UNIVERSITI PUTRA MALAYSIA

INVESTIGATION OF TOBRAMYCIN INCORPORATED CALCIUM PHOSPHATE BEADS IN PREVENTING METHICILLIN-RESISTANT Staphylococcus aureus INDUCED OSTEOMYELITIS IN RABBITS

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By

LULU GODDAY ANEBOW

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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DEDICATION

Dedicated to God the Father, the Son and the Holy Spirit

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

INVESTIGATION OF TOBRAMYCIN INCORPORATED CALCIUM PHOSPHATE BEADS IN PREVENTING METHICILLIN-RESISTANT *Staphylococcus aureus* INDUCED OSTEOMYELITIS IN RABBITS

By

LULU GODDAY ANEBOW

August 2016

Chairman : Professor Fauziah Othman, PhD
Faculty : Medicine and Health Science

The persistence rise in bacteria resistant to antibiotics and reoccurrence of infections, has led to the involvement of new aminoglycosides class of antibiotics which serves as a promising alternative to overcome the escalating antibiotic resistance. The aim of this study is to investigate the efficacy of tobramycin beads in preventing osteomyelitis in rabbit model.

Tobramycin (30mg/mL) was incorporated into calcium phosphate (CaP) beads, by dipping method as a local drug delivery system. For juxtaposition, beads incorporated with calcium phosphate and tobramycin, and beads incorporated with calcium phosphate alone (without tobramycin, control) were prepared. A total of 20 New Zealand white rabbits were involved. Animals were divided into five groups as follows: group I were the sham (operated but without treatment), group II were inserted with tobramycin incorporated calcium phosphate bead without bacteria inoculums, group III were inoculated with bacteria only, group IV were inserted with calcium phosphate bead and bacteria inoculums, and group V were inserted with tobramycin incorporated calcium phosphate bead with bacteria inoculums. For the groups involving beads implantation, bacterial culture was inoculated immediately after surgery at the mid-shaft of tibia. After 28 days, they were euthanized and the presence or absence of osteomyelitis as well as the extent of the destruction of the bone architecture were investigated by radiology and histology. Microbiological analysis was carried out for the enumeration of bacterial load present in the bone and surrounding tissues. Blood samples were collected before surgery and weekly after surgery for confirmation of infection and inflammation. Hematological counts were analyzed by automated hematology analyzer, while erythrocyte sedimentation rate (ESR) by ESR fast detector. Blood smears were made and blood samples were analyzed through automated hematology analyzer for confirmation of infection. ESR fast detector which

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reads the rate of inflammation of one hour in 30 min was used to detect the rate of inflammation. Changes in body temperature and weight were also done to see its association with infection.

Bone samples were harvested from a transverse saw cut made in the middle of the tibia at the site of infection and the samples were subjected to scanning electron microscope (SEM) to confirm the presence of *S. aureus*. It was observed by SEM that once inoculation was introduced to the tibia bone, *S. aureus* at this site of infection started colonization, through a combination of cell division and recruitments, and undergoes changes in shapes and sizes, allowing them to become more resistant to antibiotics and antibodies. Presence of *S. aureus* colonies was observed within the site of infection and bacterial cells were distributed in numerous ways that permit them to infect new tissues.

Tobramycin incorporated with calcium phosphate bead group, potentially minimized bacterial growth in the tibia compared to control group, where there is potential bacterial growth and bone destruction, and there was significant difference between these groups. Chronic osteomyelitis with enhanced bacterial growth and marked changes in the bone architecture were noticed in the group infected with *S. aureus*. No bacterial growth and pathological changes were noticed in the group implanted with tobramycin-coated CaP without bacteria and the sham group. Hematological parameter showed the presence of infection as well as inflammation in groups III, IV and V respectively. No infection or inflammation was noticed in groups I and II. Hematological parameters especially ESR, is sensitive in detecting inflammation and infection and have good correlation with respond to treatment. Changes in temperature and body weight were not fully correlated with the rate of infection, in confirmation of their relationship to infection and inflammation.

In conclusion, SEM observations confirmed the presence of *S. aureus* in bone samples which further underscores that *S. aureus* is the major cause of wound suppuration and osteomyelitis formation. Tobramycin incorporated with CaP beads is found to be an effective prophylaxis in this model. CaP bead is an excellent biodegradable biomaterial, especially in local administration/delivery of antibiotics. It is effective in prevention of osteomyelitis in rabbit model and degrades after 28 days. White blood cell (WBC) count and ESR are promising markers of infection and inflammation associated with osteomyelitis formation.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

SIASATAN TERHADAP GABUNGAN ANTIBIOTIK TOBRAMYCIN DENGAN SILIKA KALSIUM FOSFAT DALAM MENCEGAH OSTEOMIELITIS AKIBAT JANGKITAN BAKTERIA METHICILLIN TAHAN *Staphylococcus aureus* DALAM ARNAB

Oleh

LULU GODDAY ANEBOW

Ogos 2016

Pengerusi : Profesor Fauziah Othman, PhD
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Kebangkitan rintangan bakteria terhadap antibiotik dan pengulangan jangkitan, telah membawa kepada penglibatan antibiotik kelas aminoglikosida yang berfungsi sebagai alternatif untuk mengatasi rintangan antibiotik yang kian meningkat. Tujuan kajian ini adalah untuk mengkaji keberkesanan gabungan antibiotik tobramycin dengan Silika kalsium fosfat *in vivo*, dalam model arnab osteomielitis.

Tobramycin (30 mg/mL) telah digabungkan ke dalam silika kalsium fosfat (CAP), dengan kaedah celupan sebagai sistem penghantaran ubat. Untuk saling bertindih, gabungan silika dengan kalsium fosfat dan tobramycin dan gabungan silika dengan kalsium fosfat sahaja (tanpa tobramycin, kawalan) telah disediakan. Sebanyak 20 arnab putih New Zealand telah digunakan dalam kajian ini. Kesemua arnab telah dibahagikan kepada lima kumpulan seperti berikut: Kumpulan pertama sebagai kawalan tanpa kajian. Kumpulan kedua disuntik gabungansilika kalsium fosfat tobramycin tanpa inokulum bakteria. Kumpulan ketiga telah disuntik dengan bakteria. Kumpulan keempat disuntik dengan silika kalsium fosfat dan inokulum bakteria, manakala kumpulan kelima disuntik dengan gabungan silika kalsium fosfat tobramycin dengan inokulum bakteria. Bagi kumpulan yang melibatkan penempelan silika, kultur bakteria telah disuntik selepas pembedahan di pertengahan tulang tibia. Selepas 28 hari, arnab telah dieutanasia dan diperhati kehadiran osteomielitis serta kemusnahan seni bina tulang telah disiasat melalui radiologi dan histologi. Analisis mikrobiologi telah dijalankan dalam penghitungan bakteria di dalam tulang dan tisu sekitarnya. Sampel darah telah dikumpul sebelum pembedahan dan setiap satu minggu selepas pembedahan untuk pengesahan kadar jangkitan dan pemendapan eritrosit (ESR). Calitan darah telah dijalankan dan sampel darah dianalisis melalui penganalisis hematologi automatik bagi mengesahkan jangkitan. Pengesan pantas

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pemendapan eritrosit yang merekod kadar keradangan satu jam dalam masa 30 minit telah digunakan untuk mengesan kadar keradangan. Demam telah dikenalpasti melalui pengukuran suhu badan, serta perubahan dalam berat badan yang berkaitan dengan jangkitan dan pembedahan telah dijalankan dengan mengukur berat badan arnab.

Sampel tulang dikumpul di pertengahan tibia dengan memotong secara melintang pada pertengahan tibia di lokasi jangkitan dan sampel diimbas melalui mikroskop elektron imbasan (SEM) untuk mengesahkan kehadiran bacteria *S. aureus*. Pemerhatian mikroskop elektron imbasan menunjukkan bahawa inokulasi *S. aureus* di tulang tibia memulakan kolonisasi jangkitan, melalui gabungan pembahagian sel dan pengambilan, serta mengalami perubahan dari segi bentuk dan saiz, yang membolehkan *S. aureus* untuk menjadi lebih tahan terhadap faktor-faktor luaran. Kehadiran koloni *S. aureus* diperhatikan padalokasi jangkitan dan sel-sel bakteria telah disebarkan melalui pelbagai cara yang membolehkannya untuk menjangkiti tisu baru.

Gabungan tobramycin dengan kumpulan silika kalsium fosfat, berpotensi menghalang pertumbuhan *S. aureus* dalam tibia berbanding dengan kumpulan kawalan, di mana terdapat potensi pertumbuhan bakteria dan kemusnahan tulang, dan terdapat perbezaan yang ketara di antara kumpulan-kumpulan ini. Osteomielitis kronik dengan pertumbuhan bakteria dan perubahan ketara dalam seni bina tulang telah diperhatikan dalam kumpulan yang dijangkiti *S. aureus*. Tiada pertumbuhan bakteria dan perubahan patologi diperhatikan dalam kumpulan yang ditempel dengan kalsium fosfat bersalut tobramycin tanpa bakteria dan kumpulan kawalan tanpa kajian. Parameter hematologi menunjukkan kehadiran jangkitan serta keradangan dalam kumpulan III, IV dan V. Tiada jangkitan atau keradangan telah disedari dalam kumpulan I dan II. Perubahan suhu dan berat badan tidak berkaitan sepenuhnya dengan kadar jangkitan.

Kesimpulannya, pemerhatian mikroskop elektron imbasan mengesahkan kehadiran *S. aureus* dalam sampel tulang yang membuktikan bahawa *S. aureus* adalah punca utama luka bernanah dan pembentukan osteomielitis. Gabungan tobramycin dengan silika kalsium fosfat didapati sebagai model pencegah yang berkesan dalam ujikaji ini. Silika kalsium fosfat merupakan bahan biomaterial biodegradasi yang baik, terutamanya dalam pemberian/penghantaran antibiotik. Penghitungan sel darah putih dan pemendapan eritrosit adalah penanda penting dalam jangkitan dan keradangan yang berkaitan dengan pembentukan osteomielitis.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

ACP	Amorphous calcium phosphate
ATP	Adenosine triphosphate
β -TCP	Beta-tricalcium Phosphate
Ca^{2+}	Calcium ion
CaP	Calcium Phosphate
CFU	Colony forming unit
CPD	Critical point drying
CRP	C-reactive protein
DCPD	Dicalcium phosphate dehydrate
DCP	Dicalcium phosphate
DDS	Drug delivery system
ESR	Erythrocyte sedimentation rate
HA	Hydroxyapatite
HT	Hematocrit
kGγ	Kilogray
LB	Luria Bertani
MCPM	Monocalcium phosphate monohydrate
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
MSA	Mannitol salt agar
nHA	Nano hydroxyapatite
NZW	New Zealand white
OD	Optical density

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PBS	Phosphate buffered saline
PMMA	Polymethylmethacrylate
PO_4^3	Orthophosphate
rpm	Revolution per minute
SEM	Scanning electron microscope
TCP	Tricalcium Phosphate
TTCP	Tetracalcium phosphate
WBC	White blood count

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CHAPTER 1

INTRODUCTION

Osteomyelitis in bone is associated with varieties of challenges depending on the infection's distinct features and the host. Over some period of time, tremendous progress in preventing this disease as the major factor that account for the recurrence and persistence of infection have been identified, and a variety of antibiotics with different spectrums of activity against osteomyelitis causing pathogens have been developed. Also, new operative techniques such as debridement and muscle flaps, and innovative delivery systems for antibiotics have been added to curb this problem (Ferguson *et al*., 2014). In spite of these advances, however, osteomyelitis remains difficult to be treated, with considerable morbidity and costs (Cosgrove *et al*., 2005; Engemann *et al*., 2003).

Previous studies have confirmed that the major cause of this infection is *Staphylococcus aureus* (Gordon and Lowy, 2008; Harris and Richards, 2006). It is the most common cause of this infection after trauma or surgery (Giannoudis *et al*., 2006), and also the most common organism isolated in bone infection that is associated with osteomyelitis *(*An *et al*., 2006). Bone infections are treated by parenteral administration of antibiotic, but this treatment does not provide high local bone concentration of antibiotic, due to the poor vascularization of the cortical bone and low penetration of antibiotics at this local site of infection (Dombrowski and Winston, 2008). Moreover, antibiotic can lead to system side effect and poor venous tolerance due to long term parenteral antibiotic administration (Gentry and Rodriguez, 1990) and in some cases, bead incorporated with antibiotics are often used, example, polymerized polymethacrylate (PMMA) beads and series of failures of these beads as a non-biodegradable material has been identified, and the non-biodegradable nature of these beads, often result in the secondary removal of the implants (Anagnostakos *et al*., 2006; Hsu *et al*., 2014).

Since the delivery of antibiotics at a sufficiently higher concentration by the intravenous route to the target site is difficult and recurrence of osteomyelitis is high, drug delivery system (DDS) is currently under study and has shown to be a promising alternative for osteomyelitis treatment or prevention. The merit of drug delivery system (DDS) lies on the provision of high concentrations of antibiotics in a limited blood circulation area and equally on the infected bone. In this present study, it involves the incorporation of calcium phosphate with tobramycin in order to investigate the effect of tobramycin incorporated calcium phosphate in the prevention of osteomyelitis. This study is serving as the first study of this nature in Malaysia.

Significance of the study

High local levels of antibiotics are achieved through temporary implantation of non-biodegradable beads, despite their antibiotic release, these beads act as a biomaterial surface to which bacteria preferentially adhere, grow and potentially develop antibiotic resistance and recurrence of osteomyelitis, therefore it is important to develop biodegradable high local antibiotic-loaded bead as an antibiotic delivery system and bead without antibiotics for comparison.

Main Objective

To understand *in vivo* activities and efficacy of tobramycin-coated calcium phosphate as a local antibiotics in prevention of osteomyelitis.

Specific Objectives

1) To determine the histological changes in the pathological and normal tissue environment.
2) To determine the radiological changes in the associated tissue.
3) To determine the microbiological status in relation to treatment.
4) To qualify the relationship of physiological and physical parameters to infection.

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BIODATA OF STUDENT

Lulu Godday Anebow was born in Apani, Rivers state, Nigeria. His Primary Education was in State School 1 Apani, where he obtained his first School Leaving Certificate in 1992. He proceeded to Secondary Education at Government Army Secondary School Elele, after his First Entrance Examination in 1992. After his Secondary Education ended in 1998, he furthered his education in College of Arts and Science, Rumuola, Port-Harcourt (Now Port Harcourt Polytechnics), in 2000. After his completion of the programme, he pursued his undergraduate study at University of Port-Harcourt and successfully graduated with a Bachelor of Science degree in Human Anatomy in 2006. After graduation, he later joined the National Youth Service Corps in 2008, where he obtained his NYSC certificate.

His enthusiasm towards foreign experience and to impact to the world at large after successfully served his nation, leads him to further think about studying abroad and he was grateful to develop research interest in developing a New Drug Delivery System (DDS) for incorporating new aminoglycoside antibiotics to calcium phosphate, in combating osteomyelitis. He is well-experienced and knowledgeable in vast aspect of research, including animal research and handlings, pre and post operative care, animal surgery, scanning electron microscope, microbiological and hard tissue processing, aside his vast knowledge in Human Anatomy. His future desire is to always be a successful research scientist in Medical and Health Sciences.

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