UNIVERSITI PUTRA MALAYSIA

HEALING OF SURGICALLY CREATED ULNA DEFECT TREATED WITH BONE GRAFT SUBSTITUTES IN A PIGEON MODEL

TUNIO AHMED

FPV 2013 14

HEALING OF SURGICALLY CREATED ULNA DEFECT TREATED WITH BONE GRAFT SUBSTITUTES IN A PIGEON MODEL

TUNIO AHMED

DOCTOR OF PHILOSOPHY
UNIVERSITI PUTRA MALAYSIA

2013

HEALING OF SURGICALLY CREATED ULNA DEFECT TREATED WITH BONE GRAFT SUBSTITUTES IN A PIGEON MODEL

By

TUNIO AHMED

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

December 2013

DEDICATION

I would like to dedicate this study with love and gratitude

To,

My Father and Mother, My Wife Shazia Parveen Tunio, My son Fahad, Faraz and daughter Zainab, My sisters and brothers for their prayer and moral support during my PhD study at Universiti Putra Malaysia.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

HEALING OF SURGICALLY CREATED ULNA DEFECT TREATED WITH BONE GRAFT SUBSTITUTES IN A PIGEON MODEL

By
TUNIO AHMED

December 2013

Chair: **Assoc Professor Jalila Abu, PhD**
Faculty: **Veterinary Medicine**

The use of demineralized bone matrix (DBM) avian source either in the form of powder, chip, or tubular in experimental studies on pigeon models has proven to be osteoinductive. Fracture is a break in the continuity of the bone and comminuted fractures are commonly observed in birds. In case of bone loss due to fracture, ultimately to bring the broken bones to its normal function is needed for the survival of birds. Pigeons (*Columba livia*) with a mean body weight of 283.76±17.53g were used in this study. Eighty-one pigeons were divided into Study-1 and Study-2. In Study-1, 16 and in Study-2, 65 birds were used. Extra four pigeons were sacrificed for DBM preparation for critical sized defect (CSD) healing. The hypothesis of Study-1 was that the external skeletal fixators (ESF) would stabilize the non-critical sized defect (NCSD) ulna fracture and thus leads to clinical union in a pigeon model. Therefore, the objective of this study was to evaluate the speed of the fracture healing, and quality of the union by radiology, histology and biomechanical examination in a NCSD ulna fracture stabilized with ESF in a pigeon model. In Study-1, NCSD was created in left ulna and the fracture was fixed with ESF. Sixteen birds in this study were divided into three groups. Group 1 (n=4) for 3 weeks, Group 2 (n=4) for 6 weeks and Group 3(n=8) for 12 weeks. The bone healing in these birds were evaluated through radiological, histological and biomechanical examinations. After euthanasia at each end-point, healed specimens were dissected and fixed in 10% formalin for histological examination. The hypothesis of Study-2 was that combination of osteoinductive (DBM) and osteoconductive [Bio-Oss and hydroxyapatite (HA)] biomaterials would hasten osteogenesis and clinical union in critical sized defect (CSD) in pigeon ulna. Therefore, the objective study-2 was to monitor the radiographic, histologic and biomechanical healing progress as a result of the application of osteoinductive (DBM) and osteoconductive (Bio-Oss or HA) bone grafts implanted in CSD and stabilized with ESF in a pigeon ulna. Sixty-five birds were divided into five groups namely Group 1 (DBM), Group 2 (Bio-Oss®), Group 3 (HA), Group 4 (DBM+Bio-Oss®) and Group 5 (DBM+HA). A 1 cm CSD was created at the mid-shaft of the left ulna and defects were treated as per design and stabilized with ESF. All the experiments were performed under Isoflurane inhalation anaesthesia. After euthanasia of birds at each end-point of the experiment, the grafted sites were removed.

The specimens (n=16) were evaluated by radiography, histology and biomechanics. The specimens were fixed in 10% neutral buffered formalin and decalcified in 5% formic acid for histological purpose. Results of the radiographic assessment in Study 1 showed clinical union at week 3 in 50% of the birds. At 6 and 12 weeks, all the pigeons showed good clinical union (100%) and less callus formation or primary bone union. Histological assessment on the NCSD at 3 weeks revealed bridging of callus and bone union of the callus in all of the birds except for 2 birds, where only the trabecular bone was formed. At 6 and 12 weeks, there was minimal callus, good alignment and excellent clinical union of the bone. A 3-point bending test at 12 weeks of the NCSD healed ulna (n=4) showed, maximum flexure load and the flexure stress at the maximum load were significantly (p<0.05) higher in the intact right ulna (n=4) compared to the left ulna (n=4), while the flexure stress and the flexure strain at the break were not significantly different (p>0.05). External skeletal fixators provided good mechanical strength to the operated ulna in all groups and can be used for further bone graft experiments. From Study-1, it can be concluded that bone healing proceeds more rapidly if bird fracture fixed with ESF and could be useful for avian clinical fracture management.

In study-2, radiographic results of CSD treated with DBM (Group 1), observed at 3 and 6 weeks, showed no significant (P>0.05) difference in CSD healing in all birds. At 12 weeks only 50% birds showed clinical union (n=4/4). Using DBM alone showed good cortical union at week 12. In the Bio-Oss® (Group 2) at week 3 there was no significant (P>0.05) difference was observed within the group; but at week 6, there was some evidence of periosteal callus. At week 12 Bio-Oss® showed a significant difference (P<0.05) compared to 3 and 6 weeks. In HA (Group 3) after 3 and 6 weeks of graft healing, radiographs showed no significant difference (P>0.05) was observed, but there was a significant (P<0.05) difference in CSD healing at week 12 with HA graft. Fifty percent of the CSD healing occurred at week 12. In DBM®+Bio-Oss (Group 4), after 3 weeks, healing there was no significant (P>0.05) bone formation. After 6 weeks, CSDs were completely filled with radiodense material but there was no new bone formation with this combination. At week 12 there was improved bone graft healing but it was no difference when the same bone grafts were used alone. Demineralised bone matrix + Bio-Oss® did not lead to faster clinical union. In DBM+HA (Group 5), there was no change in the defect healing at 3 and 6 weeks post-grafting. At 12wks there was CSD healing and radiodensity, but no clinical union.

Birds were sacrificed at each end-point using 0.3 ml of Pentobarbital (Lure Cedex, France) and the implant sites were dissected and processed for histology. Histological results of CSD healing with DBM showed formation of fibrous connective tissue and there was no significant (P>0.05) difference or similarly at week 6. Critical skeletal defect healed with fibrocartilage union and formation of new bone next to the bone graft. At week 12 of DBM healing, few sections revealed a cartilage area and some bone formation in the matrix, and mesenchymal cells had developed on the surface. With Bio-Oss® at 3 weeks of healing no bone formation was seen. After 6 weeks of healing, birds showed that 100% of the CSDs were filled with Bio-Oss® and formed trabecular bone and small new bones. After 12 weeks, woven bone formation was observed.

In HA group at 3 and 6 weeks, the graft was encapsulated with fibrous and immature bone. After 12 weeks there was early bone ingrowth. Treatment with DBM+Bio-Oss® showed little cortical development at 3 and 6 weeks. However, at week 12 good graft incorporation with the host bone occurred, but no clinical union. Demineralized bone matrix + HA treatment produced healing with fibrous connective tissue and there was very little graft incorporation at week 3. At week 6 there was no clinical union, but at week 12 the bone grafts showed excellent graft incorporation in the CSD. Healed ulna CSD with bone grafts at week 12 did not shown clinical union in all groups and all the grafted sites were too unstable to proceed with a biomechanical evaluation.

In conclusion, ESF is the best choice for NCSD fracture fixation and provided excellent clinical union in pigeon ulna. The application of ESF is effective and easy to fix in cases of ulna fractures in birds and can be used for bone graft repair studies. Results based on the radiological evaluation showed that the combination of DBM with Bio-Oss® as well as with HA did not hasten osteogenesis or produce faster clinical union in CSD healing in pigeon ulna. Results from histological evaluation showed that the addition of Bio-Oss® and HA to DBM enhanced the tendency of bone healing in the pigeon models. This study also confirmed that ESF is crucial to attain CSD healing along with bone grafts as it had stabilized the ulna bone in birds. This study showed that DBM alone provided bone union and there was evident callus formation in the CSD at 12 week post-grafting in pigeon ulna. Demineralized bone matrix (DBM) implant could be beneficial for faster fracture healing in birds since it contains bone morphogentic proteins (BMPs).

Keywords: Pigeon, NCSD, CSD, DBM, Bio-Oss®, HA, ESF, clinical union.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENYEMBUHAN KECACATAN YANG DIWUJUDKAN SECARA PEMBEDAHAN SELEPAS DIRAWAT DENGAN GANTIAN CANTUMAN TULANG DALAM MODEL BURUNG MERPATI

Oleh

TUNIO AHMED

Disember 2013

Pengerusi: Profesor Madya Jalila Abu, PhD

Fakulti : Perubatan Veterinar

Penggunaan sumber matriks tulang ternyah mineral (DBM) avian sama ada dalam bentuk serbuk, cip, atau tubul dalam kajian mengguna model burung merpati telah terbukti osteoindusif. Fraktura dalah tulang patah renggang dan fraktur remuk biasa dilihat pada unggas. Dalam kes di mana berlaku kelesapan tulang kerana fraktur, mengembalikan tulang patah untuk berfungsi normal adalah perlu untuk kemandirian unggas. Burung merpati (*Columba livia*) dengan berat badan min 283.76±17.53 gram telah guna dalam kajian ini. Lapan puluh satu ekor burung merpati telah diagihkan kepada Kajian 1 dan Kajian 2. 16 ekor burung diguna dalam Kajian 1 dan 65 ekor dalam Kajian 2. Empat ekor burung lagi telah disembelih untuk persediaan DBM untuk penyembuhan kecacatan saiz kritikal (CSD). Hipotesis Kajian 1 ialah penetap rangka luaran (ESF) boleh menstabilkan fraktur ulna kecacatan saiz bukan kritikal (NCSD) dan membawa kepada penyatuan klinikal dalam model burung merpati. Justeru itu objektif kajian ini ialah untuk menilai kepantasan penyembuhan fraktur, dan mutu penyatuan melalui radiologi, histologi dan permeriksaan biomekanikal pada fraktur ulna NCSD yang distabilkan dengan ESF dalam model burung merpati. Dalam Kajian 1, NCSD telah diwujudkan pada ulna kiri dan fraktur ini ditetapkan dengan ESF. Enam belas ekor burung dalam kajian ini dibahagikan kepada tiga kumpulan. Kumpulan 1 (n=4) diperlaku selama 3 minggu, Kumpulan 2 (n=4) selama 6 minggu dan Kumpulan 3 (n=8) selama 12 minggu. Penyembuhan tulang dalam burung ini dinilai melalui radiologi, histologi and pemeriksaan biomekanikal. Pada setiap penghujung kajian, spesimen yang sembuh di disek dan ditetapkan dalam 10% formalin untuk pemeriksaan histologi. Hipotesis Kajian 2 ialah gabungan bio bahan osteoinduktif (DBM) dan osteokondutif [Bio-Oss® and hydroxyapatite (HA)] akan mempercepatkan osteogenesis dan penyatuan klinikal pada ulna kecacatan saiz kritikal burung merpati. Justeru itu, objektif Kajian 2 ialah untuk memantau secara radiografi, histologi dan biomekanikal kemajuan penyembuhan hasil daripada penggunaan cantuman tulang osteoinduktif (DBM) dan osteokonduktif (Bio-Oss® atau HA) yang dimplankan pada ulna burung merpati CSD dan distabilkan dengan ESF. Enam puluh lima (n=65) ekor burung telah dibahagikan kepada lima kumpulan iaitu Kumpulan 1 (DBM), Kumpulan 2 (Bio-Oss®), Kumpulan 3 (HA), Kumpulan 4 (DBM + Bio-Oss®), and Kumpulan 5 (DBM + HA). Satu CSD 1cm diwujudkan pada

pertengahan batang tulang ulna kiri dan kecacatan dirawat seperti yang telah direka bentuk dan distabilkan dengan ESF. Kesemua ujikaji dilakukan di bawah anestesia sedutan isofluran. Selepas eutanasia burung pada titik hujung ujikaji, tapak yang dicantum dikeluarkan. Spesimen (n=12) dinilai melalui radiografi, histologi, dan biomekanik. Kemudian spesimen ditetapkan dengan 10% formalin tertimbal neutral dan dinyah kalsium dengan 5% asid formik untuk tujuan histologi. Hasil penilaian radiografi dalam Kajian 1 menunjukkan penyatuan klinikal pada minggu ketiga dalam 50% daripada burung. Pada minggu 6 dan 12, kesemua (100%) burung menunjukkan penyatuan klinikal baik, sambil kurang pembentukan kalus atau penyatuan tulang primer. Penilaian histologi terhadap DCSD pada minggu 3 menunjukkan berlakunya penyambungan kalus dan penyatuan tulang pada kalus dalam kesemua burung kecuali 2, dimana hanya tulang trabekular terbentuk. Pada minggu 6 dan 12 kalus terbentuk secara minimum, penjajaran baik dan penyatuan klinikal yang unggul pada tulang. Ujian lentur 3 tempat pada ulna NCSD yang sudah sembuh menunjukkan beban lentur dan tekanan lentur maksimum pada beban maksimum adalah lebih tinggi tererti ($P<0.05$) pada ulna kanan utuh berbanding ulna kanan, sambil tekanan lentur dan terikan lentur pada tempat patah tidak beza secara tererti ($P<0.05$). Penetap rangka luaran membekal kekuatan mekanikal baik kepada ulna yang dibedah dalam kesemua kumpulan dan boleh diguna untuk ujikaji cantuman tulang akan datang. Daripada Kajian 1, kesimpulan boleh dibuat yang penyembuhan tulang berlaku dengan lebih pantas jika burung ditetapkan dengan ESF and ini paling berguna untuk pengurusan fraktur klinikal avian.

Dalam Kajian 2, hasil radiografi CSD yang dirawat dengan DBM (Kumpulan 1) yang dilihat pada minggu 3 dan 6, menunjukkan tiada perbezaan tererti ($P>0.05$) dalam penyembuhan CSD dalam semua burung. Pada minggu 12 hanya 50% burung menunjukkan penyatuan klinikal (n=4/4). Mengguna DBM sahaja menunjukkan penyatuan korteks baik pada minggu 12. Dalam kumpulan Bio-Oss®, pada minggu 3, tiada perbezaan tererti ($P>0.05$) yang dilihat di kalangan kumpulan; tetapi pada minggu 6 ada bukti yang berlakunya pembentuk kalus periosteum. Pada minggu 12, Bio-Oss® menunjukkan perbezaan tererti ($P<0.05$) berbanding pada minggu 3 dan 6. Dalam kumpulan HA selepas 3 dan 6 minggu penyembuhan cantuman, radiograf menunjukkan tiada perbezaan tererti ($P>0.05$) yang dilihat, bagaimanapun ada perbezaan tererti ($P<0.05$) dalam penyembuhan CSD dengan cantuman HA. Lima puluh peratus daripada penyembuhan CSD berlaku pada minggu 12. Dalam kumpulan DBM + Bio-Oss® selepas penyembuhan selama 3 minggu, tidak berlaku pembentukan tulang yang tererti ($P>0.05$). Selepas 6 minggu, CSD keseluruhannya dipenuhi dengan bahan radio tumpat tetapi tidak berlaku pembentukan tulang baharu dengan penggunaan gabungan ini. Pada minggu 12 berlaku penyembuhan cantuman tulang yang lebih baik tetapi tidak ada perbezaan apabila cantuman tulang sama diguna secara bersendirian. Matriks tulang ternyah mineral + Bio-Oss® tidak membawa kepada penyatuan klinikal yang lebih pantas. Dalam kumpulan DBM + HA tidak berlaku perubahan dalam penyembuhan kecacatan pada minggu 3 dan 6 pasca-cantuman. Pada minggu 12 penyembuhan CSD dan keradio tumpatan dilihat, tetapi penyatuan klinikal tidak berlaku.

Burung yang sembelih pada setiap titik penghujung dengan menguna 0.3 ml Pentobarbital (LureCedex, France) dan tapak implan di disek dan diproses untuk histologi. Hasil histologi untuk penyembuhan CSD dengan DBM menunjukkan pembentukan tisu penyambung bergentian dan tiada perbezaan tererti (P>0.05) atau persamaan pada minggu 6. Kecacatan rangka kritikal sembuh dengan penyatuan fibro rawan dan pembentukan tulang baharu di sebelah cantuman tulang. Padaminggu 12 penyembuhan DBM, beberapa bahagian menunjukkan kawasan rawan dan sedikit pembentukan tulang dalam matriks, dan sel mesenkima telah berkembang pada permukaannya. Dengan mengguna Bio-Oss® pada minggu 3 penyembuhan, pembentukan tulang tidak berlaku. Selepas 6 minggu penyembuhan, burung menunjukkan yang 100% CSD dipenuhi dengan Bio-Oss® dan membentuk tulang trabekular dan tulang kecil baharu. Selepas 12 minggu, pembentukan tulang jalinan dapat dilihat. Dalam Kumpulan HA pada minggu 3 dan 6, cantuman disaluti dengan tulang bergentian tidak matang. Selepas 12 minggu berlaku penumbuhan tulang ke dalam.

Rawatan dengan DBM + Bio-Oss® menunjukkan sedikit perkembangan korteks pada minggu 3 dan 6. Bagaimanapun, pada minggu 12 penggabungan cantuman dengan tulang perumah baik berlaku, tetapi tanpa penyatuan klinikal. Matriks tulang ternyah mineral + HA menghasil penyembuhan dengan tisu penyambung bergentian dan tidak banyak berlaku penggabungan cantuman pada minggu 3. Pada minggu 6, tiada penyatuan klinikal berlaku, tetapa pada minggu 12 cantuman tulang menunjukkan penggabungan cantuman yang unggul dalam CSD. Kecacatan rangka kritikal ulna sembuh dengan cantuman tulang pada minggu 12 tidak menunjukkan penyatuan klinikal dalam kesemua kumpulan dan semua tapak cantuman adalah terlalu tidak stabil untuk penilaian biomekanikal dilakukan.

Kesimpulannya, ESF adalah pilihan terbaik untuk penetapan fraktur NCSD dan membekalkan penyatuan klinikal unggul pada ulna burung merpati. Penggunaan ESF adalah berkesan dan mudah untuk diperbaiki dalam kes fraktur ulna pada unggas dan boleh diguna untuk kajian pembaik pulih cantuman tulang. Hasil berasaskan penilaian radiologi menunjukkan yang penggabungan DBM dengan Bio-Oss® dan juga dengan HA tidak mempercepatkan osteogenesis atau menghasilkan penyatuan klinikal lebih pantas dalam penyembuhan CSD pada ulna burung merpati. Hasil daripada penilaian histologi menunjukkan yang penambahan Bio-Oss® dan HA kepada DBM meningkatkan kecenderungan kepada penyembuhan tulang dalam model burung merpati. Kajian ini juga mengesahkan yang ESF itu adalah kritikal untuk mencapai penyembuhan CSD bersama cantuman tulang, oleh sebab ia dapat menstabilkan tulang ulna pada burung. Kajian ini menunjukkan DBM secara bersendirian boleh menyebabkan penyatuan tulang dan bukti telah menunjukkan yang pembentukan kalus berlaku pada minggu 12 pasca-cantuman pada tulang ulna burung merpati. Implan DBM bermanfaat dalam mempercepatkan penyembuhan fraktur dalam unggas kerana ianya mengandungi tulang morphogenic proteins (BMPs).

Kata kunci: burung merpati, NCSD, CSD, DBM, Bio-Oss®, HA, ESF, penyatuan klinikal

ACKNOWLEDGEMENTS

First of all, I am immensely grateful to Almighty “Allah” for His blessings in giving me the strength and encouragement to conduct this research and everything I have.

I would like to express my special and heartiest appreciation to my main supervisor, Assoc. Prof. Dr Jalila Abu, Head Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, UPM Malaysia, for her kindness in accepting me as a graduate student, for advising me to do research on bone graft study on birds and for her encouraging guidance throughout my research. I also thank her for the financial support for this study, and for her help in writing this thesis and for teaching me about bird clinical approaches.

I would also specially like to thank my other committee members for their valuable support. My thanks goes to my co-supervisor, Assoc. Prof Dr Goh Yon Meng, Head of the Department of Pre-clinical Studies, Faculty of Veterinary Medicine, UPM, for his excellent feedback and suggestions, and who taught me statistics. All my deepest appreciation is to Dr Intan Shameha, another co-supervisor who actively helped to evaluate the histological slides, for her excellent supervision and encouragement. I also offer my sincere thanks to Assoc. Professor Shanthi Ganabadi, for her kind supervision.

My sincere gratitude goes to Dr. A. Q. Mughal, V.C, S.A.U.Tandojam, Pakistan, for his great efforts in this study and for appointing me as Assistant Professor during my PhD study at UPM Malaysia. Many thanks to Lt. Gen. (R) Moinudin Haider, the ex-Governor of Sindh, Pakistan for his sincere diplomatic help in my admission at UPM.

I would like to express my gratefulness to Dr. I.H. Kathio, DVM, Pittston Animal Hospital, USA for sending me the research material and other financial support to complete this study. Great thanks to Professor Dr A. B. Kalhoro, ex Dean, Faculty of Veterinary Medicine, SAU Tandojam. Many thanks to all staff and colleagues in Fac, Vet. Med.SAU Tandojam.

Also I offer my grateful thanks to Datin Paduka Prof. Dr. Ani Ideris for her great suggestions in my study. Thanks go to Prof. Dr. M. Hair Bejo, for his permission to use the serology lab for the histological examinations. I am also thankful to Associate Professor Dr. Siti Khariani and Associate Professor Dr. Zunita Zakaria, for her permission to conduct the lyophilisation of DBM in microbiology Lab. I would like to show my thanks to Dr Malika Wanitabe for her financial support for my PhD study. My appreciation goes to the Lab Staff; En. Usman, En. Adam, Pn. Jamilah, Pn. Latifah, Haji Zahid and Pn. Aisha.

I would like to express my sincere thanks to Higher Education Commission, Pakistan for the grant of partial scholarship for this study. I am truly thankful to the Association of Avian Veterinarians, USA, for grant of “AAV Scholarship Award 2012” for conference paper presentation. I do appreciate to Dr Jaime Samour, UAE, Dubai, for the recommendation for my “American AAV Scholarship Award 2012”. I was also lucky to attend the orthopaedic Practical Class during the AAV Conference 2012. I am deeply grateful to the School of Graduate Studies, Universiti Putra Malaysia, for providing the travel grant to participate in AAV conference, USA in August, 2012.

I also thankful to Prof. Dr. Abd Wahid Haron for his moral support during my stay at UPM. My sincere thanks go to Prof Dr Hasanah M. Ghazali, ex Dean, SGS, UPM for her special help in my admission.

Also thanks to Assoc. Prof. Dr. Gurmeet Dhaliwal, for her permission to use the graduate surgery room for my experiments. Many thanks goes to my friends who helped me in the surgeries: Dr Akeel Ahmed Memon, Dr Mahdi Ebrahimi, (Physiology Lab), Dr Gita, (Pathology lab), Dr. Reza Dr Siti Hanim (VO). Dr Ubid Kaka, Dr Asmat Kaka, Dr Atique Behan.

I also thank all friends Munir Khan Khattak, Balach Rasheed Baloch and Amdad Baloch. Lastly, I thank my family, especially my dear mother and my brothers and sisters, for their prayers. My sincere thanks also go to M.N Tunio and Tariq Tunio for taking care of my children. To my brother in-law, Captain Shaheed Iqrar Ali Tunio, 24Y, who was martyred in December 2012 for the cause. Last but not least and most importantly, I would like to thank my beloved wife, Shazia Parveen Tunio I thank my dear children, Fahad, Faraz, and Zainab, for their love. Allah Hafiz.

I certify that a Thesis Examination Committee has met on 6 December 2013 to conduct the final examination of Tunio Ahmed on his thesis entitled "Healing of Surgically Created Ulna Defect Treated with Bone Graft Substitutes in a Pigeon Model" 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 the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

Rasedee @ Mat bin Abdullah, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Tengku Azmi bin Tengku Ibrahim, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Md Zuki bin Abu Bakar @ Zakaria, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Patrick T. Redig
Professor
University of Minnesota
United States
(External Examiner)

NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 January 2014

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Jalila Abu, PhD
Associate Professor
Faculty of Veterinary Medicine
University Putra Malaysia
(Chairman)

Goh Yong Meng, PhD
Associate Professor
Faculty of Veterinary Medicine
University Putra Malaysia
(Member)

Shanthi Ganabadi, PhD
Associate Professor
Faculty of Veterinary Medicine
University Putra Malaysia
(Member)

Intan Shameha A.R, PhD
Senior Lecturer
Faculty of Veterinary Medicine
University Putra Malaysia
(Member)

____

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced
- this thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- Written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminars papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software

Signature: ________________________ Date: ____________________________

Name and Matric No.: **Tunio Ahmed, GS26796**

Declaration by Members of Supervisory committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision,
- supervision responsibilities as slated in Rule 41 in Rules 2003 (Revision 2012-2013) were adhered to.

Signature		Signature	
Name of Chairman of Supervisory Committee:	**JALILA ABU, PhD**	Name of Member of Supervisory Committee:	**GOH YONG MENG, PhD**

Signature		Signature	
Name of Member of Supervisory Committee:	**SHANTHI GANABADI, PhD**	Name of Member of Supervisory Committee:	**INTAN SHAMEHA A.R, PhD**

TABLE OF CONTENTS