UNIVERSITI PUTRA MALAYSIA

EVALUATION OF OSTEOGENIC POTENTIAL OF DEMINERALIZED BONE MATRIX IN PIGEON (Columba livia Gmelin)

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BY

MOHAMAD REZA SANAEI

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

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DEDICATION

I dedicate this thesis to my parents who never failed to provide me with financial and moral support, for their love which never stopped and to my beloved wife for her graceful patience.

I would also like to dedicate this work to all those lovable birds whose lives were sacrificed in hope to make our science a better means of recruiting their species. May this work fulfill its requirements to compensate for a small portion of what they lost.

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

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Chairman: **Jalila Abu, PhD**

Faculty: **Faculty of Veterinary Medicine**

Bone fractures are among the most frequent tragedies crippling a large number of birds every year. Unfortunately, many of these birds will not be able to get back to the wild or have to be euthanized due to a wide range of complications. Despite the dearth of cancellous bone in avian species, there are currently no reliable alternatives to autologous bone grafting techniques available. Thus this study was undertaken to evaluate implants of demineralized bone matrix (DBM) in a pigeon model. In order to do so, DBM was evaluated in both heterotopic and orthotopic sites.

In heterotopic study, two forms of allogenic DBM comprised of tubular DBM (TDBM) and chipped DBM (CDBM) were implanted bilaterally into the superficial pectoral muscles in 24 pigeons (*Columba livia*). Autologous bone marrow which is known to produce ectopic bone in mammals was implanted in a third site. A fourth intramuscular site served as a negative control and was irrigated with normal saline before closure. Birds were euthanized in batches at 1, 4, 6, 8, 10, and 12 weeks following implantations to histologically evaluate the fate of implants. Unlike previous studies in mammalian models, bone marrow failed to produce *de novo* bone formation in this pigeon model. Instead, statistical analysis of semi-quantitative and quantitative histologic data revealed a significant difference from controls in the extent of neovascularization in favor of the bone marrow after 6 and 10 weeks. All DBM explants showed new bone at retrieval with the exception of tubular implants at first week. The most reactive part of the implants was found to be their interior area between the periosteal and endosteal shells followed by the area at the implant-muscle interface. Nevertheless the latter was mostly associated with the DBM chips. On the other hand, quantitative measurements showed the overall amount of new bone to be greater at the TDBM implant sites (80.28% ± 8.94) and chipped DBM showed to be significantly inferior in this regard (57.64% ± 3.12).

In the second experiment, osseous critical sized defects were surgically created in the ulnar midshafts of 60 adult pigeons in which TDBM or CDBM implants served to augment the healing process. Autologous bone graft (ABG), harvested from the sternal keel was used in a third control group. Created fractures were stabilized using an external skeletal fixator-intramedullary tie-in device. Results were evaluated by radiography and histology after 4, 8, 12 and 24 weeks postoperatively. Although ABG group demonstrated greater improvement in comparison to DBM groups early in the course of this study, they failed to advance after the eighth postoperative week with the overall radiographic score of 2.95 ± 0.15, histological score of 6.87 ± 0.44 and bone area of 68.41 ± 3.79. Conversely, both DBM groups and particularly TDBM demonstrated promise with regards to their continuous improvement over time. Nevertheless, CDBM proved to be inferior to TDBM in overall healing qualities with overall radiographic score of 1.5 ± 0.11, histological score of 5.37 ± 0.32 and bone area of 41.32 ± 3.83. Despite the early dominance of ABG, TDBM finally overtook autografts after week 12 and such was proved to be significant by 24 postoperative week (2 fractures fully healed versus only one complete healing for ABG). However the overall average of values for this group remained lower than that of the ABG (radiographic score of 2.40 ± 0.21, histological score of 8.07 ± 0.77 and bone area of 59.3 ± 7.47).

This study showed that avian DBM is an osteoinductive, biocompatible, biodegradable, safe and osteoconductive material in pigeons. Results from the orthotopic study showed promise as TDBM implants appeared as efficacious as autografts in the healing of osseous critical sized defects. This confirmed that implant geometry (shape and size) could affect DBM associated osteogenesis in avian species. We suggested osteoconduction augments the magnitude of the effects in larger implants.

Keywords: Pigeon, bone graft, demineralized bone matrix, implant geometry

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

PENILAIAN POTENSI OSTEOGENIK DINYAHMINERAL TULANG MATRIKS DALAM MERPATI (*Columba livia* Gmelin)

Oleh

MOHAMADREZA SANAEI

Disember 2011

Pengerusi: **Dr. Jalila Abu, PhD**

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Tulang patah adalah tragedi yang paling kerap dalam jumlah yang besar dalam burung setiap tahun. Malangnya, kebanyakan daripada burung ini tidak berupaya untuk kembali ke alam semulajadi dan akan dieutnasia kerana komplikasi yang pelbagai. Di sebalik kekurangan tulang cancellous di dalam species burung, tiada alternatif kepada teknik graf tulang autologus. Oleh itu kajian ini dijalankan untuk menilai implan dinyahmineral tulang matriks (DBM) dalam model merpati. Dalam usaha untuk berbuat demikian, DBM dinilai dalam kedua-dua laman heterotopik dan orthotopik.

Dalam kajian heterotopik, dua bentuk DBM allogenik terdiri daripada tiub DBM (TDBM) dan sumbing DBM (CDBM) diimplan dua hala ke otot pectoral luar dalam 24 merpati (Columba livia). Sumsum tulang autologous yang dikenali untuk menghasilkan tulang ektopik dalam mamalia telah ditanam pada tapak ketiga. Laman intramuskular keempat berkhidmat sebagai kawalan negatif dan diairi dengan salina normal sebelum penutupan. Burung akan dieutanasia dalam kelompok pada 1, 4, 6, 8, 10, dan 12 minggu selepas implantasi untuk menilai nasib implan secara histologi. Berbeza dengan kajian sebelumnya dalam model mamalia, sumsum tulang gagal untuk menghasilkan *de novo* tulang pembentukan dalam model merpati ini. Sebaliknya, analisis statistik data semi-kuantitatif dan kuantitatif histologik mendedahkan perbezaan yang signifikan daripada kawalan setakat neovascularization memihak kepada sumsum tulang selepas 6 dan 10 minggu. Semua explants DBM menunjukkan tulang yang baru boleh diperolehi semula kecuali implan tiub pada minggu pertama. Bahagian yang paling reaktif daripada implan adalah di dapati kawasan pedalaman mereka antara cengkerang periosteal dan endosteal yang diikuti oleh kawasan di antara muka implan-otot. Walau bagaimanapun, kedua kebanyakannya dikaitkan dengan DBM sumbing. Sebaliknya, pengukuran kuantitatif menunjukkan jumlah keseluruhan tulang baru yang lebih besar ditapak implan TDBM (80.28% ± 8.94) dan DBM sumbing menunjukkan ketara lebih rendah dalam hal ini (57.64% ± 3.12).

Dalam eksperimen kedua, pembedahan tulang kecacatan saiz kritikal pembedahan dicipta dalam midshafts ulnar pada 60 merpati dewasa di mana implan TDBM atau CDBM berkhidmat untuk menambah proses penyembuhan. Graf tulang autologous (ABG), dituai dari terjungkir sternal telah digunakan dalam kumpulan kawalan ketiga. Tulang patah yang dibuat distabil menggunakan luar rangka penetap intramuskular-ikat di dalam peranti. Keputusan telah dinilai oleh radiografi dan histologi selepas 4, 8, 12 dan 24 minggu pasca operatif. Walaupun kumpulan ABG menunjukkan peningkatan yang lebih besar berbanding dengan kumpulan DBM di awal kajian ini, mereka gagal untuk memajukan selepas minggu pasca operatif kelapan dengan skor keseluruhan radiografik 2.95 ± 0.15, skor histologi 6.87 ± 0.44 dan kawasan tulang 68.41 ± 3.79. Sebaliknya, kedua-dua kumpulan DBM dan terutamanya TDBM menunjukkan janji berhubung dengan penambahbaikan yang berterusan dari semasa ke semasa. Walau bagaimanapun, CDBM terbukti lebih rendah daripada TDBM dalam kualiti penyembuhan keseluruhan dengan markah keseluruhan radiografik sebanyak 1.5 ± 0.11, skor histologi 5.37 ± 0.32 dan kawasan tulang 41.32 ± 3.83. Walaupun penguasaan awal ABG, TDBM akhirnya mendahului autograf selepas 12 minggu dan itu dibuktikan dengan ketara pada 24 minggu pasca operatif (2 jurang tulang sepenuhnya sembuh berbanding hanya satu penyembuh ABG). Walau bagaimanapun, purata keseluruhan nilai untuk kumpulan ini kekal lebih rendah daripada-ABG (radiografik skor 2.40 ± 0.21, skor histologi 8.07 ± 0.77 dan kawasan tulang 59.3 ± 7.47).

Kajian ini menunjukkan bahawa DBM burung osteoinduktif, bioserosi, terbiodegradasi, bahan yang selamat dan osteokonduktif dalam burung merpati. Hasil dari kajian yang orthotopik menunjukkan janji dimana implan TDBM muncul berkesan sebagai autograf dalam penyembuhan kecacatan bersaiz kritikal yang berhubung dengan tulang. Ini mengesahkan bahawa geometri implan (bentuk dan saiz) boleh menjejaskan DBM dikaitkan osteogenesis dalam spesies burung. Kami mencadangkan menambah osteokonduksi kesan magnitud dalam implan yang lebih besar.

Katakunci: Merpati, Burung graf tulang, DinyahmineralL tulang matriks, implan geometri

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APPROVAL SHEET NO. 1

I certify that an Examination Committee has met on **December, 06, 2011** to conduct the final examination of **Mohamad Reza Sanaei** on his **MVSc** thesis entitled "**Evaluation of Osteogenic Potentials of Demineralized Bone Matrix in Pigeons**" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirement for the degree of Master of Veterinary Science (MVSc). The members of the Supervisory Committee were as follows:

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Declaration Form

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 it is not concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

MOHAMADREZA SANAEI

Date: 6 December, 2011

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List of Abbreviations/Notations/Glossary of Terms

ABG: autologous bone graft

Biocompatibility: the property of being biologically compatible by not producing a toxic, injurious, or immunological response in living tissue.

Bone morphogenesis: the developmental cascade of skeletal pattern formation, establishment of mirror-image bilateral symmetry, initiation and promotion of endochondral bone differentiation, and growth culminating in functional weight bearing.

CDBM: chipped demineralized bone matrix

CSD: critical sized defect; a defect in tissue continuity that will not heal spontaneously.

DBM: demineralized bone matrix

***De novo* bone formation:** refers to the process of bone formation from its precursors in tissues other than bone.

Graft rejection: the immunologic destruction of transplanted organs or tissues.

Graft: any live tissue or organ for transplantation.

Heterotopic: occurring in an abnormal or unusual anatomic location.

Implant : an inanimate biomaterial for transplantation.

µm: micrometer

mm: millimeter

Orthotopic: occurring in the normal or usual anatomic location.

Osteoconduction: a process in which new bone is formed from local osseous precursors along a framework or scaffold.

Osteogenesis: bone histogenesis regardless of the mechanism involved; formation of new bone from cellular elements originated from the graft material.

Osteoinduction: a process in which new bone is formed through induction of pleuripotential stem cells and by their transformation into osseous precursor cells.

Osteointegration: the formation of a direct interface between an orthopedic or dental implant and bone, without intervening soft tissue.

TDBM: tubular demineralized bone matrix

CHAPTER 1

INTRODUCTION

While most long bone fractures lend themselves well to routine surgical interventions, some fail to be anatomically reconstructed as in the case of neoplasias and highly comminuted fractures. This fact could be appreciated not only in the mentioned circumstances, but also in instances where extensive soft tissue trauma or complications such as non-unions require surgical intervention. In some other instances, the surgeon may anticipate delayed healings or even non-unions based on the clinical presentation. Hence, it deems necessary to search for new ways of enhancing the speed of healing and preventing undue fatigue of the orthopedic implants. Additionally, the most important goal in orthopedic surgery, resuming the function, is often compromised in case of prolonged healing which should be addressed alike. This is particularly important in avian orthopedics where flight capabilities are to be restored as much as possible.

Unfortunately being a relatively young science, avian bone grafting has not received proper attention in the previous decades and only a few studies have been carried out to evaluate the contemporary methods used in other species. Autologous bone grafting techniques are described in avian species which comprises harvesting corticocancellous chips from the sternal keel[1, 2], the last two ribs[3] or the old callus (if large enough) in case of more chronic fractures.[4] In addition to the drawbacks generally ascribed to autologous bone grafting[5-7], there are even more when it comes to the avian patient which might include very little volume of bone available and substantial morbidity.[8-10] Moreover, increased anesthesia time, trauma, and pain may greatly endanger the life of the sensitive avian patient. In mammals, numerous bone substitutes and techniques are recommended to avoid such complications; however, being different in biology and structure, avian bone renders many of these ineffective or subjected to insufficient merits to apply which prompts the need for further investigations.[8, 11] Historically, allogenic and xenogenic sources have always been the primary interest for such purpose. However complications such as immunogenic reactions and transmission of infectious diseases have always plagued their versatility in clinical circumstances. To overcome such concerns various tissue processing methods have been suggested.[12, 13] Two common methods include treating the bones with high temperatures or decalcification in acids. The former is said to remove the organic matrix and preserve the three-dimensional mineral structure and thus is osteoconductive.[14] Conversely, demineralization in acids leaves the matrix intact and somehow contributes to a better availability of structural growth factors such as bone morphogenetic proteins (BMPs) and thus the quality of osteoinduction while preserving osteoconductivity.[15-21] Therefore, these biomaterials could induce bone even in heterotopic beds such as muscle or subcutaneous tissue. Such processing is inexpensive and requires no sophisticated facilities and therefore could be easily used in avian practice. However preliminary works in pigeons suggest that heterotopic implantation of allogenic and xenogenic demineralized bone matrix (DBM) in muscular beds (pectoral muscles) may not induce new bone as the results had not been consistent and in few that osteogenesis had occurred, only trivial amounts of new bone could have been detected.[22] Later orthotopic studies, provided proof that this bone graft might be able to induce osteogenesis via endochondral and intramembranous ossification as seen in other mammalian species.[23, 24] We attributed this to the substantial impact of preparation technique, the size of the implants or the time points at which evaluations were made as incriminated in other studies.[25-31] Hence this study was undertaken to reevaluate this material by using a different preparation technique and using two different implant sizes in relatively longer time frames. As it is not yet clear whether birds are capable of developing heterotopic bone, autologous bone marrow which is proved to be osteogenic in such beds, served as the control for the first set of experiments. However as the outcome turned out to be unpredictably different, this control element will be discussed separately in Chapter three.

1.1 Hypotheses

(1) If avian autologous bone marrow is osteogenic and/or vasogenic, then its implantation in heterotopic beds will result in *de novo* bone formation and/or an increased rate of vasculogenesis when compared to non-implanted control sites.

(2) If avian allogenic DBM, is osteoinductive, then it will induce *de novo* bone formation following implantation in heterotopic beds.

(3) If the heterotopic osteogenicity of DBM implants is related to implant geometry, then there will be a difference between the tubular and chipped types of implants in the extent of heterotopic osteogenesis.

(4) If avian allogenic DBM is capable of promoting orthotopic osteogenesis, then using avian DBM as a means of bone grafting in osseous critical-sized defects will be comparable in quality and speed of healing to that of autologous bone grafting.

(5) If the orthotopic osteogenicity of DBM implants is related to implant geometry, then there will be a difference between the tubular and chipped types of implants in the quality and speed of healing when grafted into osseous critical-sized defects.

1.2 Objectives

(1) Determine if implantation of avian autologous bone marrow results in *de novo* bone formation in heterotopic sites by radiographic examination.

(2) Determine if implantation of avian autologous bone marrow results in *de novo* bone formation and/or an increased rate of vasculogenesis in heterotopic sites by histological and histomorphometric evaluations.

(3) Determine if avian allogenic DBM induces *de novo* bone formation in heterotopic sites by radiographic examination.

(4) Determine if avian DBM induces *de novo* bone formation in heterotopic sites by histological evaluation.

(5) Compare the heterotopic osteogenicity of tubular and chipped type DBM implants by radiographic examination.

(6) Compare the heterotopic osteogenicity of tubular and chipped type DBM implants by histological and histomorphometric evaluations.

(7) Compare the osteogenicity of tubular DBM, chipped DBM and autologous bone graft in the healing of osseous critical-sized defects by radiographic examination.

(8) Compare the osteogenicity of tubular DBM, chipped DBM and autologous bone graft in the healing of osseous critical-sized defects by histological and histomorphometric evaluations.

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