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TOBRAMYCIN AND GENTAMICIN-INCORPORATED CALCIUM PHOSPHATE BEADS AS DELIVERY SYSTEM IN PREVENTION OF Staphylococcus aureus BIOFILM FORMATION

CHE NOR ZARIDA CHE SEMAN

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By

CHE NOR ZARIDA CHE SEMAN

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TOBRAMYCIN AND GENTAMICIN-INCORPORATED CALCIUM PHOSPHATE BEADS AS DELIVERY SYSTEM IN PREVENTION OF *Staphylococcus aureus* BIOFILM FORMATION

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Chairman : Professor Fauziah Othman, PhD

Faculty : Faculty of Medicine and Health Sciences

Staphylococcus aureus (S. aureus) is the most common organism causing osteomyelitis which is associated with biofilm formation. This infection is difficult to treat, usually requires prolong administration of antibiotics and extensive surgical procedure. In order to eradicate biofilm formation, biomaterial incorporated with suitable antibiotics can be used as a preventative measure. Therefore, this study was conducted to assess the antibacterial properties of tobramycin and gentamicin-incorporated calcium phosphate beads in prevention of *S. aureus* biofilm formation.

In this present study, the live event for development of *S. aureus* biofilm was viewed under live cell imaging system and the morphology of biofilm was viewed under scanning electron microscopy (SEM). These microscopic studies showed that the biofilm formation involved initial attachment to a solid surface, the formation of microcolonies,

and finally differentiation of microcolonies into exopolysaccharide-encased as matured biofilm.

The 3(4, 5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to assess the efficacy of tobramycin and gentamicin-incorporated calcium phosphate beads against *S. aureus* biofilm. There was a significant difference between tobramycin-incorporated calcium phosphate beads and gentamicin-incorporated calcium phosphate beads on cell viability of *S. aureus* biofilm ($p<0.05$). Gentamicin-incorporated calcium phosphate beads showed the strongest action to inhibit *S. aureus* with IC_{50} value of 0.05 mg/ml compared to tobramycin-incorporated calcium phosphate beads with IC_{50} of 0.12 mg/ml. Fluorescence staining with acridine orange and propidium iodide (AOPI) showed that the live bacterial cells appeared green and dead cells appeared red-orange.

Ninhydrin assay was used to investigate the elution of tobramycin and gentamicin from the calcium phosphate carrier. The standard graphs for tobramycin and gentamicin solution versus absorbance reading were prepared as references to identify the concentration of the antibiotics release after incorporating calcium phosphate beads at the time points of 0.5 hour, 1 hour, 2 hours, 4 hours, 6 hours, 24 hours, 48 hours, 72 hours, 168 hours (1 week), 336 hours (2 weeks), 672 hours (4 weeks) and 1344 hours (8 weeks). The release of tobramycin from calcium phosphate beads was significantly difference with gentamicin ($F_{group} = 175.54 > F_{0.05,\ 1,\ 2} = 18.51$). The release of gentamicin from calcium phosphate beads was higher than tobramycin. Futhermore, cumulative release of tobramycin and gentamicin from calcium phosphate beads showed that there was a

significant difference in the mean concentration of drug release at 1344 hours (8 weeks) between tobramycin-incorporated calcium phosphate beads and gentamicin-incorporated calcium phosphate beads ($p<0.001$). The mean cumulative release of gentamicin from calcium phosphate beads (249.3 (2.4) µg/ml) was higher than tobramycin (178.7 (4.1)) at 1344 hours.

The cytotoxicity test using MTT assay showed that osteoblast demonstrated good cell viability at the highest concentration of either 25 mg/ml tobramycin or 25 mg/ml gentamicin. There was a significant difference between tobramycin-incorporated calcium phosphate beads and gentamicin-incorporated calcium phosphate beads towards cell viability ($p<0.05$). Tobramycin-incorporated calcium phosphate beads was more cytotoxic on osteoblast than gentamicin-incorporated calcium phosphate beads. Moreover, investigation on the cell morphology and cell adherence by using SEM and CLSM showed that seeded cells were well attached to the tobramycin and gentamicin-incorporated calcium phosphate beads and continue to grow throughout the 5 days period.

In conclusion, tobramycin and gentamicin-incorporated calcium phosphate beads have the potential to be used as a new local drug delivery system in the prevention and treatment of bone infections. Furthermore, tobramycin and gentamicin-incorporated calcium phosphate beads scaffold could serve as a promising platform for the regeneration of osteoid tissues because of their slow release of antibiotic, biocompatibility and biodegradability.

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

SISTEM PENGHANTARAN TOBRAMISIN DAN GENTAMISIN – TERGABUNG KALSIUM FOSFAT DALAM PENCEGAHAN PEMBENTUKAN BIOFILEM

Oleh

CHE NOR ZARIDA CHE SEMAN

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Pengerusi : Profesor Fauziah Othman, PhD

Fakulti : Fakulti Perubatan dan Sains Kesihatan

Staphylococcus aureus (S. aureus) adalah organisma yang paling biasa menyebabkan osteomielitis di mana ia dikaitkan dengan pembentukan biofilem. Jangkitan ini adalah sukar untuk dirawat, biasanya memerlukan rawatan antibiotik berpanjangan dan prosedur pembedahan yang ekstensif. Dalam usaha untuk membasmi pembentukan biofilem, biobahan digabungkan dengan antibiotik yang sesuai boleh digunakan sebagai langkah pencegahan. Kajian ini telah dijalankan untuk menilai ciri-ciri antibakteria manik tobramycin dan gentamicin-tergabung kalsium fosfat dalam pencegahan pembentukan *S. aureus* biofilem.

Dalam kajian ini, pembentukan *S. aureus* biofilem secara lansung telah dilihat di bawah sistem pengimejan sel hidup dan morfologi biofilem telah dilihat di bawah

mikroskop elektron imbasan (SEM). Kajian-kajian mikroskopik menunjukkan pembentukan biofilem melibatkan pelekatan awal pada permukaan, pembentukan mikrokoloni dan pembezaan mikrokoloni kepada eksopolisakarida sebagai biofilem yang matang.

Asai 3(4, 5-dimethylthiazole-2-YL)-2,5-diphenyl tetrazolium bromide (MTT) telah digunakan untuk menguji keberkesanan manik tobramisin dan gentamisin-tergabung kalsium fosfat terhadap biofilem *S. aureus*. Manik tobramisin-tergabung kalsium fosfat adalah berbeza secara signifikan berbanding manik gentamisin-tergabung kalsium fosfat dari segi kemandirian biofilem *S. aureus* ($p<0.05$). Manik gentamisin-tergabung kalsium fosfat menunjukkan tindakan yang lebih kuat untuk menghalang biofilem *S. aureus* dengan nilai IC_{50} 0.05 mg/ml berbanding manik tobramisin-tergabung kalsium fosfat dengan nilai IC_{50} 0.12 mg/ml. Pewarnaan floresen dengan akridina jingga dan propidium iodida (AOPI) menunjukkan bahawa sel-sel bakteria yang hidup kelihatan hijau dan sel-sel mati kelihatan merah oren.

Asai Ninhidrin telah digunakan untuk mengesan elusi tobramisin dan gentamisin daripada manik kalsium fosfat. Graf piawai untuk larutan tobramisin dan gentamisin melawan bacaan ketumpatan optik (OD) telah disediakan sebagai rujukan untuk mengenal pasti kepekatan antibiotik yang dielusikan melalui kalsium fosfat mengikut titik masa yang telah ditentukan iaitu 0.5 jam, 1 jam, 2 jam, 4 jam, 6 jam, 24 jam, 48 jam, 72 jam, 168 jam (1 minggu), 336 jam (2 minggu), 672 jam (4 minggu) and 1344 jam (8 minggu). Pelepasan tobramisin daripada manik kalsium fosfat adalah berbeza secara

signifikan dengan pelepasan gentamisin (F_{group} = 175.54 > $F_{0.05,\ 1,\ 2}$ = 18.51). Gentamisin yang dilepaskan dari manik kalsium fosfat adalah lebih tinggi berbanding tobramisin. Selain itu, pelepasan kumulatif tobramisin dan gentamicin daripada manik kalsium fosfat pada 1344 jam (8 minggu) adalah berbeza secara signifikan (p <0.001). Pelepasan kumulatif min gentamisin daripada manik kalsium fosfat (249,3 (2.4) μg / ml) adalah lebih tinggi daripada tobramycin (178,7 (4,1)) pada 1344 jam.

Ujian bioserasi menggunakan asei MTT menunjukkan kemandirian osteoblast yang bagus pada kepekatan yang tinggi sama ada 25 mg/ml tobramisin atau 25 mg/ml gentamisin. Kemandirian sel selepas dirawat dengan manik tobramisin-tergabung kalsium fosfat adalah berbeza secara signifikan dengan manik gentamisin-tergabung kalsium fosfat (p<0.05). Manik tobramycin-tergabung kalsium fosfat adalah lebih sitotoksik terhadap osteoblast berbanding manik gentamicin-tergabung kalsium fosfat. Selanjutnya, kajian tentang morfologi dan pelekatan sel pada permukaan manik tobramisin dan gentamicin-tergabung kalsium fosfat dengan menggunakan SEM dan CLSM menunjukkan bahawa sel yang dikultur adalah melekat dan membiak pada permukaan bahan tersebut dalam tempoh 5 hari pengeraman.

Kesimpulannya, manik tobramisin dan gentamisin-tergabung kalsium fosfat mempunyai potensi sebagai sistem penghantaran antibiotik yang baru untuk mencegah dan merawat jangkitan tulang. Selain itu, manik tobramisin dan gentamisin-tergabung kalsium fosfat

boleh menjanjikan platform untuk regenerasi tisu osteoid kerana pembebasan antibiotik secara perlahan daripada kalsium fosfat, bioserasi dan biodegradasi.

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I certify that a Thesis Examination Committee has met on 15th June 2012 to conduct the final examination of Che Nor Zarida Che Seman on her thesis entitled "Tobramycin and Gentamicin-incorporated Calcium Phosphate Beads As Delivery System in Prevention of *Staphylococcus aureus* Biofilm Formation" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Asmah binti Rahmat, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Saleha binti Abdul Aziz, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Rokiah binti Mohd Yusof, PhD
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Internal Examiner)

Nurina binti Anuar, PhD
Associate Professor
Faculty of Engineering and Built Environment
Universiti Kebangsaan Malaysia
(External Examiner)

SEOW HENG FONG, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 27 September 2012

This thesis was submitted to the Senate of 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:

Fauziah Othman, PhD
Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairperson)

Arifah Abdul Kadir, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Azfar Rizal Ahmad, MBBS
Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies

Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

CHE NOR ZARIDA CHE SEMAN

Date: 15 June 2012

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LIST OF ABBREVIATIONS

ADAHF-PRC	American Dental Association Health Foundation Paffenbarger Research Center
AO	Acridine orange
AOPI	Acridine orange-propidium iodide
ATP	Adenosine triphosphate
BSE	back-scattered electrons
β-TCP	Beta-tricalcium phosphate
Ca^{2+}	Calcium ion
CaP	Calcium phosphate
CFU	Colony forming unit
CHA	Carbonated hydroxyapatite
CLSM	Confocal laser scanning microscope
CNS	Central nervous system
CPD	Critical point drying
DCPD	Dicalcium phosphate dehydrate
DDS	Drug delivery system
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMIT	Enzyme-immunoassay
EPS	Extracellular polymeric substances
GFP	Green fluorescent protein

HA	Hydroxyapatite
hFOB 1.19	Human fetal osteoblastic cell line
HMDS	Hexamethyldisilazane
IC_{50}	Inhibitory concentration of 50
IIUM	International Islamic University Malaysia
LB	Luria Bertani
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimal inhibitory concentration
MNA	Malaysian Nuclear Agency
MTT	3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazoliumbromide
NCCLS	National Committee for Clinical Laboratory Standards
OD	Optical density
PBS	Phosphate-buffered saline
PFIA	Polarization fluorescence immunoassay
PI	Propidium iodide
PMMA	Polymethylmethacrylate
PO_4^{3}	Orthophosphate
$P_2O_7^{4-}$	Metaphosphate or pyrophosphate
rpm	Revolution per minute
SEM	Scanning electron microscope
SPSS	Statistical Package for the Social Sciences
TCP	Tricalcium phosphate

TDX	Fluorescence-immunoassay
TSA	Trypticase soy agar
UiTM	Universiti Teknologi MARA
UMK	Universiti Malaysia Kelantan
UPM	Universiti Putra Malaysia
UV	Ultraviolet
VIS	Visible

CHAPTER 1

INTRODUCTION

A biofilm is a thick community of bacteria that is attached to a substratum, interface or to each other and embedded in a matrix of extracellular polymeric substances (EPS). In the biofilm, bacteria are protected against environmental stresses, antimicrobial treatment, and the host immune system (Cortes *et al.*, 2011). Biofilms have been implicated in a variety of human infections, such as endocarditis, osteomyelitis, chronic otitis media, foreign-body-associated infections, gastrointestinal ulcers, urinary tract infections, chronic lung infections in cystic fibrosis patients, caries, and periodontitis (Costerton *et al.*, 1999). Biofilm-associated infections involved the different Gram-positive species of *Staphylococcus*, *Streptococcus* and *Enterococcus* as well as Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli* and *Actinobacillus actinomycetemcomitans* (Heilman and Got, 2010).

Staphylococcus aureus is the most common organism isolated in bone infection (osteomyelitis). Methicillin-resistant *S. aureus* (MRSA) infections are particularly difficult to treat. Antibiotic treatment of osteomyelitis is traditionally administered intravenously. However, oral regimens for osteomyelitis have been successfully tested in human trials but the choice of oral antimicrobials is restricted when dealing with multidrug-resistant organisms, and this may require the use of parenteral drugs (Yin *et al*., 2005). Long-term parenteral antibiotics with multiple surgical debridements are often required for effective therapy (Lazzarini *et al*., 2004) but, repeated failures of these

therapies often result in the removal of the orthopaedic implants which is costly and traumatic to the patient.

In orthopaedics, parenteral administration of antibiotics does not provide good local bone response due to poor vascularisation of cortical bone and low drug penetration. As an alternative, antibiotic-incorporated bone cement beads are specifically designed and directly implanted to the infected bone to combat infection localized in bone and bone tissue (Le Ray *et al*., 2005). Antibiotic-incorporated bone cement beads provide high local concentration of antimicrobial agents in a limited blood circulation area and also in the infected dead space but the total dose of antibiotic applied locally are normally not sufficient to produce systemic effects. Thus, using a delivery system or carrier such as beads, a high local concentration of antibiotic can be attained and release slowly without exposing the patient to systemic toxic antibiotic levels, which could result in toxic site effect. Furthermore, due to the slow penetration, antibiotics that absorb into the biofilm matrix could have retarded bacteria penetration (Shigeta *et al*., 1997). Hence, the *in situ* drug delivery system using calcium phosphate is tested for the slow-release of antibiotics.

Tobramycin and gentamicin are used most frequently by surgeons for incorporation into bone cement in Europe and United States (Kanellakopoulou and Giamarellos-Bourboulis, 2000; Walenkamp, 1997). This choice of antibiotics arose from the early work incorporating antibiotic in bone cement for the prophylaxis infection in total hip arthroplasty in 1970 (Nandi *et al*., 2009b). Gentamicin remains the most effective antibiotic to incorporate with bone cement due to its high solubility, heat stability and

bactericidal activity at low concentration (Campoccia *et al*., 2010; Faber *et al*., 2005). This drug was selected in this study because it is widely used for the treatment of osteomyelitis. Meanwhile, tobramycin is closely related to gentamicin with a similar spectrum of activity against both Gram-positive and Gram-negative bacteria (Randelli *et al*., 2010), but less ototoxic and nephrotoxic than gentamicin (Scott *et al*., 1999) and its elution characteristics has been shown to elute at higher concentrations than gentamicin in bone cements (Sterling *et al*., 2003; Malchau *et al*., 2000). Furthermore, tobramycin is usually substituted for gentamicin, because it is available as a pharmaceutical-grade powder, whereas gentamicin is not (Wininger and Fass, 1996).

Calcium phosphate is excellent material for bone repair and regeneration because of its chemical compositions is similar to the mineral phase of natural bone (Gazdag *et al*., 1995). In fact, calcium phosphate material serve as a mineral reservoir for calcium and phosphorus as does the role of a true bone. It is containing both calcium and phosphorus ions that help to enrich the surrounding microenvironment by degradation process which stimulates osteogenesis (LeGeros, 1993). Calcium phosphate has been given considerable attention in clinical usage and reported to have bioactivity, biocompatibility, osteointegrative, osteoconductive and osteophilic nature properties (Nandi *et al*., 2009a; Mickiewicz, 2001; Moore *et al*., 2001).

Generally, orthopaedic surgeons in Malaysia use gentamycin-incorporated polymethylmethacrylate (PMMA) chain or *Septopal* in the treatment of bone and soft-tissue infections. Although the use of gentamicin-incorporated PMMA beads is

acceptable in clinical practice, but PMMA beads are preferable to microorganism adherence and growth on the biomaterial surface, despite, the release of antibiotics and it also has potential to develop antibiotic resistance (Neut *et al*., 2001). Besides that, a secondary surgery may be required to remove the cement because it is not biodegradable and may become a nidus for future infections (Liu *et al*., 2007a). Therefore, in this study, tobramycin and gentamicin-incorporated calcium phosphate beads have been designed to be used as a new local drug delivery system in prevention of *S. aureus* biofilm formation as well as a bone replacement or implant. The bacteria were chosen since it was the most common organism that caused infection in orthopaedic practice (Calhoun and Mader, 1989).

1.1 Objectives of the research

General objective:

The general objective of this study is to assess the antibacterial properties of tobramycin and gentamicin-incorporated calcium phosphate beads as delivery system in prevention of *S. aureus* biofilm formation.

Specific objectives:

- To evaluate the development of *S. aureus* biofilm on glass slide and catheter.
- To assess the efficacy of the tobramycin and gentamicin-incorporated calcium phosphate beads against common etiological pathogen of osteomyelitis namely *S. aureus.*
- To investigate the elution of tobramycin and gentamicin from the calcium phosphate carrier (*in vitro*).
- To determine the cytotoxicity effect of tobramycin and gentamicin-incorporated calcium phosphate beads on bone tissue (*in vitro*).

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