory component, which is triggered by the microorganisms. Our findings suggest that a locally delivered combination treatment based on antibiotics and anti-inflammatory drugs may, in fact, be of high value.

We were unable to reproduce the high gentamicin concentrations of the diffusion chamber and the cell cultures in our in vivo study. This supports previous evidence of other studies that in vivo results may greatly differ from in vitro findings. Although one of our objectives was to find out whether or not the polymer material would damage synovial structures, the complex nature and environment of a joint cannot be imitated or reproduced in the laboratory. It can only be speculated at this time why we did not find any clinically significant drug levels in the POE and PLGA injected joints in the in vivo study. One possible reason may be that our immunfluorescence assay to measure gentamicin concentrations may not work accurately. Although TDx has been used in previous studies to determine gentamicin levels in synovial fluid this assay procedure has not been validated for drug monitoring in synovial fluid.

Another reason, which may be responsible for the low concentrations, may be the tremendous ability of the horse joint to eliminate gentamicin from the synovial space. The rate and amount of drug elimination by the joint space by far overcomes the amount of drug and release rate from the polymer. Previous joint studies showed that the elimination half-life ($t_{1/2}$) of gentamicin in synovial fluid is approximately 260 minutes and the clearance approaches 1.22 mL/min. If one considers a therapeutic drug concentration of approximately 100 µg/mL maintained for a seven day treatment period in the synovial space, then approximately 1.22 gr of gentamicin would be necessary to incorporate into the POE. In order to reduce this dose, combination therapy consisting of intrasynovial and systemic drug administration may be a clinical alternative. Previous studies
indicated that the highest drug concentration in the synovial fluid was found when systemic drug therapy was added to intra-articular administration of gentamicin (16).

Our *in vivo* observations confirm that cell cultures do have significant limitations and results should be cautiously interpreted. This accounts in particular for our findings in the toxicity study. However, the results of our studies were encouraging in that none of the polymer materials investigated *in vitro* and *in vivo* resulted in acute or chronic detrimental tissue changes, which would prohibit their further investigation as drug delivery carriers. In fact, the observed side effects after injection of the polymer into a joint does not seem to outweigh the benefits of a sustained drug release system at this point.

Much of the initial and ongoing work in this project involved the development of an injectible material. We experienced significant problems in formulating a polymer solution (PLGA) or ointment (POE), which could be injected through a commonly used needle size (14 ga) into the joint. Preliminary work with the POE resulted in a more viscous form and showed that warming up of the material prior to injection significantly facilitates handling. Although improvements in the "compounding" of the PLGA were made and the "injectability" improved, this still requires additional work.

One aspect of the PLGA microspheres that deserves some notice is the fairly easy (but time consuming) production of this form of drug delivery device. Considering that the equine practitioner is already routinely making and using PMMA beads, the "home-cooking" of a PLGA-based biodegradable sustained drug delivery device for implantation into surgical sites may become possible in the near future.

Although our initial experiences with the PLGA and PCL in clinical case management was somewhat mixed (see case studies Appendix A) the findings of our study may open a new field of opportunities to combat infection. Future studies which may involve the controlled pulsatile release of aminoglycosides within a joint, the efficacy of an sustained drug delivery system in the treatment of horses presented with naturally occurring septic arthritis, synovitis and/or tenosynovitis, and the incorporation of other drugs such as anti-inflammatories will further elaborate the efficacy of local drug release technology in horses.
APPENDIX A. SUPPLEMENTAL MATERIALS AND METHODS

Preliminary equine-related studies with the POE

In order to determine whether or not the POE is tolerated within a synovial cavity and what degree of inflammation can be expected, a sample of poly(ortho ester) was injected into the right tibiotarsal joint of a two year old female Quarterhorse. The horse was examined prior to injection to make sure no underlying orthopedic disease was present. A thorough lameness exam was performed including flexion-tests of both rear limbs. No lameness was detected.

The polymer consisted of a sample of 10 ml poly(ortho ester) (Lot 499-50) delivered in a 12 cc syringe with a biodegradation of 7-10 days. The sample was sterilized by the linear accelerator facility using gamma-irradiation (2.5 mrad).

On day one, the right tibiotarsal joint was aseptically prepared for arthrocentesis. A 14 ga. 1 1/2 " needle was entered into the joint under sterile conditions. Prior to injecting the sample (=10 cc), a synovial fluid sample was taken and put into an EDTA-tube for analysis. The sample was then injected through the same needle (14 1/2") and the horse closely observed for any signs of discomfort.

The pre-injection synovial fluid sample was submitted for cytology. The analysis was consistent with normal synovial fluid. On day two, arthrocentesis was repeated and the synovial fluid sample analyzed for cytology. Results were consistent with a mild inflammatory response. Total cell count went up to 8,060/µl, there were 70% macrophages, 20% non-degenerated neutrophils and no lymphocytes seen.

On day six, a second post-POE synovial sample was taken and analyzed for cytology. Results showed a decrease in Total Cell Count (1,270/µl) and a normal cell-type distribution. During the trial period, the horse never became lame or appeared sore on palpation of the joint. However, there was a mild increase in joint distension noted which completely resolved over a ten day period. These very preliminary results encouraged us to pursue the POE as a drug delivery system and resulted in a research collaboration between Advanced Polymer Systems, Redwood City, CA, and Iowa State University.

Collection of synovial fluid from euthanatized horses

Synovial fluid was collected from horses euthanized for reasons other than musculoskeletal disease. Because of the size of the joint space and available amount, the tibio-tarsal, radio-carpal, and metacarpophalangeal joints were used to collect synovial fluid. All joints were aseptically prepared prior to arthrocentesis. An 18 ga 11/2 inch needle was used to collect fluid into EDTA tubes. The tubes were immediately frozen to -60°C until used for the in vitro elution studies. Because there was a concern that synovial fluid kept in EDTA tubes may by itself inhibit the growth of S. aureus, culture studies were performed prior to the bioactivity testing of the in vitro elution study involving the PLGA and PCL polymers. Mueller-
Hinton agar plates were inoculated with a *S. aureus* and 5 mm holes were punched into the agar. The holes were filled with 100 µl of EDTA synovial fluid and incubated for 24 hours at 37°C. The plates were evaluated after 24 hours and no zone of inhibition could be observed.

**Preliminary in vitro elution characteristics POE/gentamicin**

The *in vitro* release kinetics of gentamicin sulfate from the POE polymer was performed using two double diffusion chambers separated by a Millipore membrane\(^b\). Both chambers were filled with a total of 20 ml of sterile equine synovial fluid, which was harvested from horses euthanized for reasons other than musculoskeletal disease. The synovial fluid was collected in sterile heparinized EDTA tubes\(^d\) and frozen (-60°C) until used for the experiment. The donor side contained a 500 mg sample of POE loaded with 5% gentamicin sulfate, and the receiver side contained synovial fluid only. The chamber was continuously agitated on a shaker at room temperature, and 0.5 ml samples of synovial fluid from the receiver side were collected and replaced with fresh synovial fluid at 12 and 24 hours and then daily for a total of 14 days.

Drug levels in the collected samples from both chambers were quantified using an automated fluorescence polarization Immunoassay (FPIA) analyzer\(^c\). Hyaluronidase lyophilized desiccate\(^e\) was added to each sample to decrease the viscosity of the synovial fluid for analysis by the TDx-analyzer.

Pharmacokinetic studies commonly use buffer systems such as PBS (pH 7.3, 154 mmol/L) to determine the concentrations of drug released into a closed system (34). An estimate of polymer volume needed to provide therapeutic drug concentration (eg. 2-4 µg/mL) within the synovial space is difficult. In general, for the *in vitro* kinetics the amount of synovial fluid in the chamber must be large enough so that after 24 hours release, the synovial fluid is less than 10% saturated. This is known as infinite sink conditions which differs from the joint because drug is released into a finite space in the joint. Therefore, infinite sink conditions may not be representative of the *in vivo* situation.

However, we encountered problems with the *in vitro* kinetic set up. While we found that the release of gentamicin from the POE within the first 24 hours was almost linear, a burst of drug release occurred after that. Gentamicin concentration peaked at 96 hours (6500 µg/mL) and adjusted then at around 3100 µg/mL for the rest of the study period. This phenomena has been well recognized in the synthesis of polymers and constitutes one of the greatest problems in the design of controlled drug delivery systems. "Dumping" of the incorporated drug may lead to undesired toxicity of the drug and makes the release kinetics much more random. Ideally, the main mechanism responsible for the release of drug from a biodegradable polymer is surface erosion while the drug is uniformly distributed through the polymer facilitating zero-kinetic release(28). In order to obtain zero-order release a geometry, may be necessary that does not change its surface area as a function of time(29). Bulk erosion, in addition to surface erosion, as seen in both the kinetic- and the culture study, is a result of the
polymer imbibing water into the center of the matrix and has been observed with other polymers as well. Polylactic acid (PLG) and polyglycolic acid (PLGA) polymers used for biomedical application are well known for this problem which makes the controlled release in these materials particularly difficult (30,31).

Another problem we observed with the in vitro kinetic study was the fact that we found more drug than we could account for when considering the total amount of gentamicin in the POE sample. Although technically the 500 mg sample of POE contained not more than 25,000 µg total gentamicin (5 weight %), we found approximately 84,000 µg represented in the synovial fluid solution. Several circumstances may help explaining this discrepancy. First, we could have used more polymer than we initially weighed out. Second, the amount of gentamicin incorporated into the polymer during fabrication was more than 5%, and thirdly, our immunofluorescence assay (TDx) may not work accurately. One of the first things to make sure will be to evaluate the accuracy of the assay by measuring gentamicin concentrations in synovial fluid using High Pressure Liquid Chromatography (HPLC).

Figure 1. Preliminary gentamicin kinetics from POE polymer (5 w% gentamicin) in diffusion chamber

**Infection of synovial membrane for the POE efficacy study**

Synovial explants were infected with a *S. aureus*, which had been previously harvested from an infected joint and frozen at -70°C until use. Each inoculum consisted of 3 mL of standard medium that contained 135 colony-forming-units (CFU)/mL of *S. aureus*, for a total bacterial inoculum of 405 CFU. This number was selected based on previous studies of infected equine synovial explants. An inoculum of 150 CFU/mL was visually estimated by matching sample turbidity to a 0.5 McFarland turbidity standard (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD). The exact number of CFU/mL was determined by plating 10 µL of the stock solution on Mueller-Hinton agar plates and overnight incubation at 37°C. The mean number of final, viable CFU/mL was 135. After inoculation, explants were incubated for 18 hours, then infected medium was removed and replaced with fresh standard medium.
Making of microspheres:

Microspheres were made using the emulsification-solvent evaporation technique. This technique was first described by Beck et al. (1979) and is the most widely used manufacturing technique for biodegradable microspheres. Microsphere formation consists essentially of three stages: (1) droplet formation, (2) droplet stabilization, and (3) microsphere hardening.

First, a dispersed phase containing the polymer is emulsified in an immiscible continuous phase containing a stabilizing agent. The second stage involves the diffusion of the solvent from the emulsion droplet into the continuous phase and its subsequent evaporation. Selection of an appropriate organic solvent for the disperse phase may mainly depend on toxicity, immiscibility with the continuous phase, and drug solubility. One of the most widely used organic solvents, and used in our laboratory for this purpose, is methylene chloride. In order to stabilize the dispersed-phase droplets, which are being formed during emulsification, the use of a surfactant is necessary. Surfactants are amphipathic in nature and hence will align themselves at the droplet surface. Polyvinyl alcohol (PVA) is by far the most widely used surfactant and appears to be the most effective for formation of particles below 1 µm in diameter.

The mixing conditions (e.g., stirring, sonication) primarily influence the size of the droplets and hence, the final microsphere size. By increasing the emulsification speed, there is both a decrease in the particle size and a narrowing of the size distribution. Following emulsification, the removal of remnant solvent and complete microsphere hardening is accomplished by gentle agitation of the suspension by magnetic stirring at room temperature and pressure. This step is of great importance because of toxicological concerns. Incomplete removal of solvent from the microspheres may result in tissue irritation unrelated to the microspheres themselves and give a false result concerning the biocompatibility of the polymer. Therefore, this process is typically followed by a further cleaning process in which particles are washed several times with distilled water.

After decanting, the microspheres are left behind and undergo the final step of dry freezing. This requires typically 24-36 hours and is done under vacuum.

The following is a protocol, which was used in our study for the majority of the microsphere production:

1. One gram polymer rods are dissolved in 5 ml methylene chloride. Shake as needed.
2. Five ml of methylene chloride are added with 150 mg of gentamicin sulfate (100 mg/ml), which results in a 15 % (weight percentage) drug concentration of the polymer.
3. The solution is ultrasounded for approximately 40-50 seconds until the solution is white with no flakes.
4. One hundred fifty ml of 1 % PVA (grade 70-06, MW 17,600) is added to the solution and homogenized for approximately 35-40 seconds to get an approximate microsphere size of 80-140 µm.
5. The solution is stirred at room temperature for two hours at moderate speed. To determine the size of the microspheres, a sample is put on a slide and examined under the microscope at 40x.
6. After two hours of stirring, the solution is divided up into 60 ml centrifuge tubes and centrifuged at 1500 rpm for five minutes. After that, the liquid is decanted and deionized water added. The tubes are shaken several times and centrifuged again. This step is repeated for a total of three times for each tube. Then, the tubes are sealed and dry-frozen under vacuums for 36 hours using a lyophilizer.
APPENDIX B. CASE STUDIES

Case 1 (ISU# 269175): 6 week old foal with open comminuted MT III midshaft fracture

A three months old male Quarterhorse foal was presented to the Veterinary Teaching Hospital for an open comminuted fracture of the left rear leg. The fracture site was moderately contaminated. The foal was put under general anesthesia and the wound thoroughly cleaned. 50/50 PLGA microspheres impregnated with 15 w% gentamicin were implanted subcutaneously into and around the fracture site. A transfixation cast was applied to provide stabilization. The foal recovered uneventful from anesthesia and appeared very comfortably weight bearing postoperatively.

The cast was changed two and a half weeks later which revealed a moderate amount of drainage from the fracture site. There was radiographic evidence of sequestra formation. Several small sequestra were removed and the wound thoroughly cleaned. A cancellous bone graft from the tuber coxae was harvested and put into the fracture site. No residual polymer was seen at that time and more PLGA polymer was implanted. The limb was recasted. The foal continued to bear weight on the limb and appeared comfortable.

Two weeks later the cast was changed again and another large bone sequestrum was removed. Drainage had decreased in the amount, however, there was little radiographic evidence for new bone formation at the fracture site. Because the foal was clinically doing well, the decision was made to continue and to repeat the cancellous bone graft. No further polymer was implanted at that time. A new cast was applied.

After three more weeks, the foal returned for another cast removal. It had been doing well concerning weight bearing on the cast and remained active. The foal had gained weight over the past weeks. The cast removal revealed that there was no active infection present. No significant drainage was found at the fracture site. The skin appeared to have healed over the fracture. However, there was no significant new bone formation radiographically and clinically. The fracture site remained unstable. There were radiographic signs of osteoporosis involving the distal aspect of the limb. A new cast was applied. Five days after the new cast, the foal appeared significantly more uncomfortable and was spending the majority of the time laying down. Because of this sudden deterioration, the cast was removed to inspect the limb. This showed that the skin of the site of the fracture had broken open and a significant amount of drainage and pus was present. Because of the grave prognosis, the foal was euthanized.

Case 2 (ISU# 269029): 6 week old calf with open MT III fracture

A four month old female Shorthorn calf was presented to the Veterinary Teaching Hospital for an open midshaft fracture of the right hind metatarsus. The fracture site had been cleaned and debrided and an external fixator was applied to provide stabilization.

Two weeks after the initial treatment, the open fracture site continued to drain moderate amounts of pus and debris. Radiographic evaluation revealed few small sequestra at the fracture site. The external fixator
was removed and the wound again cleaned and thoroughly debrided. 50/50 PLGA microspheres impregnated with gentamicin (40 w%) and 75/15 PLGA microspheres impregnated with Potassium Penicillin (15 w%) were implanted into the open fracture site and a cast applied to the limb. Cast removal three weeks later showed that drainage had significantly decreased and a soft tissue callus had formed.

Case 3 (ISU# 268754): 6 year old female alpaca with severe mandibular actinomycosis

A two year old female alpaca was presented to ISU for advanced mandibular osteomyelitis due to previously cultured Actinomyces bovis ("Lumpy Jaw") and Fusobacterium necrophorum. There was significant drainage and thickening at the right mandible. The infection had resulted in a pathologic fracture at the rostral aspect of the right mandible. At the time of presentation, the alpaca was able to eat but had lost a significant amount of weight. Although the prognosis for long-term survival was poor, the owner decided to initiate treatment because the alpaca was pregnant. The goal of therapy at this time was to halt the infection long enough to have the alpaca deliver the cria. Surgical curettage and debridement was performed and a type I Kirschner-Ehmer frame applied on the right mandible to stabilize the jaw. At the same time, small 1.5 mm large holes were drilled into the site of the infection. The holes were filled with microspheres of 85/15 PLGA impregnated with 20 w% Potassium Penicillin or 50/50 PLGA impregnated with 20 w% Ceftiofur (Naxel®). These antibiotics were chosen based on C&S results. Postoperatively, the alpaca appeared comfortable and was eating. She was put on broad-spectrum antibiotics because additional cultures had revealed heavy mixed growth of E. coli, Klebsiella, Bacteroides spp. and Streptomyces sp.

Five weeks later the alpaca was presented again. Radiographic evaluation at that time showed that there was no further bone lysis. The KE apparatus was still tight and providing stability to the jaw. The alpaca was again anesthetized and the polymer implantation was repeated using the same microspheres and a 50/50 PLGA impregnated with 40 w% gentamicin. Because the microspheres were difficult to inject into the holes using 14 gauge needles, polyethylene glycol (PEG) was added to increase viscosity. This slightly improved the injection technique. A transabdominal ultrasound examination was done which confirmed that the alpaca maintained its pregnancy. She was scheduled for another recheck exam in four weeks.

Nine weeks after the first PLGA microsphere implantation, radiographs of her mandible showed that new bone was filling in the osteolytic areas. The alpaca was doing well, very active and had maintained its weight. The KE apparatus was removed and a bone biopsy taken for culture. This revealed no growth on direct or enrichment cultures, aerobic and anaerobic.

Six weeks later the alpaca was still doing well, however, she continued to have a chronic draining tract at her right mandible. Culture revealed an Actinomyces species other than A. bovis.

Four months later the alpaca was represented for recurrence of swelling at her jaw and increased drainage. She was still eating, but had not gained significant weight during her pregnancy. Radiographs
showed that there was considerable bone lysis consistent with a recurrence of osteomyelitis in her mandible. Shortly after, the alpaca delivered the cria and was then euthanized for humane reasons.

Table 1. *In vitro* gentamicin elution from 500 mg POE (5 w% gentamicin) in synovial fluid

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