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Research Article

Effect of icariin on the chondrogenesis of human adipose derived stem cells on poly (lactic-co-glycolic) acid/fibrin composite scaffold

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ABSTRACT:

Objective: Icariin (ICA), a Traditional Chinese Medicine, has been demonstrated to be a promoting compound for extracellular matrix synthesis and gene expression of chondrocytes.

Design: In the present study, human adipose derived stem cells (hADSCs) were cultivated in poly (lactic-co-glycolic) acid (PLGA)/fibrin composite scaffold with a chondrogenic medium with and without icariin for 14 days; ICA was added to the same chondrogenic medium but without TGF-β3 throughout the culture period at a concentration of $1x10^{-5}$M.

Characterization techniques X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR) were performed for PLGA and PLGA/fibrin scaffolds. Cell morphology was observed using an inverted microscope. chondrogenic differentiation markers, including collagen II, aggrecan and SOX9, were detected by Real-time polymerase chain reaction (real-time PCR).Hypertrophic and Fibrous differentiation was also analyzed using gene expression type X and I collagen . Cell viability in each scaffold were evaluated using MTT chondrogenic assay.

Results: MTT results show that viability in the icariin group, is higher than TGF-β group but not significant ($P\geq0.05$). Also reveals that cell viability were significantly different ($P\leq0.05$) between Group TGF-β and ICA compared to control group.

The results of real-time PCR show that SOX9, type II collagen and aggrecan gene expression in the group having TGF-β and ICA are higher than the control group. Also SOX9, type II collagen and aggrecan gene expression in the ICA group is significantly higher ($P<0.05$) than the TGF- β group. The results of real-time PCR show that type X and I collagen gene expression in the group affected by ICA, is lower than TGF-β group. But type X and I collagen gene expression didn't have any significant differences between control and ICA group.

Conclusions: These results indicated that hADSCs containing the ICA in PLGA/fibrin composite scaffold are an effective way to potentially enhance articular cartilage regeneration with less hypertrophy.

Key words: icariin, chondrogenesis, human adipose derived stem cell (hADSC), poly (lactic-co-glycolic) acid/fibrin scaffold

INTRODUCTION:

Due to the lack of blood vessels, nerves, lymph supplies and the intrinsically poor differentiating, migratory ability of chondrocytes, damaged cartilage has only limited ability of self-healing (1). Although many methods have been developed for the restoration of defects of articular cartilage,

they have some inherent shortcomings. One of current strategies is to construct grafts derived from the expanded autologous or allogenous chondrocytes and the scaffolds which provide temporary template. Then, the grafts are used to restore the defects(2).The applications of this strategy, including matrix-supported autologous chondrocyte implantation (MACI) and cartilage tissue engineering, can provide ample grafts for the restoration, and may be promising. However, the chondrocytes are easy to dedifferentiation and hypertrophy in the in vitro culture, even seeded in a biomimetic three dimensional (3D) microenvironment (3). It leads to lower quality of new-formed chondroid tissue, worse efficacy and sometimes severe inflammation. In order to maintain the chondrocytic phenotype and promote chondrogenesis, some growth factors such as TGF- β, IGF and BMPs etc are added into the grafts (4-6). However, the high cost, rapid degradation and easy-lost activity of growth factors lessen the value of these growth factors. The security of the addition of growth factors is still doubtful, because of the widely effects of them and the worrying methods introduced (7, 8). Therefore, the identification for other accelerators with low cost, steady activity and security is really needed.

hADSCs are own to stable maintenance of undifferentiated status the pluripotency does not decrease in a growing body during the expansion in vitro, and adipose tissue is obtained by the extraction from the patient in a less invasive manner, and provides a large quantity of autologous cells, hADSCs represent a fascinating cell source for regenerative medicine and will open new avenues for therapeutic approaches (9). The use of hADSCs in cartilage tissue-engineering applications creates a need for more research in cell scaffolds.

Herb Epimedium (HEP) is a widely used traditional Chinese herbal medicine on arthritis in China, Japanand and Korea. Icariin ((2-(4′-methoxylphenyl)-3-rhamnosido-5-hydroxyl-7-glucosido-8-(3′-methyl-2-butylenyl)-4-chromanone), a natural flavonoid glucoside isolated from plants in the Epimedium family, has been proven to have various pharmacological activities. previous study suggests that icariin promote ECM synthesis and the expressions of sox9, collagen type II (Col 2) and aggrecan (AGG) genes of chondrocytes in 2D culture (10). However, in natural cartilage, chondrocytes locate in 3D lacuna. Many studies have proved that the cytobiological properties of the chondrocytes cultured in 3D environment are far different from which in 2D (10, 11). In this study, human adipose derived stem cells will be embedded by icariin in poly (lactic-co-glycolic) acid/fibrin composite scaffold.

As physical support and template, the scaffold plays an important role in tissue engineering by supplying a 3D substrate for cell growth and tissue regeneration. Poly(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. 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 (12). 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 (13).

Fibrin, an excellent natural polymer, has drawn significant interest in tissue engineering. Fibrin 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 (13). A hybrid scaffold was manufactured by filling fibrin into PLGA sponge, which integrated both the advantages of better mechanical performance of PLGA and biological performance of fibrin gel (14). The chondrocytes distributed evenly in the hybrid scaffold with a round morphology alike in their native matrix, and thereby could better maintain their phenotype in terms of Glycosaminoglycan (GAG) secretion during an in vitro culture (14).

In this study, we fabricated the hybrid scaffold from PLGA/fibrin and hypothesized that the combination of the hybrid scaffold and hADSCs was suitable for cartilage regeneration. Our study is to evaluate the capacity of differentiation of hADSCs into chondrocytes on the PLGA/fibrin scaffold with and without icariin in vitro.

MATERIALS AND METHODS:

Fabrication and characterization of the composite scaffold:

3-D PLGA(RESOMER® RG 504H, PLGA; 48/52wt% poly (lactide)/ poly (glycolide) scaffold have been prepared via solvent casting and particulate leaching (SCPL) techniques using methylene chloride, as previously described, (15). 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 particle. 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 to produce highly porous structure.

Fibrin preparation:

Fresh frozen plasma (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 bain marie for 20 min at 37 C. Finally, the equal mixture amount of thrombin and fibrinogen will be lead to fibrin clot formation (15, 16).

X-ray diffraction (XRD):

X-ray diffraction (XRD) (Philips PW1800, Holland) was used to determine the phases of the scaffolds (15).

Fourier transform infrared spectroscopy:

Infrared spectrum of any compound or drug gives information about the groups present in that particular compound. IR spectrum of Capecitabine, PLGA and PLGA/fibrin were obtained. The powder Samples were mixed with KBr to make pellets. FT-IR spectra in the absorbance mode were recorded using FT-IR spectrometer. Various peaks in IR spectrum were interpreted for the presence of different groups (17) .

Isolation& proliferation of hADSCs and Cell culture on PLGA/fibrin composite scaffold:

Subcutaneous adipose tissue samples were collected in sterile tubes having phosphate buffered saline (PBS) from theree patients (30–50year) who filled the consent form before undergoing abdominal surgery. All samples were digested with 0.075% collagenase type I (Sigma) and incubated for 30 min 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 cm_2 flasks with DMEM LG, 10% FBS, 1% penicillin and streptomycin (Gibco) and incubated with 5% CO_2, 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). Passage P3 cells were seeded in scaffolds (9, 18). 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 24 well cell calture plate, finally PLGA scaffolds were soaked in hADSCs-fibrin suspension (1 x $10^{(6)}$ cells/scaffold) and polymerized by dropping thrombin-calcium chloride (CaCl2) solution (19).

MTT assay (3, 4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide):

The viability of hADSCs was assessed by the MTT assay on 14th 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% CO2 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 (20, 21).

RNA isolation and real-time polymerase chain reaction (PCR):

Real-time quantitative RT-PCR was performed to quantitatively estimate the mRNA expression of type II, X collagens, aggrecan and SOX9 genes in hADSCs at different groups. Total RNA was isolated by RNeasy mini kit (Qiagen), treated by RNase-free DNase set (Qiagen) to eliminate the genomic DNA. The RNA concentration was determined using a biophotometer (Eppendorf). Total RNA (100 ng) was reverse-transcribed to cDNA by using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The Maxima syber Green Rox qPCR master mix kit (Fermentas) was used for real-time RT-PCR. Primer sequences are shown in Table1.1. Real-time PCR reactions were performed by using the Comparative Ct (ΔΔCt) method. The relative expression level of genes was computed by calculating the ratio of the amount of genes to that of endogenous control (GAPDH). Melting curve to determine the melting temperature of specific amplification was produced. These experiments were carried out in triplicate and were independently repeated at least 3 times (22).

Table1.1. Gene sequence 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 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

Statistical analysis:

One way ANOVA test was used to evaluate the differences between groups and the Tukey post-hoc test was operated for determination of differences between each two groups.

The term 'statistically significant' was used to signify a two-sided P-value <0.05

RESULTS:

XRD analysis:

For evaluation of Microstructure of the scaffolds, we carried out XRD analysis. The XRD patterns of the PLGA and the PLGA/fibrin scaffolds were displayed in fig1. Amorphous structure of PLGA scaffold was confirmed by XRD. In addition, the PLGA/fibrin scaffolds XRD patterns exhibited the same pattern and amorphous structure.

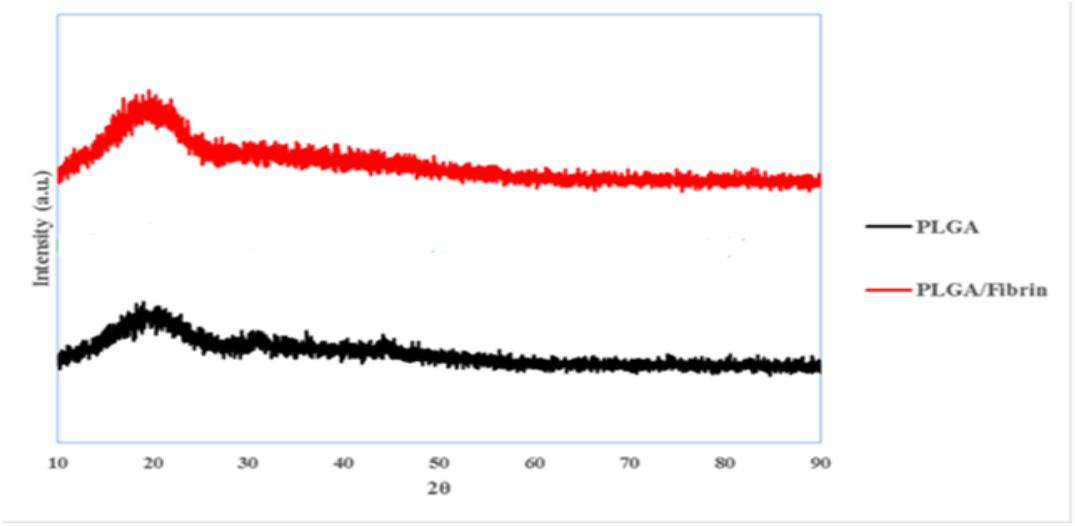
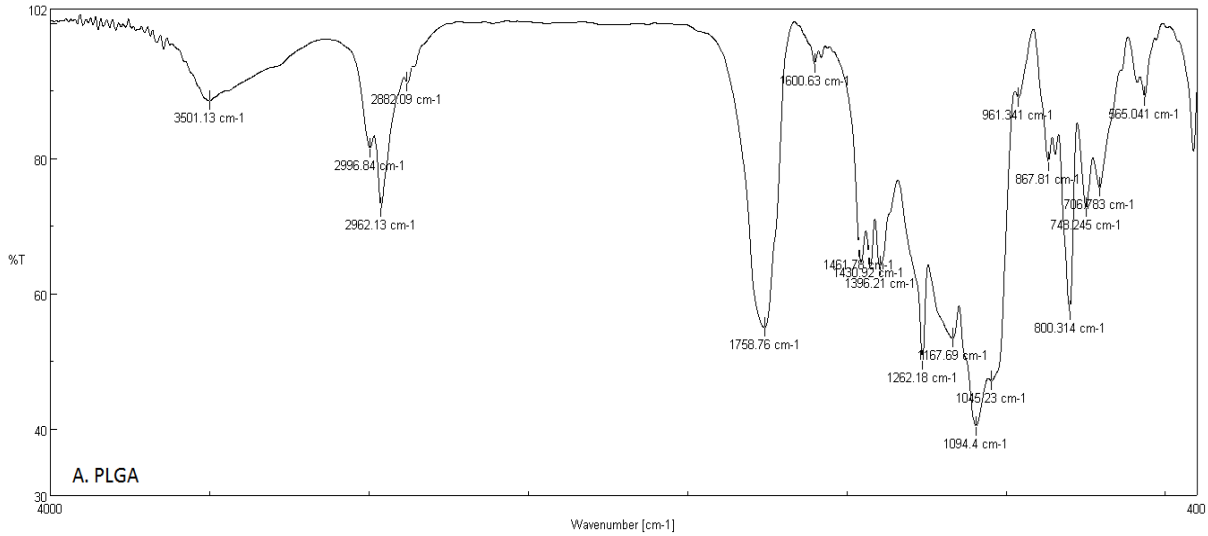


Fig.1 The XRD patterns of the PLGA and the PLGA/fibrin scaffolds.

FT-IR Spectroscopy:

FT-IR was used to characterization of PLGA/Fibrin hybrid scaffold. FT-IR spectra of the bulk PLGA, and PLGA/Fibrin are shown in Fig. 2. In bulk PLGA the main functional groups peaks represented, consist of -CH, CH2, CH3 (2800-2961 cm-1), C-O (1045-1094 cm-1), ethyl -CH2 (1420 cm-1), and OH (3500 cm-1), carbonyl -CO (1758 cm-1). On the basis of FT-IR spectra of PLGA/fibrin, the peaks at 1657, 1544 and 1261 cm-1 correspond to the characteristic peaks amide I, II, and III of fibrin, and also the FT-IR spectra showed PLGA main functional groups peaks. (Fig2)



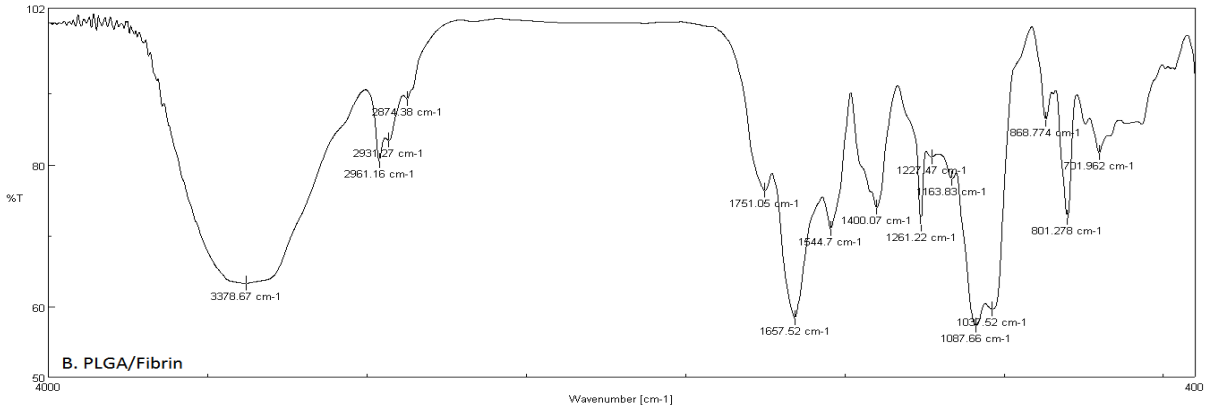


Fig2. FT- IR spectra of the bulk PLGA, and PLGA/ fibrin.

Cell viability:

MTT results on the fourteenth day show that viability in the group affected by icariin, is higher than TGF-β group but not significant (P≥0.05). Also this chart reveals that cell viability were significantly different (P≤0.05) between Group TGF- β and ICA compared to control group. (Fig.3)

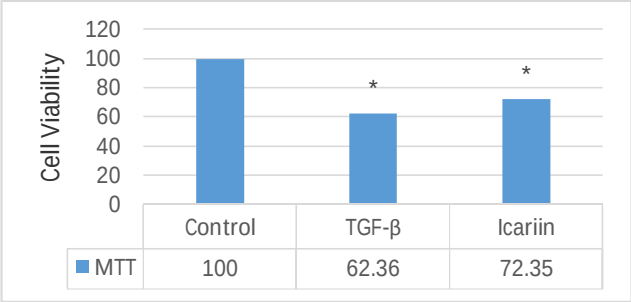


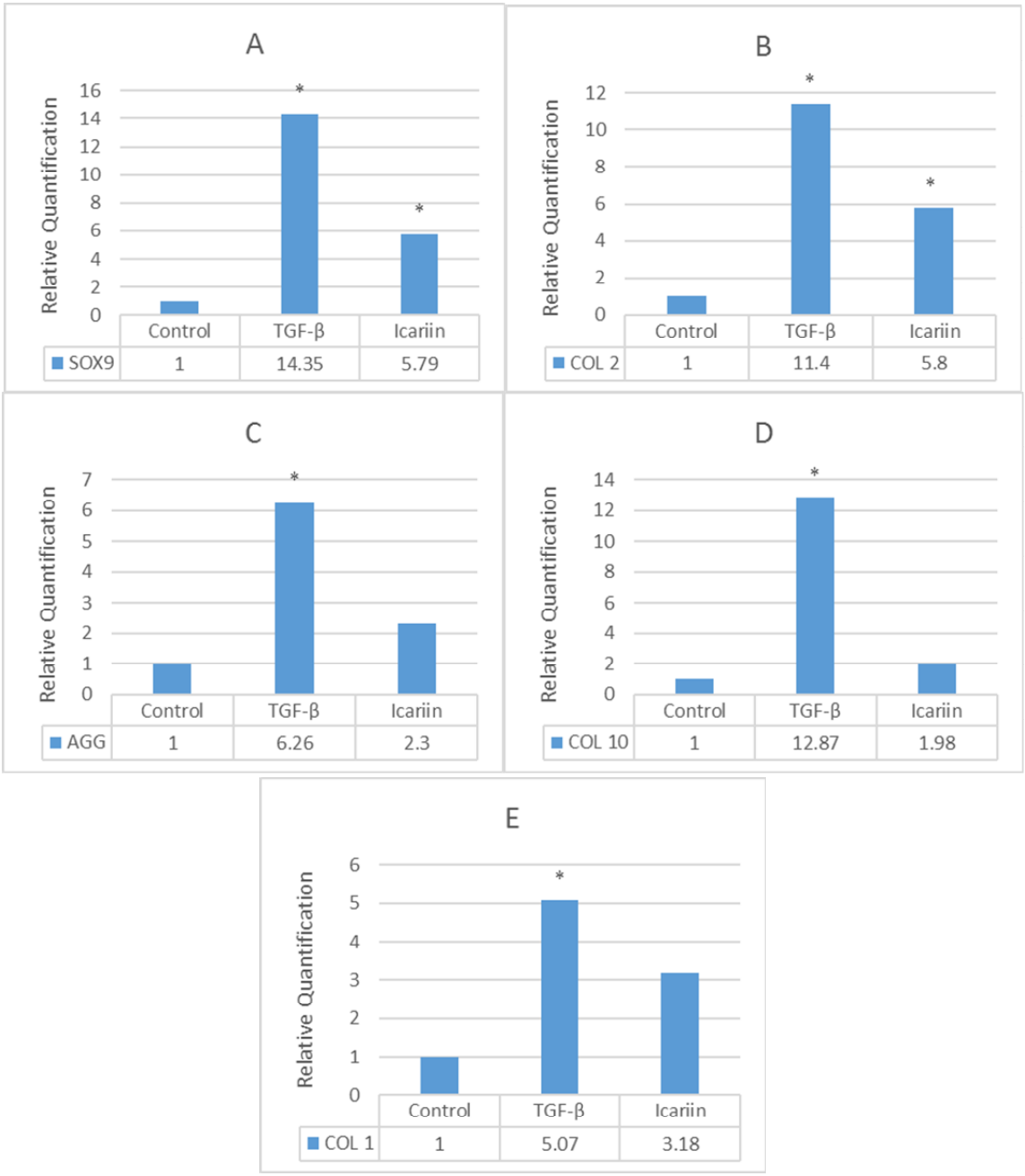
Fig.3 Comparison of MTT assay results between icariin and TGF-β groups.
*: Significant compared to control group. (P≤0.05)

Real-time PCR:

The results of real-time PCR show that SOX9 and type II collagen gene expression in the group having TGF-β and ICA are significantly higher (P≤0.05) than the control group. Aggrecan gene expression in the group affected by icariin, is higher than control group But not significant (P<0.05). Also SOX9, type II collagen and aggrecan gene expression in the ICA group is significantly higher (P≤0.05) than the TGF- β group. (Fig.1 A, B, C)

The results of real-time PCR show that type X (as hypertrophic marker) collagen gene expression in the group affected by ICA, is significantly lower than TGF-β group (P≤0.05). But type X collagen (COL 10) gene expression didn't have any significant differences between control and ICA group. (Fig.3 D)

Type I (as Fibrous marker) collagen gene expression in the group affected by icariin, is lower than TGF- β group But not significant (P≥0.05). Also type I collagen (COL 1) gene expression didn't have any significant differences between control and ICA group. (Fig.1 E)



Real-time Polymerase chain reaction for different groups. A) Sox 9, B) Collagen type II, C) Aggrecan, D) Collagen type X and E) Collagen type I.

*: Significant compared to control group. ($P \leq 0.05$)

DISCUSSION:

In this study, we constructed two groups of constructions derived from PLGA/fibrin and hADSCs under the conditions with or without icariin. By comparatively observations and evaluations of these constructions in in-vitro culture we found that the icariin could help to hADSCs to differentiate into cartilage cells and increase the synthesis of cartilage specific matrixes.

Amorphous structure of PLGA and PLGA/fibrin scaffold was confirmed by XRD and on the basis of FT-IR spectra of PLGA/fibrin, characteristic amide absorption bands were situated at 1550 cm-

1 and 1240 cm-1 that belongs to amide II and III groups of fibrin (23).

Recently, studies have indicated that stem cells from human adipose tissue contain multipotent progenitor cells that can differentiate into osteogenic, chondrogeinc, myogenic, and neurogenic cells when induced by the appropriate biological factors in vitro (9, 16). Our studies too demonstrated that adipose derived stem cells (ASCs) from human were successfully isolated and were induced to differentiate into chondrocytes on PLGA/fibrin scaffold by transform growth factor-β3 (TGF-β3) and icariin.

Growth factors play a crucial role in the regulation of Adult stem cells (ASCs) differentiation. A number of studies have demonstrated that bone morphogenetic protein (BMP), insulin-like growth factor and transforming growth factor-β (TGF-β) are able to induce chondrogenic differentiation in-vitro, and promote the formation of cartilage-like tissue in-vivo (14, 24-26). Our study also showed, growth factors such as TGF-β3 not only upregulate the expression of hyaline cartilage-specific markers, but also unavoidably lead to further hypertrophic differentiation and contribute to the development of fibrous cartilage (27). Furthermore, rapid degradation, easily-lost activity the high cost of growth factors limit their widespread use, particularly in clinical practice (27).

Several studies have shown that ICA is a safe and strong chondrocyte anabolic agent, which can enhance chondrocyte proliferation, attenuate lipopolysaccharide-induced inflammatory responses and reduce ECM degradation through the inhibition of nitric oxide, matrix metalloproteinase synthesis and cathepsin K activity (28, 29). furthermore, Zhang et al(Zhang W 2013) reported that ICA reduced the activity of the transcription factor nuclear factor-KB in an inflammatory model induced by tumor necrosis factor-α and also protected chondrocytes from damage due to osteoarthritis (OA). There are certain potential molecular mechanisms that explain these effects. For instance, ICA not only enhances the expression and secretion of various growth factors, including BMP-2 and TGF-β, but also upregulates the expression levels of Drosophila mothers against decapentaplegic (Smad) proteins, including Smad1, Smad4 and Smad5, which are key regulators specific to TGF-β activation affecting chondrogenic genes (30, 31).

D.LI and et al showed the icariin will upregulate the expressions of cartilage specific genes of seeded chondrocytes. Furthermore, icariin can increase the synthesis of cartilage matrix, accelerates and maintains the formation of chondroid tissue. Finally, icariin improves the efficiency of the restoration of supercritical-sized osteochondral defects by engineered cartilage.

ICA is a safe and effective natural anti-inflammatory drug, its partial mechanism is possible the multiple links intervention on pro-inflammatory cytokines (TNF-alpha, IL-6), inflammatory mediators (NO) and adhesion molecules (CD11b) (32).

Icariin also reduced the clinical signs of arthritis. Icariin inhibited cathpesin K activity in vitro and was effective in a mouse model of collagen-induced arthritis (CIA) similar to human RA, suggesting that this agent may have promise in the treatment of patients with rheumatoid arthritis (RA) (29).

the promotion of icariin on the synthesis of GAGs and collagen of chondrocytes, and finally exerting a potent chondrogenic effect, might be due to its ability to up-regulate the expression of aggrecan, collagen II and Sox9 genes and to down-regulate the expression of the collagen I gene of chondrocytes. These preliminary results imply that icariin might be an effective accelerant for chondrogenesis and that icariin-loaded biomaterials might have the potential for cartilage tissue engineering. 1×10^{-5} M may be a suitable concentration of icariin with chondrogenic effect for tissue engineering (33).

Icariin at the concentration of significantly increased the proliferation of chondrocytes or chondroprogenitors indicated by MTT assay (34),

our recent data show that its effects declined to the basal level when the concentration reached 10^{-5} M Similarly, other studies found that TGF-β alone led to higher collagen x and I expression. However, the presence of ICA did not potentiate the effect of the growth factors on hypertrophic differentiation while producing chondrogenic differentiating effects. Similarly, other studies found that ICA downregulated collagen x and I gene expression (35).

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