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In Vivo Engineering of the Vocal Fold ECM with Injectable HA Hydrogels -- Late Effects on Tissue Repair and Biomechanics in a Rabbit Model

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Abstract

Objectives—To determine if the utilization of injectable chemically-modified hyaluronan (HA) derivative at the time of intentional vocal fold resection may facilitate wound repair and preserve the unique viscoelastic properties of the extracellular matrix and lamina propria 6 months after treatment.

Study Design—Prospective, controlled animal study.

Methods—Twelve rabbit vocal folds were biopsied bilaterally, and the left side of vocal fold was treated with Extracel, an injectable, chemically-modified HA derivative, and the right side of vocal fold was injected with saline as control at the time of resection. Animals were sacrificed six months after biopsy and injection. Outcomes measured include transcription levels for procollagen, fibronectin, fibromodulin, TGF- β 1, hyaluronan synthase and hyaluronidase and tissue biomechanics -- viscosity and elasticity.

Results—Extracel treated vocal folds were found to have significantly less fibrosis than saline treated controls. Extracel treated vocal folds had significantly improved biomechanical properties of elasticity and viscosity. Significantly decreased levels of fibronectin, fibromodulin, TGF- β 1, procollagen I and hyaluronan synthase were measured.

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Conclusions—Prophylactic *in vivo* manipulation of the extracellular matrix with an injectable HA hydrogel appears to induce vocal fold tissue regeneration to yield improved tissue composition and biomechanical properties at 6 months.

Keywords

vocal folds; scarring; prophylactic; hyaluronic acid hydrogels; tissue engineering

Introduction

Normal vocal fold vibration is dependent upon tissue composition and viscoelasticity. When composition of the extracellular matrix (ECM) of the vocal fold cover (i.e. lamina propria – superficial and middle layers) is altered, vocal fold vibratory function can be severely disrupted due to alterations in tissue viscoelasticity.(1–4) The dysphonias that result are generally difficult to treat effectively with current surgical paradigms and available biomaterials. Treatment failures have been ascribed to poor understanding of pathologic processes in the ECM, as well as suboptimal materials that may negatively affect vocal fold biomechanical properties. Accordingly, there is a clinical need for improved understanding of the pathophysiology of disrupted ECM and the development of advanced biomaterials that appreciate the biomechanical properties of the lamina propria. The long-term aim of our laboratory's efforts is to engineer injectable products that promote wound repair and induce tissue regeneration, for treatment of scarring and other ECM defects of the lamina propria.

To date we have optimized a hyaluronic acid (HA) hydrogel Extracel, for prophylactic use as a means of improving wound healing. Extracel is gelatin-DTPH, a thiolated derivative of gelatin that is covalently co-crosslinked with CarbylanTM-S, using PEGDA as the thiol-reactive crosslinker. The hydrogel has been shown to have viscoelastic properties similar to those of human vocal fold mucosa *in vitro*(5,6). *In vivo*, Extracel has been shown to improve wound healing in a prophylactic rabbit wound healing model(6,7) as measured by histological outcomes and protein and gene expression. Furthermore it has been shown to be biocompatible(8) and noninflammatory(8). Rheologically, vocal folds treated at the time of injury with Extracel have been shown to have similar biomechanical properties (tissue elasticity and viscosity) to normal tissue and less stiffer and less viscous vocal fold tissues compared to saline treated injured vocal folds(6,7,9).

To date all *in vivo* investigations of the efficacy of Extracel have been completed with a 21 day end point. The aim of this study is to evaluate the long term (6 month) transcript and rheological changes in vocal fold tissue after intention vocal fold research, in response to the prophylactic treatment with Extracel compared to saline treated controls, to determine if improvements seen at the early time point continue into the chronic injury phase.

Materials and Methods

HA-Gelatin Hydrogel

The engineered injectable chemically-modified HA-gelatin hydrogel –Extracel (Sentrx Surgical, Inc., Salt Lake City, UT; now Carbylan BioSurgery, Palo Alto, CA) was developed in conjunction with the Center for Therapeutic Biomaterials at The University of Utah. Extracel was prepared by mixing a 1.5% solution of CarbylanTM-S in phosphate buffered saline (PBS; pH 7.4) with a 4.0% (v/v) solution of poly ethylene glycol diacrylate (PEGDA) and PBS (pH 7.4) according to a volume ratio of 4 to 1. Thiolated gelatin (gelatin-DTPH) was prepared as described previously (with PBS and PEDGA) (10) and mixed into a 1.5% (w/v) solution of Extracel solution in PBS (pH 7.4) to give gelatin weight percent at 5% (Extracel).

Surgical Procedures

Twelve New Zealand White breeder rabbits were used in this study. The University of Wisconsin Madison Institutional Animal Care and Use Committee approved this animal experiment. Rabbits were anesthetized with an intravenous injection of Xylazine 35 mg/kg, Ketamine 5 mg/kg and Acepromazine 0.75 mg/kg. Rabbits were ventilated with supplemental O₂. The larynx was visualized with a Pilling infant Hollinger pediatric endoscope (Pilling Horsham, Storz, Culver City, CA) as previously described(9). Left and right vocal fold midmembrane margins were biopsied with 2 mm Jako cup Forceps (Pilling Horsham, Culver City, CA). Extracel was prepared as above and was allowed to partially gel for 5 minutes at room temperature in a 26-gauge needle (Microfrance, Terrebonne, QC, Canada) prior to injection. The left vocal fold was injected with 0.15 ml of hydrogel into the wound that was intentionally produced. For a control, sterile saline, 0.15 ml, was injected into the right vocal fold's wound bed. A volume of 0.15 ml of hydrogel and saline had been chosen because we expected some injectant to leak out of the puncture site. Immediately after surgery, Buprenex (0.05 mg/kg) was provided for pain management. Animals were sacrificed 6 months after the initial surgery by IV administration of Beuthanasia-D (0.05 mg/kg). All vocal folds were removed from the larynges and were stored at -80 °C immediately after either being placed in RNAlater (Ambion, Austin, Texas) for 24 hours or being flash frozen. Two of the twelve rabbits were euthanized as described above, prior to 6 months because of complications related to hyperalgesia of their hind limbs.

Detection of transcription of genes by RT-PCR

Total RNA was isolated from the rabbit vocal fold tissue by using an RNA extraction kit, RNeasy Mini Kit (Qiagen, CA), according to the manufacturer's instructions. First strand cDNA was synthesized from 1 µg of total RNA by oligo(dT) priming using SuperScript II reverse transcriptase (Invitrogen Corporation, CA). The genes in mRNA level were quantified by real-time PCR method by using LightCycler System (Roche, IN), with amplification of β -actin as control. mRNA from the cDNA sample was amplified with specific primer pairs for fibromodulin, fibronectin, procollagen I, TGF- β 1, hyaluronan synthase, hyaluronidase and b-actin. The primer sequences, gene bank access number and expected PCR product sizes are listed in Table 1. Amplification was carried out for 45 cycles, each of 95°C, 10s, 55°C, 5s, and 72°C 10s in a 20µl reaction mixture containing 2µl cDNA, 0.5 µM of each primer, 1.5–2 mM MgCl₂ (dependent on the target gene), dNTPs and Tag DNA polymerase from LightCycler FastStart DNA Master SYBR Green I (Roche, IN) by the LightCycler 1.5 System. The exact amplification efficiencies of target and reference genes were assessed by LightCycler software before any calculation of the normalized gene expression was completed. The specificity of every pair of primers was confirmed by melting curves. In all of our real-time PCR experiments, the delta delta CT method was used for semi-quantification of gene expression. Results were shown by the fold change of target gene to the housekeeping gene, β -actin mRNA. Each sample was tested in duplicate.

Vocal Fold Tissue Rheology

Viscoelastic properties of the left and right vocal fold lamina propria from five animals were determined using dynamic rheometry, including elastic shear moduli (G') and viscous moduli (G'') as a function of oscillatory frequency (0.01–10 Hz), as described by Klemuk and Titze.(10) All rheological testing was carried out using a controlled stress rheometer, Bohlin CVO120 (Malvern Instruments Inc., Worcestershire, UK). All samples were stored at -80°C until testing at which time each was thawed at room temperature and hydrated with PBS. All rheological measurements were taken without knowledge of treatment condition. Gaps were 0.3 – 0.6 mm. A parallel plate set up was used, with a stationary lower plate and a rotating upper plate (8 mm diameter). Wet-dry sandpaper was affixed to both the

attachment and the base plate in order to avoid slippage between the sample and the rheometer surfaces. Temperature during testing was maintained at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ through the use of a water-jacketed base plate. Shear stress, shear strain, and strain rate associated with the oscillatory shear deformation were computed from the prescribed torque and the measured angular velocity by a computer, and viscoelastic data were obtained based on these functions.

Statistical Analysis

Gene expression values were compared between Extracel treated and the saline treated samples using a paired t-test with the SAS software procedure PROC UNIVARIATE (SAS Institute Inc., Cary, NC). A Bonferroni correction was performed because of the six concomitant t-tests. P values < 0.0083 were considered as significant. Elastic shear modulus and dynamic viscosity were compared between Extracel and saline treated groups using a repeated measures analysis of covariance assuming a first-order autoregressive error structure within a subject. In order to better meet the assumptions of analysis of covariance, elastic shear modulus, dynamic viscosity, and the covariate, frequency, were log-transformed prior to analysis. Both analyses were performed utilizing the MIXED procedure from SAS (SAS Institute, Inc., Cary, NC). P-values less than 0.05 were considered as significant.

Results

Gene transcript levels for fibronectin, fibromodulin, TGF- β 1, procollagen I, hyaluronan synthase and hyaluronidase were measured for 5 rabbit vocal folds for each condition. Results are shown in Figure 1. Compared to saline treated vocal folds, at six month post surgery, significantly less transcript levels were measured for fibronectin ($p=0.0001$), fibromodulin ($p=0.000$), TGF- β 1 ($p=0.0011$), procollagen ($p=0.0005$) and hyaluronan synthase ($p=0.000$). A non significant difference was measured for hyaluronidase ($p = 0.0248$) between groups.

Viscoelastic properties - elastic shear modulus (G') and viscous modulus (G'') -- were measured for 5 rabbit vocal folds for each condition, as a function of frequency using a stress controlled rheometer. The results of the rheological analysis (G' and G'') are shown in Figure 2. A single sample in the experimental group was removed from further analysis, because the tissue was observed as having some connective tissue, the tissue resisted relaxation under compression, and the properties were approximately 10X greater than the other four samples. The "resistance to relaxation under compression" indicated that there may have been cartilage or very dense connective tissue in the sample. In this case, rheological measurements were not valid for the intended goals of the study. The mean magnitude of the elastic and viscous moduli across frequency increased monotonically for saline and Extracel treated. Logarithmic and power law fit for G' and the dynamic viscosity η' , where η' is equal to G'' divided by 0.97 for all conditions. All tissue samples demonstrated monotonic decreases in dynamic viscosity as frequency increased, which is indicative of shear thinning. Slopes and intercepts of G' fits, a and b respectively, and intercepts of η' fits, a, decreased following the same treatment conditions as in Figure 2. The declination rate of η' fits, b, however were nearly the same for all treatment conditions. For G' , the saline treated group was significantly stiffer than the Extracel treated group ($p=0.0053$). For G'' , the saline treated group was significantly more viscous than the Extracel treated group ($p=0.0060$). Stiffer and more viscous tissue is indicative of greater fibrosis compared to controls.

Discussion

Scarring of the vocal folds following surgical resection can have adverse effects on voice production and efforts have been made to minimize the impact of surgery using microsurgical techniques.(11) Prophylaxis of the wound healing process at the time of surgery with hyaluronan (HA), an endogenous glycosaminoglycan that has been shown to aid in the healing process through to 6 months after injury, may be an approach for minimizing scar tissue formation during the acute phase of wound healing while maintaining optimal viscoelastic properties of the ECM, vital to vocal fold vibration.

The results of this study demonstrate that prophylactic use of chemically-modified HA hydrogel -- Extracel may be a way to maximize the healing benefits of HA in regenerating tissues, while circumventing the transient nature and reduced levels of endogenous HA in the wound. HA levels are significantly lower than normal during most of the acute phase of wound healing with a brief climb to normal levels around day 5.(12) In contrast, prolonged and elevated levels of HA have been measured in scarless fetal dermal wound healing.(13) The transient nature of unmodified HA in the vocal folds, where residence time has been estimated to be 3–5 days has made the use of unmodified HA as a prophylactic agent impractical.(14) Extracel is a crosslinked non-immunogenic HA hydrogel that contains additional carboxylate groups on the hyaluronan backbone and 5% gelatin. This chemical modification alters the viscosity, reduces the rate of degradation *in vitro* and *in vivo*, enhances biocompatibility, and permits additional versatility in altering biomaterial properties.

Previous research in the same animal model demonstrates that at 3 weeks post injury and prophylactic treatment, there is improved viscous and elastic moduli and less fibrosis histologically(6,7,9). The findings of present study demonstrate that the presence of Extracel in the wound bed during the early stages of repair, amplifies the normal rabbit vocal fold wound healing response, over a 6 month period of time. All significant decreased differences in transcript levels found on post op month 6, compared to saline treated control, suggesting a reduction in the production of fibrotic tissue. This difference appears to be due to the hydrogel, as previous research has demonstrated that 6 months is sufficient (15)time for the formation of a mature wound/scar in the rabbit. One of the limitations of the present study is that because we did not have normal tissue for comparison, we are unable to determine if fibrosis as described by transcript levels in either group worsens over a 6 month period compared to previously studied 21 day and 2 month time periods.

The temporal responses described in this study replicates reports from the literature describing improvement in fibrosis with treatment, in other parts of the body. Specifically, procollagens and fibronectin have been shown to be downregulated concomitantly with treatments aimed to improved fibrosis in the skin(16) and liver(17). Persistent TGF- β 1 expression affects fibrosis and ultimately scarring of skin and internal organs(18–20). Various successful treatments utilized to decrease scarring in skin have reported decreases in TGF- β 1. It appears that the decreased concerted expression of these genes may have contributed to improving the function of the healing vocal fold.

Decreased or similar levels of hyaluronan have been measured in vocal fold scar through out the wound healing period. Our findings of decreased hyaluronan synthase indicate that less HA is being made in the treated vocal folds at 6 months. We know from our previous work that HA levels did not differ with treatment with Extracel during the first 21 days after treatment(7). Nonsignificant differences with hyaluronidase indicate that there was not a difference in the amount of HA that was being enzymatically degraded between the two groups.

The 6 month post treatment impact of the HA hydrogels on tissue biomechanics was measured using a rotational rheometer. Significant differences occurred in the viscoelasticity of the treated vocal folds compared to saline treated controls signifying differences in tissue fibrosis between the groups. G' and G'' magnitudes were on the same order as those reported by (7,9) and (21) (Tables 2 and 3). Mean G' and G'' values were about 50% higher for the saline controls than for the Extracel treated vocal folds. The Extracel group values in the present study were higher than Extracel groups at the 3 week and the 2 month time points (21) but were closely aligned with saline control groups. The saline group values from the present study, however were higher than all previous studies using the rabbit vocal fold model (7,9,21). This indicates that as the scar matures, the biomechanical properties of the tissue worsened for both groups; yet the improvement measured with treatment at day 21 continue through to 6 months post treatment compared to saline treated controls.

Conclusions

Prophylactic injection of an HA hydrogel, Extracel at the time of surgical resection was evaluated in rabbit vocal folds. Extracel treated vocal folds had viscous moduli and elastic shear moduli values that were significantly lower than saline treated scarred tissue. The improved viscoelastic properties of the vocal folds measured at 6 months post treatment appears to be due to improved healing with less fibrosis of the ECM. These finds demonstrated that the early benefits of the HA injections, measured at 21 days post injury, are maintained through to the chronic stage of repair. Vocal fold scar, the cause of significant dysphonias, may be minimized in the future by the prophylactic use of chemically engineered HA gels at the time of surgery.

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Figure 1.

Expression of fibronectin, fibromodulin, transforming growth factor beta one (TGF-B1) hyaluronan synthase and hyaluronidase genes from injured vocal fold tissue treated with Extracel or saline at six months post op. Data is shown by target gene mRNA concentration (ng/μl), normalized by housekeeping gene, b-actin mRNA (ng/μl). *p<0.05

Figure 2.

A. Elastic modulus (G') and B. Viscous modulus (G'') for Extracel treated and saline treated groups.

Table 1**Primer Sequences and Products for RT-PCR**

Gene	GenBank #	Forward Primer	Reverse Primer	Size of PCR Product
Fibronectin	AF020291	5'-ATCCTGCTGGACCTGAGCTA-3'	5'-GCAGCTGGTTGTAGGAGAGG-3'	241bp
Fibronectin	AF135404	5'-GACCCCATTCAGGAAAGTT-3'	5'-CTCCTCTGGTCCCTTCAGTGC-3'	171bp
Procollagen I	S61950	5'-CTGCAAGAACAGCATTGCAT-3'	5'-TCAAGGAAGGGAACACGAGA-3'	216bp
TGF- β 1	AB020217	5'-TGCTTCAGCTCCACAGAGAA-3'	5'-CTTGTGTACTGGGTGTCCA-3'	162bp
Hyaluronan Synthase	AB055978	5'-GGACGAAAGCGTGGATTATGT-3'	5'-ATAAGACTGGCAGGCCCTTT-3'	216bp
Hyaluronidase	AY603960	5'-TCATGCTGGAGACACTACGC-3'	5'-GGTAGACGGAGGGGTAGAGG-3'	218bp
β -Actin	AF04278	5'-GGACCTGACCGACTACCTCA-3'	5'-GGCAGCTCGTAGCTCTTCTC-3'	180bp

Table 2

Coefficient a and constant b and corresponding coefficient of determination R^2 for curve fitting for elastic moduli. Historical data is included for comparison.

Material	$G' = a \ln x + b$		R^2
	a	b	
Saline Treated Vocal Folds (6 months)	3052	34237	0.99
Extracel Treated Vocal Folds (6 months)	2244	22392	0.99
Saline Treated Vocal Folds (21 days) ⁷	1665	16008	0.99
Extracel Treated Vocal Folds (21 days) ⁷	1396	13082.0	0.99

Table 3

Coefficient a and exponent b and corresponding coefficient of determination R^2 for curve fitting for dynamic moduli.

Material	$\eta = ax^b$		R^2
	a	b	
Saline Treated Vocal Folds (6 months)	933	-0.97	0.99
Extracel Treated Vocal Folds (6 months)	662	-0.95	0.99
Saline Treated Vocal Folds (21 days) ⁷	500	-0.93	0.99
Extracel Treated Vocal Folds (21 days) ⁷	391	-0.94	0.99