Original Article

The Effects of Fibrin–icariin Nanoparticle Loaded in Poly (lactic-co-glycolic) Acid Scaffold as a Localized Delivery System on Chondrogenesis of Human Adipose-derived Stem Cells

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Abstract

Background: Nowadays, cartilage tissue engineering is the best candidate for regeneration of cartilage defects. This study evaluates the effect of fibrin/icariin (ICA) nanoparticles (F/I NPs) on chondrogenesis of stem cells. **Materials and Methods:** F/I NPs were characterized by Dynamic Light Scattering DLS. Poly (lactic-co-glycolic) acid (PLGA)-F/I NP scaffold was fabricated and assessed by scanning electron microscope. Human adipose-derived stem cells (hADSCs) were seeded on scaffold and induced for chondrogenesis. After 14 days, cell viability and gene expression were analyzed by the 3-(4, 5- dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. MTT assay and real-time polymerase chain reaction (RT-PCR). **Results:** The size and surface charge of F/I NP were about 28–30 nm and − 17, respectively. The average of pore size of PLGA and PLGA–fibrin/ICA was 230 and 340 μm, respectively. Cell viability of differentiated cells in P/F group was higher than others significantly ($P \leq 0.05$). Furthermore, quantitative RT-PCR analysis demonstrated that ICA upregulated cartilaginous-specific gene expression. Furthermore, the results of the expression of type I collagen revealed that ICA downregulated this gene significantly ($P < 0.01$). **Conclusions:** The results indicated that F/I NP could be a potential factor for chondrogenesis of stem cells and downregulation of fibrocartilage marker.

Keywords: *Adipose-derived stem cells, chondrogenesis, fibrin nanoparticles, icariin, poly (lactic-co-glycolic) acid*

Introduction

Tissue engineering using stem cells, bioactive molecules, and scaffolds in order to improve biological functions of damaged cartilage is a new therapeutic strategy in regenerative medicine.[1] Scaffold structure is considered as the most important factor to create a better interaction between cells, tissue, and bioactive molecules.[2] Therefore, the use of specific scaffold with the highest porosity as well as biodegradability will have maximum performance in tissue engineering.

Poly (lactic-co-glycolic) acid (PLGA) is a copolymer with appropriate mechanical properties and biodegradability, which was used for chondrogenic differentiation.[3,4] It has been reported that PLGA is a hydrophobic composite and has a low interaction with surrounding cells and tissue.[5] As a result, PLGA cannot facilitate cell attachment. Therefore, PLGA often used in combination with other materials has optimal physical properties and creates a large and accessible surface area for cell anchorage.[6]

Fibrin is a hydrogel-forming polymer with natural origin which usually mimics key elements of normal tissue and is able to accumulate extracellular matrix (ECM) components in the space around the cells.[7] Previous studies indicated that the hybridization of synthetic and natural derived biodegradable polymers such as fibrin and PLGA is capable to increase cell attachment and proliferation and promote early chondrogenesis[8] due to increased cell seeding efficiency and homogeneity. In addition, in a similar experiment, it is reported that fibrin/PLGA hybrid scaffold can be considered as a potential delivery vehicle for cell and growth factors such as transforming growth factor-beta-3 (TGF-β3).[9] TGF-β3 as a member of TGF-β family is able

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to promote chondrogenesis process through specific receptors and with intracellular signaling.[10-12] This factor has a low half-life and high price and have some side effects such as osteophyte formation and synovial membrane inflammation.[13] Therefore, to reduce these side effects, the replacement of TGF-β with other agents is essential.

Icariin (ICA), an herbal component, widely used in order to treatment of fracture and joint diseases, also can act as a substitute for growth factors in order to promote chondrogenesis of stem cells.[14]

The main mechanism of ICA in the treatment of cartilage diseases is not clear. Nevertheless, several potential pathways such as increasing the expressions of cartilage-specific genes,[15] decreasing the expression of type I collagen,[16] increasing ECM synthesis,[15] and anti-inflammatory[17] effects may control its pharmacological effects. Since the half-life of the growth factors is short and when delivered exogenously, their efficacy is reduced; nanomaterial based systems as novel therapeutic strategies have become a primary choice for drug delivery due to unique physicochemical properties of nanoparticles.[18] Nanoparticles do not have any effect on the biological activity of growth factors and substantially prolong their biological half-life.[19] Thus; cell differentiation can be successfully supported by continuous release of growth factors. According to several current published data, nanodrug delivery system by the use of different nanoparticles such as PLGA/hyaluronic acid/fibrin/bioactive glass nanocomposite,[3] chitosan nanoparticles,[20] and TGF-β1-loaded fibrin–poly (lactide-caprolactone) nanoparticulate[9,21] is an attractive system for treatment of cartilage repair.

In this study, we prepared a novel delivery system by combining of PLGA-loaded fibrin–ICA nanoparticle scaffolds as a localized delivery system on chondrogenesis of human adipose-derived mesenchymal stem cells (hADSCs).

Materials and Methods

Preparation of fibrin–icariin nanoparticles

Thrombin was prepared by incubation of calcium gluconate (10 ml) and fresh frozen plasma (16 ml) for 90 min. 0.675 mg ICA (Sigma) was added to 5 ml fibrinogen and mixed with thrombin. Fibrin nanoparticles (FNPs) were prepared by dissolving 200 mg of fibrin in 10 ml of NaOH (1 N). To this, diluted HCl (1 N) was added dropwise under vigorous stirring (2000 rpm) which eventually led to the formation of FNPs at pH 5.5; this was milky white color. The FNP was transferred to a dialysis bag for 24 h. Later, FNPs were lyophilized and stored at −20°C.

Size and surface charge of fibrin nanoparticles

Surface charge (zeta potential) and size distribution of FNP were determined using Zetasizer Nanoseries (Malvern Instruments, USA).

Loading of fibrin–icariin nanoparticles in poly (lactic-co-glycolic) acid scaffold

At first, sodium chloride (0.3 g), as a porogen, was poured in each well up to a height of 3 mm. In the next stage, PLGA (22.5 mg) and fibrin–ICA nanoparticles (2.5 mg) were resolved in 1.5 mL of dichloromethane solvent. The prepared solution was vortexed and then was stored. The frozen solution was then transferred into a freeze-drying vessel (Labconco-Freezone, USA) for 48 h to eliminate the solvent.

Scanning electron microscope imaging

Scanning electron microscope (SEM) (Hitachi S-3400N) was used for the observation of the internal pore morphology of the scaffolds.

Contact angle measurement

The hydrophilicity and wet ability of the scaffolds were determined using a water contact angle measuring system (WCA Optima, AST Products, Inc. software, model 100-00-220, Ramé-Hart, USA).

Isolation and culturing of stem cells

hADSCs were isolated and cultured according to our previous studies.[22]

Cell seeding and differentiation

hADSCs (10^6 cells/ml) were suspended in chondrogenic medium and seeded in sterile scaffolds (PLGA/FNP = P/F and PLGA/fibrin–ICA nanoparticles = P/F/I). Each scaffold cell in chondrogenic medium with and without TGF-β3 (10 ng/ml) was incubated for 2 weeks.

MTT assay

Viability of differentiated cells was assessed by MTT assay according to protocol.[3]

Real-time polymerase chain reaction analysis

Evaluation of SOX9, COLII, COLI, and AGG genes was evaluated by real-time polymerase chain reaction (PCR) technique. Total RNA was isolated by Yekta Tajhiz Azma kit. Complementary DNA (cDNA) was synthesized by the cDNA synthesis kit (YKTA kit). Relative quantification of gene expression was measured using Maxima SYBER® Rox qPCR Master Mix kit (Fermentas). The experiments were performed three times. All primers were designed by the Allele ID software (ver. 7.6) in accordance with Table 1.

Statistical analysis

The results were analyzed by SPSS Statistics version 21.0 software. One-way ANOVA analysis and least significant

Table 1: Gene sequences of primers

Gene	Primer sequences (forward and reverse)
Collagen II-F	CTGGTGATGATGGTGAAG
Collagen II-R	CCTGGATAACCTCTGTGA
Sox-9-F	TTCAGCAGCCAATAAGTG
Sox-9-R	TTCAGCAGCCAATAAGTG
Collagen I-F	CCTCCAGGGCTCCAACGAG
Collagen I-R	TCAATCACTGTCTTGCCCCA
Aggrecan-F	CCTTGGAGGTCGTGGTGAAAGG
Aggrecan-R	AGGTGAACTTCTCTGGCGACGT
GAPDH-F	AAGCTCATTTCCTGGTATG
GAPDH-R	CTTCCTCTTGTGCTCTTG

difference *post hoc* test were operated with a significance level of $P < 0.05$.

Results

Size and surface charge of fibrin–icariin nanoparticles

DLS showed that the size of F/I NP almost is 28 nm and zeta potential is −17 mv.

Scanning electron microscope results

PLGA scaffold exhibited a porous structure and pore size varying from 210 to 250 μm [Figure 1a]. The PLGA/F/ICA scaffold had greater pore size (300–380 μm) [Figure 1c]. After cell seeding, SEM images of scaffolds indicated the differentiated cells attached and spread within the pore walls with spindle shape and cytoplasmic process in PLGA/F/ICA scaffold and in pure PLGA scaffold cells are spherical without process [Figure 1b and d].

Contact angle results

The average of contact angle in PLGA, PLGA/F, and PLGA/F/ICA is about 82°, 42°, and 27°, respectively.

Human adipose-derived stem cells

Stem cells isolated from human adipose tissue revealed spindle- and stellate-like cells in monolayer culture [Figure 2]. In the third passage, stem cells with fibroblast-like morphology increased.

MTT assay results

Viability of differentiated cells in P/F, P/F/T, P/F/ICA, and P/F/ICA/T groups was 100%, 62%, 70%, and 60%, respectively. Cell viability in P/F group was higher than others significantly ($P \leq 0.05$) [Figure 3].

Results of gene expression

The results of real time indicated that cartilage-specific (type II and I collagen and SOX9 and aggrecan) gene expression in the experimental groups is significantly higher than the stem cell group ($P < 0.01$).

Aggrecan gene expression in the PLGA/F, PLGA/F/TGF, PLGA/F/ICA, and PLGA/F/ICA/TGF groups was upregulated 5-, 21-, 19-, and 22-folds compared with undifferentiated stem cells ($P \leq 0.01$). Expression of SOX9, chondrogenic master gene, was increased in PLGA/F: 5 PLGA/F/TGF: 39, PLGA/F/ICA: 63, PLGA/F/ICA/TGF: 85 folds compared with stem cells ($P \leq 0.01$). COLII gene expression was similar in PLGA/F/ICA and PLGA/F/TGF

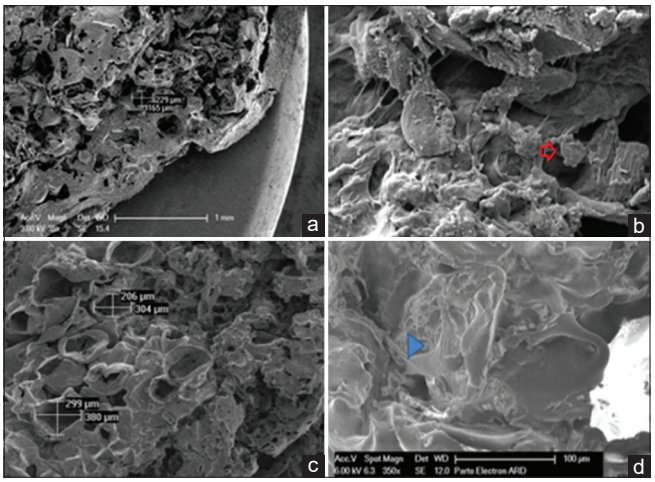

Figure 1: Scanning electron microscope images of the (a) poly (lactic-co-glycolic) acid, (b) poly (lactic-co-glycolic) acid with adipose-derived stem cells, (c) poly (lactic-co-glycolic) acid/fibrin/icariin, (d) poly (lactic-co-glycolic) acid/fibrin/icariin with adipose-derived stem cells

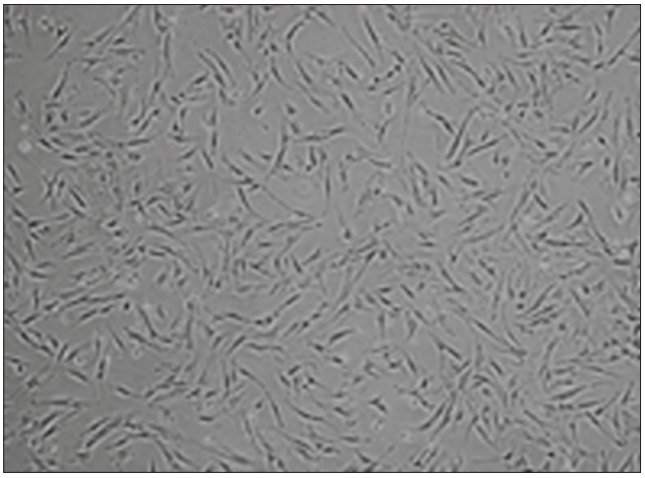

Figure 2: Human adipose-derived stem cells isolated from adipose tissue in monolayer culture. (×40)

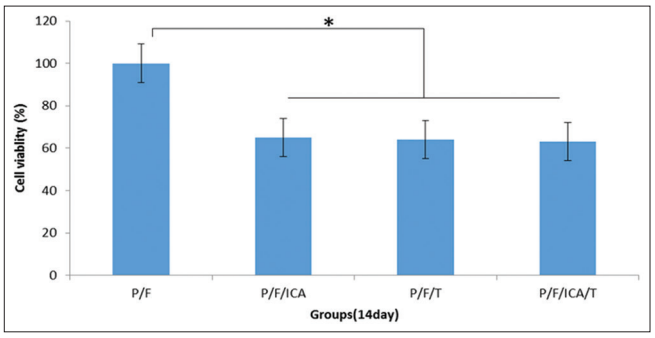

Figure 3: Comparison of MTT assay results between groups. The viability of cells in poly (lactic-co-glycolic) acid/fibrin scaffold is highest ($P \leq 0.05$)

groups (42 and 49 times, respectively) but in PLGA/F/ICA/TGF group increased 160 folds significantly ($P \leq 0.01$).

The result of real time showed that the mRNA expression of type I collagen (a fibrocartilage marker) was decreased in PLGA/F/ICA group compared with PLGA/F/TGF and PLGA/F/ICA/TGF groups [Figure 4].

Discussion

The nanoparticles are widely used in drug delivery, regenerative medicine, and tissue engineering researches. The particle size at the nanoscale allows for the study of the effects of biological and drug molecules and the transfer of them to target cells.[23-26]

In our study, fibrin-ICA nanoparticles were prepared and loaded in PLGA as a scaffold and localized delivery system for chondrogenic induction of human adipose-derived stem cells into chondrocytes.

DLS measurements revealed that FNPs exhibited particle size in the range of 22–30 nm with the zeta potential value of −17.8 mV in deionized water (pH 6.8) and −28 mV in PBS (pH 7.4). Such negative zeta potential nanoformulations prevent particle aggregation and help repel each particle in the suspension, thus maintaining their stability for a long time.

Some researchers reported different methods for preparation of FNP such as water-in-oil emulsification and cross-linking by the factor XIII or glutaraldehyde.[27,28] In our study, no cross-linking agents were used.

Vedakumari *et al*. used wet precipitation method for fabrication of FNPs and reported the FNP size in the range of 25–28 nm with the zeta potential value of −10.8 mV in deionized water (pH 6.8) and −23 mV in PBS (pH 7.4).[29] Nanofibrin preparation method and results in our study were similar to Vedakumari report.

PLGA is a Food and Drug Administration-approved material with low immunogenicity, nontoxicity, and biodegradability. However, the lack of cell attachment sites, poor hydrophilicity, and low surface energy are disadvantages of PLGA.[30,31]

By impregnating of PLGA with natural polymers such as fibrin for scaffold fabricating, cell adhesion, proliferation, and differentiation could be significantly improved.[32,33]

Several previous studies confirmed that the combination of fibrin with PLGA promoted homogeneous cell distribution, cell seeding, and chondrogenesis of stem cells *in vitro* and *in vivo*.[34,35]

In our study, the images of SEM indicated that the pore sizes of PLGA/F/ICA scaffold were greater than the PLGA) 340 and 230 μm respectively). NaCl particles with 180–220 μm in size that were employed as a porogen for the fabrication of porous PLGA/F/ICA scaffolds provided enough spaces and proper environment for cell viability and attachment. Unlike the PLGA scaffold, SEM demonstrated a stable three-dimensional and interconnected network microstructure within the PLGA/F/ICA scaffold [Figure 1]. Based on these results, we suppose that the impregnated

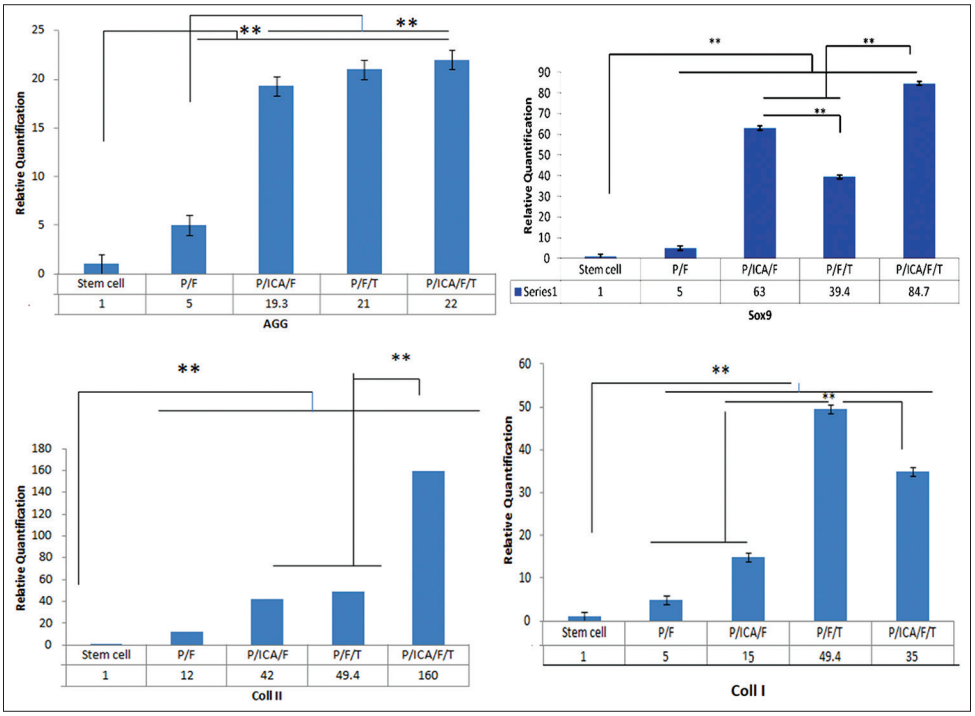

Figure 4: The results of real time-polymerase chain reaction indicated AGG, SOX9, type II collagen, and type I collagen genes were expressed in all experimental groups. Gene expression was normalized to housekeeping gene of *GAPDH* and calculated by relative expression compared to stem cells. **($P \leq 0.01$) P/F (PLGA/FIBRIN), P/ICA/F (PLGA/ICARIIN/FIBRIN), P/F/T (PLGA/FIBRIN/TGF), P/ICA/F/T (PLGA/ICARIIN/FIBRIN/TGF)

F/ICA contributes to the pores and wall surfaces of the PLGA scaffold helping them to support cell attachment and implantation of the engineered constructs for regeneration and repair of injured tissues.

Lien *et al*. showed that pore sizes between 250 and 500 μm of scaffolds are appropriate for chondrocyte proliferation and ECM secretion.[35] Cell phenotype, activity, differentiation, and ECM production vary by the size of scaffold pores. When the pore size is about 30 times, the cell diameter differentiated cells are more likely to be chondrocytes.[36-38]

Growth factors play a crucial role in the regulation of adult stem cell (ASC) differentiation. A number of studies have demonstrated that bone morphogenetic protein and TGF-β are able to induce chondrogenic differentiation *in vitro* and promote the formation of cartilage-like tissue *in vivo*.[39-42] Because the low half-time of these growth factors and the high amount of them can result in side effects, delivery system is proposed as a beneficial strategy for release of optimal growth factor or drug.[43-45]

Avocado/soybean, ICA, and pomegranate extraction were used in rheumatoid arthritis as an anti-inflammatory drug. ICA is a safe and effective natural anti-inflammatory drug.

Our studies demonstrated that adipose-derived stem cells (ASCs) from human were successfully isolated and were induced to differentiate into chondrocytes on PLGA/fibrin/ICA scaffold with and without TGF-β3.

By comparative observations and evaluations of these constructions in *in vitro* culture, we found that the ICA and TGF-β cause in hADSC differentiation into cartilage cells and increase the synthesis of cartilage-specific matrixes. ICA and TGF-β together have better chondrogenic effects than one factor alone.

Our results indicated the expression of type II collagen, aggrecan, and SOX9 genes in experimental groups. The presence of ICA in scaffold as a chondrogenic inducer compared with TGF-β in medium increased the expression of SOX9 gene. SOX9, a key gene in chondrogenesis and differentiation, promotes the expression of type II collagen and aggrecan.[46] Li has demonstrated that the expression of SOX9 significantly increased by ICA as growth factor.[14] Similarly, our results indicated that ICA with TGF-β3 enhances the expression of SOX9 considerable.

ICA enhances the expression levels of Smad proteins, including Smad1, Smad4, and Smad5, which are key regulators specific for activation of TGF-β signaling pathway and chondrogenic induction.[47,48] In addition, ICA upregulates the expression and secretion of various growth factors, including TGF-β. Some researchers have proven that ICA is an anabolic agent, which can enhance chondrocyte proliferation and reduce ECM degradation.[16,47,49]

Li and *et al*. showed the ICA will upregulate the expressions of cartilage-specific genes of seeded chondrocytes. Furthermore, ICA can increase the synthesis of cartilage matrix, accelerates and maintains the formation of chondroid tissue.[14]

Our study also showed that TGF-β3 not only upregulates the expression of hyaline cartilage-specific markers but also unavoidably leads to further hypertrophic differentiation and contributes to the development of fibrous cartilage. The expression of COL I in TGF-β and ICA groups was 35.15 times compared with stem cells, respectively. Similarly, other studies found that TGF-β3 alone led to higher expression of type I and X collagens, while ICA downregulated these genes.[50]

Conclusions

The results of this study demonstrated that ICA loaded in PLGA/FNPs could induce chondrogenic differentiation of human adipose-derived stem cells compared with TGF-β3 effectively.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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