UNIVERSITI PUTRA MALAYSIA

GENTAMICIN-COATED HYDROXYAPATITE IN PREVENTION OF BIOFILM FORMATION IN BONE TISSUE

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By

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GENTAMICIN-COATED HYDROXYAPATITE IN PREVENTION OF BIOFILM FORMATION IN BONE TISSUE

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Chairman : Professor Fauziah Othman, PhD

Faculty : Faculty of Medicine and Health Sciences

Biofilm is a multilayered complex microorganism, which attaches on any surface and is typically more resistant to the host immune response and routine antibiotic therapy. In order to limit biofilm formation, biomaterials loaded with suitable antibiotics can be used as a preventative measure. The biomaterial hydroxyapatite (HA) is an osteoconductive space filler and is produced locally by Malaysia Nuclear Agency. In this study, HA coated with the antibiotic gentamicin was explored whether it can reduce or remove biofilm formation. To assess IC_{50} values of gentamicin-coated HA, 10^8 CFU/ml of *Staphylococcus aureus* (ATCC 12600) and *Pseudomonas aeruginosa* were cultured for 48 hours in a 96-well plate for biofilm formation. MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-

diphenyltetrazoliumbromide) assays were performed to determine the effect of various doses of gentamicin (0 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml, 0.1 mg/ml and 10 mg/ml) coated on a constant number of HA particles on viability of *S. aureus* and *P. aeruginosa* biofilm. It was demonstrated that the IC_{50} values of gentamicin-coated HA were 0.1 mg/ml for *S. aureus* and 5 mg/ml for *P. aeruginosa* biofilm. Fluorescence staining with acridine orange and propidium iodide (AOPI) was also conducted to visualize viability of the biofilm. Accordingly, the doses of 0.1 mg/ml and 5 mg/ml for *S. aureus* and *P. aeruginosa* biofilm respectively decreased cell viability, as cells showed higher PI staining. In an attempt to determine the possible cytotoxic effects of gentamicin-coated HA on human cells, the human osteoblast cell line (NHOst, Lonza) was co-cultured with the doses of gentamicin (0 mg/ml, 0.1 mg/ml, 1 mg/ml and 10 mg/ml) coated on HA particles as tested above for biofilm cytotoxicity. Cell viability of osteblasts decreased with increasing doses of gentamicin when assessed at 72 hours using MTT assay (for example, 10 mg/ml gentamicin-coated HA reduced osteoblast cell viability to 75%). The efficacy of gentamicin-coated HA was also tested *in vivo*. A Teflon catheter was used to create catheter-associated biofilm segments for *in vivo* implantation. Catheter-associated biofilm were examined with scanning electron microscope (SEM) to confirm *S. aureus* biofilm formation. The catheter-associated biofilm was then implanted subcutaneously into the right flank of *Sprague Dawley* rats. Rats were sacrificed after 7 days post-implantation and the catheters were removed and assessed for bacteria count. This study showed that the gentamicin-coated HA significantly reduced *S. aureus* bacteria

count from 14.12 ± 1.09 $\log_{10}$ CFU/ml to 4.61 ± 0.49 $\log_{10}$ CFU/ml ($p \leq 0.05$). To investigate the structure of biofilm formation *in vivo* post-implantation, tissues immediately surrounding the implanted catheter was histologically assessed using haematoxylin and eosin (H&E) staining. The result obtained from H&E staining showed no inflammatory cells or tissue damage was observed. Thus, this study showed that gentamicin-coated HA is effective in reducing biofilm viability without causing overt toxicity to human osteoblasts *in vitro* or inflammation when implanted in skin.

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

KAJIAN HIDROKSIAPATIT BERSALUT GENTAMISIN DALAM PENCEGAHAN PEMBENTUKAN BIOFILM PADA TISU TULANG

Oleh

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Biofilm adalah mikroorganisma kompleks pelbagai lapisan yang melekat pada apa jua permukaan. Biofilm secara lazim mempunyai kerentangan terhadap respon imuniti perumah dan juga rawatan antibiotik rutin. Biobahan yang mengandungi antibiotik boleh digunakan dalam pencegahan pembentukan biofilm. Hidroksiapatit (HA) merupakan biobahan bersifat osteokondusif yang dihasilkan oleh Nuklear Agensi Malaysia. Dalam kajian ini, HA yang disalut dengan antibiotik gentamisin diselidik untuk menentukan samaada dapat mengurang atau mencegah pembentukan biofilm. Untuk memperoleh nilai IC_{50} HA bersalut gentamisin, 10^8 CFU/ml *Staphylococcus aureus* (ATCC 12600) dan *Pseudomonas aeruginosa* telah dikulturkan selama 48 jam untuk membentuk biofilm. Asai MTT telah dijalankan ke atas biofilm *S. aureus* dan *P. aeruginosa*

untuk menentukan keberkesanan gentamisin pada kepekatan yang berbeza (0 mg/ml, 0.02 mg/ml, 0.04 mg/ml, 0.06 mg/ml, 0.08 mg/ml, 0.1 mg/ml dan 10 mg/ml) yang disalut pada partikel HA yang tertentu. Hasil kajian ini mendapati bahawa IC_{50} HA bersalut gentamisin bagi biofilm *S. aureus* dan *P. aeruginosa* adalah masing-masing 0.1 mg/ml dan 5 mg/ml. Pewarnaan pendarfluor dengan akridina oren dan propidium iodida (AOPI) telah dijalankan untuk menilai viabiliti biofilm. Keputusan menunjukkan bahawa kepekatan 0.1 mg/ml dan 5 mg/ml gentamisin pada biofilm *S. aureus* dan *P. aeruginosa* masing-masing boleh mengurangkan kadar viabiliti biofilm. Sel osteoblas manusia (NHOst, Lonza) turut dikultur bersama HA bersalut gentamisin (0 mg/ml, 0.1 mg/ml, 1 mg/ml dan 10 mg/ml) untuk menentukan kesan ketoksikan HA bersalut gentamisin pada sel manusia. Viabiliti sel osteoblas yang telah dirawat dinilai selepas 72 jam dengan asai MTT. Viabiliti osteoblas berkurangan dengan peningkatan kepekatan HA bersalut gentamisin. Contohnya, 10 mg/ml HA bersalut gentamisin mengurangkan viabiliti sel sehingga 75%. Keberkesanan HA bersalut gentamisin juga diuji *in vivo*. Teflon kateter digunakan untuk membentuk kateter berkaitan biofilm bagi tujuan implantasi *in vivo*. Sampel kateter berkaitan biofilm telah diperiksa dengan mikroskop elektron imbasan (SEM) untuk mengesahkan pembentukan biofilm *S. aureus*. Kateter berkaitan biofilm yang seterusnya diimplan subkutaneus pada rusuk kanan tikus *Sprague Dawley*. Tikus-tikus ini dikorbankan selepas 7 hari pengimplanan dan kateter diuji untuk menentukan jumlah bilangan bakteria. Kajian ini menunjukkan bahawa HA bersalut gentamisin dapat mengurangkan bilangan bakteria *S. aureus* daripada 14.12 ±

1.09 $\log_{10}$ CFU/ml ke 4.61 ± 0.49 $\log_{10}$ CFU/ml ($p \leq 0.05$). Untuk mengkaji struktur pembentukan biofilm selepas pengimplanan *in vivo*, tisu-tisu disekitar kateter diambil dan penilaian histologi dijalankan dengan pewarnaan hematoksilin dan eosin (H&E). Hasil pewarnaan mendapati tiada sel-sel inflamasi atau kerosakan tisu yang berlaku. Oleh yang demikian, kajian ini menunjukkan bahawa HA bersalut gentamisin berkesan dalam mengurangkan viabiliti biofilm tanpa menyebabkan ketoksikan kepada osteoblas manusia secara *in vitro* atau inflamasi apabila diimplan dalam kulit.

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Serdang, June 2010

Au Lee Fong

I certify that a Thesis Examination Committee has met on 31 March 2011 to conduct the final examination of Au Lee Fong on her Master of Science thesis entitled 'Gentamicin-Coated Hydroxyapatite in Prevention of Biofilm Formation in Bone Tissue' 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 degree of Master of Science.

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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.

_____

AU LEE FONG

Date: 31 March 2011

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