Comparison of Fibrin and PLGA/fibrin Scaffolds for Chondrogenesis of Human Adipose Derived Stem Cells by Icariin

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Abstract

Background: Recently, cartilage tissue engineering is the best candidate for regeneration of cartilage defects. We evaluated the potential of fibrin and PLGA/fibrin scaffolds in providing a suitable environment for growth and chondrogenic differentiation of human adipose derived stem cells (hADSCs) in the presence of icariin.

Method: The Three-dimensional (3-D) PLGA scaffold was prepared using the solvent casting/salt leaching technique and the hybrid scaffold was fabricated by fibrin. hADSCs were isolated from human adipose tissue. 3-D PLGA/fibrin scaffolds were seeded with cultured hADSCs and analyzed 14 days later. Monolayer culture was used for the control group. The viabilities of cells in different groups were assessed by MTT. The expression of chondrogenic related genes, hypertrophic marker and Fibrotic marker were quantified by RT-PCR.

Results: MTT results show that viability in the control group was significantly higher than those in the Fibrin and PLGA/Fibrin groups. Also viability in the PLGA/Fibrin group affected by icariin was higher than that in Fibrin group. The results of the real-time PCR showed that SOX9, Agg, Coll 2, and Coll 1 gene expression in the fibrin and PLGA/fibrin groups were significantly higher than those in the control group. Coll 10 gene expression in the fibrin group was higher in comparison to the control group but not significantly. type SOX9, Coll 2 and Coll 1 gene expression in the fibrin group was significantly lower compared to the PLGA/fibrin group.

Conclusions: The study reveals that the corporation of PLGA with fibrin is an effective way to potentially enhance articular cartilage regeneration of hADSCs in the presence of icariin.

Introduction

Large cartilage defect is a well-known problem for orthopedic surgeons because the self-healing capacity of cartilage is limited (1). Tissue engineering techniques using autologous chondrocyte 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 cartilage defect site and form functional tissue
(2). However, autologous tissue transplantation can leave donor site morbidity. Furthermore, chondrocytes 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 (3). Recently, it has been shown that human adipose tissues have mesenchymal stem cells (adipose tissue derived stem cells, hADSCs) that can be differentiated into multiple cell lineages, including chondrocyte. These cells have great in vitro expansion properties and are potentially an alternative cell source for cartilage transplantation (4). 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 (5).

In tissue engineering, three-dimensional (3-D) porous biodegradable polymer scaffolds have been of great importance for in vivo as well as in vitro tissue regeneration. The primary duty of the scaffold is that it is believed to deliver specific cells into target sites in the body, serving as a mechanical support from the physiological stresses. For cartilage tissue engineering, an ideal scaffold should encourage the production of cartilage-specific extracellular matrix (ECM), type II collagen (Coll 2) and aggrecan (Agg) by the transplanted cells, and gradually degrade with the growth of regenerating cartilage tissue (6).

During the past two decades, poly lactic-co-glycolic (PLGA) biopolymer has been used as one of the most interesting candidates for fabrication of scaffolds needed in tissue engineering research (7). PLGA is a biodegradable and biocompatible polymer with optimal mechanical properties and it has been approved by the U.S. Food and Drug Administration (8). However, it has low hydrophilic properties and low water absorbency and slow degraded (9).

Fibrin is a kind of protein hydrogels derived from fibrinogen, which can be harvested and isolated from patient’s autologous blood. It is widely used in the field of medicine and materials science, such as drug delivery and cell carrier, haemostatic glue and wound repair, and tissue engineering (10, 11). Previous researchers found that fibrin could maintain the phenotype and function of chondrocytes (12, 13). Recently, cell loading in the fibrin gel was also performed, resulting in a better cell distribution and higher cell seeding efficiency (14). Yet its drawbacks of fast degradation and poor mechanical strength limit its applications as scaffolding materials alone. However, weak mechanical properties and fast degradation of fibrin have been problematic and have limited its application as a scaffold for cartilage tissue engineering (10, 15).

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 (11). Chondrocytes distributed even in the hybrid scaffold with a round morphology alike in their native matrix, and thereby could maintain better with their phenotype in terms of GAG secretion during an in vitro culture (11).

Herb Epimedium (HEP) is a widely used traditional Chinese herbal medicine for arthritis in China, Japan and Korea (16). Icariin (Ica, Molecular weight 676.65) is the major pharmacological active constituent of HEP (17). Icariin could regulate the anabolism of osteoblasts through the up-regulation of BMP-4, BMP-2 and SMAD4 expression (18). In bone tissue
engineering, icariin has been proved as an efficient accelerator. Some reports indicated that the in vivo osteoinductive effect of icariin might be expressed through the process of endochondral ossification (19). Some other reports proved that icariin was a safe and strong chondrocyte anabolic agent which could affect the proliferation of chondrocytes and reduce the degradation of extracellular matrix (ECM) (17). These suggest that Icarin may be a potential accelerator for the chondrogenesis in cartilage tissue engineering.

Due to the inadequacy of the existing methods for the treatment of cartilage injuries and on the other hand the importance of scaffolds and growth factors in tissue engineering, this study was designed to evaluate the potential of fibrin and PLGA/fibrin scaffolds in providing a suitable environment for the growth and chondrogenic differentiation of human adipose derived stem cells (hADSCs) in the presence of icariin.

Materials and Methods

Fabrication and characterization of the hybrid scaffold

3-D 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 (20). 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 (NaCl) particles (approximately 180 μm particle size) (Sigma) 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) was used for thrombin preparation. A bag of FFP was obtained from the Blood Bank of Isfahan Province (Isfahan, Iran) and its content melted in a water bath at 37 °C for 10 min. Then, the mixture of FFP (16 ml) with calcium gluconate (10 ml) was prepared and casted in falcon tube in order to be incubated 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 was extracted from cryoprecipitated antihemophilic factor (AHF) pocket by heating it in bain marie for 20 min at 37 C. Finally, the equal mixture amount of thrombin and fibrinogen led to fibrin clot formation (20, 21).

Isolation & proliferation of hADSCs and Cell culture on fibrin and PLGA/fibrin scaffolds

Subcutaneous adipose tissue samples were collected in falcon containing phosphate buffered saline (PBS) from four patients (30-50 years old) who filled the consent form before undergoing cesarean section or abdominal surgery in Isfahan Rain's beauty clinic. 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 (Sigma) containing 10% FBS (Invitrogen) was added for enzyme inactivation before being centrifuged (1200rpm, 15min). Removing supernatant, cultured cell pellet in 25 cm² flasks 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 cells were seeded in fibrin and PLGA/fibrin scaffolds. Monolayer culture was considered as the control group (4, 22). For both groups, chondrogenic medium supplemented with icarin (1 x 10^{-5} M) was used (1). The scaffold was sterilized with 70% ethanol for 60 min and disinfected via ultraviolet light for 2hrs and scaffolds were washed with PBS. The sterile scaffold was kept in a 240- well cell culture plate and finally PLGA scaffolds were soaked in chondrocytes-fibrin suspension (1x10^5 cells/ scaffold) and polymerized by dropping thrombin-calcium chloride (CaCl2) solution (23).

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

The viability of hADSCs was assessed by the MTT assay on the 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 hrs. The medium was discarded and 400 µl DMSO (Sigma) was added to each well, and was incubated in dark for 2 hrs. Next, 100 µl of the solution was transferred to a 96-well plate and the absorbance of each well was read at 570 nm with ELISA reader (Hiperion MPR4). The assays were performed in triplicate (24, 25).

### RNA isolation and real-time quantitative

Real-time quantitative RT-PCR was performed to quantitatively estimate the mRNA expression of type II collagen (coll2), aggrecan (Agg) and SOX9, type X collagen (coll10) and type I collagen (coll1) 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 SYBR Green Rox qPCR master mix kit (Fermentas) was used for real-time RT-PCR. Primer sequences are shown in Table1. 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 was produced to determine the melting temperature of specific amplification. These experiments were carried out in triplicate and were independently repeated at least 3 times (25).
**Table 1.** Gene sequence of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>(forward and reverse)</td>
</tr>
<tr>
<td>collagen II-F</td>
<td>CTGGGTAGATGATGGTGAAG</td>
</tr>
<tr>
<td>collagen II-R</td>
<td>CCTGATACCCTTGTTGA</td>
</tr>
<tr>
<td>sox-9 -F</td>
<td>TTCAGCAGCCAATAAGTG</td>
</tr>
<tr>
<td>sox-9 -R</td>
<td>TTCAGCAGCCAATAAGTG</td>
</tr>
<tr>
<td>collagen x -F</td>
<td>AGAATCCATCTGAGAAATAGC</td>
</tr>
<tr>
<td>collagen x -R</td>
<td>CCTCTTACTGCTACCTTTAC</td>
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<tr>
<td>collagen I -F</td>
<td>CCTCCAGGCTTCAACGAG</td>
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<tr>
<td>collagen I -R</td>
<td>TCAATCAGCTTGCCCA</td>
</tr>
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<td>Aggrecan -F</td>
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<tr>
<td>GAPDH -F</td>
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<tr>
<td>GAPDH -R</td>
<td>CTTCCTCTGCTGTCTTG</td>
</tr>
</tbody>
</table>

**Statistical analysis**

The comparison of MTT results and gene expression among groups was performed using Independent-samples t-test and analysis of variance. P value < 0.05 was considered as statistically significant level.

**Results**

The morphology of human adipose derived stem cells:

In the study of living and not stained cells using invert microscope, they were determined as small cells with little cytoplasm and elliptic central core (Fig. 1).

![Image of living mesenchymal stem cells isolated from human adipose tissue produced by invert microscope×40](image)

Spindle cells in the third passage are visible.
Cell viability and proliferation in different groups:

MTT results on the fourteenth day show that viability in the control group is significantly higher than that in Fibrin and PLGA/Fibrin groups but not significantly (p>0.05, Fig. 2).

Gene expression in different groups

The results of the real-time PCR showed that SOX9, Agg, Coll 2, and Coll 1 gene expressions in the fibrin group were significantly higher (P<0.05) than the same values in the control group. Coll 10 gene expression in the fibrin group, was higher than that in the control group but not significantly (p>0.05). Also SOX9, Agg, Coll 2, Coll 10 and Coll 1 gene expressions in the PLGA/fibrin group were significantly higher (P<0.05) than the corresponding values in the control group.

The results of the real-time PCR indicated that type SOX9, Coll 2 and Coll 1 (as fibrous marker) gene expressions in the fibrin group were significantly lower than those in the PLGA/fibrin group (P<0.05, Fig. 3).
Figure 3. Comparison of Real-time Polymerase chain reaction results among control, PLGA and PLGA/Fibrin groups
a: Difference between control and Fibrin groups
b: Difference between control and PLGA/Fibrin groups
c: Difference between PLGA and PLGA/Fibrin groups (P≤0.05)

Discussion
As cartilage does not have blood vessels and has limited ability for self-healing, repairing a damaged cartilage requires efficient methods (1). In this study, fibrin/hADSCs and PLGA/fibrin/hADSCs constructs loaded with icariin were fabricated. Tissue engineering techniques based on the utilization of stem cells are useful techniques with great potential in the treatment of such injuries. One of the most
important factors in successful tissue engineering is selecting an appropriate scaffold to facilitate cell growth and differentiation. As a result, in cartilage tissue engineering, an ideal scaffold seems necessary to maintain the chondrocyte phenotype in the differentiation process (26). Most of the tissue engineering studies that are based on using stem cells utilize chemical factors (such as growth factors) or signals for chondrogenic differentiation (25). Thus, less attention has been paid to the importance of scaffolds as influential factors in the regulation of tissue growth and differentiation. Since in this study, the differentiation capacity of hADSCs varied in different scaffold selections, it was concluded that scaffold is an important factor in the stem cell differentiation process.

It is necessary to provide enough space and sufficient time for cells to migrate, proliferate, and differentiate for cartilage restoration by a tissue engineering technique. The fibrin alone has been diversely used as an injectable scaffold. However, the commercially available fibrin has lower mechanical strength, tend to disintegrate in vitro and in vivo after several days, and almost completely dissolve within 3-4 weeks (27, 28). Even after optimization of the gelling parameters that determine the gel stability (27), the obtained fibrin is still not stable enough for long-term cartilage repair. In the present study, PLGA was compounded with fibrin for the purpose of enhancement of mechanical strength and stability.

It has been demonstrated that this composite scaffold, indeed, has a stronger mechanical strength (29). Intrinsically, the chemical structure of PLGA is different with that of fibrin. PLGA is a synthetic polyester with a hydrophobic and bioinert surface, whereas fibrin is highly hydrophilic, making them thermodynamically incompatible. Surface modification of the PLGA, for example by fibrin coating, is a simple but effective way to obtain a compatible composite. Here, the surface aminolysis (30, 31), following glutaraldehyde (GA) coupling was applied to covalently anchor the fibrinogen. In this process, part of the surface ester groups of PLGA is converted into -NH2 groups, which are further converted into aldehyde groups by large amount of glycolic acid. Mechanical strength of the hydrogels, especially at a dynamic state, is of critical importance for practical applications. This is most typical for cartilage restoration, since the cartilage is inevitably suffered from the dynamic force. Fibrin has remarkable and unique viscoelastic properties (32). Integrin binding sequence of arginine-glycine-aspartic acid existing in fibrin is a stimulating factor for cell binding and growing. Some studies reported that in cartilage tissue engineering, fibrin stimulates coll2 and Agg (33). Evidence show that chondrogenesis of PLGA/Fibrin compared to PLGA is recognized significantly higher in expression and accumulation of coll2 and SOX9 genes compared to Agg, coll1 and coll10. Cell viability in PLGA/Fibrin group affected by icariin, was higher than fibrin group affected by icariin but not significantly, proliferation of chondrocytes or chondroprogenitors indicated by MTT assay. Similar finding has been reported in the previous assessment of osteogenic potential utilizing human periosteum-derived progenitor cells and fibrin gel immobilization technique in PLGA scaffold (23). Similar to the present study, Lee et al. also reported that fibrin provides more uniform chondrocytes distribution during cell seeding via histology in macro-porous polyurethane scaffold (14).

Conclusion

Our study showed that PLGA/fibrin scaffold, in comparison to fibrin scaffold, can be considered as an appropriate scaffold for differentiation of hADSCs by icariin due to the higher expression of chondrogenic differentiation markers, including collagen II and SOX9. This scaffold can be used in animal studies for cartilage tissue engineering.
References


