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EVALUATION OF THREE-DIMENSIONAL POLY (LACTIC-*CO*-GLYCOLIC ACID), ATELOCOLLAGEN AND FIBRIN SCAFFOLDS FOR INTERVERTEBRAL DISC TISSUE ENGINEERING

BY

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ABSTRACT

Tissue engineering and regenerative medicine (TERM) offers an alternative approach for the treatment of intervertebral disc (IVD) degeneration. One of the important TERM principles is the application of biomaterial scaffolds. Poly(lactic-*co*-glycolic acid) (PLGA) is a US Food and Drug Administration (FDA) approved synthetic copolymer for clinical use. However, for TERM application, PLGA needs to be improved. Natural polymers, for example atelocollagen and fibrin can be used to enhance the PLGA properties. The combination of PLGA with atelocollagen and fibrin for IVD regeneration has not been explored yet. The aim of the present study is to evaluate firstly the monolayer culture of IVD cells, secondly the PLGA-based scaffolds namely PLGA alone, PLGA with atelocollagen (PA), PLGA with fibrin (PF) and PLGA with the combination of both atelocollagen and fibrin (PAF), thirdly the in vitro 3D constructs engineered from PLGA-based scaffold seeded with the annulus fibrosus (AF) cells and fourthly the in vivo constructs implanted subcutaneously on the athymic nude mice model. Important Islamic principles in relation with the IVD regeneration were also discussed as the fifth objective. Growth kinetics analysis and cellular proliferation assay were performed on the monolayer cells culture and in vitro constructs at day 4, 7, 14 and 21, respectively. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to evaluate cell morphology both in monolayer and the in vitro 3D culture. The fabricated PLGA-based scaffolds were characterized in terms of chemical bonding, porosity, water uptake, thermal, mechanical, degradation, pH level and compression modulus. Other evaluation parameters included histology, immunohistochemistry, gene expression, sulphated glycosaminoglycan (sGAG) and DNA quantification analysis. All constructs were evaluated at 1, 2 and 3 weeks in vitro and, at 4 weeks in vivo. The presence of autophagasome in AF cells was detected in all passages. Comparable morphology of cellular attachment on in vitro 3D constructs was observed. Passage 1 of AF cells showed better cellular profile and extracellular matrix production compared to all other passages thus chosen to be seeded onto PLGA-based scaffolds and form constructs. The PA scaffolds showed better swelling ratios, thermal stability, mechanical strength, degradation properties and ability to reduce the pH effect. As for in vitro 3D constructs, PAF exhibited higher cellular proliferation and depicted better histological staining than the other groups. Positive accumulation of collagen type I and type II with higher level of sGAG were also observed in PAF constructs. Comparable mRNA expression of condrogenic markers were detected in all monolayer, in vitro 3D constructs and in vivo samples. In line with in vitro 3D constructs, in vivo constructs especially PAF group showed notable distribution of cells, accumulation of sGAG, and proteoglycan with positive immunoreactivity of collagen type I and type II. In conclusion, the incorporation of atelocollagen, fibrin and both combination significantly improved PLGA scaffold properties in vitro and in vivo. The desk-based study on Islamic perspective indicated that TERM technology does not violate any principles of Islam. More works on IVD regeneration are needed to uncover its full potential. This laboratory grown IVD may serve as an alternative over conventional treatments in future clinical application.

ملخص البحث

يمكن لهندسة النسج والطب الترميمي (TERM) أن يوفرعلاجا بديلا لتآكل القرص بين الفقرات. احد مبادئ هذه الهندسة هو من خلال تطبيقات المواد الحيوية. البوليمير PLGA هو بوليمير صنعي واسع الاستخدام ومقبول من هيئة الدواء والغذاء الامريكية (FDA) للاستخدام البشري. لكن لاستخدامه في هذه الهندسة يجب تحسين مواصات سطح هذا البوليمير. البوليميرات الطبيعية مثل الاتيلوكولاجين والفبرين هي خيارات قوية لهذا الغرض. واستخدامها مع هذا البوليمير لم تتم دراسته بعد. تهدف هذه الدراسة الى تقييم (١) زرع وحيد الطبقة للقرص بين الفقرات لتحديد افضل مصدر للزرع ثلاثي الابعاد، (٢) ثلاث مواد جديدة ثلاثية الابعاد مبنية من دعائم الـــ PLGA وهي اسميا PLGA-Atelocollagen (PA)، PLGA-Fibrin (PF) و PLGA-Atelocollagen-Fibrin (PAF) من أجل ترميم القرص ما بين الفقرات (IVD)، (٣) زرع ثلاثي الابعاد في الزجاج لدعائم الPLGA المبذورة بخلايا annulus fibrosus (AF) و(٤) الزرع في الحي للدعائم تحت الجلد في نموذج فئران. تكت أيضا مناقشة إعادة ترميم القرص بين الفقرات من وجهة نظر إسلامية. تمت دراسة مواصفات الدعائم المصنعة بما يخص الارتباط الكيميائي، الخلالية، الشكل، الانتباج، الحرارية، التخرب، الباهاء، الخصائص الميكانيكية والتوافقية الخلوية. تم تقييم حركية النمو الخلوي لزرائع الطبقة الاحادية في مراحل مختلفة. في حين تم انجاز المعايرات الخلوية على التشكيلات ضمن الزجاج ي الايام ٤، ٧، ١٤ و٢١. كما تمت دراسة الخلايا احادية الطبقة لكل التكشيلات بما يخص الشكل، التنسج، الكيمياء النسيجية المناعية، التعبير الجيني، محتوى sulphated glycosaminoglycan (sGAG) وتحليل المحتوى من الـــ DNA. تم استخدام المجهر الالكتروني العابر والماسح لتقييم الخلايا وحيدة الطبقة وثلاثية الابعاد. تم تقييم جميع التشكيلات في الاسابيع ١، ٢ و٣ في الزجاج وفي الاسبوع ٤ ضمن الحي. أظهرت النتائج وجود الاوتوفاغوزوم في خلايا AF في كل المراحل. كما تمت ملاحظة شكل مقارن للالتصاقات الخلوية في التشكيلات ثلاثية الابعاد. الخلايا من الخيط الاول أظهرت افضل نتائج وتم اختيارها لاحتوائها ضمن الدعامات. أظهرت مجموعة داعمة PA أعلى قدرة على الانتباج مقارنة ببقية المجموعات وهذا ساهم باعلى درجة من التخرب. تحسنت الثباتية الحرارية والقوة الميكانيكية للـــPLGA بإضافة الاتيلوكولاجين. جميع مجموعات الدعامات كانت قادرة على إنقاص التأثير الحمضي لنواتج الـــ PLGAالثانوية. تحسن مستوى الباهاء وحيوية الخلايا بشكل هام بعد ٢١ يوما من الزرع. أظهرت تشكيلات PAF نموا خلويا اعلى مع شكل نسيجي اهم لأزرق الألسيان. أورق التولويدين وصباغ الـــ و–سفرانين. تمت ملاحظة مستويات اعلى من الـــ sGAG في تشكيلات PAF. كما تمت ملاحظة مستويات هامة لتعبير الـــmRNA للمعلمات الكوندروجينية في جميع العينات. اظهرت التشكيلات ضمن الحي توزعا مهما للخلايا، تراكم الـــ sulphated glycosaminoglycan، و proteoglycan مع تفاعل مناعي موجب للكولاجين من النوع ١ والنوع ٢ بشكل اساسي في PAF. كخلاصة، تضمين الاتيلوكولاجين، الفبرين ومجموع الاتيلوكولاجين والفبرين معا ادى الى تحسن اداء دعامات الـــ PLGA على نحو مهم في الزجاج وفي الحي. من الناحية الإسلامية لا تتعارض تقنية ال TERM مع مبادئ الشريعة الاسلامية. يؤمل من هذه الدراسة ان تشكل القاعدة لمزيد من الابحاث المستقبلية لترميم IVD، ابتداء من العلوم الاساسية الى الابحاث المتقدمة في TERM من اجل تطبيقات سريرية مستقبلي

ABSTRAK

Bidang kejuruteraan tisu dan perubatan regeneratif (TERM) menawarkan pendekatan alternatif untuk rawatan degenerasi cakera (disk) antara vertebra tulang belakang (IVD). Salah satu prinsip utama TERM ialah penggunaan biomaterial. Asid poli(laktik-co-glikolik) (PLGA) adalah kopolimer sintetik yang telah diluluskan oleh agensi Pentadbiran Makanan dan Dadah Amerika Syarikat (US FDA) untuk kegunaan klinikal. Walaubagaimanapun, PLGA perlu penambahbaikan untuk kegunaan TERM. Bahan polimer semulajadi seperti atelokolagen dan fibrin berpotensi untuk menambahbaik keupayaan PLGA. Kombinasi PLGA, atelokolagen dan fibrin untuk regenerasi IVD masih belum dikaji sebelum ini. Maka, kajian ini bertujuan menilai (1) kultur monolapisan sel IVD bagi mendapatkan sel terbaik untuk konstruk tiga dimensi (3D), (2) kerangka yang berasaskan PLGA iaitu PLGA sahaja, PLGA berserta atelokollagen (PA), PLGA berserta fibrin (PF) dan kombinasi PLGA, atelokolagen dan fibrin (PAF), (3) konstruk 3D yang dihasilkan dari kerangka berasaskan PLGA yang disemai dengan sel annulus fibrosus (AF) dan (4) konstruk in vivo yang diimplan secara subkutanius pada model tikus tanpa bulu. Kaitan antara prinsip Islam dan degenerasi IVD turut dibincangkan. Analisis pertumbuhan kinetik dan proliferasi sel dilakukan pada hari 4, 7, 14 dan 21. Mikroskop Elektron Transmisi (TEM) dan Mikroskop Elektron Imbasan (SEM) digunakan untuk melihat morphologi sel dalam monolapisan dan konstruk 3D. Kumpulan kerangka berasaskan PLGA tersebut dikaji sifat-sifatnya dari segi ikatan kimia, sifat keliangan, sifat kembang, haba, degradasi, pH, mekanikal dan modulus mampatan. Paramater lain yang dikaji termasuklah histologi, immunohistokimia, ekspresi gen, kandungan glikosaminoglikan sulfat (sGAG) dan kandungan DNA. Semua konstruk telah dikaji pada minggu 1, 2 dan 3 minggu in vitro, dan pada minggu ke-4 in vivo. Morfologi kelekatan sel pada konstruk 3D menunjukkan perbezaan bentuk sel yang ketara. AF pasaj 1 menunjukkan profil sel dan penghasilan matriks ekstraselular yang lebih baik maka dipilih untuk disemai dalam kerangka berasaskan PLGA untuk membentuk konstruk. Kerangka PA menunjukkan keupayaan kembang, kestabilan haba, kekuatan mekanikal, kemampuan degradasi dan kebolehan menurunkan pH. Konstruk PAF mempamerkan percambahan sel yang lebih tinggi berbanding kumpulan kerangka lain melalui pewarnaan histologi. Penghasilan kolagen jenis 1 dan jenis II juga positif dengan sGAG yang tinggi ditunjukkan dalam konstruk PAF. Kondrogenik mRNA juga berjaya dikesan dalam semua monolapisan, konstruk 3D dan implan in vivo. Seriringan dengan keputusan kajian konstruk 3D in vitro, konstruk in vivo khususnya PAF menunjukkkan pengagihan sel yang sekata, pengumpulan glikosaminoglikan sulfat, dan proteoglikan serta kewujudan immunoreaktif positif kolagen jenis I dan II. Secara keseluruhan, atelokolagen, fibrin dan kombinasi keduanya berjaya menambahbaikkan kerangka PLGA dalam kajian in vitro dan in vivo. Di samping itu, teknologi TERM didapati tidak melanggar mana-mana prinsip Islam. Kajian yang lebih mendalam berkenaan degenerasi IVD diperlukan. Kajian IVD yang dihasilkan di makmal mungkin menjadi salur alternatif mengatasi rawatan konvensional dalam kegunaan klinikal di masa hadapan.

APPROVAL PAGE

The thesis of Mohd Yusof Bin Mohamad has been approved by the following:

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DECLARATION

I hereby declare that this thesis is the result of my own investigation, 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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I dedicate this thesis to my beloved mother, Patimah binti K.Mohamed whom her soul had returned to the Almighty Allah on Tuesday, 27th Rajab 1435H/ 27 May 2014 at4.00 a.m. May Allah bless her soul, Ameen.

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LIST OF ABBREVIATIONS

3-D	Three Dimensional
AF	Annulus Fibrosus
ATR-FTIR	Attenuated Total Reflectances Fourier Transform Infrared Spectroscopy
$CaCl_2$	Calcium Chloride
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
ECM	Extracellular Matrix
NP	Nucleus Pulposus
H&E	Haematoxylin & Eosin staining
IVD	Intervertebral Disc
PLGA	Poly(lactic-*co*-glycolic acid)
PA	Poly(lactic-*co*-glycolic acid) incorporated with Atelocollagen
PF	Poly(lactic-*co*-glycolic acid) incorporated with Fibrin
PAF	Poly(lactic-*co*-glycolic acid) incorporated with Atelocollagen and Fibrin
PLGA-AF	Poly(lactic-*co*-glycolic acid) seeded with AF cells
PA-AF	Poly(lactic-*co*-glycolic acid) incorporated with Atelocollagen seeded with AF cells
PF-AF	Poly(lactic-*co*-glycolic acid) incorporated with fibrin seeded with AF cells
PAF-AF	Poly(lactic-*co*-glycolic acid) incorporated with Atelocollagen and Fibrin seeded with AF cells
PLGA-I	Poly(lactic-*co*-glycolic acid) construct implant
PA-I	Poly(lactic-*co*-glycolic acid) incorporated with Atelocollagen implant
PF-I	Poly(lactic-*co*-glycolic acid) incorporated with Fibrin implant
PAF-I	Poly(lactic-*co*-glycolic acid) incorporated with Atelocollagen and Fibrin implant
PCR	Polymerase Chain Reaction
sGAG	Sulphated Glycosaminoglycan
SD	Standard Deviation
S.E.M	Standard Error of Mean
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TERM	Tissue Engineering and Regenerative Medicine
T_g	Glass transition temperature

LIST OF SYMBOLS

ºC	Degree Celsius
MPa	Mega pascal
mg	Milligram
µm	Micrometer
µM	Micromolar
µl	Microliter
µg	Microgram
ng	Nanogram
±	Plus minus
=	Equal
>	More than
<	Less than