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Fibrin-Genipin Hydrogel for Cartilage Tissue Engineering in Nasal Reconstruction

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Abstract

Objectives: Nasal reconstruction is limited by the availability of autologous cartilage. The aim is to investigate an adhesive biomaterial for tissue engineering of nasal cartilage by evaluating mechanical properties of hydrogels made of fibrin crosslinked with genipin as compared to native tissue.

Methods: Hydrogels of fibrin, fibrin-genipin, and fibrin-genipin with extracellular matrix (ECM) particles were created and evaluated with mechanical testing to determine compression, tensile, and shear properties. Rabbit nasal septal cartilage was harvested and tested in these modalities for comparison. Transmission electron microscopy characterized hydrogel structure.

Results: Fibrin-genipin gels had higher compressive, tensile, and shear moduli compared to fibrin alone or fibrin-genipin with ECM. However, all hydrogel formulations had lower moduli than the rabbit nasal septal cartilage. Electron microscopy showed genipin crosslinking increased structural density of the hydrogel and that cartilage ECM created larger structural features with lower crosslinking density.

Conclusion: The addition of genipin significantly improved mechanical properties of fibrin hydrogels by increasing the compressive, tensile, and shear moduli. The addition of cartilage ECM, which can add native structure and composition, resulted in decreased moduli values. Fibrin-genipin is a bioactive and biomechanically stable hydrogel that may offer promise as a scaffold for cartilage tissue engineering in nasal reconstruction, yet further augmentation is required to match material properties of native nasal cartilage.

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Keywords

nasal reconstruction; tissue-engineered cartilage; tissue engineering; tissue engineering scaffold; fibrin-genipin

Introduction

Autologous cartilage is the most widely used grafting material for nasal reconstruction. Donor sites include the nasal septum, auricle, and rib. Nasal septal cartilage serves as the gold standard because of its ease of harvest, minimal donor site morbidity, shape, and mechanical properties for nasal support. However, there is a limited supply. Limitations to auricular and rib cartilage include curved shape and warping, respectively, as well as donor site morbidity for both. Synthetic materials have a high risk of infection and/or extrusion due to foreign body reaction in the setting of a thin soft tissue envelope and have not met with wide acceptance.^{1,2}

Tissue engineered cartilage with natural biomaterials potentially could provide a solution to the issues of donor site and biocompatibility. The mechanical properties of scaffolds of synthetic polymers can be more easily modified, but natural polymers and hydrogels have better biocompatibility. This is especially important when working with the soft tissue envelope of the nose.³

Fibrin glue has been studied as a scaffold for tissue engineered cartilage because it provides a favorable environment for chondrocytes to produce extracellular matrix (ECM) and is adhesive, but its use has been limited by fast degradation and weak mechanical properties.^{4,5} Fibrin products are commercially available and are currently in use for applications in the nose. Genipin is a naturally occurring crosslinking agent with relatively *low cytotoxicity* compared to other chemical crosslinkers.⁶ Fibrin-genipin hydrogels have been studied for use in knee articular cartilage and intervertebral disc applications. They have demonstrated biocompatibility in these settings⁷⁻⁹ and are proposed for use in nasal septal cartilage in this study. The addition of extracellular matrix components, such as collagen from decellularized matrix, can further augment biological and biomechanical performance of hydrogels.¹⁰ Mechanical stability is an essential feature of successful tissue engineering solutions. While the facial skeleton does not endure loading magnitudes as large as for articular cartilage, it does undergo a wide range of forces, including compressive, tensile, and shear due to wound contracture, facial movement, and bearing the load of the nose.¹¹ In this study, we evaluated the compressive, tensile, and shear properties of fibrin-genipin hydrogels to compare to native tissue.

To determine physiologically relevant biomechanical behaviors, we evaluate native septal cartilage. Due to scarcity of human septal cartilage for laboratory studies, septal cartilage from a rabbit model has been shown to have similar mechanical, cellular, and structural properties to its human counterpart.¹² The aim of this study was to evaluate the mechanical properties of fibrin and modified fibrin hydrogels as compared to native septal cartilage to determine its feasibility and best applications for use in nasal reconstruction.

Methods

Gel formulation

Each gel was formulated using a double-barreled syringe and mixing tip (4:1 syringe, Pacific Dental, Walnut, California, USA). Fibrinogen (Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in phosphate buffered saline (PBS) at a concentration of 200 mg/mL was placed into the large barrel, and the thrombin (Sigma-Aldrich) with PBS at a concentration of 100 U/mL was placed in the smaller barrel to form fibrin. Genipin (Wake, Richmond, Virginia, USA) was dissolved in DMSO and included in the thrombin mixture for the fibrin-genipin hydrogels at a concentration of 6 mg/mL. Decellularized ECM from bovine annulus fibrosus tissue containing type I and type II collagen was isolated and processed as previously described.¹⁰ ECM was then dissolved in DMSO and included in the smaller barrel with genipin and thrombin for the fibrin-genipin-ECM hydrogels. The mixture was injected into cylindrical or rectangular shaped molds to create mechanical test specimens, and the gels were allowed to set for 4 hours at 37°C.

Cartilage Harvest

Nasal septal cartilage was harvested from freshly sacrificed New Zealand White Rabbits obtained via tissue sharing from the Satlin laboratory after sacrifice for experiments that would not influence cartilage material properties. No live animals were used in this testing. An external lateral rhinotomy approach was made, and the entire septal cartilage was removed en bloc. The perichondrium was stripped off prior to testing. Cylindrical punch samples were taken from the mid 50% of the septum for compression and shear testing. For tensile testing, the septum was cut transversely into rectangular strip shapes for mechanical testing from the mid 50% of the septum to ensure universal thickness.¹² Calipers were used to confirm approximately 1 mm thickness throughout the samples. Samples were placed in PBS to maintain hydration prior to mechanical testing.

Mechanical Testing

Compressive, tensile, and shear mechanical tests were performed on hydrogel and cartilage samples. Individual test samples were used for each test and for each loading mode so that there were no concerns that deformation from mechanical testing would influence material properties. Compression and tensile testing were done using an Instron 8872 testing machine (Instron 8872, Norwood, Massachusetts, USA). Compressive testing on the cartilage specimens was done using an Electroforce 3200 (TA Electroforce, Eden Prairie, Minnesota, USA). Unconfined compression was performed on cylindrical gels (n = 6-7 per group) 5 mm thick with 4.75 mm diameter. Hydrogels were compressed at a rate of 0.2% strain/s to approximately 80% strain. For comparison, testing was performed on cylindrical septal cartilage samples (n = 6) approximately 1 mm thick from a uniform point in the midportion of the septum cut with 5 mm diameter punch. Cartilage samples were compressed at the strain rate of 0.2% strain/s to the point of maximal load of 15 N. Young's compressive modulus was calculated for each hydrogel and cartilage specimen as the slope of the linear portion of the stress strain curve at 1% strain, which allowed comparison with prior literature on human nasal septal cartilage.²

Tensile testing was done using an Instron 8872 testing system (Instron 8872, Norwood, Massachusetts, USA). The hydrogels were molded into $3 \times 3 \times 25$ mm rectangular specimens with 5 mm on each end contained within the grips ($n = 4$ per group). Cartilage strips were cut sized $3 \times 1 \times 20$ mm with 5 mm on each end attached in the grip ($n = 7$), and these were cut transversely for uniform thickness. Tensile testing was performed using specialized grips with sandpaper on both cartilage and hydrogels at a strain rate of 0.2% strain/s until failure. Young's tensile modulus was calculated for each hydrogel and cartilage specimen as the slope of the linear portion of the stress strain curve at 5% strain.

A parallel plate rheometer (AR2000ex, TA Instruments, New Castle, Delaware, USA) was used for shear testing of cylindrical hydrogels and cartilage samples ($n = 5$ -6 per group). Hydrogels were 3 mm thick and 5 mm in diameter while cartilage samples were 1 mm thick and 5 mm in diameter. A frequency sweep test was used (0.05-5 Hz at 1% strain), and complex shear modulus ($|G^*|$) and phase difference (δ) were calculated.

Transmission Electron Microscopy

Electron microscopy was performed on each hydrogel group (fibrin alone, fibrin-genipin, and fibrin-genipin-ECM; $n = 5$). The samples were fixed with 3% glutaraldehyde, followed by 1% osmium tetroxide and dehydrated in ethyl alcohol. Transmission electron microscopy (TEM) samples were embedded (Embed 812) and imaged on a H7650 transmission microscope (Hitachi High Technologies, Northridge, California, USA).

Statistical Analysis

Statistical analysis involved one-way analysis of variance (ANOVA) to test for effects of experimental group on compression and tensile moduli. Two-way ANOVA tested for effects of group and frequency on shear moduli and phase angle. Data were analyzed using Graphpad Prism 5 with $P < .05$ deemed significant.

Results

Fibrin-genipin gels had higher compressive, tensile, and shear moduli compared to fibrin alone or fibrin-genipin with ECM. However, these hydrogels had significantly lower moduli than the rabbit nasal septal cartilage.

Figure 1 shows a representative stress/strain curve of how the hydrogels and septal cartilage perform in unconfined compression and tension. Of the hydrogels, fibrin-genipin most closely approaches the material properties of cartilage, followed by fibrin-genipin with ECM and then fibrin alone, although all hydrogels had properties that were notably different from native cartilage. At 1% strain, rabbit septal cartilage has a significantly higher compressive modulus than fibrin-genipin ($P < .01$) and was also higher than fibrin-genipin with ECM ($P < .001$) and fibrin alone ($P < .0001$) (Figure 2). The compressive moduli of the hydrogels are also significantly different from one another at 1% strain, with fibrin-genipin having the highest modulus followed by fibrin-genipin with ECM and then fibrin alone with the lowest modulus (Figure 2).

Tensile moduli of the hydrogels were significantly different from one another, with fibrin-genipin having a modulus of 281 ± 29 kPa (Figure 3). The tensile modulus of rabbit septal cartilage was significantly higher than those of the hydrogels ($P < .001$; Figure 3). Shear testing showed significantly higher complex modulus and phase angle between rabbit septal cartilage and hydrogels ($P < .001$; Figure 4). The complex modulus of the fibrin-genipin gels was significantly higher than those of the other hydrogels ($P < .01$; Figure 4).

TEM imaging showed the microstructure of each hydrogel group (Figure 5) comparing fibrin polymer, fibrin-genipin crosslinked matrix, and fibrin-genipin crosslinking with ECM. In the fibrin-genipin with ECM hydrogels, the particles of ECM appear to interrupt the crosslinking seen in the fibrin-genipin hydrogels alone.

Discussion

Nasal reconstruction requires repair of degraded or lost tissue. Tissue engineering solutions can involve applications as a filler of smaller defects, a scaffold to promote repair with native tissue, or a full implant providing structural support. Our goals were to evaluate multiple formulations of fibrin compared to native septal cartilage to assess its best applications for tissue repair. Fibrin was used as a biomaterial because it is commercially available, has been used in this application, and is biocompatible, providing a favorable environment for chondrocytes to produce ECM. Fibrin was modified with the addition of genipin because genipin crosslinking can increase hydrogel stiffness and slow its degradation rate.⁷⁻⁹ Decellularized ECM particles were added to fibrin-genipin to evaluate how this composite formulation affected its biomechanical properties, and it is known to enhance bioactivity.¹⁰ However, the addition of cartilage ECM particles decreased moduli, and this was likely because the addition of ECM particles reduced crosslinking density. Of the materials tested, fibrin-genipin had the highest moduli values. Its values were still substantially lower than the properties of native cartilage, likely making it most amenable as a space filling material in its current form. Fibrin-genipin is a natural biomaterial where fibrin can promote healing responses and genipin crosslinking stabilizes fibrin to slow degradation rates. As a result, the strong adhesion, moderate stiffness, and stability of fibrin-genipin warrant further investigation and optimization for a variety of nasal repair and reconstruction procedures.

Septal cartilage serves as a gold standard for nasal reconstruction. However, other materials such as auricular or costal cartilage as well as allogeneic and synthetic materials have been used in nasal reconstruction applications. These materials have varying properties that also do not meet those of septal cartilage. Auricular cartilage is composed of primarily elastic cartilage, curved in nature, and used for contouring defects more often than for major structural support. Costal cartilage provides more substantial skeletal support but has a risk of warping and is limited by donor site morbidity. Synthetic materials such as silicone or polytetrafluoroethylene (PTFE, GoreTex), which have shown biocompatibility in other areas of the face for chin or malar augmentation, have high extrusion rates in the nose due to the thinner soft tissue envelope.^{1,2} Hydrogels such as the injectable dermal filler hyaluronic acid have been used for temporary nasal augmentation.¹³ Nasal reconstruction is complex, and various materials may lend themselves to variable applications.

Our design criteria for wide usage throughout the nose include mechanical properties similar to native cartilage, biocompatibility with minimal immune response, and longevity of the construct. With a tissue engineered construct, we include the ability to promote native tissue growth and a resorbable engineered scaffold. Ease of handling is key and depending on the specific application in the nose, could range from an injectable material that can be molded to an implant that can be carved. Nasal reconstruction needs are varied and range from dorsal augmentation, to improving contour abnormalities, to repairing major structural deficits. A material whose properties can be tuned lends itself to a range of reconstructive solutions.

Rabbit cartilage moduli measurements in this study approximated previously published values.^{2,12,14–16} Unconfined compression was used by Glasgold et al² on human nasal septal cartilage to obtain a similar modulus (0.41 ± 0.205 MPa vs 0.99 ± 0.319 MPa at 1% strain). Wong et al¹² used rabbit and porcine cartilage to demonstrate similar tensile moduli to human septal cartilage. The tensile modulus reported by Westreich et al¹⁴ in human specimens compares favorably to our results ($4.82\text{--}32.76$ MPa vs 4.12 ± 0.42 MPa), verifying our methods.

Fibrin gels are widely used and have been shown to be safe. Tasman et al⁴ have shown success using fibrin glue and diced cartilage for nasal dorsal augmentation. Fibrin hydrogels with genipin crosslinking are biocompatible and have been shown to reduce degradation and have enhanced mechanical properties. Schek et al⁸ and Guterl et al⁹ demonstrated cell viability and proliferation in vitro and tunability of mechanical properties (shear stiffness) with different levels of genipin concentration. Dare et al⁷ demonstrated that genipin addition reduced the in vivo degradation of the material by >29% compared to fibrin alone. Dynamic compression and shear testing showed improved mechanical properties with increasing genipin concentrations to an upper limit, after which no further improvements were obtained.⁷ The shapes and sizes of the hydrogels tested in this study were chosen based on the testing apparatus and standard test protocols for each loading configuration (compression, tension, and shear). Although thicker than human cartilage, the hydrogel specimens tested in compression and shear were 5 mm thick in order to characterize the materials up to failure without risk of damage to the testing machine. Calculated parameters normalize for this geometric difference, although it is worth noting that test specimens with high thickness may be most relevant clinically for dorsal augmentation rather than other graft types that would be too bulky, such as spreader, batten, or rim grafts. However, the shape and size of the hydrogel specimens tested in tension approximated spreader grafts.

Future modifications are required to augment fibrin-genipin to better match native septal cartilage mechanical properties and better enable its use as an implant material. While the direct placement of extracellular matrix particles disrupted crosslinking instead of improving mechanical properties in this study, other methods may be employed. This may involve electrospun nanofibrous scaffolds to provide additional tensile strength. This method has been applied to engineering of tendons and ligaments, bone, and cartilage.^{17–20} Additionally, ECM produced by surrounding cells as the hydrogels mature in vivo or from cells directly seeded into the hydrogels may alter the mechanical properties of the construct. The biocompatibility of fibrin hydrogels encourages further study for this application. In terms of

biomechanical comparison, the shear modulus of fibrin-genipin hydrogels is nearly 1000 times greater than for hyaluronic acid hydrogels that are currently in use as injectable soft tissue fillers for temporary nasal augmentation.²¹ Since fibrin-genipin has material properties approaching those of native cartilage and is a stable construct, we believe in vivo maturation of this fibrin-genipin hydrogel warrants further investigation for use as an implant or scaffold, but in its current form, it may be considered as a tissue filler.

Conclusions

Developing hydrogels with capacity to seal cartilage defects and promote tissue healing is a priority. Genipin crosslinked fibrin hydrogels improved the mechanical properties of fibrin by increasing the compressive, tensile, and shear moduli. The addition of cartilage ECM decreased moduli values due to a reduction in crosslinking density.

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Figure 1.
Representative stress/strain curves comparing behavior of nasal septal cartilage and hydrogels in unconfined compression.

Figure 2.

Compressive modulus comparing nasal septal cartilage and hydrogels in unconfined compression. Nasal septal cartilage modulus is significantly higher than each of the hydrogels. Each hydrogel formulation is significantly different from the others, with the modulus for fibrin-genipin having the highest.

* $P < .05$. ** $p < .01$. *** $P < .001$. **** $P < .0001$.

Figure 3.

Tensile modulus comparing nasal septal cartilage and hydrogels. (A) Tensile modulus of hydrogels showing significant difference between the formulations, with fibrin-genipin having the highest modulus. (B) Tensile modulus of nasal septal cartilage is significantly higher than the moduli of the hydrogels.

Figure 4.

Shear testing data demonstrating complex modulus and phase difference of nasal septal cartilage compared to hydrogel formulations. Nasal septal cartilage has a higher complex modulus than the hydrogel formulations. Of the hydrogels, fibrin-genipin has the highest complex modulus.

Figure 5.

Transmission electron microscopy of hydrogels. (A) Fibrin crosslinking. (B) Fibrin-Genipin with increased crosslinking density. (C) Fibrin-genipin + extracellular matrix (ECM) shows interruption of crosslinking by large ECM particles.