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Original Research

The effect of synthesized cartilage tissue from human adipose-derived mesenchymal stem cells in orthopedic spine surgery in patients with osteoarthritis

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Abstract: Osteoarthritis is a joint disease that causes degeneration of articular cartilage and involvement of subcutaneous bone and inflammation of surrounding tissues. It can affect any joints, but the most common joints are the joints of the hands, feet, knees, thighs, and spine. Osteoarthritis patients need surgery in acute cases. The use of methods that increase the efficiency of this surgery has always been considered by researchers and surgeons. For this purpose, in the current study, the effect of synthesized cartilage tissue from human adipose-derived mesenchymal stem cells was considered in orthopedic spine surgery in patients with osteoarthritis. Thirty patients over the age of 60 who had acute spinal osteoarthritis and required surgery were selected. The pellet culture system of human adipose-derive mesenchymal stem cells of each patient was used to construct cartilage tissue. For 15 of them, in addition to implants, cartilage grafts were transplanted during surgery. All patients were monitored by the Oswestry Disability Index questionnaire, for one year. In general, the results showed that over time, patients with transplanted cartilage tissue and implants were in a better condition than patients who underwent only implant surgery.

Key words: Cartilage tissue; Mesenchymal stem cells; Osteoarthritis; Spine.

Introduction

Osteoarthritis is a joint disease that first affects the cartilage of the joint. Cartilage is a slippery tissue that covers the ends of bones in a joint (1). Bones owe their easy movement to healthy cartilage (2). Another function of cartilage is to absorb impulses during physical movement (3). In osteoarthritis, the cartilage becomes thinner and gradually disappears in some areas, causing the bones to rub against each other, and there will be pain, inflammation, and decreased movement in the joint (4). Over time, the joint loses its natural shape and bony appendages form at its edges, which causes more pain and discomfort (5). Unlike other joint inflammations, such as rheumatoid arthritis, osteoarthritis affects only the joints and does not affect other organs (6).

Despite bone tissue, which heals on its own or with minimal medical intervention, damage to cartilage, tendons, and ligaments does not heal on its own (7). Common methods of treatment, including the use of grafts, are associated with many problems. Therefore, researchers are looking for a better alternative to damaged tissue. The science of tissue engineering in recent decades has made it possible to create a structure that can replace damaged tissue (8).

Autologous chondrocytes are considered a source of primary cells for the regeneration of defective and damaged cartilage and are used clinically. However, complications at the site of sampling and loss of its phenotype are problems with the use of these cells (9). As a result, there is a need to use methods that have high potential in the treatment of cartilage defects and help regenerate cartilage lesions (10). Recently, adipose-derived mesenchymal stem cells have received a great deal of attention in soft tissue regeneration. These cells have the ability to differentiate into cartilage, bone, muscle and fat in the presence of differentiation factors and appropriate culture conditions (11). Multipotent adipose-derived mesenchymal stem cells maintain their ability *in vitro* over several passages and increase and maintain cartilage-forming ability by cell proliferation (12). Compared to similar cells derived from other tissues, including bone marrow, these cells have many features and benefits, including the easy access of these cells from the patient in large quantities by the liposuction method. They are now considered as a means of treating tissue defects with the individual's stem cells (13).

TGF-β growth factor has been widely used to induce the process of differentiation of adipose-to-cartilage-derived mesenchymal stem cells (14, 15). Three-dimensional differentiation of cells usually takes place on three-dimensional scaffolds in a special differentiation medium. In addition to external growth factors, various scaffolds are used as a three-dimensional protective matrix for the proper growth of cells and tissues. 3D culture systems are valuable for their ability to differen-

tiate adipose-derived stem cells into cartilage because of their ability to maintain natural cell phenotypes (16).

Recently, the pellet culture method has been used to differentiate adipose-derived mesenchymal stem cells into cartilage tissue. The pellet culture method is one of the simplest three-dimensional culture systems that has been used to cartilage different stem cells (17, 18).

In this study, we aimed to investigate the effect of synthesized cartilage transplantation, from human adipose-derived mesenchymal stem cells, in the treatment of patients with osteoarthritis of the spine.

Materials and Methods

Patients

This study was conducted as a parallel randomized clinical trial. Thirty patients with severe spinal osteoarthritis requiring surgery were selected. The age of these patients was over 60 years and they were divided into two groups. In the first group, which included 15 patients, only spinal orthopedic surgery was performed by implant placement. In the second group, there were 15 patients who, in addition to surgery, underwent artificial cartilage transplantation.

In terms of inclusion criteria, the condition of these patients should have required surgery based on clinical criteria. Exclusion criteria included patient dissatisfaction with cooperation or continuation, simultaneous involvement of acute spinal osteoarthritis with degenerative processes, the impossibility of surgery for various reasons and the presence of any underlying systemic disease, comorbidity or other skeletal disorder that had a major impact on the results of the surgery.

In addition to demographic indicators (age, gender, weight, body mass index), the total number of hospitalization days from the time of preoperative hospitalization to the day of discharge was calculated. Frequency distribution of postoperative complications such as wound infection, intraoperative fractures, and systemic complications such as embolism, delirium, respiratory problems, and kidney problems was recorded.

Also, for one year, every three months, in addition to clinical examinations, patients were asked to answer the Oswestry Disability Index questionnaire to assess their recovery status. This questionnaire consists of ten sections with six options. These ten sections show how people perform in their daily activities. Each section ranks the degree of inability to function in order from zero (optimal performance without pain) to five (inability to perform the activity due to severe pain). The Oswestry disability index is equal to the sum of the scores of ten parts multiplied by 2 and has a value of zero up to 100. The Zero Disability Index indicates that the person is healthy and able to perform pain-free activities daily. 0-20 Low disability, 21-40 Medium disability, 41-60 High disability, 61-80 severe disability, and a higher score is a severe disability in which a person is unable to move (19).

Cartilage tissue synthesis

To isolate mesenchymal stem cells, 5 g of abdominal adipose tissue was obtained from 15 patients. The adipose tissue sample was placed in saline buffer phosphate solution (Gibco, Life Technologies, USA) containing the antibiotic 1% penicillin-streptomycin (Gibco, Life Technologies, USA) and transported from the hospital to the laboratory under sterile conditions. Mesenchymal stem cells were then extracted from adipose tissue by digestion with the collagenase I (0.2%) enzyme. First, 1.5mg of collagenase I was added for each gram of adipose tissue and incubated for 45 to 60 minutes at 37°C. They were then centrifuged at 1800 rpm for 10 minutes, and finally, Dulbecco's Modified Eagle's Medium (Sigma-Aldrige, USA) containing the 1% antibiotic penicillin/streptomycin and 10% fetal serum (Gibco, Life Technologies, USA) was added to the cell sediments and they were transferred to a cell culture flask and were incubated at 37°C in the incubator containing 5% CO_2 and 95% moisture. After 24 hours, the suspended cells were removed from the flask by changing the medium. The cell culture medium was changed every 3 days. After trypsinization, the cells were transferred to the third passage stage and prepared for use.

In this study, a three-dimensional culture system was used for chondrogenic differentiation. By centrifugation at 1400rpm, in 15ml tubes, 1×10^6 stem cells derived from adipose tissue were formed in pellets for 15 minutes. In chondrogenic media, there was DMEM-High Glucose with 1% Insulin-transferrin-selenium (Sigma-Aldrige, USA), 1% bovine serum albumin (Sigma-Aldrige, USA), 50µg/L ascorbate 2-phosphate (Sigma-Aldrige, USA), 5µg/L Linoleic acid (Sigma-Aldrige, USA), TGF-β growth factor (Sigma-Aldrige, USA), and 1% penicillin/streptomycin. They were stored in an incubator at 37°C and 5% carbon dioxide for 14 days and the culture medium was changed every 2-3 days.

Cell viability was assessed after 14 days of cell culture on scaffolds in the presence of a cartilage differentiation medium. 0.5ml of DMEM medium and 50µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) was added to the scaffolds. After 4 hours of incubation, 0.5ml of dimethyl sulfoxide (DMSO) was added to the resulting solution. After 20 minutes, the cell/scaffold light absorption at 570 nm was measured by a microplate reader (20).

First, all RNA samples were prepared separately from each scaffold after 14 days of chondrogenic differentiation. The scaffolds were degraded in liquid nitrogen and then RNA was extracted using AccuZol™ kit (BioNEER, Korea) according to the relevant instructions. Reverse transcription of RNA to cDNA was performed by AccPower®RT Premix (bioNEER, Korea) kit. Real-time PCR was done by SYBRGreen PCR Master Mix (Amplicon, UK) and Rotor-Gene™ 6000 Series (Corbett Life Science, UK). PCR reaction for SOX9 and Aggrecan genes was performed according to the following program. The reaction mixture was first heated at 95°C for 15 minutes followed by 15 C. It was then placed at 58°C for 40 seconds.

Also, the thermal program used in the PCR reaction of collagen I and II genes included 95°C for 15 min for initial denaturation, 95°C for 15 s for denaturation, and 58°C for 35s for annealing. The reaction was repeated from the second stage for 40 cycles. The primers were designed by the Primer-3 program for each gene. The sequences are listed in Table 1. The gene was normalized based on the beta-actin reference gene. The expression

Table1. Primer sequence of genes used in Real-Time PCR analysis.

Gene	Sequence	
Collagen I	Forward	5′-TTGTACAGACATGACAAGAGGC-3′
	Reverse	5′-CTCTACCTGGGTACTACCCA-3′
Collagen II	Forward	5′-CAGAGTGAAATCCACCAAGT-3′
	Reverse	5′-TGTCCGTGGACAAACAGGTA-3′
Aggrecan	Forward	5′-TACGACTACACGCACCACCA-3′
	Reverse	5′-TTAGGATCATCTGCGCCATC-3′
SOX9	Forward	5′-ACACAGCGCCTTGAGAAGAG-3′
	Reverse	5′-TTCTACGGTCTCCCCAGAGA-3′

level of each target gene was calculated by $2^{-\Delta\Delta Ct}$ (21).

Statistical analysis

Statistical analyzes were used by SPSS software version 17. Mean and frequency was used to report descriptive statistics and t-test, chi-square and Pearson correlation tests were used to report analytical statistics. $P<0.05$ was considered as a significant level in all tests. Also, MTT analysis was repeated 4 times for each sample.

Results

Cartilage tissue synthesis

During cell isolation and proliferation, adipose-derived mesenchymal stem cells grew as fibroblast-like or spindle-shaped with distinct nuclei and they were observed by contrast phase microscopy (BHS, OLYMPUS, USA). In the third passage, uniform cells were obtained with growth and an increase in their number. At this stage, 5×10^6 cells/ml was used to create a pellet culture system. Cartilage cell production was confirmed by the expression of Collagen I, Collagen II, Aggrecan, and SOX9 genes (Figure 1, Figure 2).

Surgery results

As mentioned earlier, group 1 included patients who received only implants during surgery. But in the second group, in addition to implants, they were transplanted with synthesized cartilage from this study. Table 2 lists all demographic and clinical information of the patients. In this table, for both groups, by gender, the mean data are presented such as age, weight, body mass index (BMI), number of underlying diseases, duration of surgery, number of hospitalization days, and recovery room time.

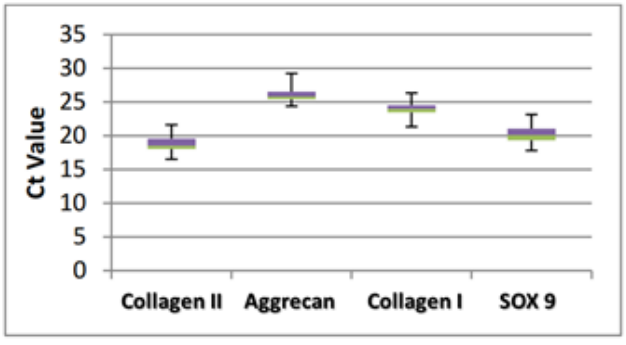

Figure 1. Box Plot for the general pattern of expression Collagen II, Aggrecan, Collagen I, and SOX 9. For each gene, the first and fourth quartiles (25%) have the maximum and minimum expression in the vertical axis.

Of all participant patients in the study, almost all of them had no side effects of surgery, except for one case with infection, and she was treated with appropriate antibiotics.

The clinical outcomes of each patient at the end of the first week, first trimester, second trimester, third trimester, and fourth trimester after surgery were assessed separately by the Oswestry Disability Index questionnaire (Table 3). The results of the questionnaire showed that patients in both groups at the end of the first week (68.25 and 69.12 in the first and second groups) were in the range of severe disability based on the degree of disability index. After three months of surgery, they were in the range of moderate disability and there was not much difference between the two groups. But after 6 months of surgery, the first group was still in the moderate disability range, but the second group entered the low disability period. Ninth-month studies showed that although the pain was significantly reduced in patients in the first group, they were still in the range of moderate disability. The second group was still in a low disability period at the end of the ninth month of surgery with a decrease in disability index. At the end of the first year of surgery, although both groups were in the range of low disability, a significant difference was observed between the two groups.

Discussion

Adult stem cells are a good source of cells for cartilage tissue regeneration and engineering. The potential for differentiation of these cells into cartilaginous lines has been evaluated in many scaffold-based and scaffold-free cell culture systems (22-24). One of the most important adult stem cells is mesenchymal stem cells, which are widely used in cartilage tissue engineering (25, 26). The process of cartilage formation from mesenchymal stem cells requires high cell density and cell-cell contact in the differentiation medium of serum-free cartilage with TGF-β and dexamethasone (27). Research reports indicate that a pellet culture system can be used to create the highest level of cell density in mesenchymal stem cells by imitating the mesenchymal densification process that occurs during the development of cartilage formation (28). Cell-cell interaction is not only an important factor in re-establishing specific functions in tissue engineering but can also play an essential role in the extracellular matrix. Numerous studies have shown that under certain conditions, adipose-derived mesenchymal

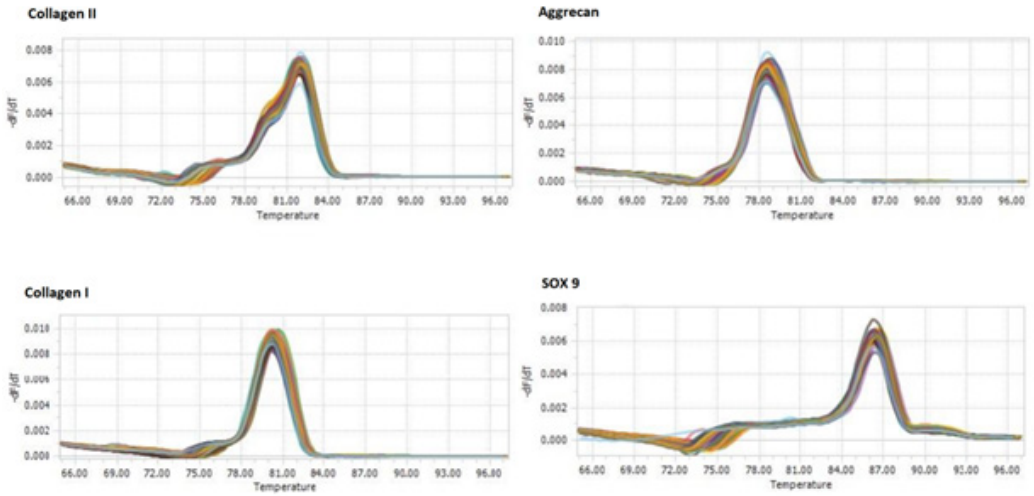

Figure 2. Melting curve of Collagen II, Aggrecan, Collagen I, and SOX 9 genes after 40 cycles of Real-time quantitative PCR.

Table2. Mean demographic and clinical information of participant patients.

Group		Gender	Age	Weight (Kg)	BMI	Number of Underlying Disease	Operation Time (min)	Hospitalization (day)	Recovery room time (min)
1	Men	9	67.31	75.3	32.13	1.66	195	5.5	113.33
	Women	6	71.29	68.2	29.13	1.33	211	6	105
2	Men	11	73.51	71.9	31.68	2.56	360	5.5	110.5
	Women	4	69.19	68.8	30.54	2.25	375	6.5	108.75

Table3. The mean clinical outcomes of patients at the end of the 1st week, 1st trimester, 2nd trimester, 3rd trimester, and 4th trimester by Oswestry Disability Index questionnaire.

Group	First week	First trimester	Second trimester	Third trimester	Fourth trimester
1	68.25	40.74	36.74	21.35	20.28
2	69.12	39.15	20.21	17.57	15.68

stem cells are able to express cartilage-specific genes and proteins, including type II Collagen and Aggrecan, without expressing hypertrophic chondrocytes markers such as type X collagen (29-31). Winter *et al.* Showed that mesenchymal stem cells derived from bone marrow and adipose tissue show the potential for cartilage differentiation in pellet culture. The results of this study showed that this method can be used to differentiate fat-derived mesenchymal stem cells into cartilage, which the results of our study also confirmed (32). The results of this study showed that cartilage tissue was synthesized correctly. If a comprehensive genetic study is done on this subject, many of its unknowns, which are controlled by genes, will be discovered (33,34).

Because cartilage tissue is destroyed in patients with osteoarthritis and the body is unable to synthesize this cartilage (4), we decided to use the cartilage synthesized in this study to treat patients with osteoarthritis of the spine. The results showed that orthopedic surgery and cartilage transplantation were successful for every 15 people and were able to greatly reduce the disability index compared to the group who underwent surgery by implant placement alone.

In this study, after synthesizing cartilage tissue from human adipose-derived mesenchymal stem cells, out of 30 patients with acute osteoarthritis of the spine, 15 of them underwent orthopedic spine implant surgery and cartilage transplantation. Fifteen other patients received only implants. Cartilage transplantation was successful in every 15 patients and the results showed that the synthesized cartilage was able to significantly reduce the disability index in patients.

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