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Original Article

Optimizing the Proliferation and Differentiation of Mesenchymal Stem Cells by Using PLLA Nanofibers for Epidermis Layer Repair

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Abstract:

Background: Tissue engineering has attracted a lot of attention, especially regarding wound and skin damage repair. PLLA is a biocompatible and biodegradable polymer. It has excellent mechanical properties and is able to support cell proliferation and growth. Electrospinnig is a fabrication method which has gained a lot of attention over the past years. As a result, a lot of studies have been carried out using it.

Methods and Materials: -In the present study, the potential of PLLA fabricated scaffolds is evaluated. Scaffolds were fabricated by electrospinning. After fabrication they were then tested for toxicity, using MTT assay. After confirming their safety, their potential for cell and stem cell proliferation and growth were examined. Additionally, another step was taken and mesenchymal stem cells were differentiated to skin cells on PLLA nanofibers. After 21 days of differentiation, PCR, SEM imaging were performed.

Results: SEM imaging showed a clear transformation of stem cells. Furthermore, PCR analysis confirmed the differentiation of relevant genes.

Conclusion: Our results undoubtedly confirmed the potential of PLLA in tissue engineering. It yielded a feasible nanoscaffold, with great mechanical properties. Furthermore, it helped support cell and stem cell growth, as well as skin differentiation of stem cells.

Key words: Nanofibrous PLLA Scaffolds, Electrospining, skin epidermal keratinocytes, Mesenchymal stem cell

Introduction

Tissue engineering is known as an interdisciplinary field that investigates the connection between structure and function of the cells in normal and abnormal tissues (1). Stem cells play and important role in tissue engineering. Over the recent years, they have helped improve tissue engineering greatly, especially regarding cell damage repair and tissue/organ regeneration (2, 3, 4). Cells are usually seeded onto artificial structures which mimic natural 3D biological structures. These structures are called nanoscaffolds and are used both *in vivo* and *in vitro*. A variety of biodegradable and biocompatible materials are used to fabricate nanoscaffolds. Fabrication of nanoscaffolds is a critical part of tissue engineering since a lot is needed to mimic ECM (5, 6). Porosity is one of the important factors of nanoscaffolds since it helps support cells and improves signaling. Structurally, ECM is made of different protein fibers with a variety of diameters, ranging from ten to hundreds of nanometer. Different techniques are used to fabricate nanoscaffolds such as phase separation, self-assembly and electrospinning. Among them, electrospinning can fabricate the most desired fibers in

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terms of being similar to ECM fibrous structure; additionally, it is cost efficient (6, 7). High surface to volume ratio of nanofibers alongside their porosity enhance cell attachment, migration and differentiation. This study has aimed to reach the desired aforementioned properties of its nanofibers to evaluate its potential in tissue engineering. Poly lactic acid (PLA) is a widely used synthetic polymer in tissue engineering. It should be noted that PLA byproducts are eliminated through normal cell metabolism and it is FDA approved [8,9]. Its biocompatibility and biodegradability make it an ideal candidate for tissue engineering purposes. The aims of this study were first, fabricating feasible PLA nanofibrous scaffolds by electrospinning and secondly, evaluating its potential in skin tissue engineering.

Material and methods

Fabrication of nanofibers

Electrospinning was used to fabricate 2D scaffolds. In this project, to make PLLA scaffolding, 7.3% by weight-volume solution of polyLactic acid polymer in chloroform and DMF solvents was prepared and placed in a syringe with a volume of 10 ml. The speed of the fiber collector was adjustable from 0 to 1000 rpm (230 rpm was used in this study) and a reciprocating motion of 50 mm per minute and the reciprocating movement was performed at a speed of 50 mm per minute. The spraying angle of the solution could be controlled and be changed from 0 to 60 degrees and as needed. It should be noted that in all experiments, a -10 degree angle was selected relative to the horizon. The maximum supplied voltage is kV30, in this case kv18 voltage was used.

Contact angle

Contact angle is in fact the angle between a drop of water and the scaffold surface. This test is performed to evaluate the hydrophilicity of the scaffolds. In order to perform the temperature, a drop of water was put on the surface of the scaffold in room temperature and after few seconds, contact angle was measured.

Flow cytometry

Flow cytometry results of fat tissue extracted cells showed that the cells were CD34 and CD45 negative (denying any blood cell-related origin), while being CD105 and CD90 positive, with an expression of 98.7% and 91.6% accordingly.

Biocompatibility

MTT assey was performed to evaluate the potential of cell survival on nanofibers. Extracted stem cells were cultured on scaffolds and their proliferation, adhesion and growth were evaluated using MTT and microscopic assays. PPLA nanoscaffolds were sterilized by 70% ethanol and then put into 12-well plates. 120 ml of stem cell suspension with a density of 12.5-104 cells/ml was cultured on the plate. Cultured cells were the incubated at 38°c with 10% FBS and 5% CO_2. After 24, 48 and 72 hours, 50 ml of MTT solution was added to each plate. In order to transform MTT to formazan crystals with the aid of dehydrogenase enzyme in living cells mitochondria, plates were incubated for 4 hours. To dissolve formazan sediment, top of the solution was removed and sufficient amount of solvent was added and then, absorbance was read at a wavelength of 570 nm by ELISA reader. The same procedure was performed for control group.

Cell culture

Extracted stem cells from fat tissue were cultured in a DMEM culture medium containing 10% FBS in 25T flasks. After sterilization of nanoscaffolds, cells were cultured on them. It was observed that nanoscaffolds supported cell proliferation and growth. Stem cells were then trypsinized and cultured in DMEM culture medium (supplemented with fetal bovine serum [FBS] 10%, 100U/mL penicillin, 100 mg/mL streptomycin, at 378C with 5% humidified CO2). After cell attachment, stem cells were trypsinized and cultured in DMEM culture medium containing 10% FBS.

Cell morphology

Nanoscaffolds were evaluated using SEM imaging. 75 ml of stem cells with a density of 105 ×2 cells/mL were added to wells that contained sterilized nanoscaffolds. After 2 to 6 days of incubation the surface liquid was removed and nanoscaffolds were washed with phosphate buffer. Finally, cells were fixed on nanoscaffolds using 2.5% glutaraldehyde (1 hour) and then, using ethanol they were dried. They were then imaged using SEM.

Molecular analysis

Stem cells that were separated from fat tissue were cultured in DMEM medium (containing 10% FBS) in 25T flasks. Epidermal differentiation was done using

a culture that contained fibroblast growth factor and epidermal growth factor, on both control group and the samples. RNA extraction was done on day 14 and 21 of differentiation (12000 rpm centrifuge, 15 min, and 4°c). After cDNA synthesis, skin related genes, Integrin B and P63, and B actin housekeeping gene were analyzed by PCR (Table.1).

Table 1: Primers used in RT-PCR (F forward, R reverse).

Primer	Sequence(F+R)	Products Length
P63	F: CCTCACTCCTACAACCATTCCTG R: ACCTCGCTAAGAAACTGACAATGC	199 bp
Integrin B	F: CCATTGGAGATGAGGTTCAATTTG R: CACTTGGGACTTTCAGGGATG	174 bp
B actin	F: GTCCTCTCCCAAGTCCACAC R: GGGAGACCAAAAGCCTTCAT	198 bp

Results

Nanofibers properties

Examination by electron microscopy shows that the synthesized scaffolds have a porous, flawless structure in their morphology. The average diameter of fibers was measured by Microstructure measurement software at about 700-600 nm. Electron microscope images are shown in Figure1.

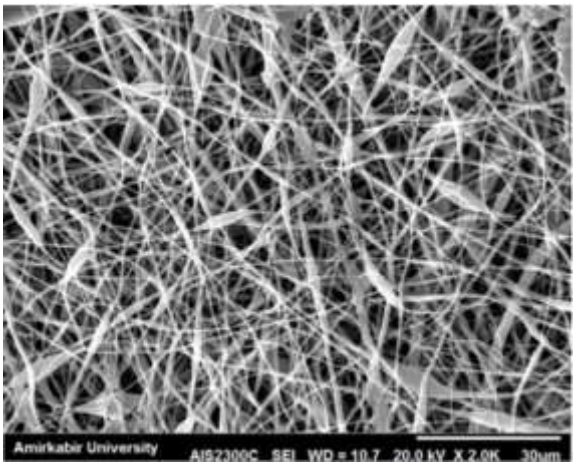

Figure 1: SEM image of PLLA nanofibers a Morphology of fabricated PLLA scaffold (2,000 times magnification)

Assessing the Water Contact Angle

After the measurement, the angle of contact with the water was estimated to be 37 degrees, as shown in the Figure2.

Figure 2: Drop of water on the PLLA scaffold

MTT assay

To assess cytotoxicity, MTT was performed. Results on day 1, 2 and 3 clearly demonstrated a high absorbance of samples compared to control group (figure3).

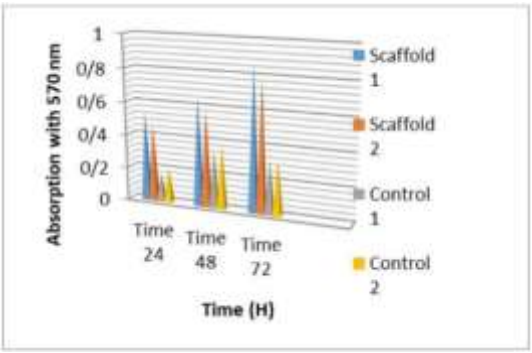

Figure 3: Biocompatibility of PLLA scaffold was investigated via MTT assay which revealed the proliferation rate and viability of MSCs on PLLA scaffold during a 24, 48 and 72 h culture period.

Post-culture cell morphology analysis

SEM images clearly showed that cell were able to attach properly on nanoscaffolds. PPLA had strengthened differentiated keratinocytes attachment. When comparing the results between day 14 and 21, a higher rate of differentiation is observed on day 21. This result can be potential of PLLA in cell attachment, proliferation and differentiation (fig. 4a-b).

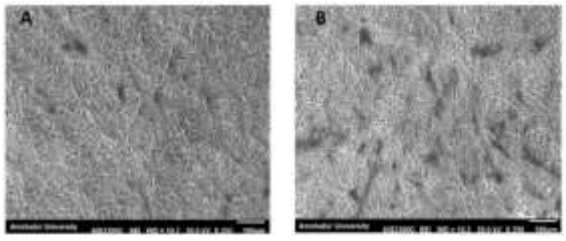

Figure 4: The cell-polymer constructs were fixed in 2.5 % glutaraldehyde, dehydrated through a gradd series of ethanol, vacuum dried, mounted onto aluminum stubs, and sputter coated with gold. Samples were examined using a SEM at an accelerating voltage of 20 kV. After a 14-21 day

culture under epidermal medium cells seeded on PLLA scaffolds after two weeks of differentiation process. An after 14 days, B after 21 days.

Analysis of electron microscopy of cells cultured on nanofiber scaffolds

The mesenchymal stem cells isolated from the fat cultured on the scaffold were photographed by an electron microscope, which shows the normal shape of the cells on the scaffolds, indicating that the cells on the scaffolds grow and multiply properly. Scaffolding has been able to provide a good environment for cells to differentiate into skin cells. Also, the rate of cell differentiation was compared on the 14th and 21st days which was increased on the 21st day.

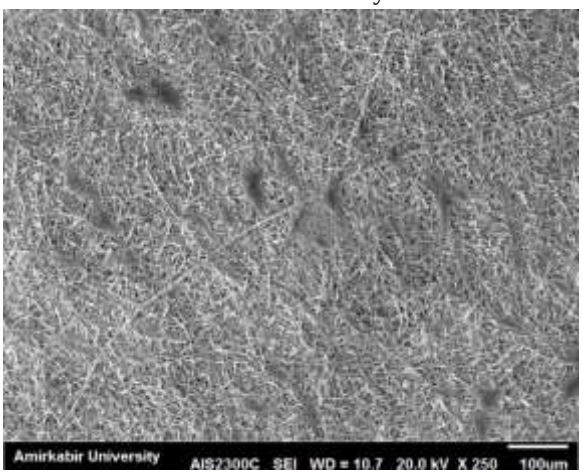

Figure 5: Morphology of cultured cells on PLLA scaffolds on day 14 (250 times magnification).

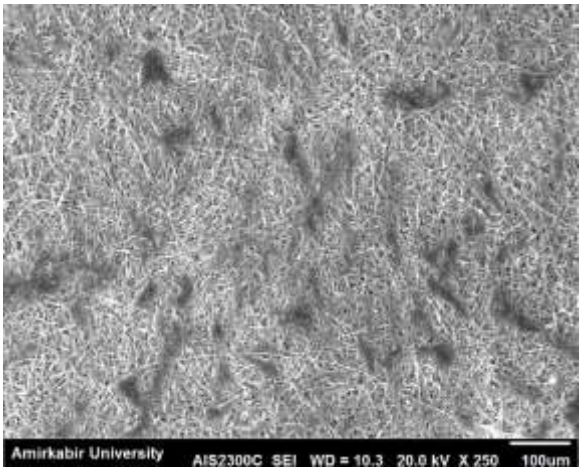

Figure 6: Morphology of cultured cells on PLLA scaffolds on day 21 (250 times magnification).

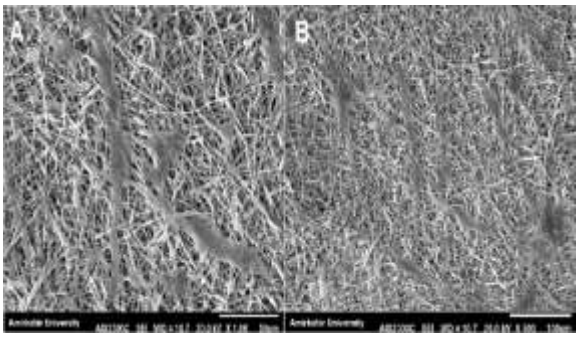

Figure 7: Morphology of cultured cells on PLLA scafolds on day 21, a magnification 1000 times, B magnification 500 times.

RT-PCR

PCR results confirmed expression of B-integrin and β-actin gene; genes that are expressed in keratinocytes of skin epidermis. All of the samples that were loaded on gels had formed a bond, confirming skin-related genes expression. PCR results are show in Fig. 8a–c. The all of 3 genes expression were elevated, but rates of elevations in PLLA seeded cells more significant expressed compare with control.

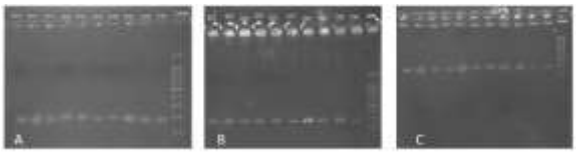

Figure 8: a-c relative gene expression of fibroblast and epidermal growth factor at day 14-21 of skin epidermal keratinocytes differentiation of mesenchymal stem cells on control samples and PLLA nanofibres. A B-actin gene, B Integrin B gene, C P63 gene.

Discussion

It could be stated that one of the main goals of tissue engineers is to regenerate body tissues. To do so, scientists are required to mimic ECM. Nanoscaffolds are good candidates to mimic ECM provided they can present a suitable environment for cell attachment, proliferation and growth (6, 7). PLLA is a suitable polymer candidate to be used in scaffolds since it supports cell attachment, proliferation and growth. Agathe Grémare et al. evaluated the characteristics of PLA for bone tissue engineering (9). It was stated that PLLA was heat resistant, biodegradable and biocompatible. It could only be assumed that PLLA would be feasible for other purposes, such as skin

tissue engineering. Porosity is one of the most important characteristics of nanoscaffolds. Asghari et al. published a study on Biodegradable and biocompatible polymers for tissue engineering application. It was concluded that PLA was a fine polymer because of its characteristics; most notable porosity and the possibility to control it (10). Santoro et al. investigated the potential of Poly (lactic acid) nanofibrous scaffolds for tissue engineering. It was stated that due to its characteristics, PLLA could be of great potential in skin tissue engineering (11). A great number of studies have confirmed the suitability of electrospinnig for tissue engineering purposes. Additionally, it is a cost-efficient and rather simple method (6,7). In this study, for the first time, PLLA nanoscaffolds were fabricated and their potential for skin cells differentiation was investigated. The scaffolds were non-toxic, which was confirmed by MTT assay. To evaluate their potential for stem cell differentiation, mesenchymal stem cells were differentiated on the scaffolds. Overall, our results confirmed that PLLA due to biocompatibility, suitable mechanical properties and porosity supported cell attachment, proliferation and growth as well as mesenchymal cell differentiation. It should be mentioned that cell migration from a layer to another layer of fabricated nanoscaffolds has been rarely reported. In has been additionally reported that after an overall refinement of electrospinning, this process was improved and cell migration was enhanced (12-14). In this study we were able to successfully fabricate PLLA nanoscaffolds in a way that they provided a suitable surface for cell proliferation and growth, in a 3D manner. This study could be further continued to help improve the process of 3D fabrication of organs and tissues.

Conclusion

Our results undoubtedly confirmed the potential of PLLA in tissue engineering. It yielded a feasible nanoscaffold, with great mechanical properties. Furthermore, it helped support cell and stem cell growth, as well as skin differentiation of stem cells.

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