Injectable Tissue-Engineered Cartilage Using a Fibrin Sealant

Jinsoon Chang, MD, PhD; Jk J. Rasamny, BA; Stephen S. Park, MD

Objective: To investigate a commercially available fibrin sealant as a vehicle for developing injectable tissue-engineered cartilage.

Methods: Fibrin glue was mixed with autogenous chondrocytes from rabbits (n = 15). This isolate was injected along their nasal dorsa using 1 of 3 different fibrin glue concentrations. The samples were harvested at 8 weeks and compared with elastin and hyaline cartilage controls.

Results: Neocartilage was created along a linear injection tract on the dorsa of the nasal bones in 5 of 15 rabbits. Higher thrombin concentrations proved to be directly correlated with successful creation of injectable cartilage. Histologically, the staining patterns of both hematoxylin-eosin and safranin O stains were identical to that of normal auricular control cartilage. The presence of elastin fibers was observed following Verhoeff staining. No foreign body reaction was observed from the host.

Conclusions: This study demonstrated a successful method for percutaneous injection of tissue-engineered cartilage as a mixture of chondrocytes suspended in fibrin glue. The thrombin concentration, along with the concentration of fibrinogen and chondrocytes, must be optimized to succeed consistently in cartilage growth.

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The biomaterials used for tissue engineering have evolved over the last few decades. To date, the most common approach for developing tissue-engineered cartilage has been to seed chondrocytes onto a 3-dimensional, biodegradable polymer. However, a study has suggested that on implantation, the synthetic polymer induces an immunologic reaction, resulting in fibrosis and resorption of any subsequent cartilage. Consequently, natural, biologic polymers have been investigated for the development of tissue-engineered cartilage. Of the natural polymers, injectable mediums have drawn optimistic attention because they hold several advantages over solid scaffolds. Most important, injectable matrices can be introduced in a minimally invasive manner while still providing bulk and many of the virtues of autogenous implants. Moreover, the viscous character may lend itself to molding and 3-dimensional configurations in situ.

The ideal liquid polymer would be malleable, stable enough to maintain a 3-dimensional structure following injection, yet porous enough to permit diffusion of nutrients to promote chondrocyte survival and proteoglycan matrix production. Of the recognized liquid polymers, fibrin glue, a well-known biocompatible hemostatic agent, has been used to fix cultured cellular components and also to serve as a matrix to repair bony defects. Ideally, injection of chondrocytes, suspended in fibrin glue, would be able to fill and repair any defect in a minimally invasive manner. Hence, fibrin glue may prove to be a promising vehicle for transporting chondrocytes and developing tissue-engineered cartilage. In fact, studies in the rabbit model have already shown that subcutaneous injection of a modified fibrin glue, seeded with chondrocytes, can result in the in vivo formation of tissue-engineered cartilage. These results validate fibrin sealants as a suitable injectable polymer for cartilage tissue engineering.

Commercially available fibrin glues (eg, Hemaseel [Haemacure Corp, Sarasota, Fla] and Tisseel [Baxter, Deerfield, Ill]) have been used as the carrier.
medium for tissue-engineered cartilage, but many unresolved questions remain. First, researchers have yet to establish whether the injectable matrix, fibrin glue plus chondrocytes, will maintain its volume and contour over prolonged periods of time. Second, although in this study we succeeded in forming tissue-engineered cartilage subcutaneously, it is unclear if the same result can be achieved on bony or cartilaginous surfaces, where the surrounding vasculature may not be as robust. Clearly, if research demonstrates that, following injection, tissue-engineered cartilage is able to develop in vivo on bony structures while maintaining its shape and size, the clinical usefulness of injectable tissue-engineered cartilage will be greatly enhanced.

In this study, we evaluated a method to contour the nasal dorsum of a rabbit via injection of chondrocytes seeded in a semisolid fibrin glue scaffold. In addition, because the rate of clot formation of fibrin glue is a function of thrombin concentration, we sought to analyze the effects of the latter on the efficiency of cartilage development in vivo.

### METHODS

The experiment isolated chondrocytes from the auricular cartilage of 15 juvenile rabbits. Chondrocytes were mixed with varying concentrations of a fibrin sealant and injected along their nasal dorsa (bony and cartilaginous portions). The 15 rabbits were separated into 3 groups of 5, each with a predetermined concentration of thrombin in the fibrin glue. Five rabbits received the chondrocytes in a fibrin glue solution with a 200-fold dilution (manufacturer’s recommendation) of thrombin. 5 rabbits received a solution with a 50-fold dilution of thrombin, and 5 rabbits were injected with chondrocytes in a 4-fold dilution of thrombin. Tissue-engineered cartilage was allowed to grow in vivo for 8 weeks, after which the rabbits were assessed for neocartilage development on the nasal dorsum, comparing variability with thrombin concentration. Assessments were made grossly and histologically, with hematoxylin-eosin, safranin O, and Verhoeff stains.

For chondrocyte isolation, the removal and preparation of auricular cartilage, along with the isolation of chondrocytes, were performed following our recently published guidelines.

### FIBRINOGEN AND THROMBIN PREPARATION

This procedure used a dual syringe injection Tisseel system (model 706332; Baxter). The first syringe for all rabbits contained a 0.5-mL solution of 0.25% fibrinogen with 750 U/mL of fibrinolytic inhibitor. The second syringe was prepared by first spinning down the 1-mL solution of chondrocytes in complete medium at 300g for 5 minutes. The chondrocyte pellet was then resuspended in 0.5 mL of thrombin containing 20mM of calcium chloride solution. The thrombin concentration in the second syringe varied among the 15 rabbits. The second syringe for the first 5 rabbits contained a 100-fold dilution of thrombin (5 U/mL), as suggested by the manufacturer. The second syringe for the next 5 rabbits contained a 25-fold dilution of thrombin (20 U/mL). The second syringe for the remaining 5 rabbits contained a 2-fold dilution of thrombin (250 U/mL). Consequently, following injection and subsequent mixing of the contents of the 2 syringes, the final thrombin concentrations among the 3 groups were 2.5 U/mL (200× dilution), 10 U/mL (50× dilution), and 125 U/mL (4× dilution). Table 1 demonstrates the concentrations of the components in each injection. The final chondrocyte concentration ranged from 4.7 × 10⁶ cells/mL to 9.3 × 10⁶ cells/mL (Table 2).

### INJECTION AND EXPLORATION

Fifteen rabbits were prepped, shaved, and anesthetized (with inhaled ketamine and acepromazine) prior to injection. The dual syringe system was then used to deliver the contents of the 2 syringes, fibrinogen and thrombin (containing the seeded chondrocytes), simultaneously. The syringe was placed beneath the rabbit’s skin at the top of its nose, along the bone, and then slid to the tip of the animal’s nose. The total of 1 mL of injectable medium was injected slowly along the entire dorsum of the animal’s nose. Next, digital pressure on both sides of the dorsum was manually and firmly applied to mold the fibrin glue on the top of the nasal dorsum for at least 5 minutes until complete gelation was achieved in a semisolid scaffold. We photographed the nasal dorsum in a standardized sequence (immediately after injection, 4 weeks after injection, and just before exploration at 8 weeks after injection) to examine the change of contour. At 8 weeks after injection, each group of rabbits was killed.

### Table 1. Summary of Concentration of Fibrinogen, Fibrinolytic Inhibitor Solution (FIS), Calcium Chloride, and Thrombin

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Fibrinogen, mg/mL</th>
<th>FIS, IU</th>
<th>Calcium Chloride, mM</th>
<th>Thrombin, U/mL (Dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>75-115</td>
<td>3000</td>
<td>40</td>
<td>500</td>
</tr>
<tr>
<td>Final*</td>
<td>9.375-14.375</td>
<td>375</td>
<td>5</td>
<td>2.5 (× 200)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 (× 50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>125 (× 4)</td>
</tr>
</tbody>
</table>

*The thrombin concentrations are given for the 3 groups of rabbits.

### Table 2. Cell Concentration of 3 Different Thrombin Dilutions

<table>
<thead>
<tr>
<th>Rabbit*</th>
<th>2.5 U/mL</th>
<th>10 U/mL</th>
<th>125 U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.7</td>
<td>7.1</td>
<td>5.9</td>
</tr>
<tr>
<td>2</td>
<td>7.5</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>3</td>
<td>6.3</td>
<td>6.0</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>9.5</td>
<td>5.4</td>
<td>4.7</td>
</tr>
<tr>
<td>5</td>
<td>4.8</td>
<td>5.0</td>
<td>5.7</td>
</tr>
</tbody>
</table>

*We labeled each rabbit 1 to 5 in each of the 3 different groups of 5 rabbits.
humanely according to the protocol created by the University of Virginia Care Advisory Committee.

**HISTOLOGIC ANALYSIS**

Changes in gross appearance and the contour of the nasal dorsum were examined and assessed through digital photographic inspection and palpation of the nasal dorsum. Histologic features were visualized by light microscopy using both hematoxylin-eosin and safranin O stains of 10% buffered formalin-fixed tissue sections. By using Image Pro software (Media Cybernetics, Silver Spring, Md), an imaging tool that allows importing, enhancement, and analysis of images seen under the microscope, the intensity of safranin O was also determined by measuring the light penetrance of the slide. Finally, Verhoeff staining was used to confirm the presence of elastin in the tissue-engineered cartilage and auricular control cartilage. Hyaline cartilage was used as a control for the Verhoeff elastin fiber stain.

**RESULTS**

**GROSS EXTERNAL APPEARANCE AND EXPLORATION**

The augmented nasal dorsum, which was clearly present immediately after injection, consistently diminished over time. This was especially true for the group that received the most diluted thrombin (200×dilution); in this group, no discernable augmentation was palpated, even at 4 weeks, for any of the 5 rabbits. Of the 5 rabbits that received an intermediate thrombin concentration (50×dilution), only 1 maintained signs of altered contour, in the form of a slightly palpable bump on the dorsum of its nose. In the group that received the highest concentration of thrombin (4×dilution), 4 of the 5 rabbits retained an augmented contour at 4 weeks after injection. However, at the time of exploration (8 weeks after injection), there was a reduction in the degree of external augmentation that could be palpated (Figure 1). On exploration, however, 5 animals clearly displayed strips of neocartilage along the length of the injection, atop their nasal dorsa (Figure 2).

**HISTOLOGIC CHARACTERISTICS**

The tissue-engineered cartilage displayed a high level of metabolic activity evidenced by the degree of extracellular matrix surrounding the chondrocytes. Furthermore, the staining pattern of the tissue-engineered cartilage with safranin O was identical to that of the auricular control cartilage (Figure 3). Verhoeff staining for the presence of elastin fibers in the tissue-engineered cartilage also showed a characteristic staining pattern similar to that of the auricular control cartilage. The linear filamentous lines in the extracellular matrix represent elastin fibers, which are not seen in the hyaline cartilage control, suggesting that the tissue-engineered cartilage came from the auricular chondrocytes and maintained their properties throughout the process of cartilage formation (Figure 4). Last, no identifiable foreign body reaction was seen in histologic examination of any of the tissue-engineered cartilage sections. A previous study noted the occurrence of immunogenic reactions following implantation of a synthetic polymer scaffold seeded with chondrocytes, as detailed in the first paragraph of this article (Figure 5). No such reaction occurred following injection of the fibrin glue-chondrocyte mixture in this study.

**EFFECT OF THROMBIN CONCENTRATION ON THE DEVELOPMENT OF THE TISSUE-ENGINEERED CARTILAGE**

The total number of chondrocytes in the group injected with the most diluted levels of thrombin (200×dilution) ranged from 4.8×10⁶ cells/mL to 9.5×10⁶ cells/mL (Table 2). None of the 5 rabbits had developed cartilage when examined 8 weeks after injection. Of the 5 rabbits that received the intermediate thrombin concentration (50×dilution), the total chondrocyte cell numbers ranged from 5.0×10⁶ cells/mL to 7.1×10⁶ cells/mL (Table 2). Only 1 of the 5 rabbits yielded tissue-engineered cartilage 8 weeks following injection. However, 4 of the 5 rabbits that received the highest thrombin concentration (4×dilu-
In this study, we demonstrated that injectable tissue-engineered cartilage using a commercially available fibrin sealant can be achieved on the dorsum of the nose in a rabbit. Histologic staining of the neocartilage resembled that of the chondrocyte cell of origin (ie, auricular cartilage). This was demonstrated by routine histologic staining (hematoxylin-eosin and safranin O) and special staining for the presence of elastin fibers. There was no histologic evidence of inflammation or other foreign body reaction when fibrin glue was used as the chondrocyte carrier platform. This finding stands in stark contrast to that of a previous experiment in which tissue-engineered cartilage from synthetic polymers induced a dramatic foreign body reaction. This finding is consistent with that of another study that suggested that fibrinogen and thrombin cause little to no immunologic reaction.

Unfortunately, our initial goal of maintaining a significant augmentation of the dorsum of a rabbit nose was not achieved. The failure to sustain the desired volume and contour might be a function of several factors, including the external pressure from the native nasal skin, the degradation rate of the polymer, the duration for extracellular matrix production, and the proliferation of chondrocytes. Although histologic examination displayed a high density of chondrocytes and abundant extracellular matrix, we cannot fully assess whether the production of extracellular matrix among the tissue-engineered cartilage was truly equal to that of the control cartilage. Therefore, a decreased rate of extracellular matrix production cannot be ruled out as the cause behind the failure of our cartilage to maintain its shape and contour.

A previous study also demonstrated that the concentration and the total number of chondrocytes are important determinants for the development of tissue-engineered cartilage. Although histologic examination displayed a high density of chondrocytes in our material, the number that remained fully viable and functional (ie, an actively producing matrix) was more difficult to determine. Consequently, a low number of functional chondrocytes may have made the tissue-engineered cartilage more susceptible to loss of its original bulk and contour.

Last, a study has also demonstrated that increased fibrinogen concentration in the fibrin glue leads to an increased mass of tissue-engineered cartilage. We set the fibrinogen concentration lower than the suggested level to increase the viscosity of and promote proper solidification of the fibrin matrix. The low level of fibrinogen may have resulted in higher rates of polymer degradation and may be responsible for the inability of the tissue-engineered cartilage to maintain its contour.
In conclusion, 3 factors—the rate of extracellular matrix production, the total number of chondrocytes, and the rate of degradation of the polymer—all may have caused the tissue-engineered cartilage to lose its shape and contour.

Our experiment also examined the effects of the thrombin concentration of the fibrin glue on the development of tissue-engineered cartilage. It is known that adequate levels of fibrinogen and chondrocytes are important factors in both the development and maintenance of tissue-engineered cartilage. We investigated the thrombin concentration levels to determine the impact they might have on neocartilage growth. We discovered that, by optimizing the thrombin concentration, tissue-engineered cartilage could still be developed despite low levels of both fibrinogen and chondrocytes. In our study, the manufacturer-suggested concentration of 200 × dilution failed to produce cartilage in any of the 5 rabbits that received that level, whereas the highest levels of thrombin (4 × dilution) produced tissue-engineered cartilage in 4 of the 5 rabbits that received that level. We hypothesize that the degree of cross-linking, which is increased in higher concentrations of thrombin,8 was insufficient in the samples with a low concentration of thrombin to form a stable matrix. As a result, the unstable matrix was unable to support a suitable environment to promote the development of chondrocytes and the production of mature cartilage. Rigid forms of fibrin sealant seem to be more useful for providing a scaffold for chondrocytes and cartilage production.

Furthermore, the concentration of chondrocytes in each group did not specifically correlate with a successful outcome. For example, the lowest concentration of chondrocytes (4.7 × 10⁶ cells/mL) in the group that received the highest thrombin concentration (4 × dilution) produced tissue-engineered cartilage, whereas the largest concentration of chondrocytes (9.5 × 10⁶ cells/mL) in the group that received the lowest thrombin concentration (200 × dilution) failed to create tissue-engineered cartilage. No statistically significant conclusions were made comparing the chondrocyte concentration and successful development of tissue-engineered cartilage owing to the low sample size in this experiment. However, the trend holds that higher concentrations of thrombin were associated with the development of tissue-engineered cartilage.

In conclusion, injectable tissue-engineered cartilage can be achieved in the animal model with autogenous auricular chondrocytes transplanted into the nasal dorsum. The gross augmentation we hoped to observe was not sustained over time, but strips of tissue-engineered cartilage were harvested after an 8-week incubation. This cartilage resembled normal cartilage in many ways. Thrombin concentration seems to be important because much higher levels yielded more consistent results. As this process is refined through further experiments, we believe percutaneous injection of tissue-engineered cartilage shows tremendous clinical promise as a means of creating facial augmentation through minimally invasive means. Its application may have an impact on treatment for such problems as saddle nose correction, nasal valve collapse, microtia refinement, and skeletal augmentation.
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REFERENCES


Call for Papers

The Archives of Facial Plastic Surgery will publish a theme issue on plastic and reconstructive surgery of the orbit and eyelid in conjunction with the Archives of Ophthalmology. These manuscripts will highlight the multidisciplinary character of this fascinating area and should be of broad interest. Robert A. Goldberg, MD, will be the guest editor for these joint theme issues, which will be published in the November/December 2007 issue of the Archives of Facial Plastic Surgery and the December issue of the Archives of Ophthalmology. We are most interested in receiving articles on the topics of Graves orbitopathy, orbital and adnexal trauma and reconstruction, lacrimal outflow disorders, aesthetic periorbital rejuvenation techniques, orbital-sinus disease, and orbital and adnexal physiology. Manuscripts received by June 1, 2007, will have the best chance for acceptance.