

THE USE OF PRIMARY CHONDROCYTES IN
COMBINATION WITH POLYCISTRONIC *SOX-TRIO*
PLASMID AND *TERT* GENE TRANSFER FOR
ARTICULAR CARTILAGE TISSUE ENGINEERING
AND ITS BIOETHICAL IMPLICATIONS

BY

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ABSTRACT

A polycistronic *SOX*-trio plasmid vector was designed using VectorBuilder free software. The combination of *SOX5*, *SOX6* and *SOX9* genes resulted in a plasmid vector of more than ten (10) kbp. It is challenging to transfect primary cells culture with a larger than two (2) kbp DNA fragment. Thus, the *SOX*-trio plasmid DNA's transfection efficiency was optimised using a lipofection method. The three experimental groups that resulted from the *SOX*-trio and *TERT* genes transfected in the chondrocytes were *SOX*-trio/*TERT* (SXT), *SOX*-trio (SX) and *TERT*. They were observed using fluorescent microscopy. The non-transfected chondrocytes served as a control group. Various histology staining procedures were used to evaluate the presence of cartilaginous pericellular and extracellular matrix (ECM). The matrices production level was evaluated further using sulphated glycosaminoglycan (sGAG) assay for confirmation. The formation of neocartilage or the *in vitro* 3D "cell-scaffold" tissue constructs was done using chondrocytes seeded in the PLGA-based scaffolds. This step resulted in three 'cell-scaffold' experimental groups, namely PLGA/Fibrin/Atelocollagen (PAF), PLGA/Fibrin (PF), PLGA/Atelocollagen (PA). The constructs were cultured for three (3) weeks. They were evaluated based on gross morphological changes, cell proliferation (MTT) assay at 7, 14, 21 days of culture, and scanning electron microscopy (SEM) at the end of the 3-week culture. Histological stainings were used to evaluate the constructs' ECM production level for three consecutive weeks. The outcome was further verified via sGAG assay. The present study's findings indicated that the monolayer cultured chondrocytes could be transfected efficiently with the >10 kbp plasmid using the optimised lipofection procedure. The transfected chondrocytes produced ECM comparable to the control group. The resulting *in vitro* 3D "cell-scaffold" constructs exhibited a newly formed neocartilage tissue. The tissue can be further developed *in vivo* to complete tissue regeneration. The study showed that the *SOX*-trio and *TERT* genes transfer is beneficial for articular cartilage tissue engineering. The goal of TERM is to be of benefit to the patient by taking positive steps to prevent and remove harm from the patient. Hence, TERM advocates must strive to provide a proper standard of TERM use and care in clinical settings to avoid or minimise the risk of harm. This practice may be best supported by commonly held moral convictions and society's laws.

خلاصة البحث

ناقل بلازميد *SOX*-ثلاثي متعدد المقارين مصممة باستخدام برنامج مجاني VectorBuilder. نتج الجميع بين جينات *SOX5*، و *SOX6*، و *SOX9* ناقل بلازميد أكثر من عشرة ألف زوج قاعدة (10) kbp. ومن الصعب لنقل ثقافة الخلايا الأولية مع جزء من الحمض النووي أكبر من اثنين (2) kbp. وبذلك، تم تحسين كفاءة تعاء DNA البلازميد *SOX*-ثلاثي باستخدام طريقة حقن الدهون. نتج النقل *SOX*-ثلاثي و جينات *TERT* في الخلايا الغضروفية ثلاث مجموعات تجريبية وهي *SOX*-ثلاثي/*TERT* (*SXT*)، و *SOX*-ثلاثي (*SX*)، و *TERT*. وقد لاحظوا باستخدام المجهر الفلوري. وكانت تمثل الخلايا الغضروفية المنقولة كمجموعة مرجعية. استخدمت إجراءات تليخ الأنسجة المختلفة لتقييم وجود محيط الخلايا الغضروفية و المصفوفة خارج الخلية (*ECM*). تم تمييز مستوى إنتاج المصفوفات بشكل إضافي باستخدام مقياس (*sGAG*) للتأكيد. تكوين بنيات أنسجة الغضاريف الجديدة باستخدام الخلايا الغضروفية المصنفة في السقالات القائمة على *PLGA*. ونتجت هذه الخطوة ثلاث مجموعات تجريبية تسمى *PLGA*/*ليفين*/*أثيلوكولاجين* (*PAF*)، و *PLGA*/*ليفين* (*PF*)، و *PLGA*/*أثيلوكولاجين* (*PA*). تمت زراعة البنى لمدة (3) ثلاثة أسابيع. يتم الحكم عليهم على التغيرات المرفولوجية الإجمالية، و فحص تكاثر الخلايا (*MTT*) في يوم 7، و14، و21 من زراعة، و الفحص المجهر الإلكتروني الماسح (*SEM*) في نهاية ثلاثة أسابيع من زراعة. استخدمت إجراءات تليخ الأنسجة المختلفة لتقييم مستوى إنتاج *ECM* التركيبات لمدة ثلاثة أسابيع متتالية. ترسخت لتحقيق النتيجة عبر مقياس *sGAG*. إشارة نتائج الدراسة الحالية إلى أن يمكن نقل الغضروفية أحادية الطبقة المستزرعة بكفاءة بالبلازميد < 10 kbp في البوصة باستخدام إجراء إزالة الدهون المحسن. أنتجت الخلايا الغضروفية المنقولة *ECM* مقارنة بمجموعة مرجعية. أظهر الناتج في بنيات الزراعة "خلية سقالة" 3D أنسجة غضروفية حديثة التكوين. ويمكن على تلك الأنسجة التطور بشكل الأوسع في الفيفو لاستكمال تجديد الأنسجة. أثبتت الدراسة أن نقل *SOX*-ثلاثي و جينات *TERT* مفيد في هندسة أنسجة الغضروف المفصلي. الهدف من *TERM* هو أن يكون مفيدا للمريض من خلال اتخاذ خطوات إيجابية لمنع وإزالة الضرر من المريض. ومن ثم، يجب أن يسعى المدافعون *TERM* لتوفير معيار مناسب لاستخدامه والعناية في الإعدادات السريرية لتجنب أو تقليل مخاطر الضرر. ويمكن دعم هذه الممارسة بشكل أفضل من خلال المعتقدات الأخلاقية الشائعة وقوانين المجتمع.

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 of Health Sciences (Tissue Engineering).

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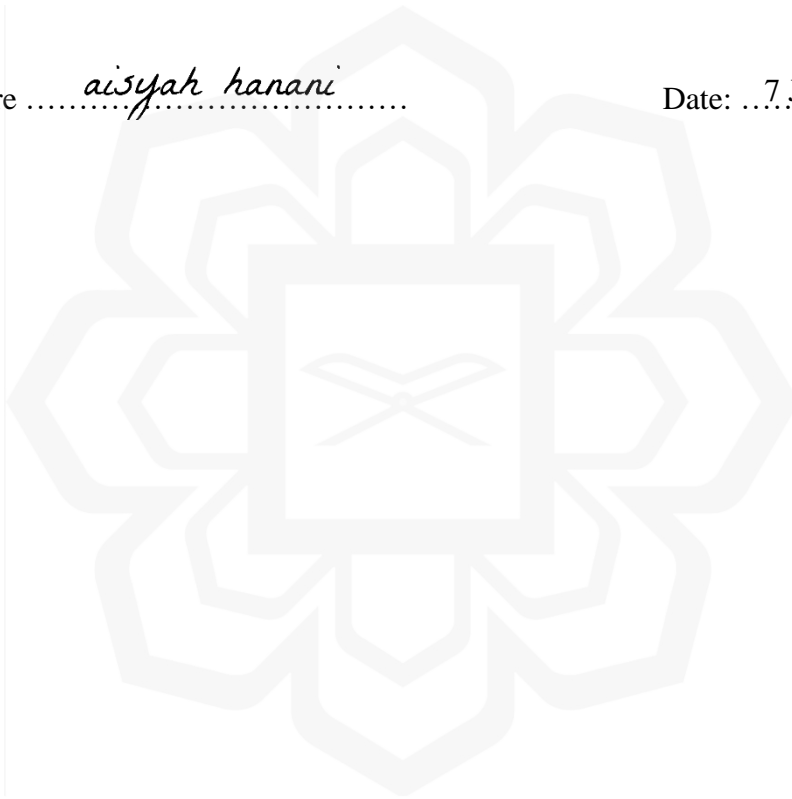
DECLARATION

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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This thesis is specifically dedicated to my late father, Hj Md Ali @ Tahir bin Mat @ Tahir; my heart and my endless prayer always there for you, Ayah. And my beloved one and only mother, Hjh. Hasiah bt Abas for your dua' and unfailing support.

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LIST OF ABBREVIATIONS

2D	Two-Dimensional
3D	Three-Dimensional
ANOVA	Analysis of Variance
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared
BSC II	Biohazard Safety Cabinet Class II
bp	Base Pair
CaCl ₂	Calcium Chloride
cDNA	Complementary DNA
<i>COL2A1</i>	Collagen Type II
CH ₂ Cl ₂	Methylene Chloride
CPRD	Clinical Practice Research Datalink
CMV	<i>Cytomegalovirus</i>
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ECM	Extracellular Matrix
<i>E. coli</i>	<i>Escherichia coli</i>
EDC	1-ethyl-(3-(3-dimethylaminopropyl) Carbodiimide Hydrochloride
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
GuHCl	Guanidine-HCl
HA	Hyaluronic Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HCl	Hydrochloric Acid

H&E	Haematoxylin and Eosin
H ₂ SO ₄	Sulphuric Acid
IACUC	Animal Care and Use Committee
IVD	Intervertebral Disc
kbp	Kilo Base Pair
LB	Luria-Bertani
MSCs	Mesenchymal Stem Cells
MES	2-ethenesulfonic Acid
MgCl ₂	Magnesium Chloride
mRFP1	Monomeric Red Fluorescent Protein-1
MTT	Modified 3-(4,5-dimethylthiazole-2-yl)- 2,5-diphenyltetrazolium-Bromide
NaHCO ₃	Sodium Hydrogen Bicarbonate
Na ₂ HPO ₄ ,	Disodium Hydrogen Phosphate
NaOH	Sodium Hydroxide
NBF	Neutral Buffered Formalin
NCBI	National Center for Biotechnology Information
NHS	Hydroxysuccinimide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OA	Osteoarthritis
ORFs	Open Reading Frames
PA	PLGA/Atelocollagen
PAF	PLGA/Fibrin/Atelocollagen
PBS	Phosphate Buffered Saline
PCR	Polymerase Reverse Transcriptase
PF	PLGA/Fibrin
PGA	Polyglycolic Acid
PLGA	Poly(lactic- <i>co</i> -glycolic acid)
PLLA	Poly-L-lactic Acid
RNA	Ribonucleic Acid
SD	Standard Deviation

SDGs	Sustainable Development Goals
SEM	Scanning Electron Microscopy
S.O.C	Catabolite Repression Medium
<i>SOX</i>	<i>Sex determining region Y (SRY)-box</i>
sGAG	Sulphated Glycosaminoglycan
SPSS	Statistical Package for Social Sciences
SXT	<i>SOX-trio/TERT</i>
SX	<i>SOX-trio</i>
TBE	Tris/Borate/EDTA
TE	Tissue Engineering
TEM	Transmission Electron Microscopy
TEMPs	Tissue-Engineered Medical Products
TERM	Tissue Engineering and Regenerative Medicine
<i>TERT</i>	Telomerase Reverse Transcriptase
UN	United Nation
WHO	World Health Organization

LIST OF SYMBOLS

cm	Centimetre
°C	Degree Celcius
et al.,	(<i>et alia</i>): and others
g	Gram
g	Gravitational Force
IU	International Unit
kg	Kilogram
µl	Microlitre
µm	Micrometre
ml	Millilitre
mg	Milligram
ng	Nanogram
%	Percentage
±	Plus Minus
rpm	Revolutions Per Minute
®	Registered Patent
™	Trademark
v/v	Volume / Volume
w/v	Weight / Volume

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

Articular cartilage is a connective tissue that is avascular, aneural, and alymphatic. This hyaline cartilage has only one cell type, namely chondrocyte. The chondrocyte has low mitotic activity and thus limits its capacity to regenerate. Due to this characteristic, articular cartilage does not heal once it is damaged. If left untreated, this condition will lead to the development of osteoarthritis. Osteoarthritis is a degenerative joint disease that results from a breakdown of articular cartilage and the underlying bone at the synovial joints (Lozada, 2016).

It is reported that almost 50 % out of 117 434 osteoarthritic patients with a complete database obtained from the Clinical Practice Research Datalink (CPRD) has undergone total knee replacement (The Lancet, 2017). The outcomes ranged from poor to satisfactory. The available drugs/pharmacotherapy, physical rehabilitation, interventional therapy, complementary medicine, and surgery help reduce disease's manifestations. However, they do not treat the root cause and cannot yet restore a typical articular cartilage structure and function (Clinical Practice Guidelines, 2013; Osteoarthritis Action Alliance, Arthritis Foundation, & Centers for Disease Control and Prevention, 2020).

To date, there is no known cure for OA. There has been a surge of research interest in engineering tissue substitutes to restore, repair, or even reverse the damaged tissue's aetiology. In this case, tissue engineering and regenerative medicine (TERM) may serve as an alternative solution in managing joint injuries. This biomedical

technology combines the three elements, namely cells, biomaterial scaffolds, and signalling factors ranged from chemicals to mechanical cues to engineer and regenerate various new tissue substitutes that structurally and functionally mimic the human body parts. The TERM triad can be used either individually or in combination to regenerate functional tissues.

The idea to introduce candidate genes into intended cells through viral or non-viral transfection methods is known as gene transfer. This protocol is included in TERM research to address the signalling factor role in cultured cells. It sees if the introduced genes could regulate and differentiate the cells from within. Viral transfer of regulatory genes/oncogenes is inherently dangerous. Other than requiring highly-skilled research personnel, viral transfection or transduction needs a specialised laboratory equipped with high biosafety level specifications. Since not every researcher has the advantage to access such facilities, non-viral or chemical transfection methods, including liposome-mediated transfection, can be used as an alternative (Chemical- and Viral-Based Transfection Methods, Bio-Rad, n.d.; Introduction to Transfection, Thermo Fisher Scientific, n.d.). Besides, the methods may minimise the safety issue, especially if it is to apply to the human. This aspect is included in the discussion of *al-Daruriyyāt*, which is *Hifz al-Nafs* (preservation of life) offered by the *Maqāṣid al-Sharī'ah* (goals or objectives of *Sharī'a* or Islamic Law).

The non-viral protocol aims at transient transfection, enabling the introduced nucleic acid to exist only for a limited period in the transfected cells. Unlike permanent transfection using viral methods, the transiently transfected nucleic acid does not integrate and interfere with the host's genome. Hence, foreign genetic material is not inherited across generations during cell division, thus preserved the lineage. This aspect is in-line with another *Maqāṣid al-Sharī'ah* (goals or objectives of *Sharī'a* or Islamic

Law), which also included in the discussion of *al-Daruriyyāt*, i.e. *Hifz al-Nasl* (preservation of lineage).

At the micro-level, articular cartilage depends on the ECM produced by the chondrocytes to maintain its biomechanical property. Chondrocytes are scarcely scattered within the ECM. In the experimental setting, other than ensuring the chondrocytes properties, a sufficient number of cells is needed for *in vitro* tissue regeneration. With a small number of starting materials (i.e. the donor tissue), the isolated cells must be grown longer in culture to achieve the workable cells number. Numerous approaches were performed to address this perennial issue using various cells sources, biomaterial scaffolds, and signalling factors.

This present study was designed to address the above three TERM principles interaction based on and in continuation of the team's previous TERM research activities. The use of specific transcription factor, *Sex determining region Y (SRY)-box 9 (SOX9)* gene transfer (Sha'ban et al. 2011) and PLGA/fibrin scaffolds have shown promising results in tissue-engineered cartilage reconstruction (Khang et al. 2008; Munirah et al., 2008; Sha'ban et al., 2008a, 2008b). The rabbit's pluripotent bone marrow mesenchymal stem cells (BMSCs) seeded in PLGA/fibrin scaffolds were reported to have successfully served as functional tissue-engineered cartilage *in vitro* (Rahman et al. 2015a) and *in vivo* (Rozlin, 2016; Rahman et al. 2015b). The *SOX9* transfected in rabbit's chondrocytes and seeded in PLGA/fibrin scaffolds improved engineered cartilaginous tissue property *in vitro* (Sukri et al., 2015) and *in vivo* (Norhamiza, 2016). The use of hybrid biomaterials combining PLGA/atelocollagen/fibrin was shown to provide a temporary framework template for intervertebral disc (IVD) cells' attachment, proliferation, and ECM synthesis better than the PLGA alone (Mohamad et al. 2020; Mohamad et al. 2017). The *telomerase reverse*