OSTEOMYELITIS TREATMENT IN NEW ZEALAND WHITE RABBIT USING HYDROXYAPATITE OR CALCIUM SULPHATE CONTAINING GENTAMICIN

BY

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A dissertation submitted in fulfilment of the requirement for the degree of Master of Orthopaedic Surgery

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ABSTRACT

Treatment of osteomyelitis is still a major problem in orthopaedic field. New Zealand White rabbit is acceptable experimental model described to develop effective local delivery of antibiotics for osteomyelitis treatment as it can mimics the disease process in human. The objectives of this study are to create osteomyelitis in rabbit and to compare the treatment given via calcium sulphate or hydroxyapatite containing gentamicin beads. In this study, osteomyelitis is created by inoculation of *Staphylococcus aureus* into rabbit's distal femur. After three weeks, local antibiotic with carrier either calcium sulphate or hydroxyapatite containing gentamicin was inserted to the affected femur with 12 animals in each group. The plain radiograph of femur and culture was taken to assess the healing at three, six or twelve weeks according to the group. Osteomyelitic changes were seen in all rabbit after inoculation of bacteria at three weeks. There was significant weight reduction after osteomyelitis (CaSO4; t=2.55, P=0.03, HA; t=2.17, P=0.05), and after treatment for 12 weeks in both groups (t=6.93, P=0.001). The radiographic results ($\chi 2=61.00$, p=0.001) and culture (t=13.85, P=0.001) also showed significant bone healing after treatment at 12 weeks in both groups. CONCLUSION: Calcium sulphate and hydroxyapatite containing gentamicin gives similar effect in the treatment of osteomyelitis in rabbit model.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Orthopaedic Surgery

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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CHAPTER ONE

INTRODUCTION

The management of chronic osteomyelitis still become a challenging issue to the orthopaedic surgeon in all over the world. The infection may resolve, become a quiescent and enduring infection, or become a chronic infection with related progressive bone deterioration and infection extension. Principle of treatment for osteomyelitis consists of antimicrobial therapy, débridement, and follow-up care that includes stabilization of the bone and management of any dead space that remains after debridement (Carek, Dickerson, Pharm, & Sack, 2001; Cierny, 2011; Mader, Shirtliff, & Calhoun, 1984; Simpson, Deakin, & Latham, 2001).

The rabbit model of osteomyelitis introduced by C.W. Norden, based on injection of an infecting solution (*Staphylococcus aureus*, sodium morrhuate) has been used extensively to study the efficacy of various antibiotic regime for the treatment of this disease (Norden & Keleti, 1980).

Because of the marked variability in presentation and management of osteomyelitis in patient, research with animal models that mimic the human disease offers a more controlled approach. Currently available animal models have been used to study pathogenesis, diagnosis, and treatment of osteomyelitis with their own advantages and disadvantages.

There are various methods available for antimicrobial therapy in treating infection. Oral antibiotics give unpredictable levels over the affected area and infrequently use. To achieve adequate therapy, 6 weeks intravenous antibiotics are commonly used but with significant relapsed (Gomes, Pereira, & Bettencourt, 2013; Lew & Waldvogel, 2004). High parenteral dose of antibiotic is needed to achieve

effective therapeutic drug concentration in the bone. Antibiotic concentration required to penetrate and kill bacteria enclosed in biofilm are 10 to 100 times the standard bactericidal concentration which makes systemic therapy unsafe (Gogia, Meehan, Cesare, & Jamali, 2009).The high dose together with prolonged course of treatment can lead to systemic toxicity of the antibiotic and therefore alternative strategies with local delivery of antimicrobial agents have been introduced. The carrier materials used can be classified as non-biodegradable and biodegradable which have been developed for effective treatment in bone infection. Recently, the research for various biodegradable delivery systems with various antimicrobial agents have been developed and evaluated for osteomyelitis treatment.

Further efforts to develop new experimental models and innovative research with present models together with local antibiotic therapy are clearly needed to achieve an ideal model of osteomyelitis in the future for the betterment of management in osteomyelitis.

CHAPTER TWO

LITERATURE REVIEW

2.1 OSTEOMYELITIS

Osteomyelitis (OM) is a progressive infection of bone,that results in inflammatory destruction of the bone, bone necrosis, and new bone formation and may progress to a chronic and persistent state. It can be divided as acute haematogenous, subacute, post-traumatic , and chronic osteomyelitis. However, there are many ways described in the literature to classify osteomyelitis (Brady, Leid, Costerton, Shirtliff, & Angeles, 2006). These classification systems help to describe the infection and determine the need for surgery.

2.1.1 Organisms in Osteomyelitis

The usual organisms in chronic osteomyelitis are *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pyogenes*, *Proteus mirabilis* and *Pseudomonas aeroginosa*. (Solomon, Warwick, & Nayagam, 2010). Staphylococci are Gram-positive 1μm in diameter. They form clumps. It can be classified as coagulase positive (*S. aureus* and *S. epidermidis*) and negative (all other staphylococci). *S. aureus* is a gram-positive, facultatively anaerobic coccus that is nonmotile and non-sporeforming bacteria. *S. aureus* is a normal commensal of the human nares (Baron, 1996). *Staphylococcus aureus*, is able to cause an acute bone infection even with a low inoculum in a healthy host. In addition, through the timed expression of its arsenal of virulence factors and aided by its ability to develop antibiotic resistance rapidly, *S. aureus* progresses to a chronic, biofilm-mediated infection. A biofilm is defined as a microbially derived sessile community, typified by cells that are attached to a substratum, interface, or to

each other, are embedded in a matrix of extracellular polymeric substance, and exhibit an altered phenotype with regard to growth, gene expression, and protein production (Donlan & Costerton, 2002; Fitzpatrick, Humphreys, & Gara, 2005).

Staphylococcus spp. can produce a multilayered biofilm embedded within a glycocalyx, or slime layer. The glycocalyx develops on devitalized tissue and bone, or on medically implanted devices, to produce an infection (Ziran B.H, 2007). These bacteria are protected from the antibiotic action and host immune system by the formation of the *glycocalyx* membrane at the surface of the implant. There is also reported that that bacteria can survive inside osteoblast and osteocytes and be released when the cells die (Bosse, Gruber, & Ramp, 2005; Solomon et al., 2010).

Once a chronic infection develops, bacterial clearance cannot be attained by the host immune system or antimicrobial therapy. At this point, surgical removal of the nidus of infection is usually necessary for complete infection resolution (Brady et al., 2006).

2.1.2 Pathology

Chronic osteomyelitis will show necrotic bone (sequestra), formation of new bone (involucrum), exudation of polymorphonuclear leukocytes joined by large numbers of lymphocytes, histiocytes and occasionally plasma cells. Cavities that contain pus and pieces of sequestra are surrounded by vascular tissue followed by area of sclerosis. This will result in the formation of chronic reactive new bone with distinct bone sheath (involucrum) surrounding the dead bone under periosteum. The involucrum is irregular and is often perforated by openings through which pus may track into the surrounding soft tissue and eventually drain into the skin surfaces forming the chronic sinus. The involucrum may gradullay increase in density to form part of the diaphysis

over few months. Endosteal new bone may proliferate and obstruct the medullary canal. After host defence or operative removal of the sequestrum, the remaining cavity may be filled with new bone, especially in children. However in adults, the cavity may persist or the space may be filled with fibrous tissue, which may connect the skin surface by means of a sinus tract. A sinus may seal off over weeks or months as if it was healed but may reopen when tissue tension rises (Ciampolini & Harding, 2000; Solomon et al., 2010).

2.1.3 Evaluation

2.1.3.1 Biochemical Investigations

There are few parameters can be used to assess the progress of bone infection which includes white blood cells, erythrocyte sedimentation rate and C-Rreactive protein. The leucocyte count may be raised in acute or normal in chronic patient. C-reactive protein (CRP) is another inflammatory index that rises in both acute and chronic patient and it will decreases faster than ESR in succesful treatment. CRP was found to be decrease dramatically in succesfully treated children after three days of antibiotics (Pääkkönen, Kallio, Kallio, & Peltola, 2010) Although a sedimentation rate that returns to normal in response to therapy is a favorable development, this laboratory determinant is not reliable in the compromised host, who may be constantly challenged by minor illnesses and peripheral lesions that can elevate the index (Calhoun & Manring, 2005).

The infected bone or discharging sinus should be tested repeatedly for antibiotic sensitivity as the organism can change their characteristic and become

resistant to the treatment given. The biopsy from the infected bone will be send for histopathology and culture to see the inflammatory changes in chronic osteomyelitis.

Even though culture and sensitivity is important to start antibiotic treatment, standard bacterial cultures still gives negative results in about 20% of cases of overt infection. Thus, a lot of new test developed such as polymerase chain reaction and identification by gel electrophoresis for organism detection which is still not available for routine test (Solomon et al., 2010). The latest pilot study done by Kobayashi et al. involves vortexing and sonication to improve detection of biofilm-formative bacteria(Kobayashi, Oethinger, Tuohy, Procop, & Bauer, 2009).

2.1.3.2 Imaging

2.1.3.2.1 Plain Radiograph

Plain radiograph is one of non-invasive investigation in evaluation of osteomyelitis. Radiographic changes in bone are often difficult to interpret, and it can take at up to 2 weeks following the onset of infection to reach the 30 to 50% loss in bone density that is often required for visualization. The earliest signs seen on a radiograph are soft tissue swelling evidence by displacement of fat planes followed by thickening of the periosteum and patchy rarefaction of the metaphysis after that, and later bone detruction with sequestra (Solomon et al., 2010) Sensitivity and specificity are only 70% and 50%, respectively which shows that it is not really reliable (Pineda, Espinosa, & Pena, 2009).

In rabbit osteomyelitis study by Norden et al., four criteria used were sequestrum formation, periosteal new bone formation, destruction of bone and the extend of disease in terms of proximal, mid or distal tibia involvements. All these

criteria were recorded and analysed to see the respond of treatment in the affected rabbit (Norden, Myerowitz, & Keleti, 1980). Ambrose et al. in comparison, assessed the radiographic response by recording the size of defect and new bone formation (Ambrose et al., 2004).

2.1.3.2.2 Computed Tomography (CT)

CT scan provides multiplanar reconstructions of the axial images allowing delineation of even the most subtle osseous changes. In chronic osteomyelitis, CT demonstrates abnormal thickening of the affected cortical bone, with sclerotic changes, encroachment of the medullary cavity, detection of pieces of necrotic bone masked by surrounding osseous abnormalities on xray and chronic draining sinus. Although CT may show these changes earlier than do plain radiographs, CT is less desirable than MRI because of decreased soft tissue contrast as well as exposure to ionizing radiation (Pineda et al., 2009).

2.1.4 Diagnosis

The diagnosis of osteomyelitis is based primarily on the clinical findings, with data from the initial history, physical examination and laboratory tests support with imaging results (Carek et al., 2001).

2.1.5 Treatment

The principle of treatment of osteomylitis includes appropriate adequate drainage through debridement, obliteration of dead space, wound coverage, specific antimicrobial therapy and stabilization of affected bone. Amputation is indicated when limb salvage and palliation are neither safe nor feasible (Cierny, 2011).

2.2 ANTIBIOTIC

Although the optimal duration of antibiotic therapy remains undefined, most investigators treated patients for about six weeks. Despite three decades of research, the available literature on the treatment of osteomyelitis is inadequate to determine the best agent(s), route, or duration of antibiotic therapy. Lazzarani et al. reviewed medical literature for article published from 1968 to 2000 to determine the most appropriate antibiotic therapy for osteomyelitis. Combined parenteral and oral regimens are usually used (Calhoun & Manring, 2005; Lazzarini, Lipsky, & Mader, 2005) If possible, culture specimens should be obtained before antibiotic therapy is initiated or after the patient has been off antibiotic therapy for at least 24–48 hours. This time is necessary for two vital reasons. First, since the half-life of many antibiotics is 12 hours, obtaining culture sooner than 24 hours after antibiotic therapy has been stopped may allow the administered antibiotic to interfere with culture growth. Second, since many antibiotics are bacteriostatic, time must be allowed for low numbers of inhibited bacteria to multiply within the host and become detectable by culture techniques (Mader et al., 1984).

2.2.1 Local Antibiotic Delivery System

Because of the altered structure of the tissues surrounding an infected site, the diffusion of antibiotics into the central part of the infected region may require high serum concentrations of the therapeutic agents. This therapy may cause side effects such as myelosuppression, renal failure, and hepatitis. Thus, local delivery antibiotics for chronic osteomyelitis are created to increase local concentration of antibiotics. They are classified as non-biodegradable and biodegradable based on the nature of delivery system. Antibiotic-impregnated polymethylmethacrylate (PMMA) beads and

self-setting bone cement have been used to treat chronic osteomyelitis allowing the local delivery of high concentrations of antibiotics, while avoiding potential systemic side effects (Belt, Horn, Mei, & Busscher, 2003; Klemm, 2001). However, it needs to be removed later as it is not resorb in the body. Belt et al., in their report found that Gentamicin-release test revealed residual antibiotic release after being 5 years in a patient with gentamicin-loaded polymethylmethacrylate. Prolonged release of subinhibitory concentration of antibiotics is worrisome in the clinical application of antibiotic-loaded bone cement, as it stimulates the introduction of gentamicin-resistant strains. This case emphasizes the importance of developing biodegradable antibiotic-loaded beads as an antibiotic delivery (Belt et al., 2003).

The biodegradable antibiotic impregnated beads are developed to overcome the disadvantage by methymethacrylate. The implant can obliterate the dead space at the initial stage and helps in the repair of bone. During the degradation phase, the antibiotic will be release. Impregnation of antimicrobial agents within osteoconductive bioceramics (calcium sulphate, tricalcium phosphate or hydroxyapatite) has been proposed for the local management of osteomyelitis and to aid dead space management mainly for the delivery of antibiotics (Gogia et al., 2009; Kumar et al., 2009).

The summary of advantages and disadvantages for biodegradable and non-biodegradable systems listed in table.

Table 2.1
Advantages and disadvantages of biodegradable and non-biodegradable local antibiotics.

Carrier type	Advantages	Disadvantages
Non-biodegradable	-Easy procedure for insertion in the body -Proven to be successful with several antibiotics	-Second surgery may be needed to remove the cement beads -Polymerization process could cause thermal damage and neutralization of the antibiotic -slow residual release of antibiotic for undefined periods, risk of resistance
Biodegradable	-Osteoconductive and osteoinductive -One stage surgery -Wider selection of of antibiotics including thermolabile antibiotic	-Do not form a firm bond with the bone -No large human trials have been published

2.2.2 Carrier System

The materials used for impregnation with antibiotic include plaster of Paris pellets (Mousset, Benoit, Delloye, Bouillet, & Gillard, 1995), Calcium hydroxyapatite (Nandi et al., 2009; Yamashita et al., 1998), lactic acid oligomer (Wei et al., 1991), polymethylmethacrylate (PMMA) (Klemm, 2001), calcium sulphate (Helgeson, Potter, Tucker, Frisch, & Shawen, 2009) etc. Ceramic materials are well known for their applications in orthopaedic surgery, particularly as a coating for joint prostheses, and for articulating surfaces such as femoral heads and acetabulae. Porous calcium

hydroxyapatite (CHA) has excellent biocompatibility, can resist mechanical forces, and is effective in filling cavities and defects in bone.

2.2.2.1 HA (Hydroxyapatite: $Ca(PO_4)_6(OH)_2$)

Hydroxyapatite is the most commonly used bone graft material because of their calcium/phosphorus ratios are close to that of natural bone. They are also relatively stable in physiological environment(Teixeira, Ferraz, & Monteiro, 2008). Hydroxyapatite (HA) granules made from local raw materials have been fabricated using a novel method by Hafiz et al., and is proven to be osteoconductive and biocompatible when implanted in rabbit and in their study for treatment of closed fracture in adults shows good bone formation(A Hafiz et al., 2008; Hafiz, A.,KA Khalid, Yusof, A., Azril, MA., Shukrimi, A., Nazri, MY., Aminudin, CA., Zamzuri, Z., 2008). Porous calcium hydroxyapatite (CHA) has excellent biocompatibility, can resist mechanical forces, and is effective in filling cavities and defects in bone (Korkuruz et al., 1992) Use of porous pieces of calcium hydroxyapatite impregnated with antibiotic as a new system for drug delivery in the treatment of chronic osteomyelitis gives good result in terms of healing and no recurrence of infection. Porous CHA as a slow release system for antibiotics after using it to fill the space left after erosion by disease or excision of dead bone (Yamashita et al., 1998).

2.2.2.2 Calcium Sulphate ($CaSO_4$)

Calcium sulphate was first introduced as plaster of Paris with osteoconductive properties. Calcium sulphate is a bioceramic that occurs naturally. Surgical grade calcium sulphate is a relatively pure alpha hemihydrate crystal, which can be hydrated to produce solid implants. It is well tolerated, nonimmunogenic and fully

biodegradable (Gitelis & Brebach, 2002). Gitelis in his study found that all six patients with osteomyelitis treated with calcium sulphate impregnated antibiotic, showed progressive repair without evidence of either residual or new osteolysis and al implants are fully biodegraded. Research done by Helgeson et al. showed that antibiotic-impregnated calcium sulphate is effective in treating severe, contaminated open fractures by reducing infection and assisting with fracture union (Helgeson et al., 2009).

2.2.3 Antimicrobial Agent in Local Delivery Systems

There are few characteristics that should be considered in choosing type of antibiotics for local delivery systems which includes (Nandi et al., 2009):

1. Active against the most common bacterial pathogens in chronic osteomyelitis
2. Locally released concentrations exceeding several times(usually 10 times) the minimum inhibitory concentrations (MIC) for the concerned pathogen
3. Unable to enter in the systemic circulation
4. Not incite any adverse effects
5. Stable at body temperature and water soluble to ensure diffusion from the carrier
6. Not produce suprainfection

The most widely use agents in local delivery systems are aminoglycosides. Mousset et al., tested the antibacterial activity of 11 antibiotics in plaster of Paris after storage at 37^{o} C using microbiological method showed aminoglycoside remained fully stable with 100% activity after 2 weeks(Mousset et al., 1995).

2.2.3.1 Gentamicin

Gentamicin sulphate is aminoglycoside group antibiotic that is frequently used in the research for local antibiotic therapy in osteomyelitis as it is thermostable. Usual intravenous dose is 1.5 to 2 mg/kg loading dose, followed by 1 to 1.7 mg/kg IV or IM every 8 hours or 5 to 7 mg/kg IV every 24 hours. Duration of therapy: 14 days, depending on the site, nature, and severity of the bacteremia.

Gentamicin in combination with bone cements have been used in success in orthopaedics since 1970's (Engesæter et al., 2003). Gentamicin has proved to be the antibiotic of choice because of its wide spectrum antimicrobial activity in low minimal inhibitory and bactericidal concentration, excellent water solubility, thermal stability, low allerginicity and resistance to it is rare (Wahlig, Dingeldein, Bergmann, & Reuss, 1978). Gentamicin incorporated calcium phosphate beads provides slow residual release of antibiotic for a definite time period and biodegradability of the carrier beads avoids the need of second surgery for the their removal after therapy (Che Nor Zarida et al., 2011). Study by Klemm (2001), produced PMMA beads containing gentamicin which is 7.5mg per bead (Klemm, 2001). Each chain will consist of 10, 30 or 60 beads. By using the reference of 30 beads used in 70kg man, the total local antibiotic use is ((30 x 7.5)/ 70) 3.21mg/kg. In this study, total antibiotic given to each rabbit was approximately 3mg/kg.

2.3 ANIMAL MODEL

Numerous animal models exist for osteomyelitis research including rabbit, rat, mouse, avian, dog, sheep, and goat (Kankilic, Bilgic, Korkusuz, & Korkusuz, 2014; Patel, Rojavin, Jamali, Wasielewski, & Salgado, 2009). However, the search for the ideal model still persists as the model must have the ability to perform multiple procedures,