Evaluation of the Mechanical Characteristics of PLGA and PLGA/fibrin Scaffolds in Providing an Appropriate Environment for Viability and Growth of Human Adipose-Derived Stem Cells

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Abstract: Tissue engineered from mesenchymal stem cells (MSCs) requires a scaffold to keep the cells in the area defect and to act as a supporter for inducing tissue formation. We developed a three-dimensional (3-D) special poly-lactic-glycolic acid (PLGA) /fibrin (F) composite scaffold that provided structural supporter and stimulated repair. 3-D PLGA scaffold have been prepared via Solvent casting/salt leaching (SC/SL) techniques and the hybrid scaffold of PLGA/F was fabricated by F. Characterization techniques such as X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FT-IR) and Scanning electron microscopy (SEM) were performed. Furthermore, mechanical properties of the PLGA and PLGA/F composite scaffolds were determined. 3-DPLGA and PLGA/F scaffolds seeded with cultured human adipose tissue-derived stem cells (hADSCs) and analyzed by MTT at 3 and 7 days. The results revealed that the scaffolds contain sufficient porosity with highly interconnected pore morphology. Increase the amount of fibrin enhanced compressive modulus and compressive strength of the PLGA scaffolds. The water absorption capacity difference for the PLGA scaffold with and without F. Our findings showed that PLGA/F scaffold in vitro, enhanced cellular viability was observed compared to PLGA scaffold in 3 and 7 days.

Keywords: PLGA, Fibrin, Scaffolds, Mechanical Characteristics, Human Adipose Derived Stem Cells.

INTRODUCTION

Tissue engineering has been demonstrated a promising approach to restore the tissue defects. The success of this technique relies critically on the seed cells and scaffolds and thereby the structure and functions of the regenerated tissue. Among the various scaffolds used, the hybrid scaffold prepared by filling soft hydrogel into hard sponge is a promising way for the tissue regeneration (cartilage, bone,…) since their advantages can be maintained while the shortcomings can be avoided(1).

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Synthetic scaffold allows a better control of shape, surface morphology, mechanical and physicochemical properties, and possesses predictable biodegradation kinetics. Poly (DL-lactic-co-glycolic acid) (PLGA), which belongs to one of the synthetic scaffolds, has been widely investigated to serve as the substitute of tissue regeneration and approved by the food and drug administration (FDA) of the US for certain clinical applications(2).However, PLGA does not present a favorable surface for cell adhesion, proliferation, and differentiation because of the hydrophobic surface properties and lack of specific cell-recognizable signals(3, 4). To overcome this drawback, an alternative approach is to create a hybrid scaffold using a multifunctional biological protein and PLGA. Because the hybrid scaffold can be used to create a biomimetic cellular environment by balancing the structural and biofunctional element, the advent of biosynthetic hybrid scaffold signifies a major achievement in the fields of tissue engineering(4).

F, an excellent natural polymer, has drawn significant interest in tissue engineering. F presents several important features for the scaffold material: 1) it is an FDA approved material and has been widely used in clinical setting due to its high affinity; 2) it possesses hydrophilicity, biocompatibility, and biogradation; 3) it is rich in fibrinogen protein, which is a well-characterized extracellular matrix (ECM) molecule with a central role in tissue remodeling and chondrocyte–ECM interaction(5).

Some tissue defects have been considered extremely difficult to repair due to the low regenerative capacity of Differentiated cells. Tissue engineering techniques using autologous cells and biomaterial scaffolds have been developed to approach this problem, and several clinical studies have shown that cells grown on biomaterial scaffolds can integrate into the tissue defect site and form functional tissue(2, 6).However, autologous tissue transplantation can leave donor site morbidity. Furthermore Differentiated cells are difficult to isolate in humans, replicate slowly and are prone to phenotypic dedifferentiation in culture. Also, this can be further affected by donor age and healthy status(7).Recently, it was shown that human adipose tissues have mesenchymal stem cells (adipose tissue derived stem cells, hADSCs) that can be differentiated into multiple cell lineages. These cells have great in vitro expansion properties and are potentially an alternative cell source for tissue transplantation(8).These cells have great in vitro expansion properties and are potentially an alternative cell source for tissue transplantation(9).hADSCs have several advantages over bone marrow stem cells, including easy accessibility and minimal invasiveness. There are also no significant differences between the yield, growth kinetics, cell senescence and gene transduction of stem cells from adipose tissue and bone marrow tissue(10).

MATERIAL AND METHODES

Materials

PLGA copolymer (RESOMER® RG 504H, PLGA; 48/52wt% poly (lactide)/ poly (glycolide); inherent viscosity 0.45-0.60 dl/g (25 ºC; 0.1% in chloroform) were purchased from Resomer Boehringer Ingelheim, Germany.sodium salt from were purchased from Sigma-Aldrich Co. Methylene chloride (CH2Cl2, M=84.93 g/mol), tetraethlorthosilicate (TEOS: $Si(OC_2H_5)_4$), triethylphosphate (TEP: $C_6H_{15}O_4P$), calcium nitrate tetra-hydrate ($Ca(NO_3)_2.4H_2O$), hydrochloride acid (HCl) were purchased from Merck Inc. The sodium chloride (NaCl) extra pure salt was purchased from Sigma-Aldrich. Cryoprecipitated Antihaemophilic Factor (Cryoprecipitated AHF) and fresh frozen plasma (FFP) was prepared from the Blood Transfusion Organization, Isfahan, Iran. Calcium gluconate 10% was purchased from pharmacy.

Fabrication and Characterization of the Composite Scaffold

3-D PLGA scaffold have been prepared via solvent casting/salt leaching (SC/SL) techniques using methylene chloride, as previously described(11). Briefly, polymer/ solvent solution (8% w/v concentration of PLGA in methylene chloride) were casted in cylindrical silicon moulds (9 mm in diameter and 3 mm in height) which was filled with sodium chloride salt particles (NaCl) (approximately 180 μm particle size) as porogen. Then, the scaffolds were dried in room temperature for 12 h. In order to leach out the NaCl particles, samples were immersed (soaked) in deionized water for 3rd in 2 days. Ultimately, the samples were freeze-dried at -80 ºC for 72 h in a freeze-dryer (Christ Alpha2_4Ld Plus, Germany) to produce highly porous structure.

FFP pocket was placed into bain marie for 30 min at 37 C. Then, mixture of FFP (16 ml) with calcium gluconate (10 ml) were prepared and casted in falcon tube in order to incubate for 90 min. Then, the mixture centrifuged with 2200 rpm for 10 min. After centrifugation, the supernatant clear liquid accumulated in falcon tube was decanted for thrombin preparation.

Fibrinogen were extracted from cryoprecipitated antihaemophilic factor (AHF) pocket by heating it in bainmarie for 20 min at 37 C. Finally, the equal mixture amount of thrombin and fibrinogen will be lead to fibrin clot formation(11, 12).

Morphology of Scaffold was Observed by SEM

Structural properties of the PLGA and PLGA/F scaffolds were determined using scanning electron microscopy (SEM) (Philips XL300, Holland).

Measurements of Mechanical Properties

The mechanical properties of scaffolds were measured by electromechanical universal testing machine (HCT 400/25, Zwick/Roell, Germany) equipment at 25 °C according to ASTM F451-86 standard. The sample (length, 10 mm; width, 10 mm) end was clipped vertically and elongated at a rate of 1mm/min, and the load-displacement and modulus curve were recorded(11).

Evaluation of Hydrophilicity

Evaluate the hydrophilicity of PLGA and PLGA/F composite scaffolds, their surfaces were characterized by the measurement of water contact angle.

Water Absorption of the Scaffold

The water absorption ratios were measured following the procedure described in a previous report(5). Briefly, each scaffold, cut into 1×1×1-cm3 pieces, wasweighed (Wi) before immersion in PBS. Then each group of the scaffolds was incubated in PBS and maintained in a humidified incubator at 37°C/5% CO2 for 14 days. The scaffolds were then removed from the PBS, gently blotted with filter paper to remove surface water, and immediately weighed (Ws). The scaffolds were dried in an oven for 2 days to completely remove the water, after which they were weighed (Wd) a third time. The water absorption ratios were calculated using the following equation: water absorption ratio= (Ws−Wd)/WI×100.

X-ray diffraction (XRD)

X-ray diffraction (XRD) (Philips PW1800, Holland) was used to determine the created phases of the scaffolds.

Fourier Transform Infrared Spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) (FT Infrared Spectroscope, JASCO, FT/IR-6300 (400-4000 cm^{-1}), Japan) was used to obtain chemical bonds of the PLGA and PLGA/Fcomposite scaffolds.

Evaluation of Porosity of Scaffolds

In order to calculate the porosity of the PLGA scaffold, liquid replacement method, were used,(11)In brief, weighed PLGA scaffold were immerged in a graduated cylinder containing of ethanol with specific volume (V_1) for 2 h. Serially evacuation-repressurization cycles at room temperature and vacuum condition were performed to penetrate the ethanol into the construct. Then, total volume of the soaked scaffold was determined as V_2. (V_2-$V_{1)}$ was the volume of the PLGA scaffold. Thereafter, V_3 was recorded by removing the ethanol-soaked scaffold and calculate the residual ethanol volume. The pore volume of the scaffold recorded by measuring the ethanol volume remains in the PLGA structure was defined as (V_1-V_3). At the end, porosity percentage of the scaffold was calculated using:(11, 13).

$$Porosity(\%) = (V1-V2)/(V2-V3)*100$$

In-vitro degradation

The discs were weighed and then incubated in 10 ml phosphate buffer (pH 7.4) in 28 ml glass vial in an incubator at 37 o C. Polymer discs were picked up at specified times and air dried to constant weight; water- uptake and weight loss were determined gravimetrical- ly. Gel permeation chromatography (GPC) was used for polymer molecular weight determination(14).

Isolation & Proliferation of hADSCs and Cell Culture on PLGA AND PLGA/F Composite Scaffold

Subcutaneous adipose tissue samples were collected in falcon having phosphate-buffered saline (PBS) from four patients (30–50year) who filled the consent form before undergoing cesarean section or abdominal surgery. All samples were digested with 0.075% collagenase type I (Sigma) and incubated for 30min at 37°C in the lab. Next, DMEM low glucose (LG) (Sigma) containing 10% FBS (Invitrogen) was added for enzyme inactivation before being centrifuged (1200rpm, 15min). Removing supernatant, cultured cell pellet in 25 cm2flasks with DMEM LG, 10% FBS, 1% penicillin and streptomycin (Gibco) and incubated with 5% CO2, 37°C. The medium was changed every 4 days.

When the cells reached 80% confluence, detached with 0.05% trypsin/0.53 mM ethylenediaminetetraacetic acid (Sigma) and passage P3-p5 cells were seeded in scaffolds(8).The scaffold was sterilizied with 70% ethanol for 60 min and disinfected via ultraviolet light for 2hrs and scaffolds were washed with PBS.

The sterile scaffold kept in 96 well cell calture plate, finally PLGA scaffolds were soaked in chondrocytes-fibrin suspension (1 x 10(4) cells/scaffold) and polymerized by dropping thrombin-calcium chloride (CaCl2) solution(15).

Cell Viability

The viability of hADSCs with EF exposure was assessed by the MTT assay (3, 4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium-bromide) on 3th and 7th day. At first, the medium of each well was removed, rinsed with PBS, and replaced with 400 μl serum free medium and a 40 μl MTT solution. Next, it was incubated at 37°C, 5% CO_2 for 4 hr.

The medium was discarded and 400 μl DMSO (Sigma) was added to each well, and was incubated in dark for 2 hr. Next, 100 μl of the solution was transferred to a 96-well plate and absorbance of each well was read at 570 nm with ELISA reader (Hiperion MPR4). The assays were performed in triplicate(2, 16).

RESULTS

Mechanical Properties

Figure 1 showed the amount of compressive strength and compressive modulus of the PLGA and hybrid scaffolds. Compressive modulus and compressive strength of the PLGA scaffold were 4.87 MPa and 0.15 MPa, respectively.

By addition of F in the hybrid scaffold, compressive strength and compressive modulus were increased to 0.38 MPa and 5.28 MPa, respectively.
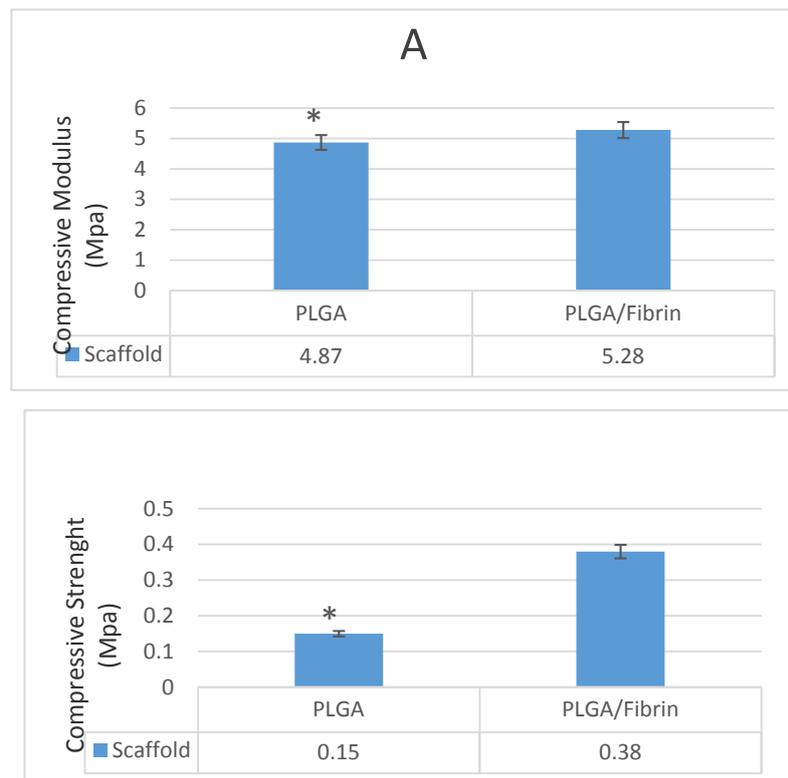

Figure 1: The amount of compressive strength and compressive modulus of the PLGA and hybrid Scaffolds. (P≤0.05*)

Morphology and Microstructure

SEM photomicrographs of the PLGA and PLGA/fibrin scaffolds are shown in Fig 2. The PLGA scaffold illustrated high porosity with well-interconnected porous structure (fig.1a).

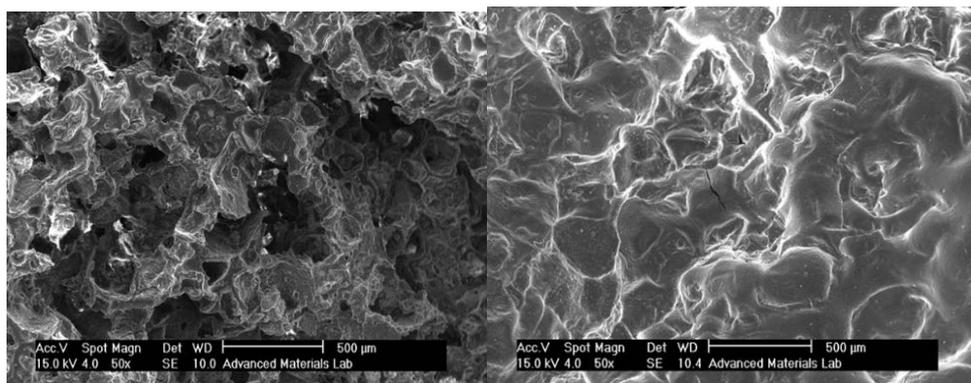The porosity percentage and the pore size of the PLGA scaffold were 87.01±03 and 100-200 μm, respectively.

Figure 2: The SEM photomicrographs of the PLGA (left) and PLGA/fibrin (right) scaffolds

XRD Analysis

To evaluate the microstructure of the scaffolds, we carried out the XRD analysis. The XRD patterns of the PLGA and the PLGA/ F scaffolds are displayed in figure. 3. Amorphous structure of PLGA scaffold was confirmed by XRD. In addition, the XRD patterns of the PLGA/F scaffolds exhibited a uniform pattern and amorphous structure.
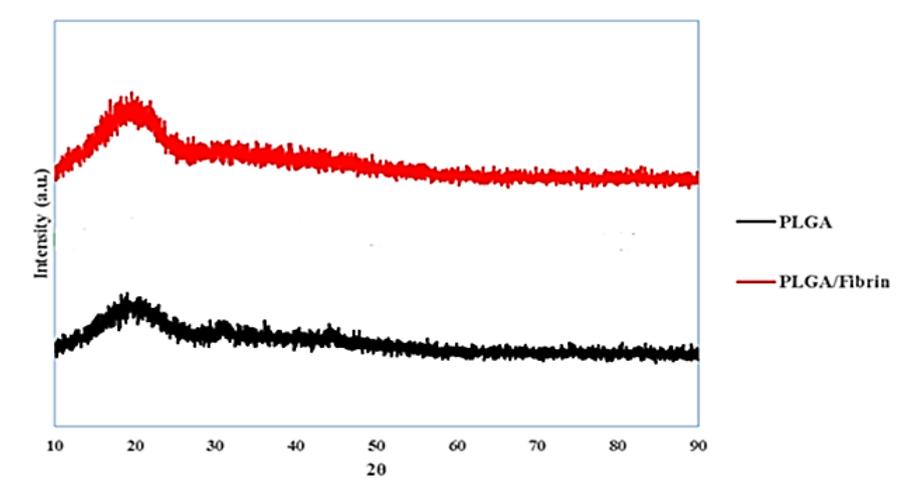

Figure 3: The XRD patterns of the PLGA and the PLGA/ F scaffolds

FT-IR Spectroscopy

FT-IR was used to characterize the PLGA/F hybrid scaffold. FT-IR spectra of the bulk PLGA and PLGA/F are shown in Figure. 4. In the bulk PLGA, the represented peaks of the main functional groups were consisted of -CH, CH2, CH3 (2800-2961 cm-1), C-O (1045-1094 cm-1), ethyl -CH2 (1420 cm-1), OH (3500 cm-1), and carbonyl -CO (1758 cm-1) groups [1]. Based on the FT-IR spectra of the PLGA/Fibrin, the peaks at 1657, 1544 and 1261 cm-1 were corresponded to the characteristic peaks of amide I, II, and III of the fibrin. Also, the FT-IR spectra showed the peaks of the PLGA main functional groups.
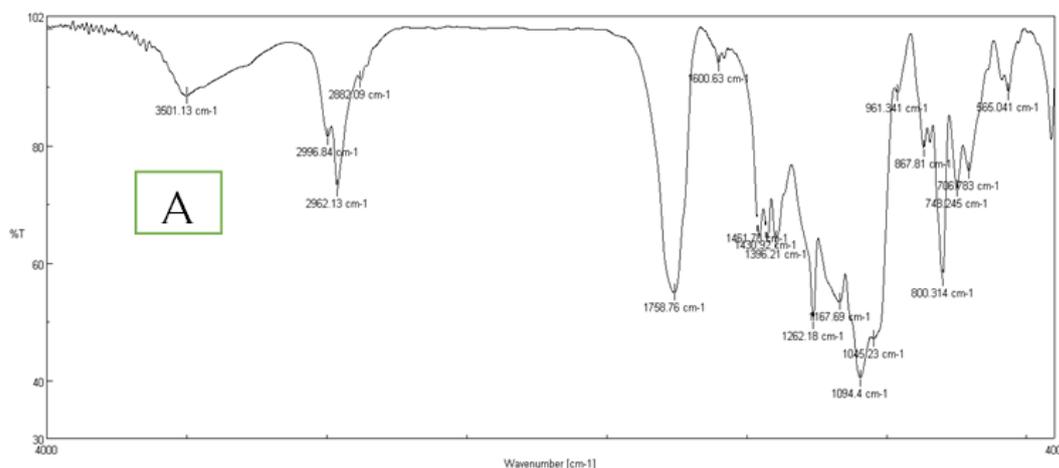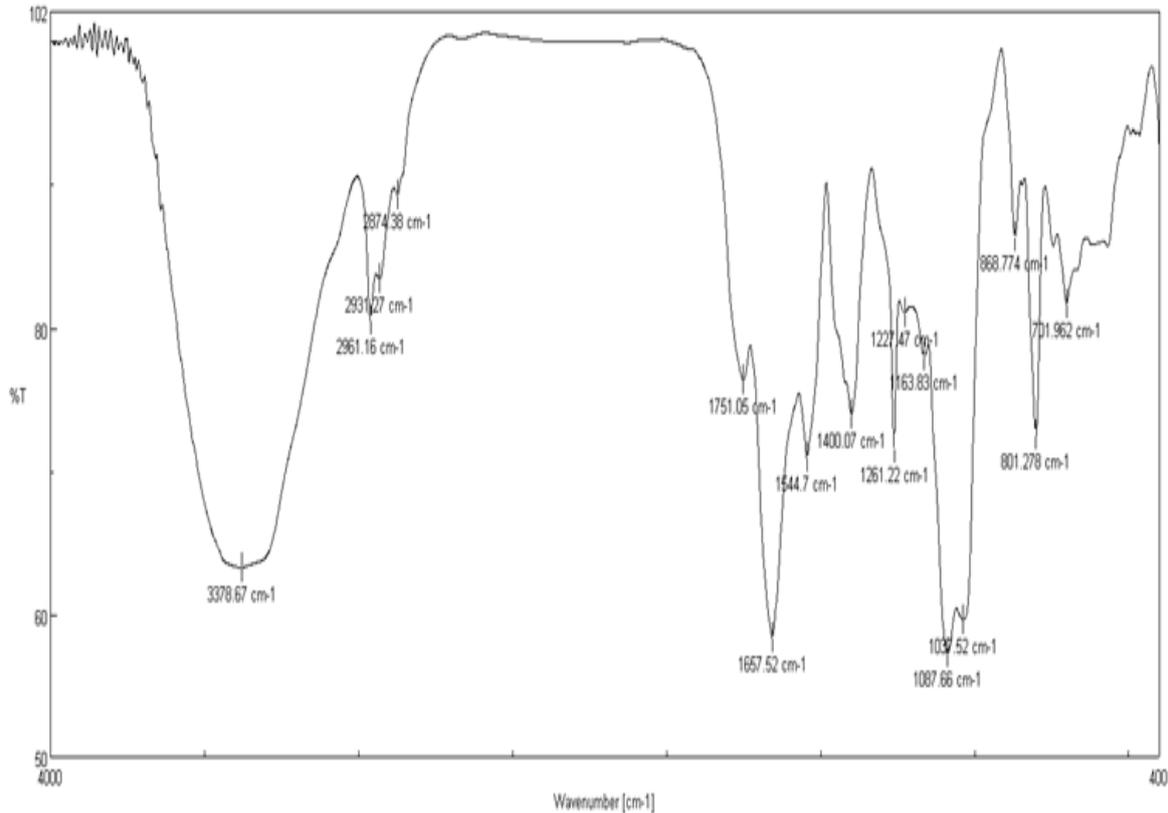
Figure 4: FT- IR spectra of the bulk PLGA (A), and PLGA/F (B)

Contact Angle Measurement of PLGA and PLGA/Fibrin

Contact angle is a measure of hydrophobicity of a material. Higher contact angle means higher hydrophobicity of the material. Here, the advancing contact angle measured for the PLGA was 73.65± 3.78◦ and that of PLGA/F was 48.54 ± 1.37◦. This result confirmed the significant difference in the hydrophobicity of two used scaffold.

Water Absorption of Scaffolds

The water absorption capacity of the PLGA and PLGA/F scaffolds were 44.45±5.88% and 88.17±5.54%, respectively. There was significant difference between the PLGA scaffolds with and without the fibrin (p<0.05).
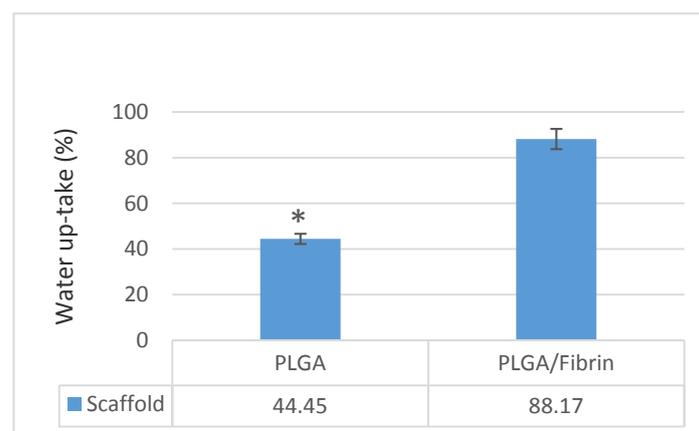

Figure 5: Comparison of Water Absorption results between PLGA and PLGA/Fgroups.

In-vitro Degradation

Figure 6 showed that the mass loss characterization of the scaffolds during the degradation intervals. The scaffold with HA composition have higher water uptake.
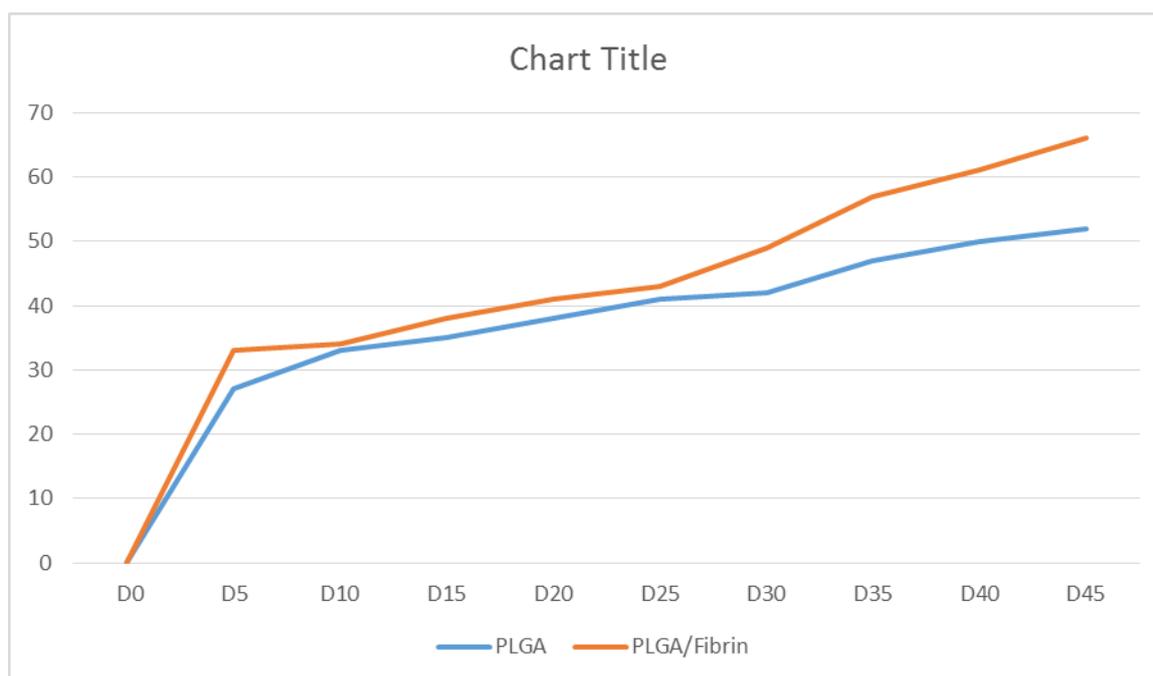
Figure 6: Comparison of Weight loss results between PLGA and PLGA/F groups

MTT Scaffold

MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay was performed on the PLGA and PLGA/fibrin scaffolds in days 3 and 7. The data indicated that on the third day, there was no significant difference between the PLGA and PLGA/fibrin scaffolds but this difference was significant on the seventh day.
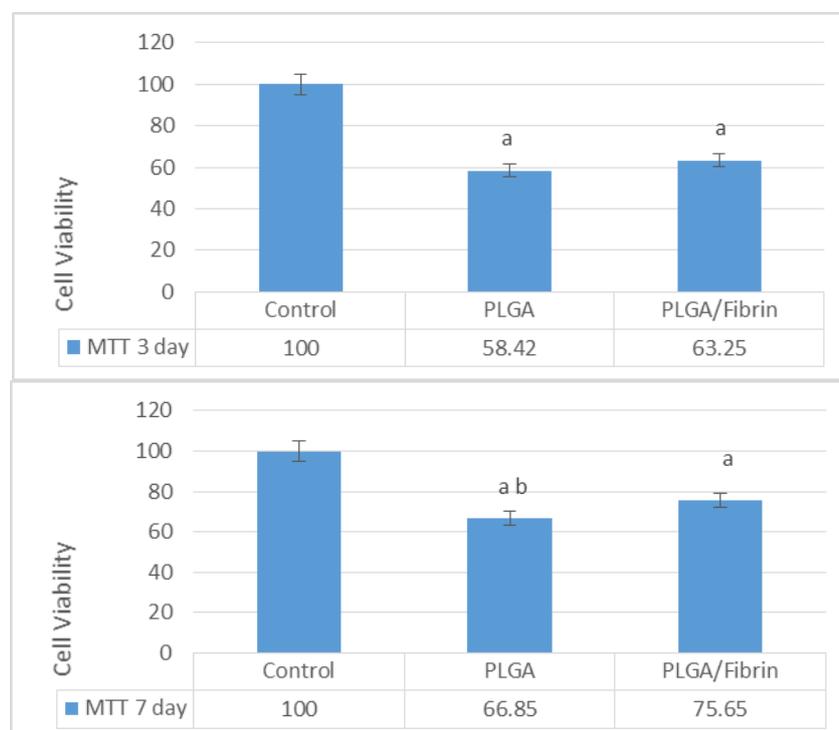

Figure 7: Comparison of MTT assay results between PLGA and PLGA/Fgroups in 3 and 7 days.
a: Significant compared to control group and b: Significant compared to PLGALF group. (P≤0.05)

DISCUSSION

Tissue engineering relies widely on the use of three-dimensional (3D) porous scaffolds to present an appropriate environment for the renewal of tissues and organs. These scaffolds fundamentally act as a template for tissue formation and are usually seeded with cells or occasionally with growth factors(17). A well designed 3-D scaffold is an important factor to guide tissue formation in vitro and in vivo. Numerous

attempts have been made for tissue reconstruction using the PLGA based studies. A number of methods, such as gas forming, freeze-drying or salt leaching have been reported to produce 3D porous matrices from natural and synthetic polymers(18). In the present study, we successfully used solvent casting and particulate leaching (SC/PL) system to fabricate the PLGA scaffold(11).

A PLGA scaffold fabricated on a SC/PL system is not so appropriate for cell attachment, which in turn affect cell proliferation and differentiation. So a hybrid scaffold is required to solve this problem. Therefore, we overcame this problem by using fibrin in combination with PLGA(5).Porosity data showed that the porosity of the scaffolds was high. This increased porosity might be because of higher interconnectivity of the incorporated PLGA scaffold as was evident from the SEM images(19).Pore distribution within the PLGA scaffold was somewhat uniform. Porous PLGA scaffold had macropores and micropores on the wall of the macropores. These structures had ideal amount of open pores and porosity due to the attendance of the porogen particles in its structure. In this regard, present research determined that the presence of interconnected open pores can modify the attachment, migration, proliferation and differentiation of the chondrocyte cells(20). Evaluation of the water absorption suggested that the hydrophilicity was significantly increased in the PLGA/ fibrin scaffold. High water absorption of the PLGA/F scaffold contributes to nutrients transport and cell growth and attachment inside the scaffold(5).The contact angle of the PLGA and PLGA/fibrin scaffolds were measured to confirm the difference in the hydrophobicity of the two polymers used in the model. The results ensured that the used PLGA is more hydrophobic than the PLGA/F.When the scaffold was immersed in the PBS buffer solution. The scaffold with fibrin composition may have higher water uptake, and higher water absorption leads to greater weight loss which results in more rapid degradation(21).Amorphous structure of the PLGA and PLGA/F scaffolds were confirmed by XRD; characteristic amide absorption bands were situated at 1550 cm-1 and 1240 cm-1 that belong to amide II and III groups of the fibrin(22).

In fact, in tissue engineering, mechanical properties (e.g., structural integrity after implantation) are necessary. Mechanical properties of the PLGA scaffold were alleviated owing to the presence of micropores and macropores and also high porosity level of its microstructure. In other samples, however, improvement of mechanical properties could be related to the reduced porosity percentage by coating the scaffolds with fibrin glue. As shown in Fig. 1 b, c, and d, many pores were filled with the fibrin glue. Researchers have confirmed that fibrin can act as a cross-linking agent to increase mechanical properties of tissue engineered porous constructs(23). Therefore, the F performs as a reinforcement agent and modifies the compressive modulus and stiffness of the PLGA scaffold.

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