EDIBLE BIRD'S NEST TREATMENT EFFECTS ON SUBCHONDRAL BONE AND ARTICULAR CARTILAGE CHANGES AND SYNOVIAL FLUID PROTEOME PROFILES IN AN OSTEOARTHRITIS RABBIT MODEL

By

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

June 2022

FPV 2022 31

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in Fulfilment of the requirement for the Degree of Doctor of Philosophy

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Osteoarthritis (OA) is characterised by progressive degeneration of articular cartilage, subchondral bone changes and synovium inflammation. Animal models are important and can demonstrate different features depending on method of induction which can resemble primary and secondary OA in humans. The choice of animal models used is based on the type and duration of research and outcome measurements. Currently, the goals for OA treatment mainly focus to alleviate disease signs and symptoms. Numerous natural products including Edible Bird's Nest (EBN) have been studied extensively in order to create potential therapies for inflammatory disorders such as arthritis. Previous studies have reported the antioxidative, anti-inflammatory and bone-strengthening effects of EBN. This study is divided into two parts. The first study is aimed to compare subchondral bone and articular cartilage changes and proteome profiles for surgically induced and chemically induced rabbit model of osteoarthritis at different time points. Based on the first study, more suitable model is chosen for the second study which is aimed to observe the effects of EBN in ameliorating OA development at different time points. For the first part of the study, New Zealand white rabbits underwent either anterior cruciate ligament transection (ACLT) procedure or injected intra-articularly with monosodium iodoacetate (MIA, 8 mg) into the right knee and were further divided into week 4 (n=5), week 8 (n=5) and week 12 (n=5) groups. The joints were subjected to micro-computed tomographic (micro-CT) analysis and histological evaluation. The synovial fluid were subjected to MALDI TOF/TOF analysis. Bone volume over tissue volume for surgically induced group increased in femur (35.35%) and tibia (32.82%) during week 12 which suggested bone remodelling whereas chemically induced group showed persistent bone resorption with decreased value of BV/TV in femur (27.12%) and tibia (26.82%). For histopathological grading of articular cartilage in femur and tibia, surgically induced group showed minimal changes during week 12 with median values of 1 and 2 in femur and tibia, respectively. As for chemically induced group, more severe changes were recorded with median values of 4.5 and 5 with significant difference with control group (p=0.0184, 0.0208) in femur and tibia, respectively. Micro-CT and histopathological analysis revealed that subchondral bone remodelling precede articular cartilage damage in surgically induced group, and vice versa in chemically induced group. Proteome profiles showed peak OA progression during week 12 for surgically induced group with upregulation of gelsolin and serotransferrin protein which involved in advanced OA. For chemically induced group, OA progression is the highest during early stage indicated by high upregulation of apolipoprotein I-IV precursor and serpin peptidase inhibitor protein during week 4 but were later downregulated. The results showed different pathogenic mechanisms for both induction method. For the second part of the study, New Zealand white rabbits were chemically induced (MIA, 8 mg) and divided into four groups; (1) negative control (n=9): non-treated osteoarthritis, (2) positive control (n=15): OA + diclofenac sodium 2 mg/kg daily orally, (3) low dosage (n=15): OA + 75 mg/kg hydrolysed EBN, (4) high dosage (n=15): OA + 150 mg/kg hydrolysed EBN. The joints were subjected to micro-CT analysis and histological evaluation and the synovial fluid subjected to LCMS/MS analysis. Micro-CT analysis showed a 22% increase in BV/TV value and 10% decrease in total porosity (PO) in treatment group that showed bone integrity improvement. Histopathological results revealed comparable changes between positive control group and EBN treatment group. There was upregulation of annexin-1, a protein involved in resolution of inflammation and downregulation carbonic anhydrase II, a protein associated with bone resorption process. Overall, morphology evaluation showed that EBN supplementation can inhibit osteoclastic activity but have no effect on cartilage damage. Protein expression showed action of several proteins involved in bone resorption inhibition and resolution of inflammation via several signalling pathways which includes toll-like receptor (TLR) and NF-κB signalling pathways.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

KESAN RAWATAN SARANG BURUNG WALIT DALAM PERUBAHAN TULANG SUBKONDRAL DAN RAWAN ARTIKULAR DAN PENGKELASAN PROFIL PROTEIN DALAM MODEL ARNAB OSTEOARTRITIS

Oleh

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Osteoartritis dikenalpasti melalui kemerosotan progresif rawan artikular, perubahan tulang subkondral dan keradangan sinovium. Model haiwan adalah penting dan boleh menunjukkan ciri-ciri tertentu bergantung kepada kaedah induksi yang boleh menyerupai osteoarthritis jenis primer atau sekunder dalam manusia. Pemilihan model haiwan adalah bergantung kepada jenis dan tempoh masa kajian dan juga hasil yang ingin dikenalpasti. Pada masa ini, tujuan rawatan OA lebih terfokus kepada pengurangan simptom dan kesan penyakit. Beberapa produk semulajadi termasuk sarang burung walit (EBN) telah dikaji secara mendalam dan berpotensi untuk rawatan gangguan keradangan seperti penyakit artritis. Kajian lepas telah melaporkan kesan EBN seperti anti-oksidatif, anti-radang dan penguatan tulang. Kajian ini dibahagikan kepada dua bahagian. Kajian pertama bertujuan untuk membuat perbandingan diantara kaedah induksi secara pembedahan dan induksi secara bahan kimia untuk menghasilkan model arnab osteoartritis dalam beberapa tempoh masa yang berbeza. Seterusnya, berdasarkan hasil kajian bagi kajian pertama, model induksi yang lebih sesuai dipilih untuk kajian bahagian kedua yang bertujuan untuk melihat kesan EBN dalam memperbaiki perkembangan OA juga dalam beberapa tempoh masa yang berbeza. Untuk kajian bahagian pertama, arnab putih New Zealand menjalani sama ada pembedahan transeksi ligament krusiat anterior (ACLT) atau disuntik secara intrasendi dengan monosodium iodoacetate (MIA, 8mg) di lutut kanan dan dibahagikan lagi kepada kumpulan minggu 4 (n=5), minggu 8 (n=5) dan minggu 12 (n=5). Tulang sendi yang diperolehi akan melalui proses mikro-CT dan penilaian histologi. Cecair sinovial akan dianalisa menggunakan MALDI TOF/TOF. Nisbah isipadu tulang dan isipadu tisu (BV/TV) dalam kumpulan induksi secara pembedahan menunjukkan peningkatan di femur (35.35%) dan tibia (32.82%) pada minggu ke-12 yang mencadangkan permodelan semula tulang manakala kumpulan induksi secara bahan kimia menunjukkan penyerapan semula tulang yang konsisten diwakili oleh pengurangan nilai BV/TV dalam femur (27.12%) dan tibia (26.82%). Bagi penskoran histopatologi rawan artikular dalam femur dan tibia, kumpulan induksi secara

pembedahan menunjukkan perubahan yang tidak ketara dengan nilai median 1 dan 2 dalam femur dan tibia pada minggu ke-12. Bagi kumpulan induksi secara bahan kimia, perubahan yang lebih ketara telah direkodkan dengan nilai median 4.5 dan 5 yang mempunyai perubahan signifikan berbanding kumpulan kawalan (p=0.0184, 0.0208) dalam femur dan tibia. Analisis mikro-CT dan histopatologi menunjukkan bagi kumpulan induksi secara pembedahan, permodelan semula tulang berlaku sebelum kerosakan rawan artikular dan perkara sebaliknya berlaku dalam kumpulan induksi secara bahan kimia. Profil protein menunjukkan progres OA yang tertinggi berlaku ketika minggu ke-12 bagi kumpulan induksi secara pembedahan dengan peningkatan ekspresi protein gelsolin dan serotransferrin yang terlibat dalam OA peringkat akhir. Bagi kumpulan induksi secara bahan kimia, progress OA adalah yang tertinggi ketika peringkat awal yang ditunjukkan oleh peningkatan ekspresi protein apolipoprotein I-IV precursor dan serpin peptidase inhibitor pada minggu ke-4 namun kemudiannya menurun. Hasil kajian menunjukkan mekanisme patogenik yang berbeza bagi kedua-dua kaedah induksi. Untuk kajian bahagian kedua, arnab putih New Zealand disuntik secara intrasendi dengan monosodium iodoacetate (MIA, 8mg) dan dibahagikan kepada empat kumpulan; (1) kawalan negatif (n=9): osteoartritis yang tidak dirawat, (2) kawalan positif (n=15): OA + diclofenac sodium 2mg/kg setiap hari secara oral, (3) dos rendah (n=15): OA + 75 mg/kg hydrolysed EBN, (4) dos tinggi (n=15): OA + 150 mg/kg hydrolysed EBN. Tulang sendi yang diperolehi akan melalui proses mikro-CT dan penilaian histologi manakala cecair sinovial akan dianalisa menggunakan LCMS/MS. Analisis mikro-CT menunjukkan peningkatan isipadu tulang sebanyak 22% dan penurunan jumlah keliangan tulang sebanyak 10% dalam kumpulan rawatan yang menunjukkan kesan EBN dalam peningkatan kekuatan tulang. Hasil analisis histopatologi menunjukkan hasil yang agak sama bagi kumpulan kawalan positif dan juga kumpulan rawatan EBN. Terdapat peningkatan ekspresi protein annexin-1 yang terlibat dalam resolusi keradangan dan pengurangan ekspresi protein carbonic anhydrase II yang terlibat dalam proses penyerapan tulang. Penilaian secara morfologi menunjukkan penambahan EBN merencatkan aktiviti osteoklas namun tidak mempunyai apa-apa kesan terhadap kerosakan rawan. Ekspresi protein menunjukkan kesan beberapa protein yang terlibat dalam perencatan proses penyerapan tulang dan resolusi keradangan melalui beberapa laluan pengisyaratan yang termasuk laluan pengisyaratan toll-like receptor (TLR) dan NF-κB.

ACKNOWLEDGEMENTS

Alhamdulillah, thanks to Allah Almighty, I finally successfully managed to complete this thesis. First and foremost, I would like to express my sincere gratitude to my supervisor Associate Professor Dr. Lau Seng Fong for the continuous support of my PhD study and research, for her patience, motivation, enthusiasm, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and supervisor for my PhD study. My utmost appreciation also to my co-supervisors, Dr. Mokrish, Prof. Suhaila, Dr. Rozaihan and Dr. Angela for their expertise and guidance throughout this research.

My sincere thanks also goes to to En. Azmil and En. Hilman from ARF for help during animal study, Puan Norasfaliza (ABI), Dr. Benjamin Lau (MPOB) and Puan Asikin (MPOB) for assisting in MALDI TOF/TOF and LCMS/MS during my proteomics study, Dr. Mohd Fauzi Md Busra for assisting in the use of micro-CT analysis, Puan Latifah Mohd Hanan and Puan Zainatulaishah Manap for preparing the histological slides, En. Hasmadi from Anatomy Laboratory, En. Ghazali from Post-mortem Laboratory and En. Jamil from Makmal Peralatan for help during my labwork.

I am also indebted to my beloved labmates and fellow postgraduates, Dr. Murshidah Mohd Asri, Dr. Amirul Nazhan, Dr. Rachel Tan, Nor Hashikin, Fadzly Salleh, Danish Adli, Dr. Mohd Akmal, Nor Shahirah Solehah, members of Biochemistry Lab, members of Nutrition Lab and my other postgraduate friends for all the fun that we had which make my PhD journey memorable. I thank all for all of our discussion and brainstorming session and I will also cherish our funny as well as awkward moments, our *'makan-makan'* session, and other activities that we spend together and hope that we can cross path again in the future.

A special appreciation to my family, Syed Sulaiman, Sharifah Zaleha, Sharifah Khadijah and Syed Alwi, and my late grandmother Sharifah Zainap for their unconditional love and moral support. I would also like to thank my beloved husband, Qayyum Latip for the never ending support and motivation during this final stage. Once again, thank you so much to everyone for all of your help and support.

This thesis was submitted to the Senate of 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:

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LIST OF ABREVIATIONS

2DGE	Two-dimensional gel electrophoresis
ACLT	Anterior cruciate ligament transection
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AGE	Advanced glycation end-products
ANOVA	Analysis of variance
BS/BV	Bone surface to volume ratio
BS/TV	Bone surface density
BSA	Bovine serum albumin
BV/TV	Bone volume over tissue ratio
COMP	Serum cartilage oligometric protein
COX2	Cyclooxygenase-2
CT	Computed tomography
DTT	Dithiothreitol
FCF	Fast green
GAG	Glycosaminoglycan
GDF5	Growth differentiation factor 5
HA	Hyaluronan
HCl	Hydrochloric acid
HIF	Hypoxia inducible factor
HMGB-1	High mobility group box-1
IAA	Iodoacetamide
IACUC	Institutional Animal Care and Use Committee

IGF-1	Insulin growth factor-1
IL	Interleukin
IL-1β	Interleukin-1-beta
JSN	Joint space narrowing
JSW	Joint space width
kDA	Kilo Dalton
LCMS/MS	Liquid chromatography mass spectrometry
m/z	Mass over charge
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen activated protein kinase
MIA	Monosodium iodoacetate
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MW	Molecular weight
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OCT	Optical coherence tomography
OPG	Osteoprotegerin
PG	Proteoglycan

PGE2	Prostaglandin E2
pH	Potential of hydrogen
pI	Isoelectric point
PO	Total porosity
RAGE	Receptor advanced glycation end-products
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
SD	Standard deviation
Tb.Sp	Trabecular space
Tb.Th	Trabecular thickness
TCA	Trichloroacetic acid
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF-α	Tumor necrosis factor alpha
TOF	Time-of-flight
US	Ultrasound
VEGF	Vascular endothelial growth factor
VOI	Volume of interest
w/v	Weight over volume
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Background and hypothesis

Osteoarthritis (OA) is characterised by degeneration of the articular cartilage, changes in the subchondral bone, and inflammation of the synovium (Li et al., 2013). OA affects up to 15% of the global human population (Johnson & Hunter, 2014) and about 20% of the canine population over the age of a year (Johnson, 2015). Since one of the traditional OA characteristics is articular cartilage degradation, OA was previously thought to be a disease affecting only articular cartilage (Buckwalter & Mankin, 1998; Pitsillides & Beier, 2011). However, recent findings suggested that subchondral bones are also importantly involved in OA pathogenesis (Mahjoub et al., 2012; Peters et al., 2018). Subchondral bones and articular cartilage are complementarily involved in biomechanical load-bearing joints (Li et al., 2013).

OA is characterised into primary (idiopathic) OA which is due to degenerative changes at the joint, mainly because of aging and secondary OA which related to risk factors that will cause OA such as obesity, joint injury, trauma and congenital disease (Chen et al., 2017). Many factors contribute to the onset of OA, including biomechanical, biochemical, genetics and also biomolecular and signalling feedback mechanisms (Shirai et al., 2011). Osteoarthritic changes are caused by cartilage matrix degradation, which culminates from disruption in the bone remodelling phase, causing further catabolic processes to outnumber anabolic processes, resulting in articular cartilage degradation, osteophytosis and functional limitations (Permuy et al., 2015).

In understanding this disease, animal models serve an important complement and simulation for human OA studies. Several small and large animal models such as horses, dogs and rabbits were developed and being used to study OA, specifically for articular cartilage and subchondral bone changes. The choice of animal models used is depending on the type and duration of research, husbandry costs and measurements of the outcome (Kuyinu et al., 2016b). While different induction method proved to successfully develop OA, the pathogeneses and progression are different in each of the model. The difference in time-dependent progression between induction methods is suggested to be caused by different pathogenesis between two types of OA. In depth knowledge of the different pathophysiology of the disease are important in planning targeted therapeutic strategies and specifically monitor disease and treatment outcome.

Current OA medications are palliative in nature, with the aim of reducing pain, which is the disease's primary symptom. Intra-articular injections of hyaluronic acids and steroids, as well as administration of nonsteroidal anti-inflammatory drugs (NSAIDs) are a few treatment strategies used in addressing this disease (Guo et al., 2021). However, these treatments come with several adverse effects such as renal toxicity, diarrhoea, vomiting, gastrointestinal disturbances, nausea, or increased cardiovascular risks (Crofford, 2013).

For the past decades, natural products are gaining a lot of attention as an alternative remedy for a variety of health problems because of their high level of effectiveness and low adverse effects (Goudarzi et al., 2018). Numerous traditional natural products including Edible Bird's Nest (EBN) have been studied extensively in order to create potential therapies for inflammatory disorders such as arthritis (Kang et al., 2019). EBN is associated with wide range of high nutritional values and health benefits such as anti-inflammatory, anti-oxidative, chondroprotective and bone strengthening effects. Therefore, EBN are hypothesized to exhibit anti-inflammatory, chondroprotective and bone strengthening effect in OA, and to reduce pain, cartilage degradation and subchondral bone alteration.

1.2 Problem statement and justification

OA is associated with high economy burden, largely attributable to the effects of disability, comorbid disease, and the expense of treatment. Additionally, OA is usually diagnosed at advanced stages where joint damage already immense and irreversible. Currently, there are no cost-effective diagnostic, treatment and prognostic method for OA in both human and veterinary medicine.

Typically, most OA studies focused only on articular cartilage changes and there is a data paucity regarding the evaluation of changes in both articular cartilage and subchondral bones for different induction methods at different time. This information is useful in understanding the pathophysiology of the disease and subsequently planning targeted treatment strategies and to specifically monitor disease and treatment outcome.

The available treatment of osteoarthritis available is based on reducing symptoms, recover function and delay the progression of the disease (Michael et al. 2010). Up to the present, it can be deduced that the treatments for OA are limited, and that the outcomes are poor due to the high occurrence of adverse side effects. The potential of new, novel non-surgical treatments approach for OA is reassuring and exciting to be explored and natural products such as EBN possess many health benefits that have the potential in ameliorating OA.

1.3 Research objectives

i. To evaluate the temporal changes of subchondral bone and articular cartilage in surgically and chemically induced osteoarthritis rabbit model using micro-computed tomography (micro-CT) and histology.
ii. To compare proteome profiles in surgically and chemically induced osteoarthritis rabbit model.
iii. To evaluate the effects of Edible Bird's Nest (EBN) treatment on subchondral bone and articular cartilage of osteoarthritic rabbit model using micro-computed tomography (micro-CT) and histology.
iv. To compare differential expression of proteins in synovial fluid of Edible Bird's Nest (EBN) treated group and non-treated group in osteoarthritic rabbit model.

1.4 Summary of the chapters

- Chapter 2 described a review on osteoarthritis, diagnostic methods, current treatment, proteomics research, Edible Bird's Nest and other related topics to increase understanding of this study.
- Chapter 3 evaluated the changes in subchondral bone and articular cartilage in surgically induced and chemically induced OA rabbit model.
- Chapter 4 compared proteome profiles between surgically induced and chemically induced OA rabbit model.

After determining that chemically induced rabbit model is more suitable in studying the effects of EBN in osteoarthritic rabbit model, we then proceeded to observe the efficacy of EBN in OA.

- Chapter 5 evaluated the effects of EBN treatment in subchondral bone and articular cartilage.
- Chapter 6 compared differential expression of proteins between EBN treated group and non-treated group.
- Chapter 7 summarized the important findings and recommendations for future research.

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