Preparation of Decellularized Bovine Tendon Scaffold and Evaluation of Its Interaction with Adipose Tissue-Derived Mesenchymal Stem Cells

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Abstract

Background: Tissue engineering is the science of tissue design and one of the main branches of regenerative medicine that aims to improve and repair tissue injuries.

Objectives: This study aimed to decellularize the tissue of bovine Achilles tendon and create a natural 3D scaffold. Then, the interaction of human adipose tissue-derived mesenchymal stem cells (hAd-MSCs) with this 3D scaffold was evaluated for use in tendon injuries.

Methods: The bovine Achilles tendon was obtained from a slaughterhouse and decellularized by the combination of ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS). Histological and biomechanical tests were used to evaluate the quality of decellularized scaffolds. Adipose-derived mesenchymal stem cells were cultured on scaffolds, and cell viability and cell behavior were evaluated by the MTT test and scanning electron microscopy.

Results: The results of histological and biomechanical tests showed the complete removal of cells with the preservation of the extracellular matrix. The results of cell culture on scaffolds also showed that optical absorption in scaffolds containing cells increased over time.

Conclusions: In general, the decellularized scaffold in this study did not undergo significant structural changes in the tendon tissue. The interaction between hAd-MSCs and the decellularized scaffold revealed that the scaffold was somehow suitable for cell culture. However, it needs to be more investigated for use in the treatment of tendon injuries of the athletes.

Keywords: Tendon, Decellularized Scaffold, Tissue Engineering, Regenerative Medicine, Stem Cell

1. Background

Tissue engineering is one of the main branches of regenerative medicine, which aims to maintain a steady-state of tissue, improve the function of the target tissue, or replace the physiological function of the tissue (1). Tissue engineering is based on three main constituents of biological tissues, including extracellular matrix, messenger molecules, and cells, which are simulated by scaffolds, growth factors, and cells, respectively. The cellular scaffold functions similar to the extracellular matrix, which provides the connectivity, proliferation, exchange of nutrients, and wastes for cells to grow. A suitable scaffold has characteristics such as non-toxicity, biocompatibility, biodegradability, lack of immunogenicity, easy preparation, suitable physical and mechanical properties such as porosity and pore size, optimum stability, and three-dimensional structure similar to natural tissue (2).

Decellularization of tissues and organs is an essential process for the production of natural scaffolds derived from the extracellular matrix. The final goal of any decellularization process is to remove cellular and nuclear materials by maintaining the mechanical and biological properties of the extracellular matrix. Decellularizing methods are divided into physical, chemical, and enzymatic methods, and these treatments are commonly used in combination. In general, decellularization involves the destruction of the cell membrane by physical treatments or ionic solutions, separation of cells from the extracellular matrix by enzymatic treatments, dissolving the cytoplasm, and com...
ponents of the nucleus by detergents, and ultimately washing and removing cell debris from the extracellular matrix (3).

Most adult stem cells are multipotent. Hematopoietic stem cells, neural stem cells, and mesenchymal stem cells are examples of adult stem cells (4, 5). For many years, the bone marrow has been the most important source of mesenchymal stem cells, but recently adipose tissue has gained a prominent place as the source of mesenchymal stem cells because these cells are more comfortable to separate from the adipose tissue under the skin and have fewer side effects (6).

The tendon is a dense connective tissue composed of parallel layers of collagen fibers. Tendon injuries often occur during exercise and other physical activities. The treatment of damaged tendons using autografts, allografts, and xenografts is not effective due to complications such as reduced transplantation, high rates of rupture, probability of infection, immune response, and limited access. Therefore, tissue engineering can be used as an alternative to treat tendon injuries (7).

Pridgen et al. (8) in their study of human tendon concluded that the combination of ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) degraded and eliminated all tendon cells, without decreasing the level of collagen, glycosylation of amino glycans, and tendon elasticity. By using Tris hypotonic buffer containing EDTA and two protease inhibitors, as well as SDS and DNase, Martinello et al. (9) successfully decellularized the human tendon. They maintained extracellular matrix integrity. These results indicate the efficacy of EDTA and SDS for the decellularization of the bovine tendon (9).

2. Objectives

This study aimed to decellularize the tissue of bovine Achilles tendon to create a natural 3D scaffold and then to investigate the interaction of human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) with this 3D scaffold in the treatment of tendon injuries.

3. Methods

3.1. Preparation of Decellularized Scaffold From Bovine Achilles Tendon Tissue

Bovine Achilles tendon was obtained from the Ardabil slaughterhouse. Approximately 5 mm × 5 mm sections were prepared for culturing hAD-MSCs. To remove cells from the tissue, the samples were first immersed in 0.1% EDTA for four hours and then in solutions containing different percentages of SDS (0.1%, 0.5%, 1%, 1.5%, and 2%). Also, EDTA 0.1% was used at different intervals, including 24 and 48 hours, and the solution was replaced every 12 hours. After decellularization, EDTA and SDS were removed from the target tissue to eliminate the damage caused by these compounds because of their toxicity.

3.2. Sterilization of Scaffolds for Cell Culture

Scaffolds were rinsed with sterile distilled water 3-4 times under a laminar hood and incubated in sterile distilled water for 24 hours in a shaking incubator. After that, they were immersed in 70% ethanol for another 4 hours. The scaffolds were removed from the incubator, washed in sterile conditions with sterile distilled water 2-3 times under a laminar hood, and then placed in sterile phosphate-buffered saline (PBS) for one hour. Finally, to prepare the scaffolds for culture, the scaffolds were sterilized in Petri dishes and placed in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Germany) containing 10% Fetal Bovine Serum (FBS, Gibco, Germany) and 1% Penstrep (Sigma, USA) was added, followed by incubation in a CO2 incubator for 4 hours.

3.3. In vitro Biocompatibility Assay of Scaffolds

For this test, hAD-MSCs were obtained from the National Center for Genetic and Biological Resources of Iran. hAD-MSCs were cultured in low-glucose DMEM (Gibco, Germany) containing 10% FBS (Gibco, Germany) and 1% Penstrep (Sigma, USA) and seeded on tendon scaffolds at a density of 250,000 cells per square centimeter. The culture media of the samples were changed every two days. On days 5, 10, 15, 20, and 25, the samples were examined for cell survival and behavior on the scaffolds by MTT and electron microscopy. The assays were done in triplicate.

3.4. Histological Analyses of Scaffolds

Histological evaluations were done to study the morphology of samples and evaluate the extracellular matrix (ECM) components. Two types of tissue fixators, including 10% formalin and 4% paraformaldehyde, were used for tissue fixation. Alcohol dehydration, xylene clearance, and paraffin embedding were done via a tissue processor (10). Then, the samples were embedded and prepared in thin sections of 5 μm thickness using a microtome machine (11). Hematoxylin and Eosin (H & E) staining and 4’,6-diamidino-2-phenylindole (DAPI) staining were used to investigate the removal of cells from the bovine tendon. To confirm the cell elimination, sections were stained with DAPI, as described previously. Briefly, tissue sections were deparaffinized by xylene and rehydrated with graded ethanol. The slides were drained and incubated with DAPI staining solution (200 mL) for 15 min in the dark. The slides
were mounted with Entellan (MilliporeSigma, St. Louis, MO) and observed under a fluorescence microscope (12). Picroindigocarmine staining was used to evaluate the ECM content in prepared scaffolds. The prepared samples were evaluated and photographed by light and fluorescence microscopy (10, 11, 13).

3.5. Scanning Electron Microscopy

This technique was used for further evaluation of tendon scaffolds, as well as the penetration and deployment of hAD-MSCs in the scaffolds. The samples were fixed with 2.5% glutaraldehyde for two hours. Then, samples were dehydrated with ascending ethanol series. After drying, the samples were covered with gold-palladium and evaluated with scanning electron microscopy (14).

3.6. Measurement of Scaffold Porosity

The porosity of the scaffold was measured using MIP4 Student software. For this purpose, five samples of scaffolds were randomly selected before and after decellularization, and the porosity was calculated in percentages.

3.7. Evaluation of Biomechanical Properties of Decellularized Tendon Scaffolds

After the preparation of decellularized tendon scaffolds, the tensile strength test (SANTAM SRT-200B, Santam Company, Iran) was used to evaluate the tensile strength of scaffolds and fresh tendons. In summary, the sample size was first calculated using a digital caliper. Then, pieces of samples with 1 cm lengths were attached to the arm of the device. The samples were stretched by the device at a constant velocity of 0.1 mm/s. The samples were kept in normal saline to prevent drying during the test.

3.8. Statistical Analysis

The normal distribution of the data was confirmed by the Kolmogorov-Smirnov test. The one-way analysis of variance (one-way ANOVA) and Tukey post hoc tests were used for the statistical analysis of data. The differences were considered statistically significant when P < 0.05. The SPSS software (version 16) was used for statistical analysis. All charts were plotted by Excel software.

Results

4.1. Histological Examination of Bovine Tendon Scaffolds by Light Microscopy

The results of histological evaluation of bovine tendon scaffolds showed the removal of cells and the maintenance of ECM in the 1.5% SDS and 0.1% EDTA solution. Observations from H & E and DAPI staining indicated the removal of cells and nuclei from the decellularized tissue, compared to a fresh tendon. In H & E staining, hematoxylin is a dispersive dye that gives negatively charged tissue components a blue to purple color while cationic components, such as proteins that contain amine groups, tend to render acid dyes such as eosin to turn pink. As a result of this staining, nuclei appear blue to purple, and the cytoplasm appears pink. The results of H & E staining showed that in the decellularized tendons, the cells were removed entirely, and no nuclei were observed (Figure 1). The DAPI dye binds specifically to DNA and is used for staining the nucleus; under UV light, the nucleus appears fluorescent blue. The results of DAPI staining also showed the complete removal of cells and nuclei so that no blue-stained nuclei were observed in the scaffold group (Figure 2).

Picroindigocarmine staining was used to evaluate the degree of maintaining collagen fibers in comparison with a fresh tendon. The results demonstrated no obvious disruption of collagen fibers. Furthermore, the collagen structure following the decellularization process by light microscopy, collagen fibers appeared blue (Figure 3).

4.2. Evaluation of Viability of hAD-MSCs Cultured on Scaffolds

The MTT assay was used to evaluate the adhesion and proliferation rate of hAD-MSCs on scaffolds. In this test, the optical absorption of each sample is directly related to the number of living and active cells in the sample. The results indicated cell viability and proliferation on the scaffolds (Figure 4). Besides, optical absorption increased in the group containing scaffolds over time. Moreover, it reached its peak on the 20th day. However, optical absorption decreased significantly on day 25 (P < 0.01) (Figure 4).

4.3. Evaluation of Behavior of hAD-MSCs Cultured on Scaffolds

On the fifth day of deployment and adhesion, a large number of hAD-MSCs were observed as a single layer in most of the scaffolds, and some of them penetrated the scaffolds. Ten days after culture, some hAD-MSCs migrated into the scaffold, and their adhesion and penetration were observed from the margin to the scaffold. The collagen nature of the scaffold was maintained during these 10 days. On the 15th day after culture, cells continued to penetrate the margin, and scaffold degradation was observed due to cell infiltration. The 20th day of culture was considered the best day because of the high density of cells infiltrated into the scaffold. High degradation of the scaffold was also observed on this day. Observations of scanning electron microscopy on the 20th day of culture emphasized cell adhesion and infiltration into the scaffold (Figure 5).
4.4. The Porosity of Tendon Scaffold

The porosity percentage was measured using MIP4 Student software. The average porosity percentage was 9.67% before decellularization, while it was 11.12% after decellularization. According to the obtained results, no significant changes in porosity were observed in scaffolds after the decellularization process.

4.5. Biomechanical Evaluation of Scaffolds

The results of comparing the mechanical strength of decellularized tendon scaffolds and the fresh tendon samples using the tensile test showed that the maximum force required ($F_{\text{max}}$) for rupturing the decellularized tendon scaffolds and fresh tendon specimens was 5.65 and 6.11 Newton, respectively. Also, strain deflection at break was 3.11 mm and 3.42 mm for tendon scaffolds and fresh tendon, respectively. Moreover, the mechanical properties of the scaffolds were restored after the decellularization process (Table 1).

5. Discussion

In this study, we used the natural decellularized ECM due to its features, such as providing an environment similar to the extracellular matrix, improving cell adhesion, homeostasis, lack of antigenic effect, and low immune response (15-18). Currently, EDTA and SDS are used to disinfect bovine tendons, which also were used in this research.
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Figure 3. A, Comparison of extracellular matrix preservation in the fresh tendon; B, and decellularized tendon after decellularization of collagen fibers and elastin (arrow) preserved in both fresh tendon and decellularized tendon.

Control

| Incubation Time (Day) | Optical Density (OD) |
|-----------------------|----------------------|
| Day 5                 | 2.5                  |
| Day 10                | 2                    |
| Day 15                | 1.5                  |
| Day 20                | 1                    |
| Day 25                | 0.5                  |

Figure 4. MTT assay for ASCs viability after culture on the scaffold. The results revealed that the rate of ASCs proliferation on the scaffold was similar to that of controls. Moreover, on the 20th day, it reached its peak (*, P < 0.05, **, P < 0.01, ***, P < 0.001).

Table 1. Comparison of Biomechanical Properties of Fresh Tendon Samples and Decellularized Tendon Scaffolds (N = 5)

| Groups                  | Fresh Tendon | Decellularized Tendon | Significance |
|-------------------------|--------------|-----------------------|--------------|
| Maximum load value Newton | 6.11 ± 0.44  | 5.65 ± 0.61           | P > 0.05     |
| Strain deflection at break mm | 3.42 ± 0.11  | 3.11 ± 0.29           | P > 0.05     |

Values are presented as mean ± SD.

The decellularization process. It is known that EDTA participates in cell adhesion to the ECM by binding to bicarbonate cations, thus facilitating the removal of cellular substances from the tissue. Moreover, SDS reacts with cell membranes due to its dual activity, breaks down cell membranes and nuclear membrane, and denatures protein; therefore, it has beneficial effects on the decellularization process (19). Bridgen et al. evaluated various methods for human tendon decellularization using EDTA in combination with various chemicals, such as Triton-X100, Tri(n-butyl) phosphate (TnBP), and SDS. Previous studies demonstrated that SDS is the only effective material for the removal of all tendon cells, without any effect on collagen content, glycosaminoglycans, and tendon elasticity and tensile (8, 20). For histological evaluation, H & E staining and DAPI staining were used to identify the cells before and after decellularization, confirming the removal of cells after the decellularization process. The maintenance of the ECM content of tissue, including the structure and density of collagen filaments after decellularization, was confirmed using picroindigocarmine staining. After the culture of hAd-MSCs on scaffolds, histological tests were done at five-day intervals. Based on the statistical data and high density of cells infiltrated into the scaffold, the 20th day of culture was identified as the best day. After the 20th day, the decrease of cell density within the scaffold indicated that probably cells that entered the scaffold might have gradually died. Studies have shown that cell movement is driven by the pattern of chemical messages, and accordingly, physical interactions between the cell and the substrate can also play a role in this pattern of movement (21). On the other hand, cells could not penetrate the deeper areas of the scaffold, and they only were observed in the superficial areas because of the high density of collagen filaments and low scaffold porosity.

In a study conducted by Hillmann et al. (22) in 2002, the migration of cells into scaffolds with dense collagen networks was less than that of poly(glycolic acid) (PGA) and...
poly(l-lactic acid) (PLLA) scaffolds; thus, after culturing fibroblasts on the collagenized scaffold, the cells could adhere to the surface and proliferate after two weeks, but few cells penetrated the scaffold due to the high density of collagen filaments. According to the evaluations performed by SEM on the 20th day of culture, the presence of cells probably is due to the presence of collagen fibrils in the scaffold, and this may be considered as one of the possible factors in the adhesion and migration of hAd-MSCs into the tendon scaffold. As cells infiltrated, degradation was also observed. The onset of ECM degradation on the 15th day of culture caused cleavages in parts of the scaffold. Possibly, the cells began to deform themselves to penetrate the complex and dense matrix of tissue. After cell migration into the scaffold, the matrix was degraded. Cellular proteolytic and matrix infiltration activities of metalloproteinases gave them the ability to open the migration pathway into the scaffold, and the portions of the scaffold were gradually degraded, and cell penetration occurred through integrins. The initial cleavage of collagen fibrils is most likely caused by MT1-MMP, which leads to cell migration from the scaffold margin (24). Mizuno et al. (25) study demonstrated that MMPs activity increased apoptosis of myofibroblasts in the lung. The production of RGD (Arg-Gly-Asp) peptides after collagen hydrolysis by MMPs activates caspase-3 in fibroblasts, which, in turn, induces apoptosis in these cells. In this study, MMPs activity, followed by matrix hydrolysis and activation of caspases, may have induced apoptosis in cells and decreased cell density on the 20th day of culture. However, the reassurance of apoptosis and MMPs’ participation in this study needs more investigation. Injury during sports activities is the most crucial problem that leads to the obligatory retirement of athletes. Tendon injuries are one of the most common injuries, and if they are acute, they can cause the inability to move, endanger physical health, increase medical costs, and reduce athletes’ motivation (26). Therefore, the preparation of natural tendon scaffolds for therapeutic activities is of great importance in this field (27).

5.1. Conclusions

Based on the results, we could obtain a natural scaffold from the tendon tissue by treatment with SDS and EDTA. These chemicals could remove cells and their debris from the tissue without altering the tendon tissue. The evaluation of the interaction between hAd-MSCs and the decellularized scaffold indicated that the scaffold was somewhat suitable for cell culture. More research is needed to develop a more appropriate tendon scaffold by improving cell penetration into deeper parts of the scaffold for the treatment of tendon injuries of the athletes.

Footnotes

Authors’ Contribution: Arash Abdolmaleki, Asadollah Asadi, and Hussein A. Ghanimi participated in research design. Arash Abdolmaleki, Leila Taghizadeh-Momen, and Hussein A. Ghanimi conducted the experiments. Arash Abdolmaleki and Asadollah Asadi performed data analysis. Arash Abdolmaleki, Asadollah Asadi, and Hussein A. Ghanimi wrote or contributed to the writing of the manuscript.
**Conflict of Interests:** The authors declare no conflicts of interest.

**Ethical Approval:** All experimental procedures were done following the guidelines of the National Institute of Health for the Care and Use of Laboratory Animals (NIH Publications no. 8023, revised 1978) and the guidelines of the Ethics Committee of Mohaghegh Ardabili University of Ardabil (Et-0025452-Iraq-2019).

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