A STUDY ON GENTAMICIN IMPREGNATED BIOMATERIALS FOR TREATING INDUCED-OSTEOMYELITIS IN THE NEW ZEALAND WHITE RABBIT ANIMAL MODEL

BY

AHMAD HAFIZ ZULKIFLY

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy in Medical Sciences

Kulliyyah of Medicine
International Islamic University Malaysia

AUGUST 2021

ABSTRACT

The treatment of osteomyelitis is still a major challenge in orthopaedics. A study of osteomyelitis and infection requires the use of a suitable animal model. The New Zealand White Rabbit (NZWR) is an acceptable experimental model that can be used for local delivery of antibiotics in osteomyelitis treatment as it can mimic the disease process in humans. The objectives of this study were to create osteomyelitis in the rabbit femurs and to analyse the treatment given via gentamicin impregnated with biomaterials beads. Thirty-six (36) NZWRs were used in the study. They were divided into two groups [Hydroxyapatite (HA) and calcium sulphate ($CaSO_4$)] with four subgroups 3-, 6-, 12-, and 26-weeks intervals. There were two surgeries performed for each NZWR. The first was to induce the osteomyelitis by inoculating *Staphylococcus aureus* in the distal of the animal, and the second surgery was for debridement and biomaterial impregnated antibiotics implantation. The responses of the treatments (gentamicin impregnated with HA and $CaSO_4$) to were evaluated through gross appearance, radiograph, micro-CT, microbiological, and histological examination. The rabbits were sacrificed accordingly to evaluate the healing process of the affected bone. The results showed osteomyelitis changes in all rabbits after the inoculation of the bacteria at 3 weeks. The rabbits' weights reduced after three weeks of bacteria inoculation following the treatment with biomaterial impregnated antibiotics, they showed significant increase in weights at 12 and 26 weeks in both groups. The microbiology analysis at 26 weeks showed that no bacteria were isolated. All the defects at the drilled site of the distal femurs of the NZWRs were united at 12 weeks interval. The histological examination revealed healing of the infected area with the appearance of a new bone formation at 6 to 26 weeks. The micro-CT results revealed increased the trabeculae numbers with the treatments. The biomaterials containing $CaSO_4$ disappeared by 26 weeks. There was completed bone healing at 26 weeks of interval for both groups. The results of the gentamicin impregnated with HA and $CaSO_4$ for all parameters are comparable. Hence, the antibiotics impregnated with biomaterials are proven effective in the treatments of osteomyelitis. In conclusion, the results of this study show that gentamicin impregnated with biomaterial has a great potential to be utilised for the treatment of osteomyelitis.

خلاصة البحث

دراسه مقارنه حول طرق علاج مبتكره لإيصال الدواء، المضاد الحيوي، موضعيا الى منطقة التهاب وخمج العظم المستحدث في حيوان التجارب الارنب النيوزيلندي الأبيض ما يزال علاج التهاب العظم الجرثومي من التحديات الكبيرة التي تواجه العاملين في جراحة العظام والكسور .ان الارنب النيوزيلندي الأبيض يعتبر حيوان تجارب مناسب لهذا الغرض حيث يمكن استعماله في تجارب ايصال الدواء الى مكان التهاب وتجرثم العظم بصورة مماثله لما يحدث في الإنسان .ان غاية هذا البحث إحداث اصابه بالتهاب العظم الجرثومي في عظم الفخذ للارنب ومن ثم علاجه بحبيبات مواد بيولوجيه مخصبه (مشبعه) بالمضاد الحيوي جنتامايسين ،وتحليل النتائج المترتبه .أحدث التهاب العظم بعمل ثقب في اسفل عظم فخذ الارنب النيوزيلندي الأبيض وبعدها زرقت البكتريا العنقوديه الذهبيه في الثقب .تراوح وزن الارانب المستعمله في البحث ما بين 4.2 – 2.5كغم وكانت جميعها بعمر اكثر من ستة أشهر .استعمل 36 حيوان لهذه الدراسه وقد قسمت الحيوانات الى مجموعه خادعه من اربع حيوانات بدون علاج، ومجموعتين أخريين من اربع أرانب لكل منهما خضعتا للعلاج، احداهما عولجت بهيدروكسيد الابتايت والأخرى بسلفات الكالسيوم المخصبتين بالجنتامايسين .قسمت كل من المجموعتين من الحيوانات التي خضعت للعلاج الى أربعة مجاميع صغرى لكل منهما ،احتوت الواحده على 4 ارانب ضحي بها على فترات هي 12 ,6 ,3 و 26اسبوع بعد بدأ العلاج .أجريت عمليتين جراحيتين لكل ارنب، الأولى لاحداث التلوث الجرثومي والأخرى للتنظيف الجراحي وزرع حبيبات المواد البايولوجيه المشبعه بالمضاد الحيوي جنتامايسين .تم تقييم نتائج العلاج بحبيبات هيدروكسيد الابتايت ومقارنته بالعلاج بكبريتات الكالسيوم المشبعتين بالمضاد الحيوي عن طريق دراسة الأعراض المرضيه في الجسم، التصوير بأشعة اكس، التصويرالشعاعي المقطعي الدقيق بالاضافه للدراسه الجرثوميه والنسيجيه في نهاية الأسبوع الثالث، السادس، الثاني عشر والسادس والعشرون من بدأ العلاج، قبيل التضحية بها لتقييم سيرعملية شفاء الخمج العظمي .بينت النتائج حصول التهاب العظم الجرثومي في الاسبوع الثالث في جميع مجموعات الارانب التي خضعت لعملية حقن البكتريا في العظم .نقص وزن في الاسبوع الثالث بعد الاصابه ولكن الارانب المصابه بدرجة ملحوظه وذات مغزى احصائيفي الاسبوع 12 مجموعتي المعالجه ازداد وزنهما بدرجة ملحوضه وذات مغزى احصائيو 26 بعد العلاج بالمادتين البيولوجيه المشبعه بالمضاد الحيوي .تم التأكد مختبريا من القضاء كليا على البكتريا المخمجه للعظم في الأسبوع 26 من بدأ العلاج .وقد تبين نسيجيا ان جميع ثقوب العظام قد التئمت في الأسبوع 12 في الحيوانات المعالجه ,وقد كانت هناك بوادر شفاء في المناطق المصابه من خلال تكون نسيج عظمي جديد منذ الأسبوع السادس واستمر حتى نهايته التجارب في الأسبوع السادس والعشرين .بينت نتائج الفحص الشعاعي المقطعي الدقيق ان عدد الترابيق العظميه ازداد بنسب ذات مغزى احصائ في مجاميع المعالجه مقارنة بالمجموعه الخادعه .ان الماده العضوية المستعمله في العلاج قل تواجدها بمرور الوقت واختفت في الأسبوع .26 لم نجد فروقات ذات مغزى احصائي بين نتائج الفحوصات الشعاعيه ،النسيجيه التصويرالشعاعي المقطعي الدقيق الخ بين مجموعتي العلاج بكبريتات الكالسيوم والأخرى بهيدروكسيد الابتايت المشبعتين بالجنتامايسين حيث ان كلاهما أعطى نتائج متماثلة في علاج التهاب وخمج العظم المستحدث في حيوان التجارب الارنب النيوزيلندي الأبيض .نستنتج من هذه الدراسه ان العلاج بهيدروكسيد الابتايت المشبع بالجنتامايسين له الامكانيه بأن يستعمل سريريا كطريق لإيصال الدواء موضعيا لمعالجة الخمج البكتيري للعظم، وذلك بعد إجراء فحوصات مقارنه أخرى والتأكد من سلامته من المضاعفات

APPROVAL PAGE

The thesis of Ahmad Hafiz Zulkifly has been approved by the following:

_____
Mohammed Imad A. Mustafa Mahmud
Supervisor

_____
Nasser Mohammad Amjad
Co-Supervisor

_____
Naznin Muhammad
Internal Examiner

_____
Mohamed Abd Razak
External Examiner

_____
Habsah Hassan
External Examiner

_____
Zarina Zainuddin
Chairman

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 Hafiz Zulkifly

Signature……. Date……...

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

A STUDY ON GENTAMICIN IMPREGNATED BIOMATERIALS FOR TREATING INDUCED -OSTEOMYELITIS IN THE NEW ZEALAND WHITE RABBIT ANIMAL MODEL

Affirmed by Ahmad Hafiz Zulkifly

……..…….…… ……..……..

Signature Date

ACKNOWLEDGEMENT

With the Greatness and Mercy of ALLAH S.W.T and the grace and blessing from ALLAH S.W.T, Selawat and Salam for our beloved prophet Muhammad (SAW) and his companion. It was a very long journey to complete this study. It was very challenging. A lot of events took place and beyond the control.

I would like to thank my beloved mother Pn. Hajjah Maimunah bt. Yacob, my dearest, respected and loved wife Dr. Hajjah Rosmaliza bt. Ismail, my lovely, cute, inspirational son Ahmad Rayyan who was there during this long journey and unconditional love.

I wish to thank Prof. Dato' Dr. Tahir Azhar, our pioneer Dean of Medicine IIUM, who always inspire others to pursue knowledge. Gratitude to Prof. Dr. Nasarudin bin Abdullah, who believes that this journey can be successfully run although tough that the writer may not need extra qualifications. He agrees to be my supervisor when others denied. Thank you, Prof., Dr. Imad, my current supervisor who agree to continue with me the journey left by Prof. Nasa.

Similar gratitude to all my teachers since early days until now they have shown the path for a student to pursue and be success. My colleagues who give their trust and support during this study and journey. Thank you.

Special gratitude to my research team at my ISO 17025 Orthopaedic Advanced Research Lab esp: Mohd Zulfadzli Ibrahim, Nurul Hafiza Mohd Jan, Che Nor Zarida Che Seman, Rosnani Abdul Jalil, Dr. Aziah Aziz and others. I am also grateful to my students where I learned things that is not in the literature.

All the great occasions were by the willing of the Almighty ALLAH S.W.T. I would like to say ALHAMDULILLAH with all His Mercy, kindness and blessings, this study was completed. Thank you all, Thank you ALLAH. Selawat and Salam to Prophet Muhammad S.A.W and Alhamdulillah.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D	2-Dimension
3D	3-Dimension
AG	German Aktiengesetz
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BEM	Bone Extracellular Matrix
BMD	Bone Mineral Density
BR	Basic Research
BS	Bone Surface
BS/TV	Bone Surface Ratio
BV	Volume of Trabeculae
BV/TV	Bone Volume
$CaSO_4$	Calcium Sulphate
CD	Compact Disc
CHA	Calcium Hydroxyapatite
Co.	Company
CT	Computed Tomography
DDS	Drug Delivery System
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPS	Exopolysaccharides
FOV	Field of View
HA	Hydroxyapatite
IACUC	Institutional Animal Care and Use Committee

IBM	IBM Corporation
IIUM	International Islamic University Malaysia
IQR	Interquartile Range
ISO/IEC	International Organization for Standardization / International Electrotechnical Commission
IV	Intravenous
KTX	A combination of drugs Ketamine, Tiletamine / Zolazepam and Xylazine for anaesthesia
MIC	Minimum Inhibitory Concentrations
Micro-CT	Micro-Computed Tomography
MOSTI	Minister of Science, Technology and Innovation
NBF	Natural Buffered Formalin
NZWR	New Zealand White Rabbit
OM	Osteomyelitis
OM	Osteomyelitis
PMMA	Poly (methyl methacrylate)
POP	Plaster of Paris
PPB	Part per Billion
Pty Ltd.	Propriety / Private Limited
qPCR	Quantitative Polymerase Chain Reaction
ROI	Region of Interest
S. aureus	*Staphylococcus aureus*
SPO_2	Blood Oxygen Saturation
SPSS	Statistical Package for the Social Science
Tb. N	Trabecular Number
Tb. Th	Trabecular Separation
TRAP	Tartrate-resistant Acid Phosphate

TSA	Tryptic Soy Agar
TV	Total Volume
USA	United States of America

LIST OF SYMBOLS

%	Percentage
<	Less than
=	Equal to
≠	Not equal to
>	More than
±	Standard deviation
≤	Less than or equal to
≥	More than or equal to
®	Registered patent
°C	Degree Celsius
µA	Microampere
µg/ml	Microgram per millilitre
µm	Micrometre
1st	First
CFU/ml	Colony-forming units per millilitre
cm	Centimetre
cm^2	Square Centimetre
CO_2	Carbon dioxide
d*f*	Degree of freedom
F	Fisher–Snedecor distribution
g	Gram
g/ml	Gram per millilitre
Ha	Alternative Hypothesis
Ho	Null Hypothesis

kg	Kilogram
kV	Kilovolt
M	Mean
MD	Mean Different
mg	Milligram
mg/ml	Milligram per Millilitre
ml	Millilitre
ml/kg	Millilitre per Kilogram
mm	Millimetre
mM	Millimolar
mm^3	Cubic Millimetre
mmHg	Millimetre Mercury
ms	Millisecond
NaCl	Natrium Chloride
rpm	Rotation per Minute
SD	Standard Deviation
SE	Standard Error
™	Trademark
v/v	Volume per Volume
w/v	Weight / Volume
w/w	Weight per Weight
Z	Normal Distribution Score
$\eta 2p$	Effect Size
$\chi 2$	Chi-squared Test
%	Percentage

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Osteomyelitis (OM) is a progressive bone infection that results from inflammatory destruction of the bone, necrosis of bone, and new bone formation, and may progress to a chronic and persistent state. It can be divided into acute, subacute, and chronic osteomyelitis. However, as described in the literature, there are many ways to classify osteomyelitis (Hotchen et al., 2017). These classification systems help to define the infection and determine the need for surgery. Chronic osteomyelitis is one of the most severe complications of orthopaedic open fracture treatment (Kuehl et al., 2016; Panteli et al., 2016). Osteomyelitis is usually caused by bacteria and sometimes by fungus. The infection causes the inflammation of bone and bone marrow. Thick walls surround the soft tissue of bone and inflammation at the surrounding tissues cause circulatory distractions, which can promptly lead to necrosis of various parts of the bone. These necrotic bones act as a non-living surface for the attachment of bacteria and the formation of biofilm. The bones that are most commonly affected, include the femur, tibia, and humerus, although nearly all bones in the body can be involved. As a consequence, the disease tends to be both localized and generalized because the bacteria can be carried to other organs (Hotchen et al., 2017; Kuehl et al., 2016; Panteli et al., 2016).

The predominant aetiological agents of chronic osteomyelitis are *Staphylococcus aureus* (S. aureus), Group A beta-hemolytic Streptococcus, and gram-negative bacteria, particularly Salmonella spp., Mycobacterium tuberculosis, and Pseudomonas aeruginosa (Hotchen et al., 2017; Kuehl et al., 2016; Panteli et al., 2016). Previous