Efficacy of Atelocollagen and Fibrin Glue Mixture on Articular Cartilage Defect of Rabbit

Jun-Keun Lee, Ji-Chul Yoo, Sang-Hun Woo, Sang-Bum Choi, Se-Ken Yeo, Ga-Ram Kim, Chang-Kwon Ko, Dong-Sam Suh, Cheong-Ho Chang

RMS research institute, Seoul, Korea
kck@RMSbio.net

Abstract

Microfracture is an articular cartilage repair technique that works by creating tiny fractures in the underlying bone. However, the predominant repair tissue after microfracture is fibrocartilage. CartiFill™ is cartilage cell scaffold for advanced technique of microfracture. We evaluated the osteochondral healing effect of CartiFill™ and microfracture combination on femoral condyle defect animal model. Twenty NZW rabbits were divided into four groups: 3% CartiFill™ (3% atelocollagen and fibrin glue) implant group, 3% atelocollagen implant group, fibrin glue implant group and non-treatment group. Animals had made femoral condyle defect 4 mm in diameter and 3 mm in depth, and the defect was microfractured using K-wire and immediately implanted accordingly. The animals were sacrificed at 2, 4, 8 and 12 weeks postoperatively, and the specimens were used for macroscopic observation and histological analysis. According to the macroscopic observation, 3% atelocollagen implant and 3% CartiFill™ implant showed smooth, consistent, glistening white tissues. On the contrary, no treatment group and fibrin glue group showed reddish irregular tissues. In histological observation, 3% CartiFill™ group filled the defects sites completely and proceeded to the subchondral bone and cartilage repair by 12 weeks. However, no treatment group didn’t fill the defects sites and didn’t show the subchondral bone repair. Also, all histological scores of 3% CartiFill™ implant ($p < 0.05$) at 2, 4, 8 and 12 weeks showed significant differences from control group and fibrin glue implant. For regeneration of cartilage on osteochondral lesion, CartiFill™ used in combination with microfracture has provided a structural basis and a good quality cartilage tissue and excellent repair.

Key Words: Articular cartilage defect, cartilage repair, scaffold, collagen, tissue engineering

Introduction

Articular cartilage is a highly specialized tissue, which consists of chondrocytes, some progenitor cells and an extracellular matrix (ECM). ECM is composed of a network of collagens, which gives the tissue its shape and strength, and proteoglycans, which give resistance to mechanical stress [1]. Chondrocytes in articular cartilage also reside, proliferate and differentiate inside the body within an abundant ECM [1].

When injured, the articular cartilage has a severely limited capacity for repair because of avascular nature and consequent lack of access to a pool of progenitor cells and humoral factors [2]. These limitations have provoked researchers to develop surgical procedures to restore cartilage defects.

Several studies have been used to repair cartilage lesions, including autologous perichondrium transplantation, debridement combined with microfracture or subchondral drilling, mosaicplasty, and autologous chondrocyte transplantation [3]. However, all these treatments have not always provided satisfactory results. No method has yet been able to consistently reproduce normal hyaline cartilage, and the best treatment in the long term is still unknown [4].

In clinical routine, bone marrow stimulating techniques like drilling, abrasion, or microfracture are frequently used, cost effective, and the first-line treatment options for focal cartilage defects [5]. In microfracture, the introduction of multiple holes into the subchondral bone of the cartilage defect allows mesenchymal stem and progenitor cells in the bone marrow blood to enter the defect, and induces formation of cartilaginous repair tissue. The cellular mechanisms underlying stem cell migration into the defect and subsequent tissue formation are not obvious [5, 6]. Recently, it has been reported that synovial fluid recruits mesenchymal progenitor cells from bone marrow [5, 7]. Also, many chemotactic cytokines and growth factors, which are components of synovial fluid and serum, induce migration and homing of mesenchymal stem cells [5, 8, 9] and may promote the ingrowth of progenitors into the cartilage defects in microfracture. These cells in the subchondral bone marrow have a high chondrogenic differentiation capacity [5, 10] and may form a cartilaginous repair tissue upon acceleration by growth and differentiation factors from the subchondral bone [5, 11, 12]. Clinical studies indicated that microfracture shows good results in the short term, but clinical results may vary in the long-term and have limited indication for relatively small defects [5, 13, 14]. In an attempt to repair cartilage tissue formation, collagen matrices have been studied as implants.

Collagen has been engineered to promote repair in many tissues including cartilage [15]. Collagen is a group of proteins that comprise the most abundant proteins in mammals representing 25–35% of the total body protein [16]. Collagen is a favorite material [17], is found in cartilage, bone, intervertebral discs, blood vessels, tendons, ligaments, skin, and cornea, and it is the main component of extracellular matrix. Various types of collagens are synthesized by fibroblasts, smooth muscle cells, chondrocytes, osteoblasts, endothelial cells, epithelial cells, myoblasts, neural retinal cells, and notochord cells. Specific receptors, such as integrins, discoidin-domain receptors, glycoprotein VI or specialized proteoglycan receptors mediate the interaction with collagens [18, 19]. Signaling by these receptors defines adhesion, differentiation, growth, cellular
reactivities as well as the survival of cells in multiple ways [20]. Collagen contributes to the entrapment, local storage and delivery of growth factors and cytokines, and it plays important roles during organ development, wound healing and tissue repair [21, 22]. Type I collagen has been shown to bind decorin, and thereby, it might indirectly block TGF-h action within the tissue [22]. Collagen also binds a number of other growth factors and cytokines [23].

Although there have been numerous studies of the healing of cartilage defects implanted with a variety of biomaterials, none have specifically showed the healing of microfracture-treated sites implanted with a scaffold material [6]. The theory is that an implanted biomaterial may stabilize the marrow-imbibed fibrin clot and thus favor the reparative process [6]. We hypothesize that in microfracture procedure an implanted collagen can organize the 3D structure at defect site and provide the optimal migration and adherence of cells. Therefore, collagen implant can be serving as carrier for the cells, growth factors, and regulators of cells activity.

**Purpose**

The purpose of this study was to determine the effect of treatment by microfracture and whether an advanced engineered type I atelocollagen could improve the healing when used in conjunction with microfracture for the treatment of chondral defects in a rabbit model.

**Materials and Methods**

This study was approved by the SewonCellontech Institutional Animal Care and Use Committee and was in accordance with guidelines established by the ethical principles in use of experimental animals.

**Preparation of type I atelocollagen and fibrin glue**

Type I atelocollagen (3% CartiFill™) derived from the porcine dermal skin was manufactured by SewonCellontech Co., Ltd (Korea), and commercially available fibrin glue kit (Green Cross P.D. Co., Korea) was used.

For the injection procedure, CartiFill™ was prepared as in the following procedures; two 1-mL syringes and a Y-shaped mixing catheter connected to a 20-gauge needle (inner diameter: 0.9 mm; length: 90 mm) were used. One syringe was filled with 1 mL of fibrinogen (Greeplast kit®; Green Cross P.D. Co. Yongin, Korea), and the other syringe was filled with 0.9 mL of atelocollagen (CartiFill™; SewonCellontech Co., Korea) and 0.1 mL of thrombin (50 IU).

**Experimental Design**

Twelve female New Zealand White Rabbit were used. The average age was 30 weeks, and average weight was 3.5 to 4.5 kg. They were kept in standard Rabbit cage (420W x 500D x 350H mm) and acclimated for four week to the constant study environmental condition: 12 to 12 hour light-dark cycle (lights on at 06:00), 22 ± 3°C room temperature, and 50 ± 10% humidity. All animal were divided into four groups: 3% CartiFill™ (3% atelocollagen with fibrin glue) implanted group, 3% atelocollagen, fibrin glue implanted group, and no treatment as a control.

All defect microfracture perforations were performed using K-wire drilling. Articular cartilage defect on the part of femur was performed bilaterally. They were kept in separate cages and allowed to move freely. No specific immobilization was applied after operation. Animals were sacrificed in a carbon dioxide euthanasia chamber after 2, 4, 8 and 12 weeks. The entire knee were dissected, examined macroscopically and photographed, and then, distal femurs were fixed with 10% buffered formalin. During the experiment, the animals were fed *ad libitum* with standard laboratory food pellets.

**Surgical procedure**

All surgeries were performed under sterile conditions. Each animal was premedicated according to their weight with an intramuscular injection of a mixture of Zoletil (Verbac, France) and xylazine (Bayer, Germany) to obtain effective anesthesia [24]. A 3 cm medial parapatellar incision was made over the both knee, and patellar was everted. A full-thickness defect (4 mm in diameter, 3 mm in depth) in the articular cartilage and subchondral bone was made with an electric burr applied to the femoral condyle of the femur [25]. The animal was made bilateral full-thickness cylindrical osteochondral defect. Kirschner-wire was used to induce the microfracture on defect sites [5]. Four equal defects were immediately implanted with atelocollagen+fibrin glue, atelocollagen, fibrin glue and no treatment (Fig 1). The implants were then inserted into the defect, and after reducing the patella, the capsule and muscle were closed with double 3-0 Vicryl (Ethicon, USA) suture and the skin with a continuous 3-0 Nylon (AILEE, Korea) suture.

Postoperatively, animals were allowed free cage activity. During the post-operative time, clinical parameters including appetite, activity, bleeding, infection and wound dehiscence were evaluated daily. Cefazoline (Chongkundang, Korea) was administered daily with intra-muscular injection for seven days for post-antibiotic.

**Macroscopic analysis**

Conditions of full-thickness defect in the articular cartilage were observed for 2, 4, 8 and 12 weeks after surgery. The knee joint was approached as described above, and the distal femoral condyles were dissected. The gross appearance of the defects was assessed. Especially, we have looked into the surface characteristics of the repair and its continuity with the host tissue as well as the presence of osteoarthritic changes throughout the joint. The surfaces of the grafts were inspected for color, integrity, contour and smoothness.
Histological analysis

At the termination of the experiments, the harvested tissues were fixed, decalcified, dehydrated, cleansed, and embedded in paraffin. The 5-mm-thick sections of embedded tissues were cut using a microtome, and stained with Hematoxylin-Eosin. Also paraffin sections were stained with Alcian blue to verify proteoglycan production and with Safranin O to assess negatively charged matrix proteoglycans. Immunohistochemistry with antibodies for type-I and -II collagen was performed according to a standard ABC protocol (Vector, USA). The type I antibody was provided by Abcam (USA), and the type II antibody was produced in our lab.

The specimens were graded with use of a histological scale, which is a modification of that described by Wakitani et al. [25], Pineda et al. [26] and O’Driscoll et al. [27]. The scale is composed of seven categories and assigns a score ranging from 0 to 23 points (Table 1). A lower score represents the better cartilage repair.

Statistical analysis

All data were expressed as mean–standard deviation and compared by one-way analysis of variance using SigmaPlot 10.0 software. P-values less than 0.05 were considered significant.

Table 1. Histological grading scale for the defects of cartilage.

<table>
<thead>
<tr>
<th>Category</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell morphology</strong></td>
<td></td>
</tr>
<tr>
<td>Hyaline cartilage</td>
<td>0</td>
</tr>
<tr>
<td>Mostly hyaline cartilage</td>
<td>1</td>
</tr>
<tr>
<td>Mostly fibrocartilage</td>
<td>2</td>
</tr>
<tr>
<td>Mostly non-cartilage</td>
<td>3</td>
</tr>
<tr>
<td>Non-cartilage only</td>
<td>4</td>
</tr>
<tr>
<td><strong>Matrix-staining</strong></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Slightly reduced</td>
<td>1</td>
</tr>
<tr>
<td>Moderately reduced</td>
<td>2</td>
</tr>
<tr>
<td>Markedly reduced</td>
<td>3</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td><strong>Surface regularity</strong></td>
<td></td>
</tr>
<tr>
<td>Smooth (&lt; 3/4)</td>
<td>0</td>
</tr>
<tr>
<td>Slight disruption</td>
<td>1</td>
</tr>
<tr>
<td>Severe disruption</td>
<td>2</td>
</tr>
<tr>
<td><strong>Filling of the defect</strong></td>
<td></td>
</tr>
<tr>
<td>111%+</td>
<td>1</td>
</tr>
<tr>
<td>91 - 110%</td>
<td>0</td>
</tr>
<tr>
<td>76 - 90%</td>
<td>1</td>
</tr>
<tr>
<td>51 - 75%</td>
<td>2</td>
</tr>
<tr>
<td>26 - 50%</td>
<td>3</td>
</tr>
<tr>
<td>≤ 25%</td>
<td>4</td>
</tr>
<tr>
<td><strong>Thickness of cartilage</strong></td>
<td></td>
</tr>
<tr>
<td>2/3</td>
<td>0</td>
</tr>
<tr>
<td>1/3 - 2/3</td>
<td>1</td>
</tr>
<tr>
<td>≤ 1/3</td>
<td>2</td>
</tr>
<tr>
<td><strong>Integration of donor with host adjacent cartilage</strong></td>
<td>0</td>
</tr>
<tr>
<td>Normal continuity and integration</td>
<td>1</td>
</tr>
<tr>
<td>Decreased cellularity</td>
<td>2</td>
</tr>
<tr>
<td>Gap (lack of continuity) on one side</td>
<td>3</td>
</tr>
<tr>
<td>Gap (lack of continuity) on two side</td>
<td>4</td>
</tr>
<tr>
<td><strong>Percentage replacement of subchondral bone</strong></td>
<td>0</td>
</tr>
<tr>
<td>90 - 100%</td>
<td>0</td>
</tr>
<tr>
<td>75 - 89%</td>
<td>1</td>
</tr>
<tr>
<td>50 - 74%</td>
<td>2</td>
</tr>
<tr>
<td>25 - 49%</td>
<td>3</td>
</tr>
<tr>
<td>0 - 24%</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total score</strong></td>
<td>23</td>
</tr>
</tbody>
</table>

Macroscopic Observation

All animals survived for the entire duration of the experiment. After the insertion of implants there was no death, weight loss, inflammation or abnormal reaction in animals. The defect conditions observed at 2, 4, 8 and 12 weeks post-implantation are shown in Figure 2. There were no signs of inflammation, osteoarthritic change, or obvious synovitis in any rabbits. In general, in the CartiFill™ implanted group and atelocollagen implanted group the regenerated area showed smooth, consistent, glistening white tissue which resembled articular cartilage. However, the regenerated area of the fibrin glue implanted group and no treatment group showed reddish irregular tissue with depression, and the margin of the defect was sharply differentiated from surrounding normal cartilage.

Table 2. Macroscopic appearance of femoral condyle cartilage defect at 2, 4, 8 and 12 weeks after implantation with no treatment group (A), fibrin glue implanted group (B), 3% atelocollagen implanted group (C), and 3% CartiFill™ (3% atelocollagen + fibrin glue) implanted group (D).

Results

Histological observations

At 2 weeks post-surgery, every group had many inflammatory cells and fibrous connective tissue, but they did not show the cartilage repair and smooth connection with adjacent host cartilage (Fig. 3). Additionally, the GAG component was not seen in Alcian blue or Safranin O staining pictures. 3% CartiFill™ implanted group filled the defects sites to some degree, but other groups displayed the unfilled defects sites. 3% atelocollagen implanted group appeared to have subchondral bone repair, but other groups did not.

After 4 weeks post-injection, 3% CartiFill™ implanted group, fibrin glue implanted group, and no treatment group displayed the fibrous connective tissue, which entered new blood vessels and cells, and 3% atelocollagen implanted group presented a few cartilage cells, which stained safranin O and Alcian blue, with fibrous tissues (Fig. 4). In common with the tissue of 2 weeks post-injection, all groups did not show the connection with neighboring normal tissues. Both 3% atelocollagen implanted group and 3% CartiFill™ implanted group had clear remodeling of subchondral bone, and fibrin glue implanted group had the sinking defects sites without subchondral bone repair. No treatment group was filled with only fibrous tissues.
Figure 3. Histological analyses at 2 weeks after implantation. Hematoxylin-Eosin (A), Safranin O (B), Alcian blue (C), Type II collagen (D). (CT: Connective tissue, BV: Blood vessel, FG: Fibrin glue, Arrow: Inflammatory cell, a: × 8, b: × 100)

Figure 4. Histological analyses at 4 weeks after implantation. Hematoxylin-Eosin (A), Safranin O (B), Alcian blue (C), Type II collagen (D). (CT: Connective tissue, BV: Blood vessel, NB: New bone, HC: Hyaline cartilage, a: × 8, b: × 100)

Figure 5. Histological analyses at 8 weeks after implantation. Hematoxylin-Eosin (A), Safranin O (B), Alcian blue (C), Type II collagen (D). (CT: Connective tissue, BV: Blood vessel, HC: Hyaline cartilage, a: × 8, b: × 100)
Both 3% atelocollagen implanted group and 3% CartiFill™ implanted group conducted subchondral bone reformation partially, and they revealed fibro-cartilage cells and hyaline-cartilage cells at upper classes of defects sites at 8 weeks post-implantation (Fig.5). 3% atelocollagen group observed many hyaline-cartilage cells, which were appeared strongly in the cartilage matrix stain and type II collagen stain, in bounded part between subchondral bone and cartilage. Fibrin glue group did not fill the defects sites as ever, and the control group presented a little fibrocartilage cells.

At 12 weeks post-implantation, 3% CartiFill™ implanted group displayed subchondral bone and cartilage repair, and they were smoothly linked with adjacent normal cartilage. 3% atelocollagen implanted group proceeded partial repair of subchondral bone and filled fibrocartilage in the upper defect sites, but it was incompletely connected with normal cartilage. Both the fibrin glue group and the control group displayed fibrocartilage, but didn’t fill the defects sites and didn’t show the subchondral bone repair. The GAG components were observed at 3% atelocollagen implanted group and 3% CartiFill™ implanted group.

According to the histological grading scale (Tab. 1), we analyzed the cell morphology, matrix-staining, surface regularity and filling of the repaired area, thickness of repaired cartilage, integration of donor with adjacent cartilage and percentage replacement of subchondral bone at 2, 4, 8 and 12 weeks after implantation (Tab. 2).

All histological scores of 3% CartiFill™ implant (p < 0.05) at 2, 4, 8 and 12 weeks showed significant differences from control group and fibrin glue implant. The histological assessment of 3% atelocollagen implant (p < 0.05) showed significant differences from the control group at 8 and 12 weeks, and it was significantly better than fibrin glue implant (p < 0.05) at 4 and 8 weeks. The score of fibrin glue implant showed significant differences from control group at only 12 weeks. This means that 3% CartiFill™ implanted group was the most efficient implant for cartilage repair.

### Discussion

Because spontaneous migration of chondrocytes from surrounding tissue is very limited, surgical procedures that promote a presence of chondrogenic cells in cartilage defects are needed [28]. In this study, we evaluated the effect of advanced therapeutic technique and found that type I atelocollagen could improve the healing when used in conjunction with microfracture for the treatment of chondral defects in a rabbit model.

In the marrow-stimulating procedures, perforations to the subchondral bone allow the influx of blood and marrow derived cells into the holes and defect followed by the formation of a blood clot. Their healing process consisting of an acute inflammatory response and cell chemotaxis leads to the generation of a vascularized granulation tissue and the proliferation of pluripotent mesenchymal progenitor cells with a capacity to differentiate into multiple mesenchymal cell types derived from bone marrow [29, 30]. The bone marrow stimulation techniques, such as subchondral drilling, abrasion arthroplasty, and microfracture, are the most widely used surgical procedures [30]. To improve the bone marrow stimulation techniques for cartilage repair, many studies have been carried out by modifying the perforation technique [31], combining with periosteal grafts [32], hybrid implants[33-35], scaffolds [36], or other surgical methods [37].

Microfracture is one of many methods available to treat articular cartilage lesions. This technique had been elaborated by Steadman et al. [38], and it had been applied chiefly in young athletes and young patients [39].

Recent studies have revealed promising results after microfracture. Steadman et al. described improved function in 95% of their study population with a mean follow-up of 11.3

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**Table 2.** Histological scoring at 2, 4, 8 and 12 weeks post-implantation

<table>
<thead>
<tr>
<th>Time after implantation</th>
<th>Histological scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (Fibrin)</td>
</tr>
<tr>
<td>2 weeks</td>
<td>21.3 ± 0.6</td>
</tr>
<tr>
<td>4 weeks</td>
<td>18.3 ± 0.6</td>
</tr>
<tr>
<td>8 weeks</td>
<td>18.7 ± 0.6</td>
</tr>
<tr>
<td>12 weeks</td>
<td>19.0 ± 2.0</td>
</tr>
</tbody>
</table>

(n = 3, Mean ± SD, a: P < 0.05 compared to the control group at each week, b: P < 0.05 compared to the fibrin glue group at each week, c: P < 0.05 compared to the 3% atelocollagen group at each week.)
years [38]. Scores for patients’ ability to do daily activities, strenuous work and sports were significantly improved in the follow-up. These results were astonishing since microfracture belongs to the classical marrow stimulation techniques that involve surgical access to the bone marrow spaces underlying regions of damaged articular cartilage and usually promote resurfacing with predominantly fibro-cartilaginous repair tissue of inferior quality. In this context a fibrous type of cartilage tissue has been found in rabbits [40-42] and dogs [43] that underwent abrasion chondroplasty [44].

CartiFill™ is cartilage cell scaffold for advanced technique of microfracture. It creates stable matrix structure of cell scaffold in the cartilage defects after microfracture with finely purified atelocollagen and fibrin glue.

Because collagen is a naturally occurring component of tissues, collagen-based scaffolds favor the attachment of cells normally found in joint tissues as well as exogenous cells embedded within a collagen delivery device [45, 46]. Collagen scaffolds have been used extensively for decades for the in vitro characterization of chondrocyte and stem cell behavior [47, 48]. As with fibrin gels, collagen fiber scaffolds have been used to deliver chondrocytes treated with gene therapy to injury sites [49].

In this study, we investigated the histological analysis and the histological grading scale on the cell morphology, matrix-staining, surface regularity and filling the repair area, thickness of repaired cartilage, integration of donor with adjacent cartilage and percentage replacement of subchondral bone at 2, 4, 8 and 12 weeks after implantation. At 4 weeks post injection, 3% atelocollagen and CartiFill™ implants represented subchondral bone remodeling. However, the control groups were filled with only fibrous tissues (Fig 4). This result implies that collagen has a critical role for the early to middle stage of cartilage repair. Atelocollagen may have a role as scaffold for the migrated bone marrow cells attachment and stable proliferations. Furthermore, collagen provided skeletal structure for cartilage reconstruction. The partial subchondral bone regeneration was observed on CartiFill™ and 3% atelocollagen groups at 8 weeks after surgery, and fibrocartilage cells and hyaline cartilage cells were also represented at upper classes of defected areas (Fig 5).

CartiFill™ implant regenerated intact cartilage tissue on chondral defect site after 12 weeks post-operation. Moreover, regenerated cartilage was identified as hyaline cartilage. In case of atelocollagen implant, subchondral bone was formatted, but mostly fibrocartilage was generated. On the contrary, fibrin glue and non-treatment groups had poor cartilage regenerations. In addition, the GAG components were found only at collagen-involved experimental groups (Fig 6).

The histological scoring results supported that CartiFill™ implant had a rapid and significantly better cartilage repair than other experimental groups at 8 weeks after surgery. The histological scores of 3% CartiFill™ implant during 12 weeks were significantly different from fibrin glue implant and no treatment groups. Also, 3% CartiFill™ and 3% atelocollagen implant had a reconstruction of subchondral bone, but fibrin glue and no treatment group had an insufficient reconstructed cartilage (Table 2).

In this study, we demonstrated that CartiFill™ with microfracture was excellent and effective treatment in osteochondral defects, and atelocollagen implant alone was effective alternative treatment for osteochondral lesions.

**Conclusion**

The combination technique of CartiFill™ cartilage scaffold (mixture of atelocollagen and fibrin) and microfracture promotes cellular migration, proliferation and cartilaginous tissue formation of rabbit articular cartilages. This study suggests that atelocollagen/fibrin hybrid scaffold may serve a structural basis for advanced tissue-engineered articular cartilage.

**References**