

Evaluation Avocado Soybean Unsaponifiables Loaded in Poly (lactic-co-glycolic) Acid/Avocado Soybean Unsaponifiables-Fibrin Nanoparticles Scaffold (New Delivery System) is an Effective Factor for Tissue Engineering

Abstract

Background: Growth factors and chemical stimulants have key role in cartilage tissue engineering, but these agents have unfavorable effects on cells. Avocado soybean unsaponifiables (ASU) has chondroprotective and anti-inflammatory effects. In this study, fibrin2nanoparticles (FNP)/ASU, as a new delivery system, with stem cells applied for cartilage tissue engineering in poly (lactic-co-glycolic) acid (PLGA) scaffold. **Materials and Methods:** FNP/ASU prepared by freeze milling and freeze drying. NFP/ASU was characterized by dynamic light scattering (DLS). PLGA-NFP/ASU scaffold was fabricated and assessed by scanning electron microscope (SEM). Human adipose-derived stem cells (hADSCs) were seeded on scaffold and induced for chondrogenesis. After 14 days, cell viability and gene/protein expression evaluated. **Results:** The results of DLS and SEM indicated that nanoparticles had high quality. The expression of type II collagen and SOX9 and aggrecan (ACAN) genes in differentiated cells in the presence of ASU was significantly increased compared with the control group (P and It; 0.01), on the other hand, type I collagen expression was significantly decreased and western blot confirmed it. **Conclusions:** This study indicated FNP/ASU loaded in PLGA scaffold has excellent effect on chondrogenic differentiation of hADSCs and tissue engineering.

Keywords: Avocado, Soybeans, Chondrogenesis, fibrin, nanoparticles, poly(lactic-glycolic acid), stem cells

Introduction

Lack of progenitor cells and blood vessels in cartilage tissue limits self-repair of articular injuries that causes serious problems for orthopedic surgeons.^[1] Inflammatory and chronic diseases of joints lead to severe pain and physical disability. Some treatments such as micro fracture, and autologous chondrocyte transplantation do not stop progress of degeneration.^[2] Hence, tissue engineering proposed as novel strategy for cartilage regeneration. Cells with chondrogenic potential, suitable scaffolds, and biochemical factors are required for cartilage tissue engineering. Human adipose-derived stem cells (HADSCs), a kind of mesenchymal stem cells, are an interesting candidate for cell therapy and tissue engineering with high proliferation and self-renewal capacity.^[3,4] Moreover, these cells have potential for differentiation

into various cells such as chondrocytes, osteoblasts, odontoblasts, and neurons. Easy accessibility, phenotypic stability, and less invasive nature are the most advantages of ADSCs.^[5-7]

Many scaffolds have been developed for protection of cell attachment, differentiation, and creating novel engineered tissue. Natural polymers such as alginate,^[4] collagen,^[8] and fibrin.^[9,10] Synthetic materials such as polyglycolic acid, polylactic co glycolic acid (PLGA), and polycaprolactone^[11] were produced for fabrication of composite or hybrid scaffolds. PLGA as a biodegradable synthetic polymer was approved by the US Food and Drug Administration for clinical use and drug delivery system. Appropriate mechanical properties, biocompatibility, nontoxicity, adjustability of degradation rate, especially toughness, and excellent processability are prominent advantages

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of PLGA.^[12,13] However, PLGA has hydrophobic surface, low bioactivity, and lacks specific cell-recognizable signals for cell attachment and differentiation.^[14] For overcome to disadvantages of PLGA addition of natural materials such as fibrin causes improvement of cell affinity of scaffold. Fibrin hydrogel as a natural polymer, with high potential for use in tissue engineering is achieved from blood plasma. Autologous fibrin lacks the risk of allergic reaction and microbial contaminations. Fibrin scaffold possesses biocompatibility, hydrophilicity, and biodegradability. Fibrin nanoparticles (FNP) with their small size, ease of preparation, low cost, and excellent biocompatibility are a novel material for drug delivery applications. Fabrication of PLGA/FNPs hybrid integrates the advantages of biological performance of fibrin hydrogel with excellent mechanical properties of PLGA.^[15,16]

Several extrinsic and intrinsic factors needed for chondrogenesis. Some members of transforming growth factor- β (TGF- β) superfamily such as TGF- β 1, TGF- β 3, and bone morphogenetic protein 6 (BMP6) are important factors in Marrow stromal cells condensation and cartilage development by stimulation of SOX9 transcription, N-CAM regulation, and extracellular matrix synthesis.^[17-19] Some studies shown that overexpression of TGF- β 3 in murine joints, led to inflammation, stimulation of osteophyte, and increasing of synovial fluid.^[20,21] Moreover, these growth factors are not cost-benefit with short half time and rapid degradation. Therefore, the other factors with high quality and without hypertrophic effects are needed for cartilage tissue engineering.

Nowadays, there is great interest in the function of herbal agents at regenerative medicine.^[14] Herbal extracts of avocado soybean unsaponifiables, (ASU), combine from one-third avocado oil to two-thirds soybean oil.^[22,23] ASU components have anti-inflammatory, pro-anabolic, and chondroprotective properties.^[24-26] Furthermore, *in vitro* studies recommended that ASU could stimulate the expression of TGF β 1 and production of collagen and proteoglycans in chondrocytes and animal models.^[27-29] There are not information about the effect of ASU loaded in FNPs on chondrogenesis. Thus, in the present study, the effect of ASU loaded (without any cross linker) in FNP/PLGA acid scaffold was investigated on chondrogenic differentiation of hADSCs.

Materials and Methods

Preparation of fibrin nanoparticle/avocado soybean unsaponifiables

Fibrinogen and fresh frozen plasma solutions (FFP) were obtained from the Iranian Blood Bank at AL Zahra Hospital. Thrombin prepared from FFP and fibrinogen was extracted from cryoprecipitate according to protocol. FNPs were prepared by dissolving 200 mg of fibrin in 10 ml of NaOH (1 N), to obtain a clear yellow-colored

solution. To this, dilute HCl (1 N) was added drop wise under vigorous stirring (2000 rpm) which eventually the formation of FNPs at pH 5.5. 200 mg of pure powder of ASU (Perarin Pars Co.) added to FNPs and FNP/ASU fabricated by freeze milling (6775 Freezer/Mill[®] Cryogenic Grinder). Later, FNP/ASU was lyophilized and stored at -20°C . FNPs/ASU was sterilized using ultraviolet radiation.

Surface charge and size of fibrin nanoparticle/avocado soybean unsaponifiables

Surface charge (zeta potential) and size of, FNP/ASU determined using Zeta sizer Nano series (Malvern Instruments, USA). Zeta sizer was based on dynamic light scattering (DLS) technique.

Fabrication and characterization of the scaffold

Three-dimensional PLGA (48/52wt% poly (lactide)/poly (glycolide)) (Sigma) with FNP/ASU scaffold prepared through solvent casting/and particulate leaching technique using methylene chloride, as previously described.^[16]

Contact angle measurement

The hydrophobicity of the scaffolds was comparatively evaluated by means of water contact angle (WCA) measurement using a sessile drop technique with an optical bench-type contact angle goniometer (model 100-00-220, Ramé-Hart, USA).

Scanning electron microscopy

Scanning electron microscopy (SEM, Phillips XL 30) was used to study the microstructure and morphology of the PLGA/FNP/ASU and cell attachment.

Isolation and proliferation of human adipose-derived stem cells

hADSCs were isolated from and cultured according to our previous studies.^[16]

Cell seeding and chondrogenic induction

Scaffolds were sterilized and 10⁶ hADSCs cells seeded in each scaffold. One milliliter chondrogenic medium containing Dulbecco's modified eagle medium-high glucose with ascorbate 2-phosphate 50 $\mu\text{g/ml}$ (Sigma – Aldrich), dexamethasone 10⁻⁸ M (Sigma – Aldrich), 1% insulin-transferrin-selenium (Sigma – Aldrich), 50 mg/ml BSA (Sigma – Aldrich), 5 $\mu\text{g/ml}$ linoleic acid (Sigma – Aldrich), and 1% penicillin/streptomycin was added to each well for 14 days. The groups evaluated in chondrogenic process.

PLGA/FNP/ASU + TGF (Experimental).

PLGA/FNP/ASU-TGF (Experimental).

PLGA/FNP + TGF (Experimental).

PLGA/FNP-TGF (control).

(3, 4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium-bromide) assay

The viability of differentiated cells was assessed by the (3, 4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium-bromide) (MTT) assay according to protocols.^[16]

Real-time polymerase chain reaction analysis

Real-time polymerase chain reaction (PCR) was used for the evaluation of gene expression of special cartilage matrix molecules. On day 14, scaffolds degraded and total RNA were extracted from all samples using Trizol in accordance with the manufacturer's protocol. Then, the complementary DNA (cDNA) was synthesized using the Accu Power RT PreMix (bioNEER, Daedeok-gu, Daejeon, Korea). Real-time PCR was completed using SYBR Green PCR Master Mix. All primers used in the real-time PCR were designed by the Allele ID software (ver. 7.6). Gene expression was normalized to housekeeping gene of GAPDH and calculated by relative expression compared to undifferentiated stem cells. Primer sequences included:

COLLII (CTGGTGATGATGGTGAAG-F 2 C C T G G A T A A C C T C T G T G A - R) ,
 SOX9 (TTCAGCAGCCAATAAGTG-F/
 TTCAGCAGCCAATAAGTG-R),
 COLLI (CCTCCAGGGCTCCAACGAG-F/
 TCAATCACTGTCTTGCCCA-R) ACAN
 (C C T T G G A G G T C G T G G T G A A A G G - F /
 AGGTGAACCTCTCTGG CGACG T-R)
 GAPDH (AAGCTCATTCCTGGTAT-F
 CTTCTCTGTGCTCTT-R).

Western blot

Western blot analysis was performed using standard protocols. Then, blots incubated with primary monoclonal antibody against type II collagen (diluted 1:200, NeoMarkers, Fremont, CA), and a monoclonal antibody against type X collagen (diluted 1:500, Invitrogen for overnight at 4°C. Horseradish peroxidase conjugated goat antimouse secondary antibody immunoglobulins G (H + L) (Bio-Rad Laboratories, Melville, NY), diluted 1: 5,000, was used. The goat antimouse secondary antibody conjugated with horseradish peroxidase was added at dilution of 1:2500 for 2 h at room temperature. After final washing, the proteins bands were detected with DAB (Sigma).^[30]

Statistical analysis

One-way ANOVA and the Tukey *post hoc* test used to evaluate the differences between groups.

Results

Size and surface charge of fibrin nanoparticle/avocado soybean unsaponifiables

DLS showed that the size of FNP/ASU was 129–140 nm and the zeta potential was 5.7.

Contact angle results

The average of contact angle in PLGA, PLGA/FNP, and PLGA/FNP/ASU is about 82°, 22° and 23° respectively.

Scanning electron microscopy results

For the investigation of the structure and pore size of fabricated scaffold and with ADSCs by SEM [Figure 1 left, right]. PLGA/FNP/ASU scaffold had homogeneous porous morphology and pore size about 200 μm (left). ADSCs seeded in PLGA/FNP/ASU (right).

Results of gene expression

The results of real-time indicated that cartilage specific (type II collagen and SOX9 and ACAN) genes expression in the experimental groups is significantly higher than the control group ($P \leq 0.01$). ACAN gene expression in the TGF, ASU, and TGF + ASU groups was upregulated 29, 25.5, and 56 folds in compared with undifferentiated stem cells ($P \leq 0.01$). The expression of SOX9, chondrogenic master gene, was increased 35.8 folds in PLG/F/ASU and about 40.8-fold in PLGA/F/TGF groups while in PLGA/F was 5-fold in compared with stem cells ($P \leq 0.01$). The mRNA expression of SOX9 gene up-regulated up to 55.8 folds in the group with two factors (ASU in scaffold + TGF in medium). COL II gene expression was 72 and 39 times in PLG/F/ASU and PLGA/F/TGF groups, respectively, but in PLGA/F/ASU/TGF increased 160 folds significantly ($P \leq 0.01$). The real-time showed that the mRNA expression of type I collagen (fibrocartilage marker) was decreased in ASU group in compared with all groups. This gene in TGFβ3 group was the highest but not significant [Figure 2].

22Results of (3, 4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium-bromide) assay in differentiated cells

MTT assay results indicated in three experimental groups proliferation and viability was decreased significantly in compared with PLGA/Fibrin group ($P \leq 0.05$) [Figure 3].

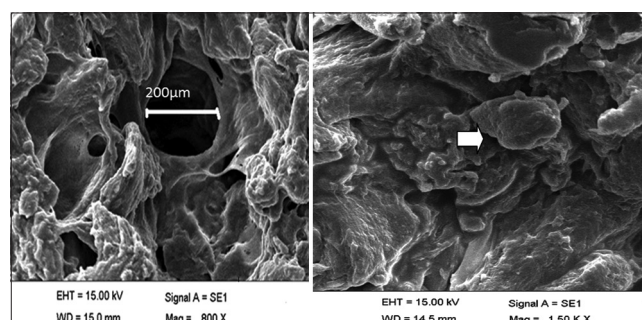


Figure 1: Scanning electron microscope images of the poly (lactic-co-glycolic acid)/fibrin nanoparticle/avocado soybean unsaponifiables (pore size, left) with adipose derived stem cells (right)

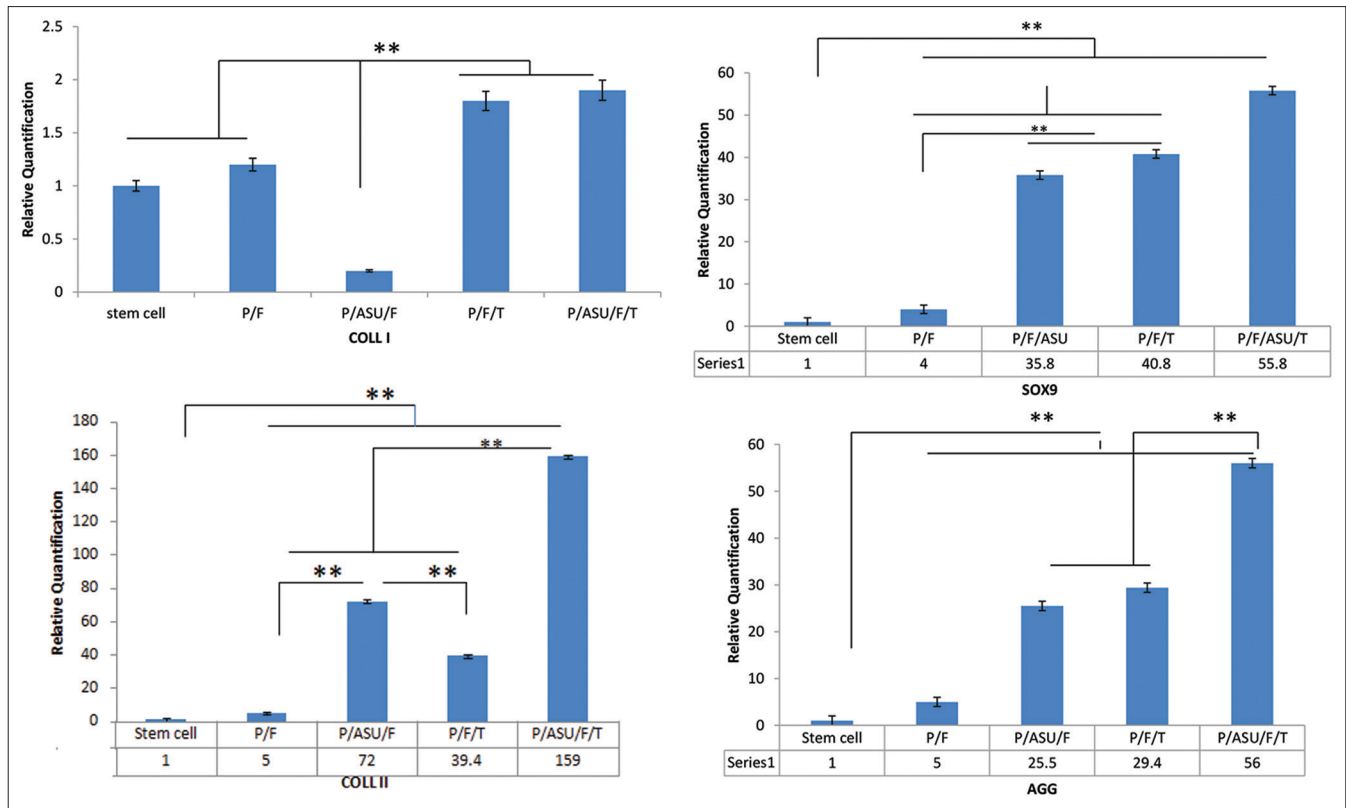


Figure 2: The results of real-time polymerase chain reaction. AGG aggrecan, Sox 9, type II collagen and type I collagen. Gene expression was normalized to housekeeping gene of GAPDH and calculated by relative expression compared to undifferentiated stem cells. The experiments were performed three times. $** (P \leq 0.01)$

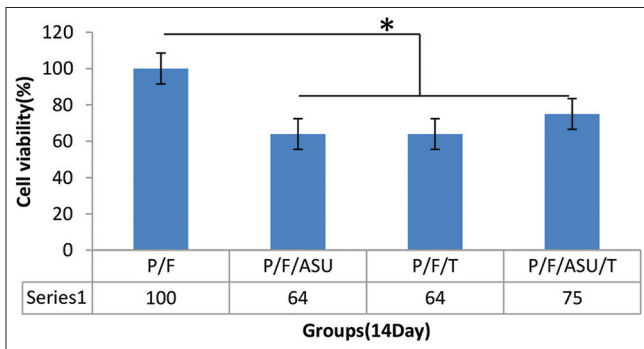


Figure 3: (3, 4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium-bromide) assay results of adipose-derived stem cells cultured in chondrogenic medium after 14 days. Viability in poly (lactic-co-glycolic) acid/F without factors was highest. There were significant differences between poly (lactic-co-glycolic) acid/F/avocado soybean unsaponifiables, poly (lactic-co-glycolic) acid/F/transforming growth factor, and poly (lactic-co-glycolic) acid/F/avocado soybean unsaponifiables/transforming growth factor groups to P/F ($P \leq 0.05$)

Western blot

The result of protein COLL II was increased and COLLX as fibrotic factor very low in PLGA/F/ASU to control (PLGA/F) significantly. These data show that ASU is effective for chondrogenic [Figure 4].

Discussion

In the present study, the performance of the ASU loaded in FNPs investigated on the chondrogenesis of

ADSCs. Fibrin-ASU nanoparticles were prepared and loaded in PLGA as a scaffold and localized delivery system (Fourier transform infrared proved FNP/ASU in PLGA). Because having small size, nanoparticles can penetrate into the cells, and when use as the delivery of the growth factors, and therapeutic agents can transfer to target organs and tissues.^[31] Smaller particles possess larger surface-to-volume ratios and present high drug loading capacity with slow rate of drug diffusion.^[25] In this research, a hybrid scaffold consisting of PLGA as the matrix and the fibrin/ASU nanoparticles as the reinforcement was fabricated for cartilage tissue engineering applications. This combination significantly improved the hydrophilicity of the scaffold and consequently, the cell response. The results of SEM indicated that incorporating fibrin-ASU nanoparticles in PLGA scaffolds significantly increased the pore sizes of scaffolds. Some researchers opposed that WCA is a good predictor for biological responses to materials.^[32] The assessment of WCA in this study indicated that the scaffolds exhibited a reduction in the WCA when loaded by fibrin and fibrin-ASU nanoparticles. This proves the beneficial effects of incorporating FNP-ASU into PLGA scaffolds. In the current study, the expression of selected chondrogenesis-specific genes (SOX9, COLLII, SMAD3, and aggrecan) was examined. Our finding revealed that there is an up-regulation in the gene expression profile

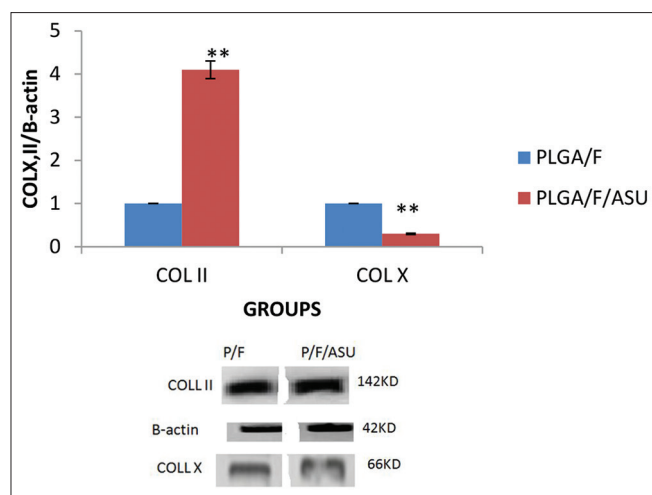


Figure 4: Comparison between poly (lactic-co-glycolic) acid/F and poly (lactic-co-glycolic) acid/F/avocado soybean unsaponifiables about protein of COLL II, X ($P \leq 0.01$)

of sox9, collagen type II, and aggrecan. Henrotin *et al.* indicated that ASU can stimulate the expression of type II collagen in chondrocytes in monolayer culture.^[33] It has been reported that chondrogenic differentiation of Wharton's jelly-derived mesenchymal Stem cells on PLGA provided a better condition for proper chondrogenesis compared to two-dimensional culture of these cells.^[34] Altinel *et al.* showed ASU treatment lead to increase TGF- β 1 and TGF- β 2 levels in the joint fluid in animal model.^[28] These data suggest that ASU induces production of endogenous TGF β s and causes more mRNA expression in chondrogenesis. We showed ASU could promote the expression of of SOX9 and COLL II genes in differentiated cells. Different studies have shown that BMP signals upregulated the expression of Sox9 which that, is a key gene in chondrogenesis, and promotes the expression of type II collagen and aggrecan.^[35] Li *et al.* demonstrated that the expression of SOX9 significantly increased by ASU.^[36] Similarly, our results indicated that ASU in combination with TGF- β 3 enhanced the expression of SOX9. Cell proliferation decreases during chondrogenic differentiation and our results indicated that in experimental groups with high differentiation, the viability of cells is lower than control group. Overall, the gene expression results of this study showed that incorporation of Avocado/soy bean in FNPs loaded in PLGA scaffolds significantly increased the chondrogenic differentiated potential of hADSCS.

Conclusions

In this study, we fabricated fibrin-avocado/soybean nanoparticles and loaded in the poly (glycolide co lactic acid) scaffold for chondrogenic induction of hADSCS. The PLGA/F/ASU scaffold that introduced in this study is a qualified and effective delivery system for *in vitro* chondrogenic differentiation.

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Conflicts of interest

There are no conflicts of interest.

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