UNIVERSITI PUTRA MALAYSIA

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

AU LEE FONG

FPSK(m) 2011 35
GENTAMICIN-COATED HYDROXYAPATITE IN PREVENTION OF BIOFILM FORMATION IN BONE TISSUE

By

AU LEE FONG

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

March 2011
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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

By

AU LEE FONG

March 2011

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 osteoblasts 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 $14.12 \pm 1.09 \log_{10} \text{CFU/ml}$ to $4.61 \pm 0.49 \log_{10} \text{CFU/ml}$ ($p \leq 0.05$). To investigate the structure of biofilm formation \textit{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 \textit{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

AU LEE FONG

Mac 2011

Pengerusi : Profesor Fauziah Othman, PhD
Fakulti : Fakulti Perubatan dan Sains Kesihatan

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\textsubscript{50} HA bersalut gentamisin, \textstyle 10^8\text{ 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<sub>50</sub> HA bersalut gentamisin bagi biofilm <i>S. aureus</i> dan <i>P. aeruginosa</i> 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 <i>S. aureus</i> dan <i>P. aeruginosa</i> 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 berkurangkan 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 <i>S. aureus</i>. Kateter berkaitan biofilm yang seterusnya diimplan subkutaneus pada rusuk kanan tikus Sprague Dawley. Tikus-tikus ini dikorbankan selepas 7 hari pengimplan dan kateter diuji untuk menentukan jumlah bilangan bakteria. Kajian ini menunjukkan bahawa HA bersalut gentamisin dapat mengurangkan bilangan bakteria <i>S. aureus</i> daripada 14.12 ±
1.09 \log_{10} CFU/ml ke 4.61 \pm 0.49 \log_{10} CFU/ml (p\leq0.05). Untuk mengkaji struktur pembentukan biofilm selepas pengimplanan \textit{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 \textit{in vitro} atau inflamasi apabila diimplan dalam kulit.
ACKNOWLEDGEMENTS

First, I would like to express my heartfelt gratitude to my honorable project supervisor, Professor Dr. Fauziah Othman, the Chairman of my Supervisory Committee, for her invaluable advice, guidance, constant support and encouragement.

I would also like to extend my appreciation to my co-supervisor, Dr. Sharmili Vidyadaran, Department of Pathology, Faculty of Medicine and Health Sciences, for her generous input, constructive criticism, advice and support throughout the course of this study.

I am grateful to all the staff of Institute Medical Molecular Biotechnology (IMMB), Universiti Teknologi MARA (UiTM) and Institute of Bioscience (IBS) for their kindness in giving me the opportunity to the access of the laboratory facilities.

In addition, I would like to thank all the staff of media preparation laboratory, microbiology laboratory, cell signalling laboratory, multipurpose laboratory 3 and the animal house facility of Faculty of Medicine and Health Sciences for their kind, excellent and continuous technical assistance.
Subsequently, I would like to extend my thanks and appreciation to Dr. Idris Besar and Puan Rusnah Mustaffa, Malaysian Nuclear Agency, for providing the biomaterials (Hydroxyapatite, HA) for this research project.

I would also like to record my utmost thanks and appreciation to Dr. Fuzina Nor Hussein, Department of Veterinary Pathology & Microbiology, UPM for her invaluable guidance and technical help in this research project.

I would like to extend gratitude to my colleagues and friends for their advices and opinions. Last but not least, I would like to acknowledge and honour my family for their sacrifices, moral support and constant love all this time.

Thank you to all who had contributed to the success of this project.

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.

Members of the Examination Committee were as follows:

**Chairman, Ph.D.**
Professor Dr. Asmah Rahmat,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia
(Chairman)

**Examiner 1, Ph.D.**
Professor Dr. Saleha Abdul Aziz,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia
(Internal Examiner)

**Examiner 2, Ph.D.**
Associate Professor Dr. Rokiah Mohd Yusof,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia
(Internal Examiner)

**External Examiner, Ph.D.**
Associate Professor Dr. Noraziah Mohamad Zain,
Faculty of Health Sciences,
Universiti Kebangsaan Malaysia.
(External Examiner)

_____________________________________
BUJANG KIM HUAT, Ph.D.
Professor and Deputy Dean,
School of Graduate Studies,
Universiti Putra Malaysia.

Date:
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment 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)

Sharmili Vidyadaran, PhD  
Lecturer  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

_______________________  
HASANAH MOHD GHAZALI, 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.

______________________
AU LEE FONG
Date: 31 March 2011
# TABLE OF CONTENTS

| ABSTRACT                                      | ii  |
| ABSTRAK                                       | v   |
| ACKNOWLEDGEMENTS                              | viii|
| APPROVAL                                      | x   |
| DECLARATION                                   | xi  |
| LIST OF TABLES                                | xvi |
| LIST OF FIGURES                               | xvii|
| LIST OF PLATES                                | xix |
| LIST OF ABBREVIATIONS                         | xx  |

## CHAPTER

1. **INTRODUCTION**
   1

2. **LITERATURE REVIEW**
   2.1 Tissue Engineering
   2.1.1 Biomaterial
   2.1.2 Hydroxyapatite (HA)
   2.1.3 BoniPor
   2.2 Biofilm
   2.2.1 *Staphylococcus aureus*
   2.2.2 *Pseudomonas aeruginosa*
   2.3 Antibiotic
   2.3.1 Gentamicin
   2.3.2 Drug Delivery System
   2.4 MTT Assay
   2.5 Safranin Staining
   2.6 Microscopy Study
   2.6.1 Scanning Electron Microscope
   2.6.2 Fluorescence Microscope
   2.7 Haematoxylin and Eosin (H&E) Staining

---

xiii
3 MATERIALS AND METHODS
3.1 Experimental Design 29
3.2 Cell Culture Study
  3.2.1 Hydroxyapatite (HA) 34
  3.2.2 Human Osteoblast Cell Line 34
  3.2.3 MTT Assay 35
  3.2.4 Scoring Method of HA Porosity 36
3.3 Bacterial Strain 38
  3.3.1 Microtiter Plate Assay 39
  3.3.2 Catheter-associated Biofilm 40
3.4 Microscopy Study
  3.4.1 Fluorescence Microscope 44
  3.4.2 Scanning Electron Microscope (SEM) 45
3.5 Antibiotic Susceptibility Test
  3.5.1 Minimal Inhibitory Concentration (MIC) assay 47
  3.5.2 Antibiotic Disk Diffusion Technique (Kirby-Bauer Disk-Diffusion) 48
3.6 Animal Study
  3.6.1 Animal Husbandry 49
  3.6.2 Animal Grouping 50
  3.6.3 Experimental Model of Infection (In Vivo) 50
  3.6.4 Sample Collection 53
3.7 Light Microscopy 53
  3.7.1 Histological Scoring 54
3.8 Statistical Analysis 55

4 RESULTS
4.1 Cell Culture Study
  4.1.1 Osteoblast Cell Line 56
  4.1.2 Microscopic Study of Gentamicin-coated HA 58
  4.1.3 Scoring of HA Porosity 64
4.2 Bacterial Strain 66
  4.2.1 Bacterial Cell Count 66
  4.2.2 Optical Density of Biofilm Stained with Safranin 66
  4.2.3 Microtiter Plate Assay 67
  4.2.4 Quantification of S. aureus and P. aeruginosa Isolated from the Catheter-associated Biofilm 70
  4.2.5 Scoring of Biofilm Formation on Catheter 71
4.2.6 Microscopy Study 73
  4.2.7 Antibiotic Susceptibility Test 81
4.3 *In Vivo* Study

4.3.1 Extraction and Quantification of Bacteria 85
4.3.2 Histological Analysis 86
4.3.3 Histological Scoring 89

## DISCUSSION

5.1 High concentration of gentamicin-coated HA will reduce the osteoblasts viability. 91
5.2 Effectiveness of gentamicin-coated HA on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm. 94
5.3 Microscopy study of biofilm formation 97
5.4 Catheter-associated biofilm implantation of *in vivo* study 98

## CONCLUSION

6 REFERENCES 104
APPENDIX I 117
BIODATA OF STUDENT 118
LIST OF PUBLICATIONS 119
AWARDS 120