8-1-2010

Evaluation of in vitro serial antibiotic elution from meropenem-impregnated polymethylmethacrylate beads after ethylene oxide gas and autoclave sterilization

Leonardo Alfredo Baez

EVALUATION OF In Vitro SERIAL ANTIBIOTIC ELUTION FROM MEROPENEM-IMPREGNATED POLYMETHYLMETHACRYLATE BEADS AFTER ETHYLENE OXIDE GAS AND AUTOCLAVE STERILIZATION

By

Leonardo Alfredo Báez

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medicine
in the College of Veterinary Medicine

Mississippi State, Mississippi

August 2010

EVALUATION OF In Vitro SERIAL ANTIBIOTIC ELUTION FROM MEROPENEM-IMPREGNATED POLYMETHYLMETHACRYLATE BEADS AFTER ETHYLENE OXIDE GAS AND AUTOCLAVE STERILIZATION

By

Leonardo Alfredo Báez

Approved:

Ron McLaughlin Professor & Chief, Small Animal Surgery Head, Department of Clinical Sciences (Director of Thesis)	Robert C. Cooper Associate Dean, Professor and Chief Operating Officer (Committee Member)
Cory Langston Professor of Pharmacology Department of Clinical Sciences (Committee Member)	Sumalee Givaruangsawat Assistant Research Professor Department of Basic Science (Committee Member)

Kent Hoblet
Dean, MSU College of Veterinary Medicine

Name: Leonardo Alfredo Báez

Date of Degree: August 7, 2010

Institution: Mississippi State University

Major Field: Veterinary Medicine

Major Professor: Ron McLaughlin

Title of Study: EVALUATION OF In Vitro SERIAL ANTIBIOTIC ELUTION FROM MEROPENEM-IMPREGNATED POLYMETHYLMETHACRYLATE BEADS AFTER ETHYLENE OXIDE GAS AND AUTOCLAVE STERILIZATION

Pages in Study: 39

Candidate for Degree of Master of Science

The objective of this study was to determine elution properties of meropenem and compare the elutions of meropenem impregnated PMMA beads without sterilization (PMMA-Cont) to those sterilized with steam (PMMA-Auto) and ethylene oxide gas (PMMA-EO). Four groups of beads were produced: one group without antibiotic and three groups of meropenem impregnated beads: PMMA-Cont, PMMA-Auto, and PMMA-EO. Antibiotic concentrations in eluent samples were determined using a microbiological assay at different time intervals.

The microbiological assay resulted in no zone of inhibition at all time periods for the PMMA-Auto samples and the samples of PMMA without antimicrobial. The meropenem concentration on the eluent remained above 4 mcg/ml for 15 days in the PMMA-Cont group and until day 18 for PMMA-EO group. The meropenem incorporated in the PMMA beads elutes effectively and gradually decreases after the 24 hour peak. Ethylene oxide does not adversely affect meropenem's elution.

DEDICATION

I would like to dedicate this work to my family (Alexandra, Isabella, Nydia, Alejandro and Myrna) and friends. I would not have been able to do it without your unconditional support. Thank you for allowing me to pursue my dream.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all the people that contributed and made this thesis possible. I would like to thank all the thesis committee members, namely, Dr. McLaughlin, Dr. Langston, Dr. Givaruangsawat, and Dr. Cooper, for your support. I will forever be indebted to Dr. McLaughlin for the great opportunity that he has given me. This thesis would not have been possible without Dr. Langston's guidance and assistance with the methodology and pharmacokinetics. Words can not describe how grateful I am for Dr. Langston's guidance during this study. Finally, I would like thank Dr. Givaruangsawat for her assistance with statistics.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE Page

LIST OF ABBREVIATIONS

PMMA = Polymethylmethacrylate

PMMA-Cont = PMMA-control with no sterilization

PMMA- Auto = PMMA- autoclave

PMMA- EO = PMMA- ethylene oxide

$AUC_{0-\infty}$ = Area under the curve from time zero to infinity

$MRT_{0-\infty}$ = Mean residence time from time zero to infinity

λz = First-order rate constant of the terminal elution phase

t1/2 = Time required for the amount of drug to decrease by one half, or 50%

DRC = Drug response concentrations

SD = Standard deviation

AR = Autoregressive

MIC = Minimum inhibitory concentration

CHAPTER I

INTRODUCTION

Local antibiotic use for treatment of infections dates back to 1939 with Jensen's discovery that instilling sulfonamide crystals, along with surgical debridement, hemostasis, primary closure, and immobilization, resulted in a reduced infection rate for open fractures.[1] As systemic antibiotics became available the interest in local use of antibiotics decreased, but the management of chronic osteomyelitis remained a challenge.[1] A decrease in the blood supply and barriers preventing penetration of antibiotics at the site of infection make treatment difficult. High antibiotic doses required to reach therapeutic bactericidal concentrations at the infection site may put the patient at risk of dose-related toxicity.[2] In the 1960's, the treatment with closed wound irrigation-suction was established as a method of delivering high antibiotic concentrations, decreasing hematoma formation, promoting influx of leukocytes and tissue fluids and achieving primary closure to prevent contamination.[1, 3] Another method used for delivery of high antibiotic concentrations to the lower extremities was isolation perfusion. The affected site was debrided; cannulae were inserted into an artery and vein; a tourniquet was applied proximally; and oxygenated blood containing high concentrations of antibiotic was pumped through the distal limb.[1] The use of wound irrigation-suction and isolation perfusion decreased because of the complexity of the procedure and the introduction of new systemic antibiotics which were more potent against the

staphylococci and gram-negative bacilli causing orthopedic infections. With the introduction of prosthetic joint replacement, the local use of antibiotics resurfaced in Germany, with the goal to prevent devastating complications as a result of infection in elective surgical procedures.[1] In 1970, Buchholz and Engelbrecht reported antibiotic elution to local tissues when penicillin, erythromycin and gentamicin were incorporated into the cement used to attach the total hip prostheses.[4] In response to the success in reducing early postoperative arthroplasty infections, Klemm formed gentamicin-impregnated beads and utilized them to temporarily fill the bone defect after debridement of the infected bone. He reported a 91.4% cure rate in 128 patients treated for chronic osteomyelitis.[1, 5]

Local implantation of antibiotic impregnated polymethylmethacrylate (AIPMMA) for treatment of orthopedic infections, wound infections and prophylaxis in joint arthroplasty has gained popularity because of the ability to attain high concentrations of antibiotic at the site while avoiding adverse effects of systemic toxicity.[6, 7] Local therapy allows use of antibiotics that may be cost prohibitive for long-term systemic administration.[8] Antibiotic tissue concentrations in wounds treated locally with AIPMMA beads can be as much as twenty times the therapeutic levels obtained in serum with systemic administration. [9] The use of beads provides a filler for the dead space left after debridement of bone in patients with chronic osteomyelitis or compound fractures and allows elution of high concentrations of antibiotic in the surgery site. Increased local antibiotic concentration may improve the spectrum of susceptible organisms.[9] A previous study comparing systemic antibiotic treatment and AIPMMA treatment did not show a significant difference in success rate. The same study revealed a 100% success rate when

AIPMMA was used in combination with systemic antibiotic treatment. Although this finding was not statistically significant it suggests that a higher success rate could be achieved with a treatment combination.[10] A multicenter study of six infected total knee and twenty-two total hip arthroplasties by *Nelson et al.* documented a similar outcome in patients treated with debridement, conventional systemic antibiotic therapy and a two stage delayed reconstruction and in patients treated with debridement, gentamicin-PMMA bead implantation and two stage delayed reconstruction.[11] No study has compared the efficacy of systemic antibiotics plus AIPMMA with that of either treatment alone.[1, 11]

POLYMETHYLMETHACRYLATE CEMENT

Polymethylmethacrylate bone cement is a high-density polymer formed by mixing a liquid monomer with a powder polymer. PMMA is porous and does not elicit an immune response making this material suitable for implantation in the body.[1, 7] The PMMA has no antimicrobial properties, but it is a great vehicle for local antibiotic delivery.[7] Prefabrication of AIPMMA beads reduces surgery time, cost, and results in a more consistent product. The methylmethacrylate monomer is known to have toxic effects to musculoskeletal tissue and is still present during the first two hours after mixing the beads.[8] Storage of the product will prevent tissue exposure to the methylmethacrylate during this period of time. Polymerized PMMA may be cytotoxic if particles are phagocytized resulting in inhibition of DNA synthesis and cell growth. PMMA may also stimulate tumor growth in rodents and result in bacterial infection, but there is no evidence that it affects reactivity of IgA, IgG, or IgM and does not alter the body's

response to bacterial chemotactic factors. Even though it does not affect immunity, it may have negative effects on bacterial inhibiting factors, lymphocyte function, late-acting components of the complement sequence and polymorphonuclear cell function. Release of small amounts of monomer into the systemic circulation may occur, but it has not been associated with any toxic effects.[8] The most commonly used commercial PMMA cements are Palacos® (Merck KGaA, Darmstadt, Germany), used primarily in Europe and Simplex™ P (Howmedica, Rutherford, N.J.) used in the United States and the United Kingdom. Palacos® PMMA cement (Palacos-Refobacin) and beads (Septopal®) containing gentamicin are manufactured and available commercially in Germany.[1] Prefabrication of antibiotic beads and gas sterilization for "off the shelf" availability has been reported, but no product has been approved for commercial use in the United States.[6] The surgeon has three bead manufacturing options which include commercially manufactured (Septopal®; Kulzer, Wehrheim, Germany), mold-made or hand-rolled beads. Currently, PMMA beads are made intraoperatively by hand, a method that prolongs surgery time and is both inefficient and inconsistent. *Seligson et al.* compared the amount of aminoglycoside eluting from the beads manufactured with the three different methods and found no statistically significant differences in antibiotic elution.[6] The authors concluded that elution rate is independent of the fabrication mode as long as the bead is similar in size and is composed of the same material. [12] To add an antibiotic to the PMMA, the antibiotic is mixed with the powder cement polymer before addition of the liquid methylmethacrylate. The antibiotic used should be a broad-spectrum antibiotic if empirical treatment is instituted, active against the etiologic pathogen and must be available as a powder. Mechanical studies demonstrated that the admixture of

gentamicin, oxacillin, and cefazolin powders did not have any effects on the color, viscosity, set times or compressive and diameter tension strengths of the PMMA.[1] The use of antibiotic solutions is not recommended since they have been shown to reduce the mixing and hardening properties of the cement by interference with prepolymerization.[8] A study by *Weisman et al.* evaluating the mechanical strength of antibiotic free cement and antibiotic impregnated cement using compression and elongation revealed a 32% decrease in compressive and tensile strength when the antibiotic powder was added to the liquid monomer compared to a 7% decrease in both when the antibiotic powder was added to the powdered polymer. [13] The mechanical strength of the PMMA is of outmost importance when used for arthroplasties, but not for AIPMMA bead production.

ANTIBIOTIC SELECTION

There are several important factors to consider when selecting an antibiotic for incorporation in PMMA. The ideal antibiotic should be bactericidal, have broad-spectrum activity, be effective at low concentrations, have a high water solubility, be hypoallergenic, be biocompatible, have low tissue toxicity, be stable at temperatures up to 100 C, have reduced effects on mechanical strength of the PMMA, and result in low serum concentrations, but high concentrations in the target tissue. Most of the research focuses on gentamicin-impregnated PMMA because of its commercial availability in Europe. [8] Antibiotics such as colistimethate, polymixin B, tetracycline, and chloramphenicol do not elute from the PMMA because of the inability to retain activity after the heat produced during the exothermic reaction. [4, 14, 15, 16] Elution characteristics from PMMA for multiple antimicrobials with good antibiotic release rates such as

aminoglycosides, β-lactams, metronidazole, and macrolides have been described. [7, 17, 18] No antibacterial activity has been found in PMMA alone. [14, 15, 19, 20] Gentamicin blood serum levels post-implantation are measurable, but not high enough that toxic side effects should be a concern. Levels of 1 to 3ng/ml were measured immediately post-implantation and dropped to less than 1ng/ml by six hours in one study. Low drug levels are measurable in serum and urine for several months if the beads remain in the patient. In human patients with normal renal function, the toxic daily dose is 3.4 mg/kg, and the non-toxic dose is 2.6 mg/kg. Serum concentrations below 8 ng/ml appear to be safe in preventing ototoxicity. [2] There have been no reports of ototoxicity associated with gentamicin impregnated PMMA, and there is probably less risk of ototoxicity than with long term systemic treatment with aminoglycosides. [2, 21]

ANTIBIOTIC ELUTION

There are many factors that affect antibiotic elution from the beads such as type of bone cement, antibiotic, concentration of the antibiotic, surface area of the bead, size of the bead, shape of the bead, surrounding tissue and fluid flow around the bead.[18] The ideal cement bead would have an initial bactericidal antibiotic release followed by a continuous bacteriostatic antibiotic release until the tissue has healed to a point where the host's immune system can kill any residual bacteria.[14] The elution rate of antibiotics vary depending on the type of cement used, and more complete antibiotic elutions have been documented with Palacos® cement. [1, 19, 22] The difference in elution rates among different cements is because of differences in surface pore size of the hardened cement. Palacos® cement has a larger pore size compared to the others. [1, 14]

ANTIBIOTIC ELUTION RATE

Results from previous studies revealed that the elution amounts and rates for different antibiotics and antibiotic combinations vary. Antibiotic elution is bimodal, with a fast elution rate within hours to days after implantation and a slow elution rate with continued release during a period of weeks to months after implantation. Up to 5% of the total amount of the antibiotic may elute during the first 24 hours. [8, 23] Antibiotic elution from the bead surface may be responsible for the initial peak in the elution rate and subsequent elution from the center matrix of the bead follows.[1] Elution rate is directly proportional to the surface area of the bead, the greater the surface area the greater the elution. A small rough bead will elute better than a large smooth bead simply because the small rough bead has a larger surface area per volume.[8] A study evaluating the relationship between bead geometry and elution of the antibiotic tobramycin revealed that the best elution was from numerous, small and elliptically shaped beads.[18] Antibiotic tissue concentrations in wounds treated with local AIPMMA could reach as much as twenty times the therapeutic levels obtained in serum after parenteral administration. The high concentration reached locally may increase the spectrum of susceptible organisms in spite of culture and sensitivity results reported. [9, 23, 24]

The ratio of antibiotic to PMMA used to fabricate the bead also affects the amount and rate of elution. Current recommendations based on biomechanical studies indicate a limit of 10% of the weight for the antibiotic if the cement is used for implant fixation. [14] The addition of more than 4.5 g of an antibiotic to 40g of PMMA reduces compressive strength below 70 Mpa (mega pascal), which is the minimum acceptable strength for weight-bearing implants. [8] As the amount of antibiotic is increased, the surface pores

become larger, and the surface becomes rougher increasing the amount eluted. The ratio for bead production is more forgiving since mechanical strength is not a concern, but should not exceed an antibiotic to PMMA ratio of 1:5. If this ratio is exceeded the beads will not harden. [14] Elution is also dependent on the tissue surrounding the beads and the fluid flow. Vascular tissue has a higher absorption of the eluted antibiotic and a higher elution rate than tissue with low vascularity. Granulation tissue and muscle being highly vascular tissues have a higher elution rate than bone, which is a less vascular tissue.

BACTERIAL ADHERANCE

Neut et al. documented the retrieval of gentamicin-impregnated PMMA beads after implantation for 5 years that were still eluting subinhibitory antibiotic concentrations.[25] This prolonged release of antibiotic at subinhibitory concentrations could result in antibiotic resistant strains. *Konstantinos et al.* reported a 22% persistence of bacterial growth on AIPMMA bead chains removed from patients with a history of implantation because of an orthopedic infection.[26] The mean duration of implantation in these cases was 89 days. Once bacteria adhere to the implant, they could produce an extracellular proteoglycan matrix or glycocalyx. The glycocalyx creates the perfect environment for bacterial growth, inhibition of host immunity and antibiotic activity.[26] An *in vivo* study evaluating the bacterial adherence to plain and tobramycin-laden beads revealed bacterial adhesion on plain beads and no organisms on tobramycin impregnated PMMA beads. Antibiotics eluting from the beads decrease exponentially over time, eventually reaching subtherapeutic levels and allowing bacterial adherence. Bacterial adherence post implantation may result in bacterial resistance. Therefore, it is

recommended to select an antibiotic based on culture and sensitivity results and remove the beads after the antibiotic elution reaches subtherapeutic levels, which can vary from weeks to months depending on the antibiotic. [27] Even though antibiotic bead removal is the standard of care there are reports of prolonged implantation of Septopal® beads with no evidence of complications. [23]

CLINICAL APPLICATIONS

A randomized prospective study by *Blaha et al.* comparing the clinical efficacy of gentamicin PMMA beads versus systemic therapy and a combination of PMMA beads and systemic therapy for osteomyelitis did not prove any statistical superiority among these treatments.[21] The treatment cost of AIPMMA alone was significantly reduced when compared with the systemic and combination treatments.[21] A prospective, randomized, controlled, closed study by *Calhoun et al.* comparing patients with infected nonunions treated with intravenous antibiotics (Group I) or Septopal® beads and perioperative broad-spectrum parenteral antibiotics (Group II) revealed a good success rate with both treatments.[28] Both groups were treated with surgical debridement and appropriate reconstruction. The success rate for infection quiescence was 83.3% in Group I and 89.3% in Group II, and the success rate for uniting the nonunion was 83.3% in Group I and 85.7% in Group II. In this same study cases that needed retreatment with debridement were considered failures. If the cases that required repeated debridement and deemed failures in this study are taken into consideration, the Septopal® beads group had a success rate of 94.2%. Success rates were better for the Septopal® beads group, and cost for treatment was less when compared to the parenteral antibiotic group.[28]

Reported use of AIPMMA in animals is limited but has been reported for prophylaxis in arthroplasties, limb-sparing procedures, treatment of septic arthritis in a dog and treatment of cellulitis in an American black bear.[29] A 4 year old, male, intact English Bull Terrier with a history of septic arthritis and a positive culture for *E. coli* in the right stifle after surgical correction of a cranial cruciate ligament rupture was treated with intra-articular Septopal® beads for 20 days with excellent results.[30] In a clinical report by *Dernell et al.*, 67% of dogs treated with tobramycin and vancomycin AIPMMA for severe osteomyelitis post allograft replacement for osteosarcoma had resolution of the condition within a median of 4 weeks post implantation.[31] In the same study, 25% of dogs had a recurrence with a median of 17 weeks post implantation. Even though the success rate for resolution of osteomyelitis in this case series is low compared to previous reports, no surgical debridement was performed because of the nature of the procedure and the presence of internal fixation. A 6 year old bear with a history of abscessation and skin necrosis in the right shoulder, forelimb and pectoral region was originally treated with wound debridement and intermittent antibiotic administration without success. A second surgical procedure with wound debridement, lavage, cefazolin impregnated PMMA placement and primary closure was performed, and the wound was completely healed 8 weeks after surgical repair.[29]

Local concentration and duration in which the concentration remains above the break point susceptibility concentration (BPSC) post-implantation of the beads depends on how fast the antibiotic elutes. Break point susceptibility concentration is the concentration used to determine susceptibility or resistance of a particular bacterial organism. Drug concentrations of 4 to 8 times the minimum inhibitory concentration

(MIC) for systemic therapy are recommended because the concentration of antibiotic reaching the target tissue may be 20% to 25% of the plasma level.[30] Treatment for an existing infection should encompass use of effective antibiotic concentrations, preferably based on culture and sensitivity results, for 3 to 4 weeks or longer depending on response to treatment. The exact duration of time with an effective concentration needed for local antibiotic treatment has not been documented to date.[8] The activities of cefazolin and meropenem are time dependent medications, and the amount of time that an antibiotic concentration is above the MIC is of outmost importance. A concentration of one to five times the MIC should be maintained for at least half of the dosing interval when time dependent drugs are given systemically.[31] The same guidelines are followed for local treatment with time dependent antibiotics since the concentration needed for this purpose is unknown, but it is assumed that the same rule applies.[32]

Local antimicrobial concentration and duration of elution after implantation of AIPMMA beads varies depending on the antibiotic chosen.[6, 33] An *in vivo* study in which AIPMMA beads were implanted in a tibia of a dog revealed that antibiotic concentrations in seroma fluid at the implantation site were above the MIC breakpoints for 3 to 28 days for the antibiotics tested. The antibiotic concentrations were above the MIC breakpoints through at least the following sampling days for the antibiotics listed: cefazolin, day 14; ciprofloxacin, day 3; clindamycin, day 28; ticarcillin, day 9; tobramycin, day 21; and vancomycin, day 3.[1] Eventually the PMMA becomes encapsulated with fibrous tissue, and antibiotic concentrations are limited to a 2 to 3mm area around the implant.[1] *Ramos et al.* reported that neither polymerization, gas sterilization, nor 2 month storage of

PMMA impregnated with gentamicin and metronidazole affected the bioactivity of the antibiotic against *E. coli* and *B. fragilis,* respectively.[9]

OSTEOMYELITIS

Osteomyelitis is an infection of bone that can affect the bone medullary cavity, cortex or periosteum. Bone infection is most commonly post-traumatic and caused by bacteria, but viral and fungal osteomyelitis can also occur. Infections are acquired by direct inoculation, extension from soft tissue infections or by hematogenous spread.[34] Approximately 70% of open fractures and 40% of closed fractures have bacterial contamination, but only a small percentage of these cases will develop infection. Factors that may increase the risk of bone infection include impaired blood supply, foreign material, devitalized bone, bone sequestration, unstable fracture fragments and severe bacterial contamination.[35] Even though several organisms can cause osteomyelitis, the most common bacteria is *Staphylococcus intermedius*, which is responsible for 50% to 60% of bone infections in dogs. Other bacteria found in osteomyelitis are *Streptococcus spp., Actinomyces, Clostridium, Peptostreptococcus, Bacteriodes and Fusobacterium*. Anaerobic bacteria can be isolated in up to 70% of bone infections in dogs and cats if proper sample collection methods are used. Caywood *et al*. found that the most common source of bacteria leading to osteomyelitis was surgical technique accounting for 58% of the cases.[36] Clinical signs commonly associated with osteomyelitis include pyrexia, erythema, swelling, local pain and lameness. Treatment of osteomyelitis can be challenging, costly and can have variable success rates sometimes ending in failure.[10] Treatment includes surgical debridement of devitalized bone, lavage, fracture

stabilization, grafting of bone deficits and a prolonged course of systemic antibiotics for 4 to 6 weeks up to several months, as well as implantation of local antibiotic delivery systems. Disadvantages of systemic antibiotic therapy include owner non-compliance, systemic toxicity and antibiotic degradation or subtherapeutic antibiotic concentrations at the infection site.[17] One of the most important factors for successful treatment is to attain high antibiotic concentrations at the infection site. However, this is not always possible with systemic therapy.[37] In addition, bacteria have the ability to protect themselves by growing in colonies encased in an extracellular matrix of carbohydrate or glycocalyx that increases bacterial adhesion and protection from phagocytes and antibodies.[32,35] Organisms such as *Staphylococcus epidermidis* produce a slime that coats the implant and results in an increase in bacterial resistance and forms a barrier against the host immune response and antibiotics. Once a biofilm is present on the implant other organisms can also adhere to the implant surface. This biofilm creates the perfect environment for the organism, typically an organism of low virulence, to have a high infective power with a low inoculum.[39] The implants should be removed at the end of the therapeutic period in order to prevent infections in the future.

Multidrug resistant (MDR) organisms have been an increasing cause of nosocomial infections in human hospitals. Recently, there has been documentation of increased MDR infections in veterinary facilities.[40] As bacterial antibiotic resistance continues to evolve, novel therapeutic strategies are needed to fight against MDR bacteria.

ANTIBIOTIC STABILITY

Stability and elution of antibiotic after incorporation in the cement bead have been reported for a gamut of antibiotics, but there are no reports, to date, evaluating the elution characteristics of antibiotics in the carbapenem class. Meropenem is an intravenous beta-lactam antibiotic that belongs to the subgroup of the carbapenems. This antibiotic has the widest spectrum of all carbapenems and is highly resistant to degradation by beta-lactamases, cephalosporinases and it is stable to dehydropeptidase-1. Meropenem's spectrum includes most aerobic and anaerobic gram positive and gram negative bacteria with the strongest affinities for *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Meropenem exhibits in vitro MIC_{90} of 4 mcg/ml or less for most organisms including the organisms previously mentioned and anaerobes such as *Bacteroides spp., Clostridium spp*., and *Fusobacterium spp*. Carbapenems are expensive antibiotics that require intravenous administration and could affect renal and hepatic function when administered parenterally.[41] Even though there have been reports of bacterial resistance to antibiotics in the carbapenem class in humans, to our knowledge there are no documented reports of bacterial resistance in veterinary medicine.[39] Staphylococci species that are resistant to methicillin or oxacillin should be considered resistant to meropenem.[41] Because of the wide spectrum of activity against gram-negative and gram-positive organisms the use of carbapenems could lead to superinfections, and therefore, are used as a last-line treatment in human medicine. Their use is limited in veterinary medicine because of cost and to prevent bacterial resistance.

Cephalosporins are also incorporated into PMMA because this group of antibiotics has a broad spectrum of activity. Cefazolin can withstand temperatures of

190°C before decomposition.[30] The exothermic reaction of the polymerization of PMMA does not exceed 120°C.[42] Regular cycle steam and gas sterilization temperatures at our hospital reach 135°C and 55°C, respectively. Neither of the temperatures used for the sterilization process reaches high enough temperatures to result in antibiotic decomposition. In an *in vitro* study evaluating the cefazolin elution from PMMA after steam and gas sterilization no deleterious effects in the activity of the antibiotic were observed using HPLC and disk agar diffusion methods.[30] Cefazolin's MIC for S. aureus is 0.25 to 1.0 mcg/ml. This same study revealed the antibiotic may be available in local tissues for a period of 7 to 10 days, but elution samples were only assayed intermittently for 9 days.[30] *Traub and Leonhard* evaluated the stability for sixty-one antibiotics at 56˚C for 30 minutes and 121˚C for 15 minutes and found that both cefazolin and meropenem were heat-labile antibiotics.[44] Treatment of both antibiotics with 121˚C for 15 minutes raised the MIC for common organisms more than 16-fold when compared with the no treatment and the 56˚C treatment groups. Ethylene oxide treatment at 56˚C failed to raise the MICs of any of the antibiotics tested by more than 2 fold with the exception of tobramycin, which had a 4 fold increase when assayed against *E. coli* ATCC 25922.[44]

A freshly constituted solution of meropenem maintains satisfactory stability at the 95% level for up to 2 hours at a controlled room temperature of 15 to 25 ˚C, or for up to 12 hours under refrigeration at 4 ˚C. A study by *Walker et al.* found that stability of meropenem was related to concentration and temperature. *Walker et al.* found that the higher the concentration and the higher the temperature the faster the degradation of the meropenem. A meropenem solution stored at -20 ˚C retains 90% of the meropenem for at least 11 days, and a meropenem solution stored at room temperature looses more

than10% within a 24 hour period. To date no studies have documented the degradation of meropenem in a solution stored at -80 ˚C. [40]

To our knowledge no research has been published evaluating the *in vitro* elution characteristics of meropenem impregnated PMMA beads. The objective of this study was to determine the elution properties of meropenem and to compare the elutions of meropenem impregnated PMMA beads without sterilization to those sterilized with steam and ethylene oxide gas.

CHAPTER II

MATERIALS AND METHODS

A commercial metal cast polytetrafluoroethylene coated bead mold (University of Vermont, Instrument & Model Facility, Burlington, VT, USA) was used. Each mold produced one chain of 25 beads that were 6.4 mm in diameter. Surgical Simplex™ P (Howmedica Inc, Rutherford, NJ, USA) radiopaque polymethylmethacrylate cement was used to make the beads as previously described.[1] Under a biocontainment hood, the sterile polymethylmethacrylate powder was combined with commercial grade meropenem powder (Merrem®: AstraZeneca Pharmaceutical LP, Wilmington, DE, USA) at a ratio of 1:5 (4 g antibiotic:20 g PMMA) in a plastic bowl and thoroughly mixed with a tongue depressor for 2 minutes.[7, 45] The powder was then divided into four equal, 6 gram portions (1 gram antibiotic, 5 grams PMMA) utilizing a laboratory scale and placed into specimen cups. Two and one half milliliters of the sterile polymethylmethacrylate liquid was mixed with 6 grams of the powder mixture, then stirred for 30 seconds and placed into two 10 cc syringes. A 22 gauge stainless steel cerclage wire was placed along the slot inside the bead mold, and the mixture was injected into each hole of the bead mold with gentle pressure. The beads were allowed to harden for 30 minutes, removed from the bead mold and allowed to harden over 24 hours at room temperature (68-70°F). This process was repeated four times to make five strands with a total of 125 beads of which 120 were used. After 24 hours, the bead strands were cut into strands of 5 beads

each. Negative control beads were made in the same fashion without the addition of antimicrobial.

Forty beads were placed in an autoclave (PMMA-Auto) and sterilized for 15 minutes at 121°C. Forty beads underwent gas sterilization using ethylene oxide (PMMA-EO) for 3 hours at 48.9°C. The final forty beads contained meropenem, but did not undergo sterilization (PMMA-Cont) and served as a positive control group. Three groups of forty PMMA beads without antimicrobial were treated in the same fashion and served as negative controls.

Evaluation of the beads consisted of placing one strand of five beads in individual test tubes (8 total test tubes per group) with 5 mls of sterile phosphate buffered saline solution at pH 7.4. The beads were maintained at normal body temperature (37°C) and constant horizontal agitation at 15 cycles per minute.[45] The phosphate buffered saline from experimental and control beads was sampled at all time periods by complete evacuation of the 5ml eluent fluid. The eluent samples were placed in 7ml plastic vials and stored at -80°C until assayed. Concentrations of the antibiotic in eluent samples from the two sterilized groups and the control beads was determined using a microbiological assay (MBA) at 1, 3, 6 and 12 hrs and at 1, 2, 3, 6, 9, 12, 15, 18, 22, 26, and 30 days.[46] The disc diffusion method was performed as previously described in other studies.[47] If the amount of antimicrobial exceeded the highest standard curve, the eluted samples were diluted with PBS before testing and adjusted accordingly.

Kocuria rhizophila, formerly classified as *Micrococcus luteus* ATCC 9341, was cultivated on Antibiotic Medium No. 1 agar and stored at -80°C. It was thawed and inoculated on a blood agar petri dish 24 hours before the bioassay test and was kept in an

incubator at 37°C until used the next day. *Kocuria rhizophila* has been previously used to assay a variety of antibiotics and fungicide residues. [48, 49, 50, 51] A BBL™ Prompt™ inoculation system (Beckton Dickinson Microbiology Systems, Sparks, MD, USA) was used to prepare standardized suspensions adhering to the standards set by the National Committee for Clinical Laboratory Standards. This inoculation system achieves the desired inoculum density, equivalent to the 0.5 McFarland turbidity standard. All eluent samples were thawed at room temperature and agitated before dilution or application to the blank paper discs.

DIFCO™ antibiotic medium 11 (Becton, Dickinson and Company, Sparks, MD, USA) was prepared following the manufacturer recommendations, autoclaved and poured at 225 mls per Nunc plate (Thermo Scientific Nunc 245mm Square BioAssay Dish: Fisher Scientific, Houston, Tx, USA) in a biocontainment hood, allowed to cool and refrigerated until used. Each Nunc plate was inoculated with *Kocuria rhizophila* using the Kirby-Bauer method and all the elution samples and quadruplets of the standard curves (8, 16, 32, 64 and 128 mcg/ml) were randomized by drawing sample numbers from a box. Each plate had duplicate internal standards of meropenem 16 mcg/ml that were used to adjust for interplate variability in zone size. Concentrations reported below the lower limit of the standard curve are because of division by a dilution factor and not extrapolation beyond the lower limit. The plates were allowed to dry after inoculation for 10 minutes, 24 BBL™ 6mm diameter blank paper discs in duplicates were applied on the surface of the agar plate equidistant from each other using a sterile technique, and 20µl of the designated sample was applied to each disk and allowed to dry for 15 to 20 minutes before inverting the plate. The plates were incubated at 37°C for 24 hours. The zone

diameters of the growth inhibition were measured using an electronic digital caliper 24 hours after inoculation and results were recorded for analysis. Areas of inhibition that were faint or less than the diameter of the disc were recorded as no zone or not reliably measured. The unknown released meropenem concentration for the *in vitro* samples was determined by comparison of their respective zone size means with the standard curve. [52]

The zone of inhibition is directly associated with drug response concentrations (DRC). As the concentration of the drug increases the zone of inhibition should increase if the organism is susceptible. This linear relationship between the diameters of the zone of inhibition and the concentration of the drug allows use of the assay as a method for quantification of a drug.

CHAPTER III

RESULTS

The microbiological assay resulted in no zone of inhibition at all time periods for the PMMA-Auto samples and the samples of PMMA without meropenem. Based on Cartesian and semi-log plots, it was determined that the elution follows a first-order process. The pharmacokinetic moment parameters are shown in Table 1. $AUC_{0-\infty}$ (mcg/ml hour) was 3,390.0 and 3,631.6 for PMMA-Cont and PMMA-EO, respectively. $MRT_{0-\infty}$ (hours) was 20.7 and 27.6 for PMMA-Cont and PMMA-EO, respectively. There was no statistical difference in $AUC_{0-\infty}$ ($P< 0.318$), however significance did occur for MRT ($P<0.005$) when comparing PMMA-Cont and PMMA-EO with the later being higher.

Table 1. Pharmacokinetic moment parameters for meropenem PMMA control and sterilized by ethylene oxide gas

Parameter	PMMA-Cont	PMMA-EO
$AUC_{0-\infty}$ (mcg/ml hour)	3390.0 ± 593.15	3631.6 ± 288.76
*$MRT_{0-\infty}$ (hours)	20.7 ± 4.19	27.6 ± 3.67
λz ($hour^{-1}$)	0.012 ± 1.253	0.013 ± 0.003
$t^1/_2$ (hours)	57.2	51.8

Table 1. Average ± one standard deviation of pharmacokinetic moment parameters for meropenem PMMA controls (PMMA-Cont) and meropenem PMMA sterilized by ethylene oxide (PMMA-EO). λz is the first-order rate constant of the terminal elution phase. Half-life ($t^1/_2$) was derived by dividing 0.693 by the terminal rate constant. The SD of the rate constant reflects the variability of the half-life. * Denotes a value that has statistically significant difference.

The cumulative drug elution released over 30 days for PMMA-Cont and PMMA-EO totaled 2.6% ± 0.4% (±SD) and 2.9% ± 0.2% of the total amount incorporated in the beads. The 24 hours elution for PMMA-Cont and PMMA-EO were 77.6% ± 4.6% and 66.6% ± 5.3%, respectively, of the total elution. The elution rapidly declined during the remaining time period with a total elution for PMMA-Cont and PMMA-EO of 22.4% ± 4.6% and 33.4% ± 5.3%, respectively, of that total drug elution. The meropenem

concentration on the eluent remained above 4 mcg/ml for 15 days in the PMMA-Cont group and until day 18 for PMMA-EO group.

CHAPTER IV

STATISTICAL ANALYSIS

A pharmacokinetic analysis was performed as in previous studies by converting the concentration of eluted drug versus time into rate of elution versus time.[53] A model-independent pharmacokinetic analysis using statistical moment theory was performed using established equations based on area-under-the-curve calculations.[53] In order to calculate the area under the curve from time zero to infinity ($AUC_{0-\infty}$) and the mean residence time from time zero to infinity ($MRT_{0-\infty}$) parameters, the terminal rate constant (λz) was determined by simple linear regression of all non-zero data points beyond 48 hours.

The analysis of Repeated Measures data was performed by using procedure MIXED with the REPEATED statement on SAS PROC MIXED 9.2 computer software (SAS Institute Inc, Cary, NC). The SUBJECT was TUBE (TREATMENT). The solution in each tube was identified as subject, treatment (BIOASSAY) as between subject, TIME as the categorical variable, and AR(1) (autoregressive of order 1) covariance structure to specify the property of correlation of each tube being larger for nearby time point than far-apart time point. Statistical analysis was performed using the concentration of drug eluted versus time and the rate of elution versus time. A one-way ANOVA was used for comparison of the pharmacokinetic parameters. The level of significance was set at P<

0.05. All samples were performed in eight replicates to allow meaningful interpretation of results.

Statistical analysis of least squares means for the mean concentrations of meropenem showed a statistically significant difference between PMMA-Cont and PMMA-EO at 6, 12, and 24 hours and at 2, 3, 6, and 9 days. No statistically significant difference was found at 1 and 3 hours and 12, 15, 18 and 22 days. Statistical analysis of least squares means for the buffer concentrations showed a statistically significant difference between PMMA-Cont and PMMA-EO at 3 hours and the baseline. No statistically significant difference was found at 9, 12, 15, 18, 21, and 24 hours and at 5, 10, 15, 20 and 25 days.

CHAPTER V

DISCUSSION

This *in vitro* study provides information about the potential clinical use of local meropenem therapy and the feasibility of bead prefabrication and sterilization for future use. Results of our study indicate that meropenem elutes effectively from PMMA, and the meropenem concentration on the eluent remained above 4 mcg/ml for 15 days in the PMMA-Cont group and until day 18 in the PMMA-EO group. No growth inhibition zone was observed on any of the samples after steam sterilization, indicating a complete loss of antibacterial activity. Because of the loss of antibacterial activity for PMMA-Auto samples no statistical comparison to the other treatments was possible. A mean cumulative drug elution for PMMA-Cont and PMMA-EO during 30 days totaled 3,210 mcg and 3,397 mcg, respectively (Fig. 1). This represents 2.6% ± 0.4% (±SD) of the total initial amount incorporated in the beads for PMMA-Cont and 2.9% ± 0.2% for PMMA-EO (Fig. 2 and 3). This finding is in accordance with previously reported antibiotic elution percentages from PMMA ranging from 2.3% to 11% for penicillins, cephaloridine, clindamycin, sodium fusidate and gentamicin.[54] Our study also supports the previous finding that PMMA alone does not have any antibacterial activity under the conditions studied. [10, 14, 19, 54]

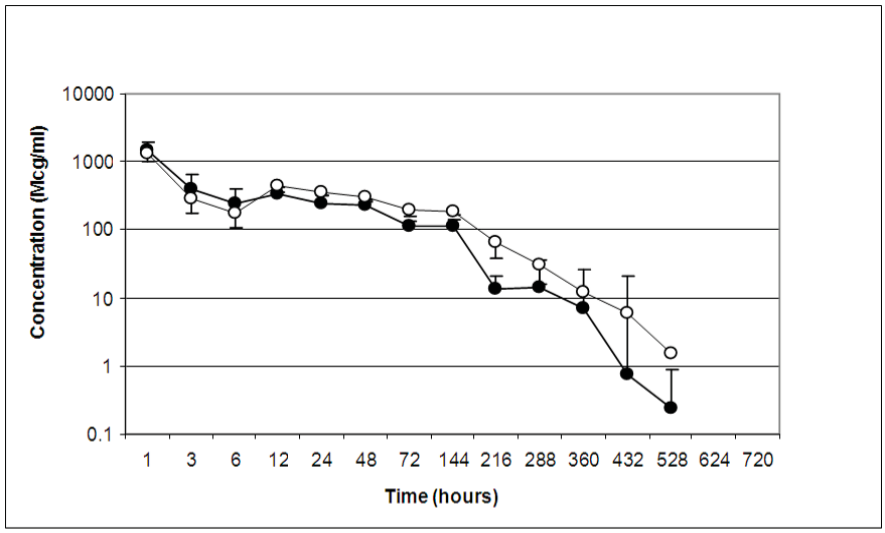

Figure 1 Meropenem mean concentration- Ethylene Oxide and Steam Sterilization

PMMA-Cont-● PMMA-EO-○ The graph shows that the mean concentration for meropenem remained above 4mcg/ml for 15 days (360 hours) in the PMMA-Cont treatment group and until day 18 (432 hours) for PMMA-EO.

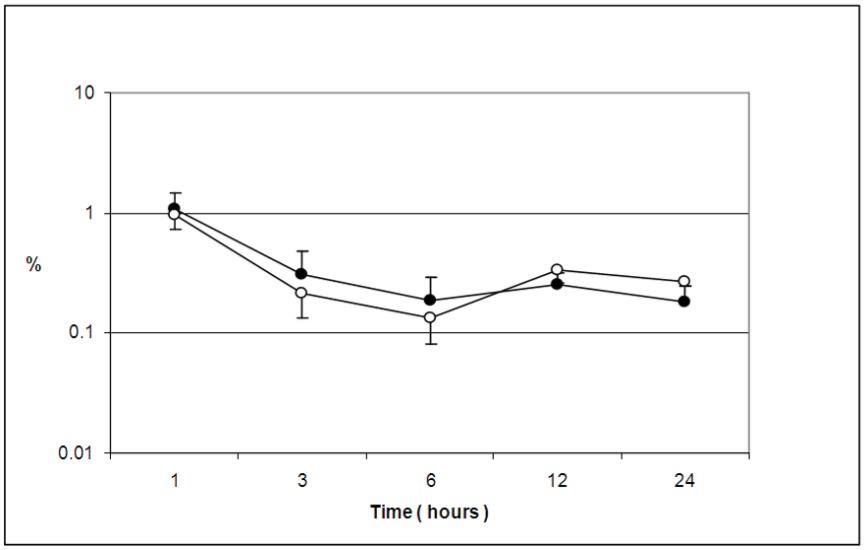

Figure 2 Meropenem percent for the 1st phase elution

PMMA-Cont-● PMMA-EO-○ The graph shows the rapid decline of the total percentage of antibiotic eluting from the PMMA beads during the 1st elution phase (first 24 hours).

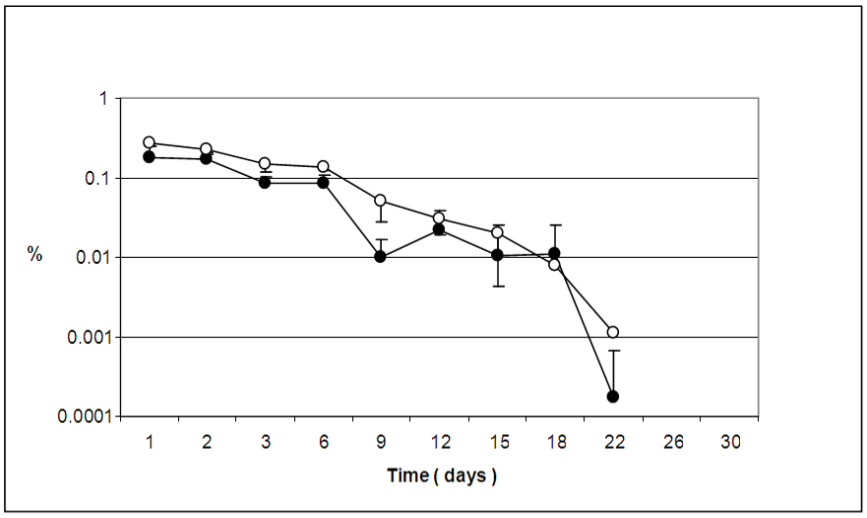

Figure 3 Meropenem percent for the 2nd phase elution

PMMA-Cont-● PMMA-EO-○ The graph shows the declining percentage of antibiotic eluting from the PMMA beads during the 2nd elution phase in the group treated with ethylene oxide and the group with no sterilization treatment.

Meropenem's elution pattern is biphasic, which is similar to patterns reported for other antibiotics.[10] This biphasic elution is suspected to be the result of initial antibiotic elution from the surface of the bead and a sustained release during the second phase of elution from the bead matrix.[54] Approximately 40% of the cumulative antibiotic elution occurred during the first hour.

There was no statistical difference in area under the curve comparisons of PMMA-EO and PMMA-Cont indicating that the total release of meropenem was equivalent. This finding confirms that gas sterilization of meropenem impregnated PMMA beads with ethylene oxide did not adversely affect antibiotic activity nor elution. Previous studies describe similar results with other antibiotics, and gas sterilized AIPMMA beads have been used successfully in the treatment of horses with localized infections. [55, 56, 57] *Ramos et al.* reported that neither polymerization, gas sterilization, nor

2 month storage of AIPMMA with varied antibiotics affected the bioactivity of the antibiotic against a common pathogen.[10] In our study the MRT did differ significantly, suggesting that ethylene oxide sterilization resulted in slightly longer elution time, though there would likely be no clinical significance in this increase (7 hours).

The minimum inhibitory concentration (MIC_{90}) for the most commonly encountered pathogens in cases of osteomyelitis and soft tissue infections sensitive to meropenem is 4 mcg/ml.[58] The activity of meropenem is time dependent, and the amount of time that an antibiotic concentration is above the MIC is important. In the study reported here, the meropenem concentration in the eluent remained above 4mcg/ml for 15 days in the PMMA-Cont group and for 18 days in the PMMA-EO group. When addressing an existing infection, it is recommended that treatment involve the use of effective antibiotic concentrations for 3 to 4 weeks or longer depending on response to treatment. [59, 60] It is also recommended that a concentration of one to five times the MIC should be maintained for at least half of the dosing interval when time dependent drugs are given systemically.[32] However, the concentration that should be maintained over time when a time-dependent antibiotic is used for local treatment is unknown. This should be considered when using meropenem impregnated beads for treatment of existing infections.

Antibiotic elution rate varies depending on the type of cement used.[14, 54] Simplex™ P radiopaque polymethylmethacrylate cement was selected for this study because it is the most commonly used cement in the United States.[7] However, Palacos® (Heraeus, Kulzer GmbH, Hanau, Germany) bone cement has been found to release most antibiotics in larger amounts and for longer periods of time than other bone cements

because of a larger surface pore size. [16, 19, 61] Also, a previous *in vitro* study evaluating vancomycin elution from PMMA and biodegradable beads reported extended antibiotic elution times when biodegradable beads were used.[61]

Implant size also affects antibiotic elution, with smaller beads having a greater surface area and as a result a better elution than larger beads.[54] The PMMA beads used in this study were 6.4 mm in diameter as described in previous studies.[6, 7] Though biomechanical studies on antibiotic impregnated PMMA indicate that no more than 10 percent of the total weight should be antibiotic if the cement is used for implant fixation, the percentage can be much higher for bead production. The limit of antibiotic to PMMA ratio recommended for bead production is 1:5. If the amount of antibiotic surpasses this ratio the PMMA will not harden into beads.[54]

The volume of PBS used and the elution rates affect the antibiotic concentration in the eluent. The PBS volume chosen for this study was the same as used in previous studies.[45] The use of serum instead of PBS has not been found to affect elution rates. [45]

Meropenem is an intravenous beta-lactam antibiotic that belongs to the subgroup of the carbapenems. It is highly resistant to degradation by beta-lactamases, cephalosporinases, and it is stable to dehydropeptidase-1. Meropenem's spectrum includes most gram positive and gram negative bacteria with the strongest affinities for *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Meropenem exhibits *in vitro* MIC_{90} of 4 mcg/ml or less for most organisms including the organisms previously mentioned and anaerobes such as *Bacteroides spp., Clostridium spp*., and *Fusobacterium spp*. Though there have been reports of bacterial resistance to antibiotics

in the carbapenem class in humans, to our knowledge there are no documented reports of bacterial resistance in veterinary medicine.[62]

Beta-lactam antibiotics have been classified as heat-labile antibiotics. The exothermic reaction during polymerization of PMMA can reach temperatures of 100˚C and may result in degradation and inactivation of certain antibiotics. [4, 14, 15, 16, 44, 63, 64] In a study of meropenem, the MIC for various organisms increased about 16-fold after autoclaving at 121 ˚C for 15 minutes, presumably from alteration of a key portion of the molecule necessary for antimicrobial activity. Autoclave treatment increased the MIC for all the organisms tested, but did not result in complete destruction of the antimicrobial activity. No change in MIC was observed after heat treatment with 56˚C for 30 minutes in a water bath.[44] Our study did not support the use of meropenem impregnated PMMA beads after autoclave sterilization since no growth inhibition zone was observed on any of the PMMA-Auto samples. The exothermic reaction combined with the exposure to 121 ˚C for 15 minutes during autoclaving may have rendered the meropenem incorporated into the cement bioactively inert. Whereas the PMMA-Cont beads with no sterilization treatment did have a growth inhibition zone and the results were not significantly difference than samples sterilized with ethylene oxide gas.

Imipenem, another antibiotic in the carbapenem subgroup, has been incorporated in PMMA pellets approximately 12mm in length and 7 mm in diameter resulting in short-term, less than adequate elutions, but antibiotic elution was only evaluated for the first 72 hours.[63] In the same study a 3:1 mix of polymer and monomer was used for preparation of the PMMA pellets to produce a lower peak temperature during polymerization of the cement, resulting in a 65 ˚C compared with a 75˚C in a 2:1 mixture. Imipenem's MIC for

common pathogens increased more than 256 fold when autoclaved at 121 ˚C for 15 minutes in a previous study evaluating heat stability.[44]

A potential limitation of this study is that the eluted samples were stored at -80˚C for up to 3 months prior to testing. Meropenem serum concentrations are reported to be stable during -20˚C freezing for greater than 20 days, though no published reports describe stability of higher concentrations for longer periods of time.[46] It is possible that the antibiotic degraded during storage. If this occurred, meropenem activity may be longer than identified in this study. Additional studies would be needed to determine degradation rate for meropenem during storage at -80˚C.

CHAPTER VI

CONCLUSION

Our study found that meropenem incorporated in the PMMA beads elutes effectively and gradually decreases during the second elution phase, but remains above concentration of 4mcg/ml for 15 days in the PMMA-Cont group and until day 18 for PMMA-EO group. Meropenem impregnated PMMA beads can be sterilized using ethylene oxide without adversely affecting antibiotic activity, though the shelf-life of prefabricated meropenem beads for "off the shelf" use needs further investigation. Several factors affecting the antibiotic elution could be used in order to increase meropenem elution such as bead size, decrease in the amount of monomer, increase the amount of antibiotic incorporated in the cement, use of a more oblong bead shape to increase bead surface area and use of biodegradable materials such as hydroxyapatite, calcium sulfate hemihydrate and polylactide-polyglycolide. The effects and pharmacokinetics of meropenem impregnated PMMA bead implantation should be evaluated *in vivo* to determine clinical applications.

REFERENCES

1. Wininger DA, Fass RJ: Minireview: Antibiotic-impregnated cement and beads for orthopedic infections. *Antimicrob Agents Chemother.* 1996; 2675-2679.

2. Haydon RC, Blaha JD, Mancinelli C, Kazanuri K. Audiometric thresholds in osteomyelitis patients treated with gentamicin-impregnated methylmethacrylate beads (Septopal). *Clin Orthop Relat Res*.1993; 295:43-46.

3. Drombrowski ET, Dunn AW. Treatment of osteomyelitis by debridement and closed wound irrigation-suction. *Clin Orthop Relat Res*.1966; 43:215-231.

4. Buchholz HW, Engelbrecht H. Uber die Depotwirkyng einiger Antibiotica bei Vermishchung mit dem Kunstharz Palacos. *Chirurg.* 1970; 41:511-515.

5. Klemm K. Gentamycin-PMMA-Kugeln in der Behandlung abszedier-ender Knochen- und Weichteilinfektionen. Zentralbl. *Chir.* 1979; 104:934-942.

6. Seligson D, Popham GJ, *et al*. Antibiotic-leaching from polymethylmetharcyrlate beads. *J Bone Jt Sur Am.* 1993; 5:714-720.

7. Cunningham A, Demarest G, Rosen P, DeCoster TA. Antibiotic bead production. *Iowa Orthop J.* 2000; 20:31-35.

8. Phillips H, Boothe DM, Shofer F, *et al.* *In vitro* elution studies of amikacin and cefazolin from polymethylmethacrylate. *Vet Surg*. 2007; 36(3):272-8.

9. Tobias KM, Schneider RK, Besser TE. Use of antimicrobial-impregnated polymethylmethacrylate. *J Am Vet Med Assoc.* 1996; 208:841-845.

10. Ramos JR, Howard RD, Pleasant RS, Moll HD, Blodgett DJ, Magnin G, Inzana TJ.: Elution of metronidazole and gentamicin from polymethylmethacrylate beads. *Vet Surg*. 2003; 32(3):251-61.

11. Evans RP, Nelson CL. Gentamicin-impregnated polymethylmethacrylate beads compared with systemic antibiotic therapy in the treatment of chronic osteomyelitis. *Clin Orthop Relat Res*. 1993; 295:37-42.

12. Nelson CL, Evans RP, Blaha JD, Calhoun J, Henry SL, Patzakis MJ. A comparison of gentamicin-impregnated poymethylmethacrylate bead implantation to conventional parenteral antibiotic therapy in infected total hip and knee arthroplasty. *Clin Orthop Relat Res.* 1993; 295:96-101.

13. Weisman DL, Olmstead ML, Kowalski JJ. *In vitro* evaluation of antibiotic elution from polymethylmethacrylate (PMMA) and mechanical assessment of antibiotic-PMMA composites. *Vet Surg.* 2000; 29:245-251.

14. Calhoun JH, Mader JT. Antibiotic beads in the management of surgical infections. *Am J Surg.* 1988; 157:443-449.

15. Chapman MW, Hadley WK. The effect of polymethylmethacrylate and antibiotic combinations on bacterial viability. An *in vitro* and preliminary *in vivo* study. *J. Bone Joint Surg Am.*1976; 58:76-81.

16. Walenkamp GHIM. Gentamicin-PMMA beads. A clinical, pharmacokinetic and toxicological study. Darmstadt, FR Germany, E. Merck, 1983.

17. Lee CG, Fu Uin-Chih and Wang Chi-Hwa. Simulation of gentamicin delivery of the local treatment of osteomyelitis. Wiley InterScience. www.interscience.wiley.com March 2005.

18. Seeley SK, Seeley JV, Telehowski P *et al*. Volume and surface area study of tobramycin-polymethylmethacrylate beads. *Clin Orthop Relat Res*. 2004; 420:298-303.

19. Marks KE, Nelson CL, Lautenschlager EP. Antibiotic-impregnated acrylic bone cement. *J Bone Jt Sur Am.* 1976; 58:358-364.

20. Petty W, Caldwell JR. The effect of methylmethacrylate on complement activity. *Clin Orthop Relat Res*. 1997;128:354-360.

21. Blaha JD, Nelson CL, Frevert LF, Henry SL, Seligson D, Esterhai JL, *et al*. Use of Septopal (polymethylmethacrylate beads with gentamicin) in the treatment of chronic osteomyelitis musculoskeletal sepsis. In Green, W. (ed): AAAOS Instructional Course Lectures, vol. 39. St. Louis, C. V. Mosby, 1990.

22. Levin PD. The effectiveness of various antibiotics in methylmethacrylate. *J Bone Jt Surg Br.* 1975; 57:234-237.

23. Henry SL, Hood GA, Seligson D. Long term implantation of gentamicin-polymethylmethacrylate antibiotic beads. *Clin Orthop Relat Res*. 1993; 295:47-53.

24. Henry SL, Seligson D, Mangino P, *et al*. Antibiotic-impregnated beads, Part 1: Bead implantation versus systemic therapy. *Orthop Rev.* 1991; 20:242-247.

25. Neut Daniëlle, Van de Belt Hilbrand, Stokross Ietse *et al*. Biomaterial- associated infection of gentamicin-loaded PMMA beads in orthopaedic revision surgery. *Journal of Antimicrobial Chemotherapy* 2001; 47, 885-891.

26. Anagnostakos K, Hitzler P, Pape D, Kohn D, Kelm J. Persistence of bacterial growth on antibiotic-loaded beads: Is it actually a problem? *Acta Orthopaedica* 2008; 79 (2): 302-307.

27. Lyons VO, Henry SL, Faghri M, Seligson D. Bacterial adherence to plain and tobramycin-laden polymethylmethacrylate beads. *Clin Orthop Relat Res*. 1992; 278:260-264.

28. Calhoun JH, Henry SL, Anger DM, Cobos JA, Mader JT. The treatment of infected nonunions with gentamicin-polymethylmethacrylate antibiotic beads. *Clin Orthop Relat Res*. 1993; 295:23-27.

29. Tobias KS, Charles TR, William TF. Treatment of cellulitis in an American black bear (*Ursus americanus*) with antibiotic-impregnated implants. *Journal of Zoo and Wildlife Medicine.* 1996; 27(1): 109-114.

30. Florey K. Analytical Profiles of Drug Substances. New York: Academic Press, Inc, 1975; pp 2-20.

31. Dernell WS. Treatment of severe orthopedic infections. *Vet Clin North Am Small Anim Pract.* 1999; 29 (5):1261-1274.

32. Craig WA. Interrelationship between pharmacokinetics and pharmacodynamics in determining dosage regimens for broad-spectrum cephalosporins. *Diag Microbiol Infect Dis.* 1995; 522:89-96.

33. Klemm, KW. Antibiotic bead chains. *Clin Orthop Relat Res*. 1993; 295:63-76.

34. Johnson KA. Osteomyelitis in dogs and cats. *J Am Vet Med Assoc.* 1994; 205:1882-1887.

35. Stevenson S, Olmstead M, Kowalski J. Bacterial culturing for prediction of postoperative complications following open fracture repair in small animals. *Vet Surg.* 1986; 15:99-102.

36. Caywood DD, Walace LJ, Braden TD. Osteomyelitis in the dog: A review of 67 cases. *J Am Vet Med Assoc.* 1978; 172:943.

37. Krasko MY, Golenser J, Nyska A, *et al*. Gentamicin extended release from an injectable polymeric implant. *Journal of controlled release.* 2007; 117:90-96.

38. Olson ME, Ceri H, Morck DW, *et al*. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *The Canadian Journal of Veterinary Research.* 2002; 66:86-92.

39. Chang, Che Chang, Merrit Catherine. Effect of *Staphylococcus epidermis* on adherence of *Pseudomonas aeruginosa* and *Proteus mirabilis* to polymethyl methacrylate and gentamicin-containing PMMA. *Journal of Orthopaedic Research.* 1990; 284-288.

40. Gibson JS, Morton JM, Cobbold RN *et al*. Multidrug-resistant *E. coli* and *Enterobacter* extraintestinal infection in 37 dogs. *J Vet Intern Med.* 2008; 22:844-850.

41. AstraZeneca Pharmaceuticals LP. Merrem® Drug Insert.

42. Jeffries C, Lee A, Ling R. Thermal aspects of self-curing polymethylmethacrylate. *J Bone Joint Surg Br*. 1975; 57:511-518.

43. Bogaerts P, Naas T, Wyb I, *et al*. Outbreak of infection by carbapenem-resistant *Acinetobacter baumannii* producing the carbapenenamase OXA-58 in Belgium. *Journal of Clinical Microbiology.* 2006; 4189-4192.

44. Traub WH, Leonhard B. Heat stability of the antimicrobial activity of sixty-two antibacterial agents. *J Antimicrob Chemother.* 1995; 35:149-154.

45. Ethell MT, Bennett RA, Brown MP, *et al*. *In vitro* elution of gentamicin, amikacin, and ceftiofur from polymethylmethacrylate and hydroxyapatite cement. *Vet Surg.* 2000; 29(5): 375-382.

46. Ip M, Au C, Cheung SW *et al*. A rapid high-performance liquid chromatographic assay for cefepime, cefpirome, and meropenem. *J Antimicrob Chemother.* 1998; 42(1): 121-123.

47. Welch, AB. Antibiotics in acrylic bone cement, *'in vitro'* studies. *J Biomed Mater Res* 1978; 12:679-700.

48. AOAC, Association of Analytical Chemists. In US FDA Bacteriological Analytical Manual, 8th edition, Gaithersburg, MD 1995; pp. 20.01- 20.04.

49. British Pharmacopoeia Commission, London: The Stationery Office. Biological assay of antibiotics. In British Pharmacopoeia, vol. 2, appendix XIV, 1993; pp. A165-A169.

50. Tang, Jane S, Gillevet PM. Reclassification of ATCC 9341 from *Micrococcus luteus* to *Kocuria rhizophila*. *International Journal of Systematic and Evolutionary Microbiology* 2003; 53:995-997.

51. Wichelhaus TA, Dingeldein E, Rauschmann M, *et al*. Elution characteristics of vancomycin, teicoplanin, gentamicin and clindamycin from calcium sulphate beads. *J Antimicrob Chemother.* 2001; 48, 117-119.

52. Adams K, Couch L, Cierny G, *et al*. *In vitro* and *in vivo* evaluation of antibiotic diffusion from antibiotic-impregnated polymethylmethacrylate beads. *Clin Orthop Relat Res* 1992; (278):244-252.

53. Yamaoka K, Nakagawa T, Uno T. Statistical moments in Pharmacokinetics. *J Pharmacokinet and Biopharm.* 1978; 6 (6), 558-574.

54. Picknell B, Mizen L, Sutherland R, *et al*. Antibacterial activity of antibiotics in acrylic bone cement. *J Bone Joint Surg Br.* 1977; 59(3):302-307.

55. Butson RJ, Schramme MC, Garlick MH, *et al*. Treatment of intrasynovial infection with gentamicin-impregnated polymethylmethacrylate beads. *Vet Rec.* 1996; 138(19):460-464.

56. Flick AB, Herbert JC, Goodell J, *et al*. Non-commercial application of AIPMMA beads. Technical note. *Clin Orthop Relat Res*.1987; 223:282-286.

57. Holcombe SJ, Shneider RK, Bramlage LR, *et al*. Antibiotic-impregnated beads, Part 1: Bead implantation versus systemic therapy. *Orthop Rev.* 1991; 20:242-247.

58. Merrrem® Drug Information Insert, AstraZeneca Pharmaceuticals LP, Wilmington, DE 19850.

59. Buchholz HW, Elson RA, Heinert K. Antibiotic-loaded acrylic cement: current concepts *Clin Orthop Rel Res.* 1984; 190:96-108.

60. Tobias KM, Schneider RK, Besser TE. Use of antimicrobial-impregnated polymethylmethacrylate. *J Am Vet Med Assoc.* 1996; 208(6):841-845.

61. Wahlig H, Dingeldein E. Antibiotics and bone cements: Experimental and clinical long-term observations. *Acta Orthop Scand.* 1980; 51(1):49-56.

62. Mader JT, Calhoun J, Cobos J. *In vitro* evaluation of antibiotic diffusion from antibiotic-impregnated biodegradable beads and polymethylmethacrylate beads. *Antimicrob Agents Chemother.* 1997; 41(2):415-418.

63. Bowyer GW, Cumberland N. Antibiotic release form impregnated pellets and beads. *J Trauma.* 1994; 36 (3):331-335.

64. Hoff SF, Fitzgerald RH, Kelly PH. The depot administration of penicillin-G and gentamicin in acrylic bone cement. *J Bone Joint Surg Am.* 1981; 63(5):798-804.