Injectable Tissue-Engineered Cartilage Using a Fibrin Sealant

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

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<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>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 (× 50)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>125 (× 4)</td>
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</table>

The experiment isolated chondrocytes from the auricular cartilage of 15 juvenile rabbits. Chondrocytes were mixed with varying concentrations of fibrin glue plus chondrocytes, were performed following our recently published guidelines.1

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-fold dilution), 10 U/mL (50-fold dilution), and 125 U/mL (4× dilution). Table 1 demonstrates the concentrations of the components in each injection. The final chondrocyte concentration ranged from 4.7×10^6 cells/mL to 9.3×10^6 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
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 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.

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, 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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Author Contributions: Study concept and design: Chang, Rasamny, and Park. Acquisition of data: Chang and Rasamny. Analysis and interpretation of data: Chang and Rasamny. Drafting of the manuscript: Chang, Rasamny, and Park. Critical revision of the manuscript for impor-
tual content: Chang, Rasamny, and Park. Statistical analysis: Rasamny. Administrative, technical, and material support: Chang and Rasamny. Study supervision: Chang and Park.

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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.