DEVELOPMENT, *IN-VITRO* AND *IN-VIVO* EVALUATION OF GENTAMICIN AND NIGELLA SATIVA OIL PLGA MICROSPHERES

BY

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ABSTRACT

Introduction: Antibiotics are amongst the highly studied and researched candidates for sustained drug release formulation. In this current study, gentamicin was formulated in the form of microspheres using poly(lactic-co-glycolic acid) (PLGA) to be used as prophylactic approach in eradicating osteomyelitic condition in rabbits. Together with *Nigella sativa* oil (NSO) where its potentials are well known, the ability of the PLGA microspheres loaded with gentamicin and NSO was evaluated for *in-vitro* and *in-vivo* studies.

Objective: The main objective of this study was to formulate a sustained release microspheres containing gentamicin and NSO intended to treat osteomyelitis.

Methodology: Gas chromatography mass spectrometry (GC-MS), high performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), thin layer chromatography (TLC) and cell viability study (MTT assay) were utilized to evaluate the compatibility as well as the stability for pre and post gamma irradiation. 27 formulations for both gentamicin and NSO microspheres were fabricated. Other than observing the external morphology of the microspheres using scanning electron microscope (SEM), the size distributions were also being analyzed. For quantification purposes, two method validations were completed by using UV-spectrophotometry following the requirements listed under ICH Q2 (R1) guidelines. For the *in-vivo* study, 16 White New Zealand rabbits were divided into 4 groups (Control, Gentamicin, NSO and Fusion) before the osteomyelitic condition was induced in the tibiae of the animals. The compressed selected microspheres were administered *in-situ* and all the animals were closely monitored for any physiological changes over a course of 6 weeks. X-ray images, hematological evaluation (white blood cell differentiations) and post surgical bacterial culture were evaluated to confirm the prophylactic ability of the microspheres to halt the infection.

Results: The size distribution ranging from 463.67 nm ± 52.54 to 4602 nm ± 113.58 and 409.67 nm ± 37.45 to 6568.00 nm ± 147.22 for gentamicin and NSO microspheres respectively. Five best formulations from gentamicin and NSO microspheres with the highest drug loading capacity (ranging from 67.32% ± 3.18 to 83.39% ± 4.22 for gentamicin microspheres and from 62.94% ± 4.84 to 73.42% ± 2.14 for NSO microspheres) were further analysed for the *in-vitro* release profile over a course of 4 weeks study. The best formulation was selected from gentamicin microspheres and NSO microspheres based on the *in-vitro* release data before being used for *in-vivo* study (compressed microspheres from formulation 6 for both gentamicin and NSO group).

Conclusion: Based on the data collected with 95% confidence level ($p<0.05$), gentamicin and NSO microspheres indicated the ability to treat osteomyelitis when compared to the untreated group. The replacement of current conventional treatment using PMMA beads is possible by the microspheres fabricated.

خلاصة البحث

ا لــمـقــدمــة :علم ايتاء الدواء هو علم دائم التطور رغم كل المعوقات التي تحد من نجاحه. يتطلب ايتاء الدواء في العصر الحالي تحقيق ثلاثة اهداف عامة: حماية الدواء اثناء وصوله الى المنطقة المستهدفة، امكانية تأمين افضل فعالية مع اقل سمية اضافة الى افضل مطاوعة من قبل المريض تجاه الدواء. تعتبر المضادات الحيوية من اكثر الادوية المناسبة لتطويرها بشكل مطول التأثير. في ههذ الدراسة تم تطوير الجنتامايسين بشكل مكروسفيرات باستخدام بوليمير PLGA لكي يتم استخدامها وقائيا ضد انتان العظام عند الارانب. مع زيت الحبة السوداء المعروف بميزاته المتعددة، تم تقييم فعالية المكروسفيرات المحملة بالزيت مع الجنتامايسين في الزجاج وفي الحي. تم استخدام اشعة غاما لتعقيم المنتج والمواد الاولية قبل الاستخدام في الحي. لتقييم التوافق والثباتية قبل وبعد الاشعة تم استخدام عدة تقنيات مثل GC–MS، HPLC، FTIR، DSC، TLC، MTT. تم تحضير 27 تحضيرة من مكروسفيرات الجنتامايسين والزيت ودراستها من ناحية المظهر الخارجي باستخدام المجهر الالكتروني الماسح وقياس الابعاد. تراوح قياس الابعاد بين 52.54 ± 463.67 nm الى 113.58 ± 4602 nm و37.45 ± 409.67 nm الى 147.22 ± 6568.00 nm لمكروسفيرات الجنتامايسين والزيت على التوالي. من اجل متابعة دراسة التحرر في الزجاج خلال 4 اسابيع تم اختيار 5 افضل تحضيرات والتي امتلكت اعلى نسبة تحميل (تراوحت بين 3.18 ± 67.32% الى ± 83.39% 4.22 لمكروسفيرات الجنتامايسين و4.84 ± 62.94% الى 2.14 ± 73.42% لمكروسفيرات زيت الحبة السوداء). من اجل التحليل الكمي تم تطوير طريقتين باستخدام UV–spectrophotometry وتقييمهما حسب توصيات ICH Q2 (R1). ومن اجل الدراسة في الحي تم اختيار افضل التحضيرات وفقا لدراسة التحرر في الزجاج. تمت الدراسة على 16 ارنب نيوزلندي قسمت الى 4 مجموعات (شاهد، جنتامايسن، زيت الحبة السوداء والمزيج من الاثنين) قبل تحريض حالة انتان عظمي في عظم الظنبوب. تم اعطاء المكروسفيرات المضغوطة اعطاء موضعيا ومراقبة جميع الحيوانات عن كثب خلال مدة 6 اسابيع لملاحظة اية تغيرات فيزيولوجية. تم تقييم صور اشعة اكس، التقييم الدموي (تمايز الكريات البيض) والزرع الجرثومي بعد الجراحة لتأكيد القدرة الوقائية للمكروسفيرات لايقاف الانتان. بناء على النتائج التي تم الحصول عليها هناك ثقة كبيرة بان مكروسفيرات الجنتامايسين وزيت الحبة السوداء يمكن ان توفر بديلا لمعالجة انتان العظام في المستقبل.

ABSTRAK

Pengenalan: Antibiotik adalah salah satu ubatan yang sangat dikaji dalam menambahbaikan proses penghantaran di dalam badan kita. Dalam kajian ini, gentamicin diformulakan dalam bentuk butiran mikro dengan menggunakan poly(lactic-co-glycolic acid) (PLGA) untuk digunakan sebagai rawatan bagi penyakit osteomyelitis. Keupayaan butiran mikro ini yang diformulakan bersama minyak *Nigella sativa* (NSO) terus dikaji sehingga ke tahap *in-vitro* dan *in-vivo*.
Objektif: Objektif utama peyelidikan ini adalah untuk formulasikan satu bentuk rawatan bagi penyakit osteomyelitis dengan menggunakan mikrosfera mengandungi gentamicin dan NSO yang mampu melepaskan kandungannya secara perlahan-lahan.
Kaedah: Radiasi gamma diperlukan untuk menyahkuman produk yang akan digunakan bagi kajian *in-vivo*. Atas sebab ini, beberapa pendekatan pengkajian telah digunakan seperti kromatografi gas (GC-MS), kromatografi cecair berkemampuan tinggi (HPLC) dan penilaian kelangsungan sel (MTT assay) untuk menilai kesan radiasi gamma ke atas bahan mentah yang digunakan. Hanya 5 formulasi terbaik yang dipilih berdasarkan nilai kadar pengisian ubatan tertinggi sebelum hanya 1 formulasi (formulasi 6 dari kedua-dua kumpulan) yang terpilih yang akan dikaji lebih mendalam dari skop *in-vivo*. 16 arnab putih New Zealand telah didedahkan dengan kuman untuk mendapatkan kesan jangkitan kuman semisal osteomyelitis. Arnab-arnab ini dipecahkan kepada 4 kumpulan (kawalan, gentamicin, NSO dan Fusion) sebelum rawatan diberikan. Jangkitan kuman ke atas arnab-arnab tersebut dibuat ke dalam tulang tibia sebelum rawatan dengan menggunakan butiran mikro yang dimampatkan diberikan. Imej sinar-X, kadar pengiraan sel darah putih dan juga pengkulturan bakteria selepas pembedahan telah digunakan untuk menilai kadar keupayaan rawatan yang diberikan.
Keputusan: 27 formulasi butiran mikro telah dibuat dan keadaan luaran butiran tersebut dinilai dengan menggunakan mikroskop pengimbasan elektron (SEM) selain taburan saiz dan kadar pengisian ubatan turut dinilai. Taburan saiz sekitar 463.67 nm ± 52.54 ke 4602 nm ± 113.58 dilihat untuk gentamicin; dan 409.67 nm ± 37.45 ke 6568.00 nm ± 147.22 dapat dilihat untuk butiran mikro NSO. Butiran mikro tersebut terus dikaji dan dinilai dengan melihat kepada kadaran pelepas ubatan untuk kajian selama 4 minggu.
Kesimpulan: Daripada data terkumpul, amat jelas kelihatan di mana rawatan yang diberikan berkesan untuk menghalang berlakunya jangkitan kuman di dalam tibia arnab tersebut yang boleh merebak menjadi osteomyelitis. Kami percaya butiran mikro yang diformulasikan ini mampu memberi satu pendekatan alternatif dalam merawat jangkitan kuman pada tulang dan menjadi salah satu rawatan yang berkesan pada masa hadapan.

APPROVAL PAGE

The thesis of Ahmad Fahmi Harun Ismail has been approved by the following:

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, 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.

Ahmad Fahmi Harun Ismail

Signature ..... Date .....

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..for you, mom..

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"The acknowledgement of our weakness is the first step to the endless world of knowledge"

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CTAB	Cetyl trimethylammonium bromide
CFU	Colony Forming Unit
DSC	Differential Scanning Calorimeter
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas Chromatography-Mass Spectrometry
HLB	Hydrophile-Lyphophile Balance
HPLC	High Performance Liquid Chromatography
LE	Drug Loading Efficiency
LOD	Limit of Detection
LOQ	Limit of Quantification
MAA	Methacrylic Acid
MSDS	Material Safety Data Sheet
MIC	Minimum Inhibition Concentration
MTT	Microculture Tetrazolium Assay
NSO	*Nigella sativa* oil
O.D.	Optical Density
PBS	Phosphate Buffer Saline
PMMA	polymethylmethacrylate
PDLA	poly D-lactic acid
PLLA	poly L-lactic acid
PGA	polyglycolic acid
PLA	poly(D,L-lactic) acid
PVA	Poly vinyl alcohol
PLGA	Poly (D,L-lactide-co-glycolide) acid
Rf	Retention Factor
S.D	Standard Deviation
SEM	Scanning Electron Microscopy
*T*g	Glass Transition Temperature
TLC	Thin Layer Chromatography
US FDA	United State Food Drug Administration
MW	Molecular Weight
WBC	White Blood Cell

CHAPTER ONE

THE OVERVIEW

1.1 INTRODUCTION

The discovery of penicillin as one of the early most antibiotics in 1940s has become the most remarkable breakthrough in medical history. This huge discovery has later become the starting point for more discoveries of antibiotics originated from microbes especially from the members of actinomycetes and fungi (Abed & Couvreur, 2014; Peláez, 2006). The world nowadays has benefited healthy living and enjoyed the control over the infectious diseases for many years. The quality of life has drastically improved and more new methods of combating the infections have been introduced.

However, many bacteria have shown to develop resistance towards the antibiotics available. Many scientific reports have raised the concern of inadequate availability of new antibiotics to address the problem posed by the bacterial resistance that has been growing by day (Bradley et al., 2007; Baucher et al., 2009; Freire-Moran et al., 2011). Resistant and multi resistant bacteria are no doubt posing huge danger to everyone. The fact that their resistance genes can be spread easily, faster and further has further made the emergence of antibiotic resistance to become more complicated (Cars et al., 2011)

Although the requirement for new compounds to fight the infectious organisms is growing as the result to the increase number of antibiotic-resistant pathogens, surprisingly just a handful of new antibiotics have been approved for the clinical use in the past decades (Wright, 2014).

This situation that has been called an "apocalyptic" threat to the mankind has drawn a huge concern amongst the scientists while the pharmaceutical sectors seem to

unable to counter the threat effectively (Wright, 2014). Other than in favor to the large libraries of synthetic molecules, the difficulties to identify new natural compounds that possess antimicrobial property have become the reason for the pharmaceutical industry to focus more on developing new method of administrating the existing drugs to increase the efficacy.

The development of new agents with antimicrobial property is much needed when the discovery of new antimicrobial natural compounds could not keep up with the resistance which is emerging in a very fast manner. The development in drug discovery and pharmaceutical technology has proven that natural compounds are no longer the only citizen in the world of antibiotics. The formula modification and combination with other potent existing drugs has proven to be the most efficient way to develop novel drugs active against resistant strains (Fernebro, 2011).

The novel drug combinations coupled with the new strategies in controlling the release of the drugs at the specific area are amongst the factors which improve the efficacy of the existing antibiotics. Many formulations are being developed by taking into consideration the effectiveness and the optimization of the drugs by manipulating the release patterns and the synergistic effect obtained through combinations with foreign compounds. Despite of having such "apocalyptic" threat, the new drug development and formulation seem to give a new opening to huge opportunities in solving the crisis faced by healthcare personnel and scientists around the world.

1.2 RESEARCH BACKGROUND

Staphylococcus aureus is one of the bacteria that are responsible for most medical infectious cases especially related to the bone. In general, the most isolated pathogen from the patients in the field of orthopedics was *S. aureus* (Nelson & Williams, 2014;