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ANTIBIOFILM STUDY ON OSTEOMYELITIC BACTERIA USING NEW GENTAMICIN-*NIGELLA SATIVA* FUSION EMULSIONS

BY

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ABSTRACT

The treatments of chronic osteomyelitis are difficult, time-consuming and relatively expensive due to the presence of bacterial biofilm that is highly resistant to antibiotics. This study aimed to assess synergistic antibacterial activities of gentamicin-*Nigella sativa* fusion towards the most common biofilm-bacteria in osteomyelitic infection. Briefly, a total 57 samples (prostheses, bones, tissues and swabs) were taken from 17 cases of osteomyelitic infection at Hospital Tengku Ampuan Afzan, Kuantan. The samples were processed, isolated species were identified, as well as biofilm identification and antibiotic sensitivity assays were performed. Fusion of gentamicin and *N.sativa* were formulated in 4 different types of emulsions (A, B, C, and D) consisting of constant 0.1% (w/v) gentamicin and different *Nigella sativa* oil concentrations from 32.5% to 46.6% (v/v). Antimicrobial activities of the emulsions were evaluated using disc diffusion assay and determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Then, the assessment of antibiofilm activities was carried out as pre- and post-biofilm assays. The pre-biofilm consists of biofilm formation inhibition and minimum biofilm inhibition concentration (MBIC). The post-biofilm assay was done to evaluate the effects of the emulsions on the biofilm, using biofilm penetration test and confocal laser screening microscope (CLSM) analysis. It was found that prosthesis (89%) and bone (66.7%) samples produce the most bacteria growth and *Staphylococcus aureus* (10 out of 16) was the most frequently identified. In the disc diffusion assay, significant synergistic effect of emulsions was seen only in resistant *S.aureus* (clinical isolate) (Tukey's test $p < 0.05$). Additionally, emulsions MIC values were up to 10 times lower than gentamicin alone against all *S.aureus* while MBC values of emulsions were up to 3 times lower towards sensitive *S.aureus* (clinical isolate and control). No bactericidal activity was exhibited by all compounds tested on resistant *S.aureus* (clinical isolate). In pre-biofilm evaluation, there were significant differences in biofilm formation inhibition in comparison between these emulsions with *N.sativa* and gentamicin alone in both clinical isolate *S.aureus* (sensitive and resistant) (Tukey's test $p < 0.05$). MBIC values of emulsions were up to 10 times lower than gentamicin against all *S.aureus*. In contrast, *N.sativa* alone was lesser than emulsions and gentamicin. For post-biofilm assessment, no significant difference in penetration rate was found between emulsions and gentamicin. As opposed to *N.sativa* which showed little penetration. In the CLSM analysis, only emulsion C was used. Results revealed that emulsion C significantly reduced the biofilm thickness compared to gentamicin and *N.sativa* alone (Tukey's test $p < 0.05$). Furthermore, the surface percentage (%) of non-viable bacteria of emulsions is significantly higher than gentamicin and *N.sativa* alone (Tukey's test $p < 0.05$). In conclusion, this new fusion of gentamicin-*N.sativa* have synergistic antimicrobial and antibiofilm properties towards different strains of *S.aureus* including resistant strains, thus, can be developed as a new, and customized, gram-positive-specific treatment for ostoemyelitic infection.

خلاصة البحث

العلاجات من التهاب العظم المزمن صعبة، تستغرق وقتا طويلا ومكلفة نسبيا بسبب وجود بكتيريا بيوفيلم التي هي شديدة المقاومة للمضادات الحيوية. هدفت هذه الدراسة إلى تقييم الأنشطة المضادة للبكتيريا المستمرة لانصهار الجنتاميسين–حبة البركة نحو الأكثر شيوعا لبكتيريا بيوفيلم في عدوى التهاب العظم . باختصار، تم اتخاذ مجموع 57 عينة من ال (prostheses والعظام والأنسجة ومسحات)أخذت من 17 حالة من حالات عدوى التهاب العظم في مستشفى تنكو امبوان أفزان، كوانتان. تم تجهيز العينات، تحديد الأنواع المعزولة، وكذلك تم تنفيذ تحديد بيوفيلم وفحوصات الحساسية للمضادات الحيوية. صيغت مزيج من الجنتاميسين وحبة البركة في 4 بأنواع مختلفة من المستحلبات (أ ،ب ،ج ،د) ويتألف من تراكيز ثابتة 0.1٪ (و / ح) من الجنتاميسين وتركيزات مختلفة من زيت حبة البركة من 32.5٪ إلى 46.6٪ (ح / ح) . تم تقييم الأنشطة المضادة للميكروبات من المستحلبات باستخدام فحص القرص نشرها وتحديد الحد الأدنى لتركيز المثبط (MIC) و الحد الأدنى لتركيز مبيد الجراثيم .(MBC) ثم جرى تقييم الأنشطة المضادة للبيوفيلم باجراء فحوصات ما قبل وما بعد بيوفيلم. تتكون فحوصات ما قبل بيوفيلم تثبيط تشكيل بيوفيلم و تثبيط الحد الأدنى لتركيز بيوفيلم (MBIC)وقد تم فحص ما بعد بيوفيلم لتقييم الآثار المترتبة على المستحلبات على بيوفيلم، وذلك باستخدام اختبار الاختراق للبيوفيلم ومتحد البؤر مجهر فحص الليزر (CLSM) وقد تبين أن (89٪) prosthesis عينة و(66.7 ٪) عينة من العظام تنتج معظم النمو للبكتيريا والمكورات العنقودية الذهبية (10 من 16) كان الأكثر تحديدها بشكل متكرر. في قرص نشر الفحص، كان ينظر إلى تأثير متناغم كبير من المستحلبات فقط في مقاومة البكتريا (عزل السريري) (اختبار توكي $p<0.05$). بالإضافة إلى ذلك، كانت القيم المستحلباتMIC تصل أقل إلى 10 مرات من الجنتاميسين وحده ضد كل البكتريا بينما كانت قيم مستحلبات MBC تصل إلى 3 مرات أقل تجاه البكتريا الحساسة (بالعزل السريري والمراقبة). وقد عرضت نشاط الجراثيم لجميع مركبات واخضعت للاختبار على مقاومة البكتريا (بالعزل السريري). في تقييم ما قبل بيوفيلم، كانت هناك اختلافات كبيرة في تشكيل تثبيط بيوفيلم بالمقارنة بين هذه المستحلبات مع حبة البركة وجنتاميسين وحدها في كل من عزل البكتريا السريري (الحساسية والمقاومة) (اختبار توكي $p<0.05$). وكانت قيم MBIC للمستحلبات أقل تصل إلى 10 مرات من جنتاميسين ضد كل البكتريا. في المقابل، كانت حبة البركة وحدها أقل من المستحلبات ومن الجنتاميسين. لتقييم ما بعد بيوفيلم، لم يتم العثور على اختلاف كبير في نسبة الانتشار بين المستحلبات وجنتاميسين. في مقابل حبة البركة التي أظهرت اختراقا قليلا. في تحليلCLSM ، وكان يستخدم فقط مستحلب و.كشفت النتائج أن مستحلب د أدى إلى خفض كبير في سماكة بيوفيلم مقارنة مع الجنتاميسين وحبة البركة وحدها (اختبار توكي $p<0.05$). وعلاوة على ذلك، فإن النسبة المئوية السطحية (٪) من البكتيريا غير قادرة على البقاء من المستحلبات هي أعلى بكثير من الجنتاميسين وحبة البركة وحدها (اختبار توكي $p<0.05$). في الختام، هذا الانصهار جديد من الجنتاميسين–حبة البركة يكون لهذا التآزر خصائص مضادة للميكروبات ولبيوفيلم نحو سلالات مختلفة من البكتريا بما في ذلك السلالات المقاومة، وبالتالي، يمكن تطويرها باعتبارها العلاج لغرام الإيجابية ومخصصة لعدوى التهاب العظم المزمن.

ABSTRAK

Rawatan untuk kronik osteomielitis adalah sukar, memakan masa dan mahal kerana bakteria 'biofilm' yang tidak berkesan dengan antibiotik. Kajian ini bertujuan untuk menilai aktiviti sinergi gabungan gentamicin dan *Nigella sativa* terhadap bakteria 'biofilm' di dalam jangkitan osteomielitis. Sebanyak 57 sampel (implan, tulang, tisu dan 'swab') telah diambil daripada 17 kes osteomielitis di Hospital Tengku Ampuan Afzan, Kuantan. Sampel diproses dan spesis bakteria bersama dengan biofilm dikenal pasti dan ujian keberkesanan antibiotik telah dijalankan. Formulasi gabungan gentamicin dan *N.sativa* telah dihasilkan di dalam 4 jenis emulsi (A, B, C, dan D) yang terdiri daripada sama kepekatan gentamicin (0.1% w/v) dan pelbagai kepekatan *N.sativa* daripada 32.5 % sehingga 46.4% (v/v). Aktiviti anti-mikrob emulsi dinilai dengan menggunakan 'disc diffusion assay', 'minimum inhibitory concentration' (MIC), dan 'minimum bactericidal concentration' (MBC). Kemudian penialaian aktiviti 'antibiofilm' dijalankan dengan 'pre-biofilm' dan 'post-biofilm'. 'Pre-biofilm' menganalisis kesan agen antimikrobial untuk merencat penghasilan 'biofilm' dengan ujian seperti 'biofilm formation inhibition' dan 'minimum biofilm inhibition concentration' (MBIC). Sementara itu, 'post-biofilm' bertujuan menganalisis kesan agen antimikrobial terhadap 'biofilm' matang menggunakan parameter seperti 'biofilm penetration' dan analisis menggunakan 'confocal laser screening confocal microscope' (CLSM). Keputusan menunjukkan bahawa sampel yang paling banyak menghasilkan bakteria adalah implan (89%) dan tulang (66.7%). Kebanyakan bakteria yang didapati daripada sampel adalah *Staphylococcus aureus*. Ujian 'disc diffusion' telah menunjukkan semua kesan sinergi emulsi hanya pada 'resistant *S.aureus* (clinical isolate)' (Tukey's test $p < 0.05$). Selain itu nilai MIC semua emulsi adalah sehingga 10 kali lebih rendah berbanding gentamicin terhadap semua *S.aureus* dan 3 kali lebih rendah pada kedua-dua 'sensitive *S.aureus* (clinical isolate dan control)'. Tiada 'bactericidal' aktiviti diperhatikan pada semua agen yang ke atas 'resistant *S.aureus* (clinical isolate)'. Untuk 'pre-biofilm', ujian 'biofilm formation inhibition' menunjukkan perbezaan signifikan ke atas semua emulsi diantara gentamicin dan *N.sativa* terhadap kedua-dua 'clinical isolate *S.aureus* (resistant dan sensitive)' (Tukey's test $p<0.05$). Nilai MBIC semua emulsi adalah sehingga 10 kali lebih rendah berbanding gentamicin terhadap semua *S.aureus*. Untuk penilaian 'post-biofilm', tiada perbezaan signifikan antara emulsi dan gentamicin. Untuk analisis CLSM hanya emulsi C digunakan. Keputusan menunjukkan emulsi C berjaya mengurangkan ketebalan 'biofilm'dan nilai peratus (%) bakteria yang tidak berdaya maju juga adalah paling tinggi serta perbezaan dengan gentamicin dan *N.sativa* adalah signifikan (Tukey's test $p<$value 0.05). Kesimpulannya, gabunagn antra gentamicin dan N.sativa mempunyai kesan sinergi antimikrobial dan 'antibiofilm' terhadap pelbagai jenis *S.aureus.* Gabungan ini berpotensi untuk dibangunkan sebagai agen baru untuk disesuaikan khusus pada gram- positif bakteria untuk rawatan osteomeiliti.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Health Sciences (Biomedical Sciences).

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DECLARATION

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

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For my late father

...My hero and my inspiration…

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BA	Blood Agar
BCS	Biopharmaceutical Classification System
CA	Cetrimide Agar
CLSM	Confocal Laser Screening Microscope
EPS	Extracellular Polymeric Substances
FDA	Food and Drug Administration
HLB	Hydrophilic Lypophilic Balance
IREC	IIUM Research and Ethics Committee
MBC	Minimum Bactericidal Concntration
MBIC	Minimum Biofilm Inhibitory Concentration
MHA	Muller Hinton Agar
MIC	Minimum Inhibition Concentration
MREC	Medical Research and Ethics Committee
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
MSSA	Methicillin Sensitive *Staphylococcus aureus*
NB	Nutrient Broth
PBS	Phosphate Buffer Saline
PI	Propidium Iodide
PJI	Prosthetic Joint Infection
PMMA	Polymethyl methacrylate
SOP	Standard Operating Procedure
SVC	Small Colony Variant
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSBglu	Tryptic Soy Broth with Glucose
VBNC	Viable but Non-Culturable

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Biofilm formation is one of the important characteristics and mechanisms for survival in certain bacteria. Its formation is essential for their survival (Donlan & Costerton, 2002) as it also act as virulence factor that make it highly pathogenic and resistant to antimicrobial agents (Mah & O'Toole, 2001). Although biofilm-producing bacteria may present in many types of chronic diseases, it is highly prevalent in osteomyelitis especially in prosthetic related infections. The increase of orthopaedic implantation contributes to high cases of failed and prolonged treatment (Brady et al. 2008). There is currently no antibiotic that is effective in the treatment against bacterial biofilm. To eradicate biofilm, high concentrations of antibiotic are needed. However, at this high, toxicity become a problem (Mah & O'Toole 2001).

One of the approaches to overcome this issue is by combining antibiotic with natural product to achieve synergistic antibacterial effects (Chanda & Rakholiya 2011). Many natural products are known to contain active metabolite that is effective against bacteria. Even though the natural product alone is not great as antibiotic, the combination is helpfulin term of expanding antimicrobial spectrum, prevent emergences of resistant, increase antibiotic effectiveness and minimize the toxicity (Chanda & Rakholiya 2011). This new strategy have focused on the different mechanism of actions, enhanced antibiotic, reverse resistance mechanism and inhibit antibiotic-induce toxicity (Chanda & Rakholiya 2011; Chait et al. 2011).

1.1.1 Research Background and Justification of Study

Osteomyelitis is an inflammation of bone caused by infection. . It is the common problem that is encountered in the orthopaedic settings. Even though bone is not very likely to be susceptible to an infection, high exposure to large number of bacteria inoculation will cause osteomyelitis (Lew & Waldvogel 2004). The presence of foreign bodies such as prosthetic implants also often lead to the high risk of bacteria colonization at the bone (Brady et al. 2008). This infection can occur in acute and chronic stages. Acute stages most commonly happened in children occurring from hematogenous infection while chronic stage is the severe form of osteomyelitis that often take place in adult occurred from contiguous focus or secondary infection (Carek, Dickerson, & Sack, 2011; Cierny & Mader, 1984). Also, high risk of infection in orthopedic setting could occur notably by direct inoculation during open fracture surgery (Brady et al. 2008).

The most common pathogencausing osteomyelitis is *Staphyloccocus aureus*, followed by *Enterobacteriaceae* spp. and *Pseudomonas* spp. Osteomyelitis associated with implant is 90% caused by *Staphylocccoccus epidermidis.* Patients who are immunocompromised, generally infected by polymicrobial organisms, mixed between gram-positive and gram-negative bacteria (Ciampolini & Harding 2000). The infection usually characterized by necrotic dead bone tissue that acts as a non-living surface for bacterial attachment and biofilm formation (Mader et al. 1996; Brady et al. 2008). The existence of prosthetic implants also increase high chances of bacteria adherent (Brady et al., 2008).

Microbial film is a community or an aggregation of interactive bacteria attached to a solid surface or to each other and encased in an exopolysaccharide matrix (Toole et al. 2000). These matrixes acts as protective barrier for bacteria and

prevents the access of antibiotics and make it highly resistant. To access the bacteria encased within biofilm, high concentration of antibiotic is required and that often lead to toxicity (Mah & O'Toole 2001).

The treatments of osteomyelitis are so difficult because of biofilm resistant to antibiotic and systemic antibiotic treatment is completely ineffective. Additionally, poor blood flow in the bone and slow healing process increase complication of this disease and Due to these, local implantation of antibiotic is commonly applied during the surgery for extended period of time to optimize healing rate (Barth et al. 2011). However, current available antibiotic bead for implant are very expensive and requires another minor surgery to remove the non-biodegradable beads. Indeed, there is also a possibility of biofilm growth on the surface of the antibiotic beads (Ciampolini & Harding 2000). Due to complicated treatment of osteomyelitis, it decreases patient quality of life.

The use of antibiotic alone is insufficient and not suitable for biofilm-producing bacteria. Combination with natural products might produce better outcome. Plants-derived antibacterial are source for new therapeutic approach. In the nature, infrequency of infective diseases in wild plants is an indication of the successful defence mechanism that is possess by the plant (Hemaiswarya et al. 2008). Plants are also containing many of active compounds that are useful for therapeutic application. The antibiotic combination is effective as the treatment because it has different mechanism of actions, enhance antibiotic effects, neutralise resistance, suppress antibiotic-induce toxicity and low degradation (Chait et al. 2011). Thus, it is beneficial as therapy in terms of expand antimicrobial spectrum, effective at low dosage with low toxicity, and also reduce incidence of bacteria resistant (Chanda & Rakholiya 2011).