

THE POTENTIAL OF SRY (SEX DETERMINING
REGION Y)-BOX 9 AND TELOMERASE REVERSE
TRANSCRIPTASE GENES TRANSFECTION FOR
ARTICULAR CARTILAGE TISSUE ENGINEERING

BY

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ABSTRACT

This study incorporates gene transfer with tissue engineering to evaluate the feasibility of cartilaginous tissue formed using SRY(Sex Determining Region Y)-Box 9 (*SOX9*) and Telomerase Reverse Transcriptase (*TERT*) genes transfected chondrocytes. The aim of this research is to improve on the current cartilage treatment strategies with undue limitations and to work toward an alternative treatment for cartilage damage. The experimental settings involve monolayer cell culture, *in vitro* three-dimensional (3D) culture and *in vivo* ectopic implantation. The cells were isolated from six rabbit's articular cartilage and cultured until passage-1 (P1). The P1 cells were transfected with *SOX9/TERT*-, *SOX9*-, and *TERT*-gene. The non-transfected chondrocytes serve as the control group. For monolayer study, the cells were sub-cultured until P3 and evaluated in each serial passage. The *in vitro* 3D construct was formed by seeding the P1 cells in poly(lactic-*co*-glycolic acid) (PLGA) and PLGA/fibrin hybrid scaffolds with cells density of 1×10^5 per scaffold. The resulted cell-scaffold constructs were evaluated at week-1, -2 and -3 of culture. For *in vivo* study, the week-3 *in vitro* constructs formed by *SOX9/TERT*-transfected chondrocytes were subcutaneously implanted at the dorsum of the athymic mice. The constructs were evaluated at week-2 and -4 post-implantation. The analyses include growth kinetics profile, cell proliferation analysis, compression-stress analysis, macroscopic, microscopic visualisation, histological stains, quantitative sulphated glycosaminoglycan (sGAG) content analysis and gene expression study using real-time polymerase chain reaction (RT-PCR) of cartilaginous markers (*SOX9*, *COL2A1*, *ACAN*), *COL1A2*, *TERT* and collagenolytic marker (*MMP13*). A total of 60.4% transfection efficiency can be achieved using Lipofectamine® 3000 reagent. The upregulation of the transferred genes was noted in the cell groups indicating the effectiveness of the procedure. The monolayer cultured cells were unable to retain their cartilaginous phenotype. However, the *in vitro* 3D culture successfully exhibited the cartilaginous tissue formation. The cells and extracellular matrix (ECM) were densely distributed in the constructs at week-3. The cell number was increased in the constructs. The ECM components (sGAG, proteoglycan and collagen type-II) were visualised in the constructs. The cartilaginous genes expression was upregulated in the *SOX9/TERT*-transfected chondrocytes constructs group. Hence, this group was selected for the *in vivo* study. The *in vivo* constructs have the appearance which resembles cartilage. In terms of the construct's rigidity, there are no changes in the groups from week-2 to week-4 post-implantation. The cells and ECM distribution were homogenous in the *in vivo* constructs, which is better than the one observed in the *in vitro* constructs. The presence of ECM components was noted in the constructs indicates the cartilaginous tissue development. The cartilaginous genes expression was particularly upregulated in *SOX9/TERT*-PLGA/fibrin construct. The *SOX9/TERT*-PLGA/fibrin construct has the potential to be developed into a functional cartilaginous tissue and translated into clinical application. Since the end goal of this present study is to benefit the humankind, proper research guidelines to ensure safety and efficacy of the engineered tissue must be followed with good intention and values. The approach is in-line with the teaching of Islam – there should be neither harming nor reciprocating harm.

خلاصة البحث

استخدمت هذه الدراسة نقل الجينات المتضمن لطرق هندسة الأنسجة لتقييم إمكانية تكوين الأنسجة الغضروفية باستخدام خلايا غضروفية تم تعداؤها بجينات SRY (المنطقة المحددة للجنس Y)-بوكس 9 (SOX9) وجين التيلوميراز المنتسخ العكسي (TERT). هدف هذا البحث إلى تحسين الاستراتيجيات الحالية المحدودة لمعالجة الغضاريف والعمل على علاج بديل للغضاريف المتضررة. تضمنت الإعدادات التجريبية كلا من المستنبتات الخلوية أحادية الطبقة، والمستنبتات المختبرية الثلاثية الأبعاد، والزرع المنتبذ داخل الجسم الحي. تم عزل الخلايا من الغضاريف المفصليّة لستة أرناب واستنبتاتها حتى الطور 1 (P1). تم تعداء خلايا P1 بجينات SOX9/TERT، و SOX9، و TERT. وضعت الخلايا الغضروفية التي لم يتم تعداؤها في المجموعة الضابطة. لدراسة الطبقة الأحادية، تم استنبتات الخلايا ثانويا حتى الطور 3 (P3) وتقييمها في كل طور تسلسلي. تم تكوين البنية المختبرية الثلاثية الأبعاد عن طريق زرع خلايا P1 في بولي(حمض اللاكتيك-كو- حمض الجليكول) (PLGA) والسقالات الهجينة بـ PLGA/فيبرين بكثافة خلوية قدرها $10^2 \times 1$ لكل سفالة. تم تقييم "السقالات الخلوية" التي تم انتاجها في الأسبوع الأول والثاني والثالث من الاستنبتات. أما بالنسبة للدراسة داخل الجسم الحي، تم زرع التركيبات المختبرية من الأسبوع الثالث المكونة بالخلايا الغضروفية التي تم تعداؤها بجينات SOX9/TERT تحت الجلد على ظهر فئران عديمة الغدد الزعترية. تم تقييم التركيبات في الأسبوع الثاني والرابع بعد الزرع. شمل التقييم على بروفايل النمو الحركي، وتحليل تكاثر الخلايا، وتحليل الضغط والإجهاد، والتصوير العياني والمجهري، والبقع الهيستولوجية، وتحليل محتوى الجليكوزامينوجليكان الكمي (sGAG)، ودراسة التعبير الجيني باستخدام تفاعل البوليميراز المتسلسل اللحظي (RT-PCR) للمعلومات الغضروفية (SOX9، COL2A1، ACAN، COL1A2، TERT، والمعلومات الكولاجينية (MMP13). كان بالإمكان تحقيق نسبة 60.4% من كفاءة التعداء باستخدام كاشف Lipofectamine® 3000. تم ملاحظة التنظيم الرفعي للجينات المنقولة في مجموعات الخلايا مما يشير إلى فعالية العملية. لم تكن الخلايا الأحادية الطبقة المستنبتة قادرة على الحفاظ على النمط الظاهري للغضروف. ومع ذلك فقد أظهرت المستنبتة المختبرية الثلاثية الأبعاد بنجاح تكون الأنسجة الغضروفية. كانت الخلايا والمصفوفة خارج الخلية (ECM) موزعة بشكل كثيف في التركيبات في الأسبوع الثالث. وارتفع أيضا عدد الخلايا في التركيبات. وتم تصوير مكونات المصفوفة خارج الخلية (sGAG، وبروتيوجليكان، وكولاجين نوع 2) في التركيبات. تم تنظيم الجينات الغضروفية بشكل رفعي في مجموعة تركيبات الخلايا الغضروفية التي تم تعداؤها بجينات SOX9/TERT، وبالتالي فقد تم اختيار هذه المجموعة لدراستها في الجسم الحي، حيث يوجد في التركيبات داخل الجسم الحي مظهرا مشابها للغضروف. أما بالنسبة لصلابة التركيبية، فلم يكن هنالك أي تغير في المجموعات من الأسبوع الثاني إلى الأسبوع الرابع بعد الزرع. كانت الخلايا وتوزيع المصفوفة خارج الخلية متجانسة في التركيبات داخل الجسم الحي، وذلك بالطبع أفضل من تلك الملحوظة في التركيبات خارج الجسم الحي. كان وجود مكونات المصفوفة خارج الخلية ملحوظا في التركيبات وبدل ذلك على تطور الغشاء الغضروفي. كان لدى مجموعة SOX9/TERT-PLGA/fibrin القدرة على التطور إلى نسيج غضروفي وظيفي وتحويله إلى التطبيقات الإكلينيكية. بما أن الهدف النهائي لهذه الدراسة الحالية هو نفع البشرية فإنه من الواجب اتباع الإرشادات البحثية المناسبة لضمان سلامة وفعالية الأنسجة المهندسة بنية وقيم حسنة. توافقت طرق البحث مع التعاليم الإسلامية التي تستوجب عدم وجود الضرر والضرار.

APPROVAL PAGE

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DECLARATION

I hereby declare that this dissertation 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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LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AA	Antibiotic antimycotic
AAV	Adeno-associated virus
ACI	Autologous Chondrocyte Implantation
ADA	Adenosine deaminase
BMP	Bone morphogenetic protein
BSC	Biosafety cabinet
CaCl ₂	Calcium chloride
CDC	Cartilage-derived cell
cDNA	Complementary deoxyribonucleic acid
CDMP	Cartilage-derived morphogenetic protein
CGTPs	Cellular and Gene Therapy Products
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EPC	Epiphyseal chondroprogenitor cell
F-12	Ham's F-12 nutrient mixture
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GCP	Good clinical practice
H&E	Haematoxylin and eosin
HA	Hydroxyapatite
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IACUC	Institutional animal care and use committee
IREC	Institutional research Ethic committee
IGF	Insulin-like growth factor
IHH	Indian hedgehog
IL	Interleukin
ITS	Insulin transferrin selenium
Lico A	Licochalcone
LB	Luria Bertani
MACI	Matrix-induced autologous chondrocyte implantation
MREC	Medical research and ethics committee
mRNA	Messenger ribonucleic acid
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
Na ₂ HPO ₄	Disodium hydroxyphosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NMRR	National medical research register
OA	Osteoarthritis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGA	Polyglycolic acid
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic) acid
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
S.E.M	The standard error of the mean
SEM	Scanning electron microscopy
sGAG	Sulphated glycosaminoglycan
SOX9	SRY (sex determining region Y)-box 9
TAE	Tris-acetate EDTA
TERM	Tissue engineering and regenerative medicine
TERT	Telomerase reverse transcriptase
TKR	Total knee replacement

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Osteoarthritis (OA) is a global common health condition. Approximately 85% of the OA burden is contributed by knee OA, worldwide (Hunter & Bierma-Zeinstra, 2019). According to World Health Organization (WHO), it is estimated that 9.6% of men and 18% of women aged above 60 years suffered from OA. The 80% of the sufferers will experience difficulties during movement, another 25% of them are totally incapable to perform their daily routine activities. Besides physical disability, the disease also affects the sufferer's psychological state that may cause several mental health conditions. For instance, it has been reported that the mental conditions such as suicidal ideation and memory loss have been associated with OA (Vina & Kwoh, 2019). Besides that, OA is also known as a one of the factors that contribute to the development of cardiovascular disease (Wang, Bai, He, Hu, & Liu, 2016). The never-ending drawbacks of this disease can threaten the sufferer's life quality, entirely. These possible OA impact lead to the technological advancement in treatment modalities related to cartilage degenerative disease. According to disease severity, the current available treatment modalities such as prescribed medications, total knee replacement (TKR) (Sarda & Alshryda, 2017), autologous chondrocytes implantation (ACI) or matrix-induced autologous chondrocytes implantation (MACI) (Ebert et al., 2017) are being administered to relief the pain. For now, the treatments are able manage the symptoms, but unable to address the root cause of the disease.

The high economic burden of OA is another aspect that affects sufferer's life. Based on the previous reports, the total cost for OA treatment per patient was

estimated to be around RM50,000 (Salmon et al., 2016) or RM61,000 (Dibonaventura, Gupta, McDonald, & Sadosky, 2011) and the number is expected to increase each year.

Therefore, tissue engineering is perceived as a hope to provide an alternative treatment for cartilage-related disease. Tissue engineering field offers organ or tissue replacement to human by replacing the person's own regenerated tissue at the damaged site (known as the autologous implantation) (Langer & Vacanti, 1993). The concept of autologous is described as the use of biological substances taken from the same individual. The use of autologous tissue is able to minimise immune responses as compared to the tissue taken from a different individual. This concept has been applied in the ACI and MACI procedures to restore a functioning tissue with minimal immunoreactivity effect. In the ACI procedure, cell culture technique is used to prepare a sufficient number of cells prior to the implantation (Davies & Kuiper, 2019; Ogura, Bryant, Merkely, & Minas, 2019), whereas MACI uses three-dimensional (3D) tissue implants made up of the autologous cells seeded in a scaffold (Erickson, Strickland, & Gomoll, 2018; Jones & Cash, 2019).

The use of tissue engineering principles has been recognised to improve the existing medical intervention. Despite that, the incorporation of other approaches such as gene transfer with tissue engineering is being explored in search of a better treatment option. Gene transfer (gene therapy) approach is capable to facilitate the genetic materials delivery into the mammalian cells, plant cells and bacteria. This approach has been around for years since early 1960s and its first clinical trial was performed in 1990. Until now, a numerous research publications combining gene transfer with tissue engineering have been made, showing the incorporation of the approaches is reliable.

1.2 PROBLEM STATEMENT

According to the World Health Organization (WHO), organ transplantation is always the end-state organ failure treatment. Organ shortage is a known health-related issue because the available donated organ could not accommodate the increasing demand from the patients who need the organs. One of the examples of the organ that can be donated is articular cartilage.

The injured articular cartilage has the potential to progress into cartilage degenerative disease and OA if it is left untreated. Several other factors include obesity, ageing and overuse of the joints could also disrupt the cartilage morphology and its function. This painful event could eventually limit the sufferers' physical activity. The available treatment modalities could not completely cure the disease and only promises a temporary recovery effect. Other than that, the emotional state of a patient that receives continuous treatment may be affected by the expensive treatment cost.

Efforts are being made through cartilage tissue engineering application in finding an alternative, non-invasive and less expensive treatment modality. Despite that, the growth of tissue engineering research is also contributed by other technique including gene transfer. The incorporation of gene transfer with cartilage tissue engineering has been practised for years. The researchers have been tested several cartilage related genes such as *SOX9*, cartilage derived morphogenetic protein, (*CDMP*), and bone morphogenetic protein (*BMP*) to find the suitable signalling cues for cartilage repair. This study chooses *SOX9* and *TERT* genes to be transfected into chondrocytes as the genes are directly involving in cartilage formation and maintaining the cells lifespan, respectively. In the previous studies, the transfer of *SOX9* gene in the human osteoarthritic chondrocytes has been tested in the monolayer

culture (Sha'ban, Osman Cassim, Mohd Yahya, Saim, & Hj Idrus, 2011) and 3D culture (Mohamad Sukri et al., 2015). To the best of our knowledge, there has been no study used the combination of *SOX9* and *TERT* genes transfected in chondrocytes. Hence, it is hoped that this study could provide some information regarding cartilage regeneration.

1.3 RESEARCH OBJECTIVES

1.3.1 General Objectives

The study aimed to evaluate the chondrogenic properties of the *SOX9* and/ or *TERT* genes transfected chondrocytes in monolayer culture, *in vitro* 3D culture and *in vivo* ectopic implantation.

1.3.2 Specific Objectives

The study aimed to achieve the following objectives:

- 1- To optimise the transfection efficiency of three transfection reagents.
- 2- To evaluate the chondrogenic properties of *SOX9* and/ or *TERT* transfected chondrocytes in monolayer culture.
- 3- To evaluate cartilaginous properties of the cell-scaffold construct formed using *SOX9* and/ or *TERT* transfected chondrocytes in 3D culture.
- 4- To evaluate cartilaginous properties of the cell-scaffold construct formed using *SOX9/TERT*-transfected chondrocytes implanted in an *in vivo* ectopic implantation model.
- 5- To review the safety and efficacy issues of gene transfer application in cartilage TERM from the Islamic perspective.