Biointerface Research in Applied Chemistry
Platinum Open Access Journal (ISSN: 2069-5837)

Article
Volume 11, Issue 4, 2021, 11525 - 11534
https://doi.org/10.33263/BRIAC114.1152511534

Impact of Fibrin on the Chondrogenic Avocado Soybean Unsaponifiables on Poly (Lactic-co-Glycolic) Acid Scaffold

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Scopus Author ID 55613068100
Received: 4.11.2020; Revised: 5.12.2020; Accepted: 9.12.2020; Published: 12.12.2020

Abstract: Synthetic and naturally derived-biodegradable polymers have been applied extensively for scaffold fabrication for cartilage tissue engineering. Human adipose-derived stem cells (hADSCs) seeded in poly (lactic-co-glycolic) acid (PLGA) and PLGA/fibrin scaffolds and cultured in chondrogenic media containing Soybean Unsaponifiables (ASU). All constructs were cultured for 14 days. Cell viability was measured by MTT assay. Chondrogenic differentiation markers, including type II collagen (Coll II), Aggrecan (AGG), and SOX9, were detected by real-time PCR. Hypertrophic and Fibrous differentiation was also analyzed using gene expression type X (Coll X) and I (Coll I) collagen. The MTT results on the 14th day showed that the viability of hADSCs in the PLGA group was higher than the PLGAL/Fibrin group, but it was not significant. Real-time PCR results demonstrated that SOX9, Coll II, and AGG gene expression in the PLGA and PLGA/Fibrin groups are higher than the control group. The real-time PCR results indicated that Coll X in the PLGA/Fibrin group is lower than the PLGA and control groups. Also, Coll I gene expression in the PLGA group was higher in contrast with the control group. Administrating fibrin with a PLGA scaffold can induce chondrogenesis in hADSCs on chondrogenic media containing ASU.

Keywords: poly (lactic-co-glycolic) acid; fibrin, avocado/soybean; human adipose-derived stem cell; scaffold.

1. Introduction

Articular cartilage injury is an increasingly emphasized topic around the world. Kurtz *et al*. estimate that the total number of knee replacements will elevate from 700,000 to 3.48 million yearly by 2030 [1]. Fibrin is a usual biopolymer matrix used for surgical homeostasis and tissue sealing [2]. Due to its good biocompatibility, intrinsic affinity, and good biodegradability for multiple biological levels, fibrin-based biomaterial preparation has been widely used in tissue engineering [3]. Fibrin is a potent three-dimensional scaffold biomaterial for cell proliferation and differentiation to conduct tissue organization [4, 5]. However, fibrin's

mechanical resistance to preserve performed tissue appearance, structures, and textures is negligible. Various synthetic degradable polymers, like poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and their copolymer, poly (lactic acid-glycolic acid) (PLGA), have been developed as scaffolds for tissue engineering [5]. Due to PGA's rapid degradation and the weak affinity of PLA for biological levels, we selected PLGA embedded in a fibrin scaffold in this survey. PLGA is a degradable synthetic polymer that is commonly used for cell seeding [5]. Biodegradation is done to lactic acid and glycolic acid, and eventually to H_2O and CO_2, excreted by normal metabolism. PLGA is gradually degraded, and its diverse contents may have variant effects on mechanical proliferation and strength of cells [6].

There is much agreement on the use of herbal ingredients in rheumatoid arthritis and osteoarthritis disorders. Soybean oils and Avocado contain a class of biologically active compounds classify as Unsaponifiables lipids [7]. The main components of avocado/soybean Unsaponifiables (ASU) In terms of weight, phytosterols are beta-sitosterol, stigmasterol, and campesterol. Preliminary studies have demonstrated the primary beneficial efficacy of phytosterols and their potency to prevent cholesterol uptake and intercede in endogenous cholesterol biosynthesis [7, 8]. Sterol extracts from various plant origins demonstrate their familiar dispensation. An experiment trial on animals revealed their anti-inflammatory outcomes [9]. Avocado/soybean stimulates types II collagen and Aggrecan synthesis while inhibiting stromelysin activity in osteoarthritis chondrocytes [10, 11]. In tissue engineering, hADSC has been found to have a high potential for cell differentiation of various types, including osteogenic, lipid, chondrogenic, and myogenic strains under a convenient situation. Numerous experiments have been performed by hADSC *in vivo* or *in vitro* to persuade the regeneration of various tissues [12].

The purpose of this study is to determine the impact of PLGA incorporated into fibrin scaffold and the chondrogenic ASU medium on hADSCs differentiation.

2. Materials and Methods

PLGA copolymer (RESOMER® RG 504H, PLGA; 48 / 52wt poly (lactide) / poly (glycolide); with substantial viscosity 0.45-0.60 deciliters per gram (25ºC; 0.1% in chloroform) from Resomer Boehringer Ingelheim Germany was purchased. Hasodium salt was purchased from Streptococci Equi from Sigma-Aldrich. Methylene chloride (CH_2Cl_2, M = 84.93 g per mole), trisylphosphate (TEP: $C_6H_{15}O_4P$), calcium nitrate tetrahydrate ($Ca(NO_3)$ $2.4H_2O$), hydrochloric acid (HCl) were purchased from Merck. The antiviral agent Cryoprecipitate (Cryoprecipitate AHF) and Fresh Frozen Plasma (FFP) was obtained from the Blood Transfusion Organization, Isfahan, Iran. Calcium gluconate 10% was purchased from a pharmacy. Avocados/ soybeans were purchased from Sigma-Aldrich.

2.1. Morphology and microstructure of scaffolds.

The morphological and surface, and internal characteristics of PLGA and PLGA / fibrin scaffolds were determined using scanning electron microscopy (SEM). (Philips XL300, Holland).

2.2. Fibrin preparation.

The FFP pocket was put at 30ºC for 30 min in a Bain Marie. A mixture of FFP (16 ml) with calcium gluconate (10 ml) was poured into a falcon tube to incubate for 90 minutes. Mixed

centrifugation was performed at 2200 rpm for 10 min. Afterward, the clear plane fluid accumulates in the flask tube was isolated to produce the thrombin. The fibrinogen was extracted from the pocket of the cryoprecipitate antihaemophilic factor (AHF) at a temperature of 37ºC for 20 minutes by heating in a bain-marie. Finally, an equal mixture of fibrinogen and thrombin resulted in the formation of a fibrin clot [5].

2.3. Poly (lactic-co-glycolic) acid scaffold preparation and Fabrication of the hybrid scaffold.

The 3-D PLGA scaffold is made by solvent casting and particle washing (SCPL) methods using methylene chloride. Polymer/solvent solution (8% by weight concentration in PLGA volts in methylene chloride) was poured into cylindrical silicone molds (diameter 7 mm and height 3 mm) with sodium chloride (NaCl) salt particles (particle size 180 μm) filled as progeny particle. The scaffolding was then dried at room temperature for 12 hours. To remove NaCl, the samples were immersed in deionized water for 3 days. The samples were frozen at -80 72ºC for 72 hours in the refrigerator-freezer (plus Alpha2_4Ld plus Germany) to produce a very porous structure. The scaffolds were then sterilized with 70% ethanol for 60 minutes, disinfected by ultraviolet light for 2 hours, and washed with PBS. Sterile scaffolding was placed on 24 well cell culture plates. Finally, PLGA scaffolds were saturated in fibrinogen hADSCs suspension and polymerized by dissolving calcium chromium thrombin solution ($CaCl_2$) [5, 13].

2.4. Isolation and proliferation of human adipose-derived stem cells.

Human ADSCs were extracted from abdominal subcutaneous adipose tissue taken from women who undergo cesarean section (30-40 years). The adipose tissue was mechanically mixed and washed using PBS (Sigma) and then digested by IA collagenase (1 mg per 1 g). The cell solution was centrifuged at 1500 rpm for 10 min. The bullet was suspended in a chondrogenic culture medium containing DMEM-LG with 10% FBS, 1% penicillin, streptomycin (Gibco), and 10 μg/ml ASU and then cultured and stored at 5% CO2 and 37ºc, monolayer culture used to control [14]. To examine the cells' morphology, the photographs were taken by reverse microscopy at disparate intervals [15].

2.5. MTT assay.

Survival of hADSCs was assessed on the fourteenth day using MTT (3, 4, 5-dimethylthiazol-2-yl), (2, 5-diphenyltetrazolium-bromide). Initially, each well's perimeter was removed, washed with PBS, and replaced with 400 μl of the serum-free solution and 40 μl of MTT solution. Then, it was incubated for 4 hours at 4ºC, 5% CO2. DMSO is discarded, and 400 microliters of DMSO (Sigma) are added to each well and incubated in the dark for 2 hours. In the next step, 100 microliters of the solution transferred to a well plate 96. The absorption of each well at 570 nm was read by the ELISA (Hiperion MPR4) reader. The measurements were performed in triplicate [16, 17].

2.6. RNA extraction and real-time polymerase chain reaction (PCR).

First, the scaffolds were washed in different groups with PBS and digested by trisol (Invitrogen) reagent. Afterward, complete RNA isolation was performed by RNase Minikit (Qiagen). The RNA concentration was determined by applying biophotometer (Eppendorf). 100ng of extracted RNA exerted to reverse-transcribed to cDNA synthesis kit (Fermentas) as

stated by manufacturers' instruction. Comparative quantification of the expression of SOX9, type II, X, and Aggrecan collagen was measured applying the principal Maxima SYBER BER Rox qPCR (fermentation) kit. The starting sequences are shown in Table 1. The genes were normalized to the reference gene (GAPDH). Computation of every target gene's expression level was carried out as characterized previously [5, 18, 19].

2.7. Gene sequence of primers.

Table 1. Primer sequence of a gene (forward and reverse)

Gene	Primer sequences (forward and reverse)
collagen II-F	CTGGTGATGATGGTGAAG
collagen II –R	CCTGGATAACCTCTGTGA
sox-9 –F	TTCAGCAGCCAATAAGTG
sox-9 –R	TTCAGCAGCCAATAAGTG
collagen x –F	AGAATCCATCTGAGAATATGC
collagen x – R	CCTCTTACTGCTATACCTTTAC
collagen I-F	CCTCCAGGGCTCCAACGAG
collagen I-R	TCAATCACTGTCTTGCCCCA
Aggrecan-F	GTGGGACTGAAGTTCTTG
Aggrecan-R	GTTGTCATGGTCTGAAGTT
GAPDH-F	AAGCTCATTTCCTGGTATG
GAPDH-R	CTTCCTCTTGTGCTCTTG

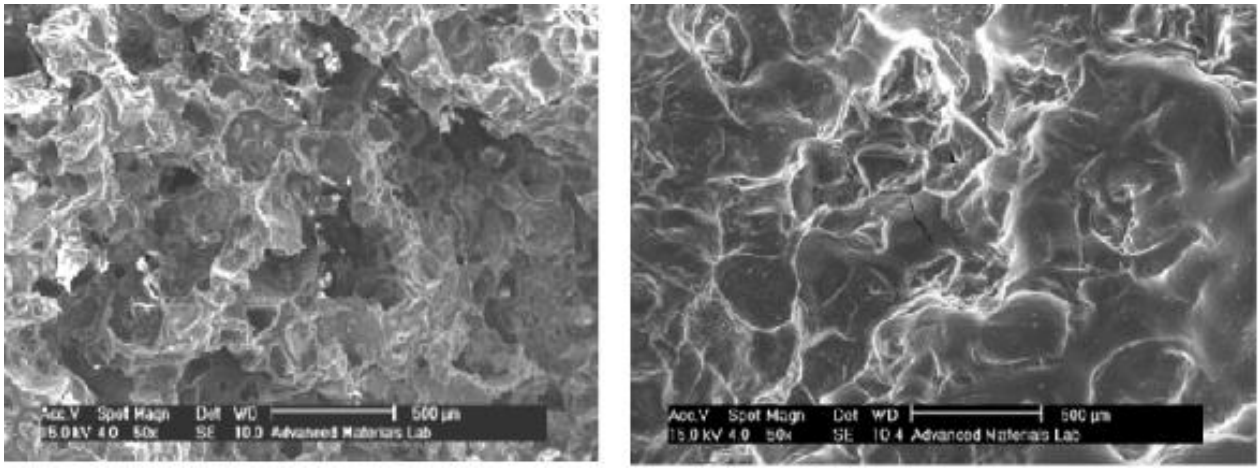
2.8. Statistical analysis.

One-way analysis of variance test was used to assess the differences between different groups, and Tukey follow-up test was performed to determine the difference between the two groups. The term "statistically significant" was used to indicate the bilateral P-value of <0.05.

3. Results and Discussion

3.1. Morphology and microstructure.

SEM photomicrographs of the PLGA and PLGA/fibrin scaffolds are shown in Figure 1. The PLGA scaffold illustrated high porosity with a well-interconnected porous structure. The porosity percentage and the pore size of the PLGA scaffold were 100-200 μm.



(**a**) (**b**)

Figure 1. shows the SEM photomicrographs of the PLGA (**a**) and PLGA/fibrin (**b**) scaffolds. As shown in the figure, fibrin has made the surface of the PLGA scaffold smoother.

3.2. MTT results.

The MTT results on the fourteenth day showed that the viability of hADSCs in the PLGA group was higher than the PLGAL/Fibrin group. However, it was not significant (P>0.05) (Figure 2).

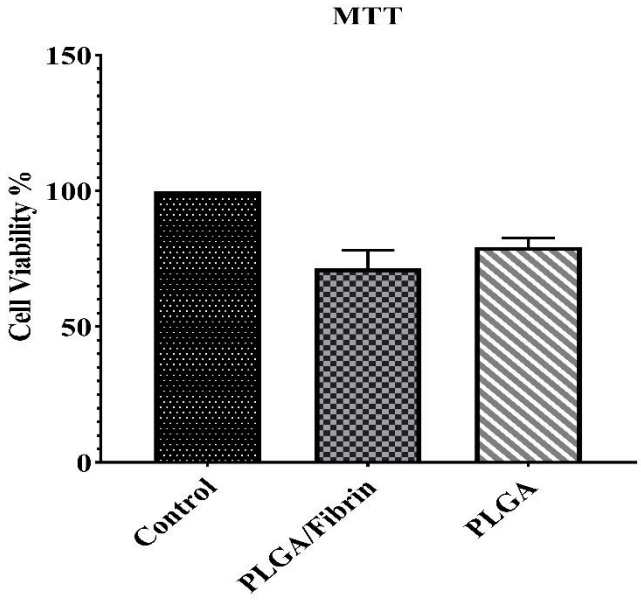


Figure 2. Comparison of MTT assay results between control, PLGA, and PLGA/Fibrin groups. a: Significant compared to the control group. b: Significant compared to the PLGA group. (P≤0.05).

3.3. Real-time PCR.

The results of PCR in real-time indicated that the expression of SOX9, Coll II, and AGG genes in PLGA and PLGA / fibrin groups was significantly higher than the control group (P <0.05). Real-time PCR results showed that Coll X (as a hypertrophic marker) was lower in the PLGA/ fibrin group than in the PLGA and lower control group (P <0.05) (Figure 3- d). Also, the expression of the Coll I gene (as a fiber indicator) in the PLGA group was significantly higher than in the control group. (P<0.05) (Figure 3- e).

In this survey, Porosity data demonstrated a high porosity of scaffolds. This increased porosity may be due to further binding to the PLGA scaffold enclosed by SEM micrographs [20, 21] (Figure 1). The distribution of pores in the PLGA scaffolding was more or less uniform. The PLGA porous scaffold had large pores and micropores in the wall. Due to the presence of germ particles in the structure. In this regard, research has shown that the presentment of morphological open pores can play a role in the binding, migration, proliferation, and differentiation of chondrocyte cells [22, 23].

According to studies, bone morphogenetic protein (BMP), growth factors such as insulin and β-growth factor conversion (TGF-β) can differentiate cartilage in the laboratory and promote cartilage-like tissue formation *in vivo* [24-26]. However, growth factors such as TGF-β not only regulate specific markers of hyaline cartilage but also lead to more significant hypertrophic differentiation and contribute to the growth of fibrous cartilage [27, 28]. Also, rapid degradation, loss of activity, and high costs of growth factors limit their widespread use, especially in clinical applications [29].

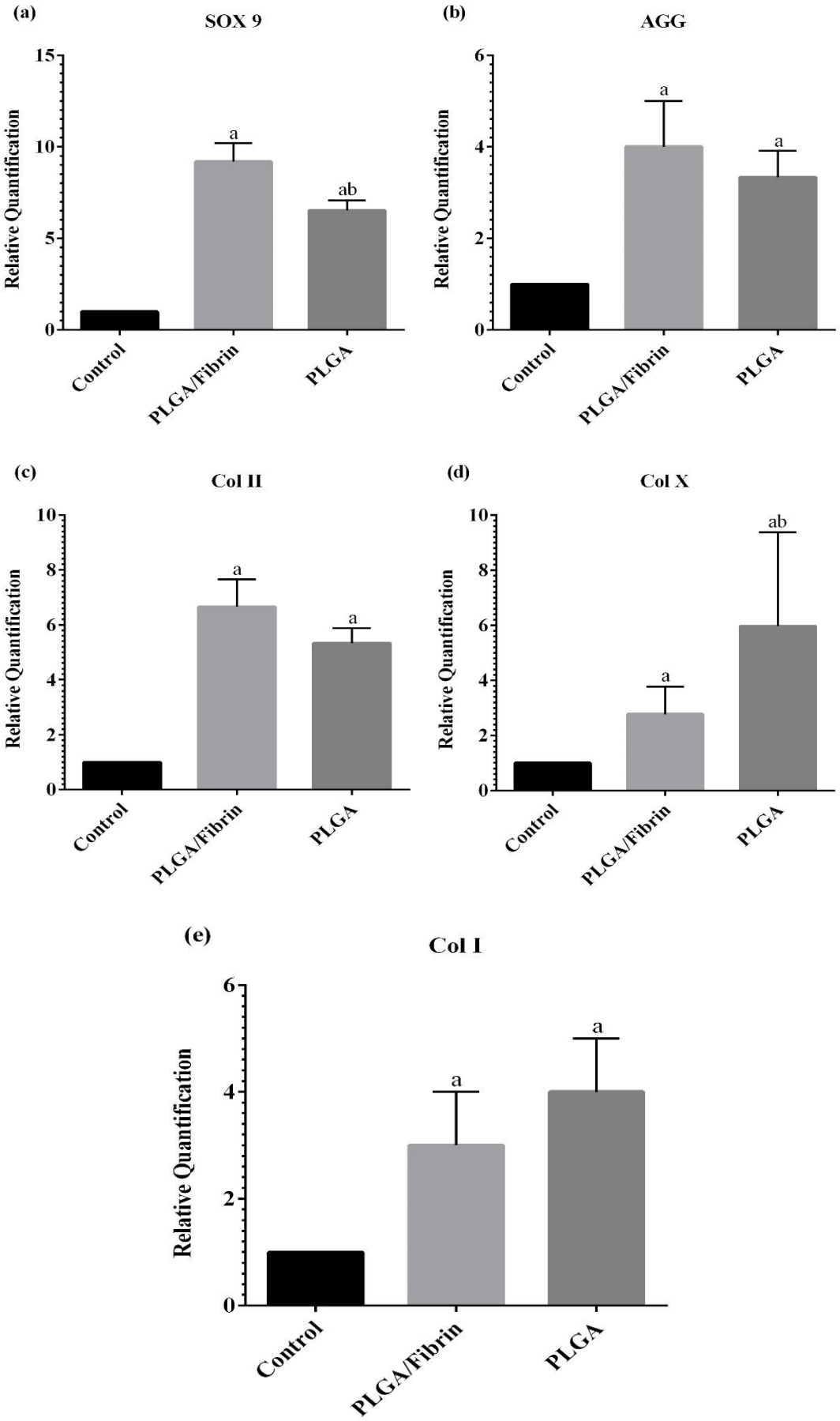


Figure 3. Real-time Polymerase chain reaction for different groups. *Sox 9***(a)**, *AGG* **(b)**, *Coll II***(c)**, *Coll X* **(d)** and *Coll I* **(e).** a: Significant compared to the control group. b: Significant compared to the PLGA group. (P≤0.05).

ASUs are natural vegetable extracts made of avocado and soybean oils in a proportion of one-third to two-thirds [10]. Previous studies have revealed that ASU significantly affects the expression levels of cartilage-specific genes Coll II, AGG, and SOX9 [30, 31]. Similarly, it was seen in this study that ASU notably up-regulated the expression levels of these genes. Some mechanisms can affect the metabolism of laundry cells. For example, ASU increases TGF-β synthesis [10, 32], prohibits metalloprotease activity and synthesis of eicosanoid [31, 33]. In addition, Sterols are immediately incorporated into cells leading to an increment in a cellular antioxidant situation [34].

Fibrin is a biocompatible, biodegradable, and non-immunogenic natural substance [35]; therefore, it explicated this material as an appropriate scaffold for cell carriers that assist in supplying homogeneous distribution of cells with no considerable cells missing through the seeding procedure [36]. Immobilization of chondrocytes in fibrin leads to the homogeneous distribution of cells in PLGA scaffolds, where the cells are quickly delivered and managed [37]. A similar finding has been investigated in further studies of ossification potential administrating human periosteum-derived progenitor cells and the fibrin gel immobilization method in PLGA scaffold [36]. Furthermore, the researchers reported that fibrin dispenses a more identical distribution of chondrocytes through cell seeding by histology in macro-porous polyurethane scaffolds [37, 38]. In this study, the following cells seeding onto scaffolds, cells proliferated significantly in fibrin/PLGA and PLGA. Due to their growth, cartilage cells can secrete the right ECM molecules and secrete chondrocyte-chondrocyte interactions to form clusters of different sizes as well as a three-dimensional structure while maintaining the original shape of the cell.

Although there were significant differences in SOX9 gene expression in the PLGA / Fibrin hybrid scaffold and the PLGA group, there was no significant difference in the semi quantification of gene expression for Coll II and AGG. Interestingly, suppression of cartilage dedifferentiation marker, collagen type I can be observed in the *in vitro* constructs. That's the *Coll I* (as a Fibrous marker) gene expression in the PLGA group is significantly higher than the control group ($P<0.05$). Similar to the present study results, Lee *et al*. reported that the fibrin hydrogels-polyurethane hybrid scaffold system promotes higher levels of cartilage gene expression in the early stage of culture [37]. In this study, fibrin may also act as an ECM by penetrating the pores of the PLGA, further promoting stem cells toward chondrocytes [32].

Accordingly, we suggest that the fibrin added to the PLGA scaffold is an ideal composite scaffold and can amplify laboratory differentiation of HADSCs in the laboratory using cartilage differentiation markers similar to natural hyaline cartilage.

4. Conclusions

PLGA/Fibrin hybrid scaffold promotes early *in vitro* chondrogenesis of hADSCs proven through chondrogenic differentiation markers, including Coll II, AGG, and SOX9 with Less hypertrophy and fibrosis. This study suggests that the PLGA/Fibrin hybrid scaffold may serve as a potential structural basis for *in vitro* tissue-engineered articular cartilage construct.

Funding

This research received no external funding.

Acknowledgments

This research was conducted at Isfahan University of medical sciences, Isfahan, Iran.

Conflicts of Interest

The authors declare no conflict of interest.

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