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An In Vivo Study of Composite Microgels Based on Hyaluronic Acid and Gelatin for the Reconstruction of Surgically Injured Rat Vocal Folds

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Abstract

Purpose—The objective of this study was to investigate local injection with a hierarchically microstructured hyaluronic acid–gelatin (HA-Ge) hydrogel for the treatment of acute vocal fold injury using a rat model.

Method—Vocal fold stripping was performed unilaterally in 108 Sprague-Dawley rats. A volume of 25 ml saline (placebo controls), HA-bulk, or HA-Ge hydrogel was injected into the lamina propria (LP) 5 days after surgery. The vocal folds were harvested at 3, 14, and 28 days after injection and analyzed using hematoxylin and eosin staining and immunohistochemistry staining for macrophages, myofibroblasts, elastin, collagen type I, and collagen type III.

Results—The macrophage count was statistically significantly lower in the HA-Ge group than in the saline group ($p < .05$) at Day 28. Results suggested that the HA-Ge injection did not induce inflammatory or rejection response. Myofibroblast counts and elastin were statistically insignificant across treatment groups at all time points. Increased elastin deposition was qualitatively observed in both HA groups from Day 3 to Day 28, and not in the saline group. Significantly more elastin was observed in the HA-bulk group than in the uninjured group at Day 28. Significantly more collagen type I was observed in the HA-bulk and HA-Ge groups than in the saline group ($p < .05$) at Day 28. The collagen type I concentration in the HA-Ge and saline groups was found to be comparable to that in the uninjured controls at Day 28. The concentration of collagen type III in all treatment groups was similar to that in uninjured controls at Day 28.

Conclusion—Local HA-Ge and HA-bulk injections for acute injured vocal folds were biocompatible and did not induce adverse response.

Keywords

injectable biomaterial; composite hydrogel; tissue engineering; wound healing; vocal fold lamina propria; scarring

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Vocal fold scarring may be caused by injury or laryngeal disease. *Scarring* involves the replacement of normal tissue by fibrotic tissue, which is much stiffer and often causes voice hoarseness and roughness. Surgical treatments have variable success and may lead, in extreme cases, to additional scarring. Behavioral modifications and therapeutic treatments require time, with unpredictable outcomes. Voice disorders affect health, resulting in decreased quality of life, reduced social interactions, poorer lifestyle, and employment difficulties (Aronson, Peterson, & Litin, 1966; Krischke et al., 2005).

Normal human vocal folds have a unique laminar structure, often conceptualized as three layers: (a) the epithelium, (b) the lamina propria (LP), and (c) the vocalis muscle. The LP may be further subdivided into the superficial, the intermediate, and the deep layer, based on its fibrous protein density and composition (M. Hirano, 1974). All three layers undergo viscoelastic deformations during phonation, with amplitude generally inversely proportional to their mechanical stiffness. The extracellular matrix (ECM) composition is directly related to biomechanical tissue properties (Chan, Fu, Young, & Tirunagari, 2007; S. Gray, Bielamowicz, Titze, Dove, & Ludlow, 1999; S. D. Gray, Alipour, Titze, & Hammond, 2000). Collagens are known to regulate, in part, the stiffness of the vocal fold LP and its nonlinear elastic response (Miri, Heris, Tripathy, Wiseman, & Mongeau, 2013). Collagen type I is mainly found in the basement membrane and the deep LP (Miri, Tripathy, Mongeau, & Wiseman, 2012); it is distributed sparsely in the superficial LP (Hahn, Kobler, Zeitels, & Langer, 2005; Tateya, Tateya, & Bless, 2007). Collagen type III is found throughout all layers; it helps to maintain the structural integrity of the LP and enhances flexibility and elasticity (Tateya, Tateya, Sohn, & Bless, 2006). Elastin, mainly found in the intermediate layer (Miri et al., 2012), is also believed to determine, in part, the elasticity of the vocal folds, for example their elongation under tensile loading forces (Chan et al., 2007).

Previous histological studies have reported structural changes that result from scarring of the vocal fold LP in humans (S. Hirano et al., 2009) and animals (Rousseau et al., 2003, 2004; Tateya, Jin, Tateya, & Bless, 2005; Tateya et al., 2006). Scarred tissue is characterized by an increased, organized, and thick bundled collagen matrix (S. Hirano et al., 2009; Rousseau et al., 2004; Tateya et al., 2005, 2006) and a defragmented and disorganized elastin fiber network (Rousseau et al., 2003, 2004). The number and strength of molecular interactions within the ECM affect the relative displacement and slippage between molecules in the tissue (Chan & Titze, 1999a). Vocal fold scarring increases resistance to elongation and slippage, resulting in increased stiffness and dynamic viscosity (Chan & Titze, 1999b; Rousseau et al., 2003, 2004; Thibeault, Gray, Bless, Chan, & Ford, 2002). Changes in protein density and distribution due to scar formation in the LP affect its viscoelasticity, with direct consequences for voice quality.

Tissue engineering treatment therapies have aimed at restoring the ECM composition of scarred tissue to its natural, uninjured state, thereby improving glottal competency. Tissue replacement using, for example, flaps and grafts (Benninger et al., 1996; Hansen & Thibeault, 2006; Hansen, Thibeault, Walsh, Shu, & Prestwich, 2005), Teflon (Arnold, 1963), silicone (Koufman, 1986), fat (Brandenburg, Kirkham, & Koschke, 1992), or cross-linked collagen (Ford, Martin, & Warner, 1984) allows vocal fold augmentation and medialization to temporarily restore glottal competency through augmentation of the LP volume with soft

substances. The main problem with all presently used biomaterials is that they degrade rapidly over time. They can thus only offer short-term benefits (Benninger et al., 1996; S. Hirano, 2005). Newer biomaterials are presently being investigated to permanently reengineer mature scar tissue or to prevent scar formation at the time of injury (Hansen et al., 2005; Thibeault, Rousseau, Welham, Hirano, & Bless, 2004).

Hyaluronic acid (HA) hydrogel is one common biomaterial used to treat vocal fold scarring. Hyaluronic acid plays a substantial role in wound healing processes (Thibeault et al., 2002; Ward, Thibeault, & Gray, 2002); it facilitates fibroblast migration in early phases, and it reduces wound contracture in later stages (Weigel, Fuller, & LeBoeuf, 1986). The concentrations of HA have been found to decrease after vocal fold injury (Thibeault et al., 2004). It is generally believed that timely HA injections may help minimize scar formation.

Injectable materials based on HA have been found to be biocompatible, noninflammatory, and biodegradable (Hallén, Johansson, & Laurent, 1999). Following HA injection, rabbit vocal folds were found to have significantly less fibrosis, with improved elasticity and viscosity (Hansen et al., 2005). The introduction of gelatin into HA hydrogels significantly improved the attachment and migration of fibroblast cells (Shu, Liu, Palumbo, & Prestwich, 2003) and has yielded improved viscoelastic properties in rabbit vocal fold tissue (Duflo, Thibeault, Li, Shu, & Prestwich, 2006). Further improvements to traditional macroscopic bulk hydrogels have resulted in hierarchical doubly cross-linked networks (DXNs), which are more effective for soft-tissue regeneration (Sahiner, Jha, Nguyen, & Jia, 2008). In contrast to traditional HA bulk gels, the hierarchical hydrogels can be cross-linked to decrease their degradation rate, and thus increase their residence time in situ, thereby minimizing the need for reinjection. Modifications of the particle size and of the inter- and intraparticle cross-linking density allow the tuning of the mechanical properties of reconstructed tissue to match those of natural vocal folds (Jha et al., 2009). Results so far suggest that DXNs possess a hierarchical organization and display structural integrity, which should lead to improved cell infiltration and neovascularization during the regeneration of scarred vocal fold tissue.

In the first phase of the present study, gelatin was incorporated in DXNs with the goal of combining favorable mechanical properties and bioactive tissue repair and engineering (Heris, Rahmat, & Mongeau, 2012). These in vitro characterization results support the potential effectiveness of newly created HA-based hierarchical hydrogels as a scaffold material for vocal fold regeneration. In vivo testing was then needed to verify the tissue-regenerating capabilities. To this end, a rat model was used to assess the biocompatibility of the same hierarchically structured HA-Ge hydrogel, and to evaluate its potential for tissue reconstruction in acute vocal fold scarring. It was hypothesized that the biomaterial would not cause an excessive inflammatory response, would provide a scaffold to facilitate neo-tissue regeneration, and would modulate the wound healing process to mitigate fibrotic tissue formation in the long term.

Unmodified HA-based bulk hydrogels have many favorable biomechanical properties for phonation (Hansen et al., 2005). Injected bulk hydrogel effectively restores phonation in patients with glottal insufficiency (Hertegård et al., 2002). Yet no quantitative analysis of the

concentrations of important ECM proteins during tissue remodeling and regeneration has been reported. In a related goal, the remodeling of the vocal fold LP following acute injury and subsequent injection with a hierarchical HA-Ge gel was studied. Our in vitro studies using human vocal fold fibroblast cells have shown that the gelatin-to-HA ratio of 1:3 is adequate to ensure cell adhesion to microgels. Increasing the volume fraction of gelatin did not improve cell proliferation and viability.

Method

HA-Based Hydrogels

Thiolated HA (CMHA-S or Glycosil), thiolated Ge (Gtn-DTPH or Gelin-S), and polyethylene glycol diacrylate (PEGDA) were purchased from Glycosan Biosystems. Saline solution was purchased from Baxter Corporation. Dioctyl sulfosuccinate sodium salt (Aerosol OT, AOT, 98%), 2, 2, 4-trimethylpentane (iso-octane, anhydrous), 1-heptanol (1-HP), fluorescamine, acetone, and isopropyl alcohol were obtained from Sigma-Aldrich.

Bulk hyaluronic acid (HA-bulk) preparation—Fifty μL of 1% thiolated HA and 50 μL of 1% cross-linker (PEGDA) were loaded into a syringe. The syringe was placed in a sonicator for about 1 min to mix the constituents. Twenty-five μL of the mixture was injected into the rat vocal fold 4–6 min after preparation.

Hierarchical hyaluronic acid–gelatin (HA-Ge) composite preparation—Detailed preparation procedures for the HA-Ge biomaterial can be found in an earlier publication (Heris et al., 2012). In brief, dense HA-Ge microgels were prepared using reverse emulsification. The liquid phase was composed of a mixture of 1% HA and 1% gelatin with volume ratio of HA:Ge = 3. The organic phase was comprised of 0.25 M AOT and 0.05 M 1-HP in iso-octane, with a volume ratio of organic phase:liquid phase of 15. The particles were cross-linked using PEGDA. The ratio of the weight of cross-linker and the volume of the liquid phase was 250mg/ml. The particles were agglomerated using a centrifuge at the speed of 14,000 RPM for 30 min after precipitation in acetone. The HA-Ge particles were repeatedly washed using water, ethanol, and isopropyl alcohol to remove excess surfactants. The composite hierarchical material was prepared by dispersing 1-mg HA-Ge particles in 0.5% thiolated HA solution. The mixture was dispersed by sonication of the solution for 5 min. A solution of 50 μL of HA-Ge particles in thiolated HA with 50 μL of 1% PEGDA cross-linker was loaded into a syringe. The syringe was placed in a sonicator for 1 min for mixing. As for HA-bulk, 25 μL of HA-Ge was injected into the vocal fold of each animal 4–6 min after preparation.

Animals

One hundred and eight male Sprague-Dawley rats (4–6 months old), each with a body weight of 400–500 g, were used. All rats were housed at the Montreal Children's Hospital Research Institute animal care facility. All experimental procedures were approved by the Facility Animal Care Committee at the Research Institute of McGill University Health Centre (RI MUHC), in accordance with the National Institute of Health guidelines for care and use of laboratory animals.

Study Groups

Rats were randomly assigned into one of the three treatment groups (i.e., $n = 36$ for each group): (a) saline controls, (b) commercially available HA-bulk hydrogel, and (c) hierarchically structured HA-Ge hydrogel. For all animals, the right vocal fold was injured. The assigned biomaterial was injected into the same vocal fold 5 days after surgery. The injected volume was 25 μl for all animals. This study was designed to allow comparisons with the uninjured left vocal fold for each animal, in addition to comparisons between the two treatment methods and the placebo control group (saline injection). The 36 rats (12×3 treatment groups) were sacrificed on Days 3, 14, and 28 after injection.

Procedure

Rats were anesthetized using inhalational isoflurane (2%–3% delivered at 0.8–1.5 L/min; Abbott laboratories, Montreal, Canada) followed by an intraperitoneal injection of ketamine (90 mg/kg; Bioniche, Ontario, Canada) and xylazine (0.4–0.6 mg/kg; Bayer Healthcare, Ontario, Canada). To minimize saliva secretion and maintain a dry larynx, the rats were injected intraperitoneally with atropine sulfate (0.05 mg/kg; Alveda Pharma, Toronto, Canada). Each anesthetized animal was placed on an operating platform in a near vertical, supine position. A mouth gag was used to open the mouth and the vocal folds were visualized using a custom-fabricated, 1-mm diameter laryngoscope at a 25-degree angle and a 1.9-mm diameter endoscope connected to an external light source and video monitor. An additional local anesthetic was applied to the oral and laryngeal mucosa using lidocaine (0.4%; Astra Zeneca, Ontario, Canada). A unilateral lesion was created on the right vocal fold by stripping the mucosa with a 25-gauge needle and micro-forceps (Welham et al., 2009). Small cotton pledgets were used to maintain homeostasis when needed. The animals were allowed to recover from anesthesia, kept warm with a heating lamp, and monitored every 10 min postoperation until recovery was complete. Recovery was assessed based on a complete return to normal recumbence and posture. Five days after vocal fold stripping, the animals were anesthetized following the aforesaid procedures, and the right vocal fold was injected with 25 μl saline, HA-bulk gel, or hierarchically structured HA-Ge gel using a 50- μl syringe with a 27-gauge needle.

On Days 3, 14, and 28, animals were humanely euthanized in a carbon-dioxide chamber. Each animal was monitored until the absence of a heartbeat. Immediately following euthanasia, larynges were excised, fixed in 4% paraformaldehyde overnight, and then embedded in paraffin. Five-micron-thick coronal sections of the vocal folds were prepared and collected on InkJet Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Coronal sections were obtained at a vocal fold location approximately 200–400 microns anterior to the arytenoid cartilage. The mid-membranous portion of the mucosa was identified, selected, and processed for subsequent staining procedures and image analysis; this region is reported to be the injury-prone site affecting vocal fold oscillation in humans. Routine hematoxylin and eosin (H&E) staining was performed to identify the mid-membranous region of the vocal folds and to evaluate the overall vocal fold morphology over time. In addition, immunohistochemistry (IHC) staining was performed to evaluate the location of macrophages, myofibroblasts, collagen type I, collagen type III, and elastin. Spleen tissue was used as positive control for myofibroblasts and macrophages. Skin tissue was used as

positive control for collagen type I and elastin. Phosphate-buffered saline (PBS) was used as negative control. Digital image analysis was performed to quantify the density of cells and ECM markers in IHC images.

IHC staining of cell markers—Tissue sections were first subjected to paraffin removal and rehydration. Antigen retrieval was then performed using a water bath at 96 °C. Slides were placed in a plastic rack immersed in a container with preheated antigen retrieval buffer (Tris/EDTA pH9.0) for 25 min. The slides were then removed from the water bath and allowed to cool for 20 min. They were then washed with Tris-buffered saline (TBS) Plus 0.025% Triton×100 and pre-incubated in 10% bovine serum albumin (BSA) in TBS for 1 hr. The BSA was used to block unwanted, nonspecific binding to epitopes, which causes high background staining.

After pre-incubation, excess serum was tapped off and the sections were incubated with monoclonal mouse anti-rat CD68 to stain for macrophages (AbD Serotec, Raleigh, NC) at 1:100 for 24 hr at 4 °C. The glycoprotein CD68 was used to detect the presence of macrophages. This marker is expressed predominantly on the lysosome membrane of myeloid cells and the majority of tissue macrophages. After incubation with the CD68 antibody, the tissue section was washed and then incubated with 3% H₂O₂ in TBS for 15 min to block endogenous peroxidases. Detection tests were carried out using a horseradish peroxidase (HRP) detection system in combination with 3-amino-9-ethylcarbazole (AEC), which was converted into a red precipitate by HRP (Polink-2 Plus HRP Mouse-NR with AEC kit, IHC World, Woodstock, MD). The sections were counterstained with hematoxylin.

Alpha (α)-smooth muscle actin (α-SMA) was used to stain myofibroblasts. Myofibroblast microfilaments exhibit neoexpression of α-SMA, therefore they are considered a reliable marker. The α-SMA staining was performed in a way similar to the CD68 staining, except with rabbit polyclonal α-smooth muscle actin antibody (Abcam Inc., Cambridge, MA) at 1:800 used as the primary. An HRP-AEC detection system (Polink-2Plus HRP Rabbit with AECKit, IHCWorld, Woodstock, MD) was used for α-SMA.

Immunohistochemistry (IHC) staining of extracellular matrix proteins—Antigen retrieval was performed using a water bath at 80 °C. Slides were placed on a plastic rack immersed in a container with preheated antigen retrieval buffer (Sodium Citrate Buffer, pH6.0) for 2 hr. Slides were then removed from the water bath and allowed to cool down for 15 min in the container. The samples were washed with TBS Plus 0.05% Tween20 and pre-incubated with 10% BSA in TBS. After pre-incubation, excess serum was tapped off and the sections were incubated with rabbit polyclonal collagen I antibody (Abcam Inc., Cambridge, MA) at 1:50 at 4 °C overnight. On the second day, the samples were washed and incubated with goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA) at 1:1,000 for 35 min at room temperature, protected from light. Sections were washed and incubated for 1 min with DAPI (Invitrogen, Carlsbad, CA) at 1:10,000. Finally, samples were washed, mounted with Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA) and stored at −20 °C until image analysis.

The staining procedure for elastin staining was similar to that for collagen I, but using rabbit polyclonal elastin primary antibody (Abcam Inc., Cambridge, MA) at 1:100. Goat anti-rabbit Alexa Fluor 594 (Invitrogen, Carlsbad, CA) was used as a secondary antibody.

For collagen type III, primary rabbit polyclonal collagen III antibody (Fitzgerald, Acton, MA) at 1:750 and a secondary anti-rabbit Ig horseradish peroxidase polymer (Vector, Burlingame, CA) were used. Diaminobenzidine chromogen (Vector, Burlingame, CA) was used to detect horseradish peroxidase before counterstaining with hematoxylin and mounting.

Image analysis—Most images (all but for collagen III) were analyzed by the same examiner, who was blind to the treatment group and the time point. H&E-stained sections were imaged using a Zeiss microscope equipped with a digital microscopy camera for the gross morphological examination. Digital images of the IHC-stained slides were captured using a Zeiss Axioskop microscope (Carl Zeiss Inc., Thornwood, NY) equipped with an MRc color camera for bright field imaging and a HRm camera for fluorescence imaging. The Metamorph Image Analysis Software (Universal Imaging, West Chester, PA) was used to quantify cell numbers and protein levels within the LP region of each section.

Image analysis of myofibroblasts and macrophages was performed using bright-field microscopy. The LP region was identified by the examiner as the area between the epithelium and the thyroid muscle. The image was separated into three hue-saturation-intensity (HSI) layers and converted from 8-bit to 16-bit. Cell density was obtained with automatic nuclei count and area calculation.

Image analysis of collagen I and elastin was performed under fluorescence microscopy. Grayscale images with 16-bit resolution were captured. To compensate for possible non-uniform illumination in the Zeiss Axioskop, a baseline 16-bit grayscale image of the illumination pattern without any tissue was used. The pixel values of all images were arithmetically divided by those of the background image, a process known as *shading correction* (Gonzales & Woods, 2001). The extent of the LP was assessed by the same examiner, and the minimum threshold was determined by the maximum intensity value in the LP of the negative control. Then, the relative density of the protein of interest was calculated as the ratio of pixels with intensity above the threshold and the total number of pixels within the LP of the right vocal fold from the same animal.

Statistical analysis—Differences in cell density and protein density for the three treatment groups across the three time points were analyzed using analysis of variance (ANOVA) along with Fisher's least significant difference (LSD) post hoc tests. The homogeneity of group variances was tested with Levene's test for all time points. In addition, differences between the right (injured and treated) and left (uninjured) of each treatment group were compared at each time point using a Wilcoxon matched-pairs signed rank test. Bonferroni correction was applied for each test due to multiple comparisons ($\alpha = 0.05 / 9$). All statistical analyses were conducted using the SPSS 18.0.3 software (SPSS, Chicago, IL). Data are shown as $M \pm SD$. The overall α -level for these tests was set at .05. We did not plan to adjust for alpha inflation because we opted to protect from Type II (β) and Type I (α) error

at this early stage of inquiry in our attempt to quantify cross-section area, cell count, and matrix protein in injected vocal folds.

Results

General Observations

On Day 3 postinjection (8 days postinjury), the vocal fold LP was completely covered with epithelial cells. The newly formed epithelial layer was composed of large cells and was thicker than uninjured vocal folds in all study groups. Immediately under the epithelium, a distinct zone of disorganized granulated tissue was observed (Figure 1, black arrows). Fourteen days postinjection, the thickness of the epithelial cell layer and the size of the epithelial cells were reduced. The epithelium had an irregular corrugated surface. The disorganized granulated tissue had apparently been remodeled to form mature scar tissue. The H&E-stained histological slides showed no distinct differences in the degree of fibrotic tissue formation between the two treatment groups and the control group. At 28 days postinjection, the thickness of the epithelium and the epithelial cell size returned to the state of the uninjured vocal fold epithelium in all groups. In uninjured vocal folds, the superior part of the epithelium contains stratified squamous cells, and the inferior part of the epithelium contains ciliated pseudocolumnar cells. In contrast, the newly formed epithelium had an irregular shape and was covered inferiorly and superiorly with stratified squamous cells. After 28 days, newly formed blood vessels were found in the LP of all study groups (Figure 4, black arrows).

Inflammatory Response and Cell Proliferation

Cell counts were performed on CD68 and α -SMA stained tissue for macrophages and myofibroblasts, respectively (Figure 2). Levene's test of equality of variance confirmed the validity of the ANOVA assumptions. At Day 3 postinjection, macrophages were observed in the entire LP, with a majority of them found in the superior and intermediate layers in all groups (Figure 3). In uninjured vocal folds, the macrophage density was two cells / $10^4 \mu\text{m}^2$. Both the saline control and HA-bulk groups showed macrophage density at least three times higher than the uninjured controls at all time points: Saline (Day 3: 11 cells / $10^4 \mu\text{m}^2$; Day 14: six cells / $10^4 \mu\text{m}^2$; Day 28: six cells / $10^4 \mu\text{m}^2$); HA-bulk (Day 3: 14 cells / $10^4 \mu\text{m}^2$; Day 14: seven cells / $10^4 \mu\text{m}^2$; Day 28: six cells / $10^4 \mu\text{m}^2$). The macrophage density for HA-Ge was the closest to that of the uninjured controls at Day 28 (Day 3: 10 cells / $10^4 \mu\text{m}^2$; Day 14: six cells / $10^4 \mu\text{m}^2$; Day 28: four cells / $10^4 \mu\text{m}^2$). The differences were statistically significant in all groups at Day 3 and Day 28 (Table 1). The macrophage count was significantly lower in the HA-Ge treatment group ($p = .05$) than that of the saline group at Day 28. No significant differences were found between the saline and the HA-bulk groups in the case of macrophages, at all three time points.

Myofibroblasts were stained because of their significant role in wound contraction and production of ECM proteins during the remodeling process (Figure 4). Myofibroblasts were located mainly in the intermediate layer of the vocal folds. In uninjured vocal folds, the myofibroblast density was two cells / $10^5 \mu\text{m}^2$. At Day 3, all treatment groups showed a greater myofibroblast density than the uninjured controls (all treatment groups: three cells /

$10^5 \mu\text{m}^2$), although the differences were insignificant (Table 1). All treatment groups also approximated the uninjured controls at Day 14 (Saline: two cells / $10^5 \mu\text{m}^2$; HA-bulk: two cells / $10^5 \mu\text{m}^2$; HA-Ge: two cells / $10^5 \mu\text{m}^2$) and Day 28 (Saline: two cells / $10^4 \mu\text{m}^2$; HA-bulk: three cells / $10^4 \mu\text{m}^2$; HA-Ge: two cells / $10^5 \mu\text{m}^2$). No significant differences in myofibroblast cell counts across time points and treatment groups ($p > .05$) arose from the ANOVAs (Figure 2).

ECM Protein Distribution

The density of matrix proteins in the LP was estimated using sections stained for elastin, collagen type I, and collagen type III. Levene's test of equality of variance, again, confirmed the validity of ANOVA assumptions. Figure 2 shows the relative elastin protein density for each treatment group and time point. In uninjured rat vocal fold LP, elastin was mainly found in the superficial layer and to a lesser extent in the intermediate and deep layers (Figure 5). The relative protein density of elastin in uninjured vocal folds was 13%. For the saline controls, the elastin densities were elevated and relatively stable throughout the 28-day follow-up period (Day 3: 21%; Day 14: 22%; Day 28: 21%). For both HA groups, the elastin densities were greater than those in the uninjured controls across all time points (Day 3: HA-bulk = 18%, HA-Ge = 22%; Day 14: HA-bulk = 20%, HA-Ge = 18%; Day 28: HA-bulk = 25%, HA-Ge = 26%). The HA-bulk group, in particular, had significantly greater elastin levels than the uninjured controls at Day 28. The changes of elastin densities were statistically insignificant across treatment groups and time points ($p > .05$).

The relative density of collagen type I was 16% in uninjured vocal folds. At Day 3, the density of collagen I was increased in all treatment groups (29%, 30%, and 39% for saline, HA-bulk, and HA-Ge, respectively), whereas significant differences were found in the saline and HA-Ge groups relative to the uninjured samples. At Days 14 and 28, collagen I densities were back to 18% and 17% in the saline group, respectively; they were statistically insignificant for uninjured samples. Collagen type I densities, on the other hand, were greater than in the uninjured controls (16%) in both HA groups at Days 14 (HA-bulk = 34%, HA-Ge = 29%) and 28 (HA-bulk = 31%, HA-Ge = 27%). Among HA groups, only the HA-bulk samples showed significantly higher collagen type I density than the uninjured controls at Day 28 (Table 1). Among treatment groups, the overall collagen type I densities were statistically greater in the HA-bulk and HA-Ge groups than in the saline controls ($p < .05$) at Day 28. Collagen I was found to form organized bundles in uninjured rat vocal folds (Figure 6). Regardless of the treatment, a thick layer of unorganized collagen I was formed directly under the epithelium, along with larger bundles in the intermediate and deep layers (Figure 6, after surgery).

The relative density of collagen type III was 1% in uninjured vocal folds. Densities of collagen III for all treatment groups were similar to the uninjured samples at all time points. No significant differences in collagen type III density were found across time points and treatment groups ($p > .05$) from the ANOVAs (Figure 2). A qualitative decrease in collagen type III was observed for HA-Ge from Day 3 to Day 28.

Discussion

The HA hydrogels used in this study were found to be biocompatible, because no observable inflammatory reactions were observed. The macrophage densities were not significantly greater in HA-injected vocal folds than in the control group, suggesting little or no initiation, maintenance, or resolution of inflammation. Our results are consistent with previous studies in which no or only mild inflammatory reactions to HA injections were observed (Hallén et al., 1999; Perazzo, Duprat, & Lancellotti, 2009). The macrophage count was statistically significantly lower in the HA-Ge group ($p < .05$) than in the saline control group at Day 28. This result suggests that HA-Ge may have long-term effects in attenuating inflammation, thereby promoting the repair of damaged tissue by the host tissue (Fujiwara & Kobayashi, 2005). The consistently higher macrophage densities in all groups on Day 3 compared to the uninjured controls indicated an ongoing, active inflammatory response resulting from the surgery or injection procedures.

The myofibroblast cell counts were not significantly different during the first 28 days after injection. Differentiated myofibroblasts with stress fibers containing α -SMA are key cells for proper tissue remodeling (Hinz et al., 2007). These cells provide tissue with tensile strength. They also synthesize elevated levels of ECM proteins. The accumulation of myofibroblasts in the LP, however, can lead to excessive scarring and undesirable mechanical properties (Gabbiani, 2003). The differentiation of fibroblasts toward myofibroblasts results from the combined actions of mechanical forces and/or deformations and surrounding chemical signals such as cellular fibronectin (ED-A splice variant) and the transforming growth factor- β (TGF- β). Injury normally results from intense mechanical forces. Fibroblasts evolve into protofibroblasts 2–4 days after mechanical injury on the connective tissue (Gabbiani, 2003). Stimulation from growth factors produced by monocytes and macrophages causes protofibroblasts to further differentiate into α -SMA-expressing myofibroblasts. In turn, myofibroblasts generate greater contractile force and exhibit increased collagen synthesis (Desmouliere, Geinoz, Gabbiani, & Gabbiani, 1993; Tomasek, Gabbiani, Hinz, Chaponnier, & Brown, 2002). Usually, a decrease in myofibroblasts starts when re-epithelialization is complete (Desmouliere, Redard, Darby, & Gabbiani, 1995). At Day 3 postinjection, myofibroblast densities were qualitatively higher in all groups than in the uninjured controls. Cell densities, however, were approximately similar to those of uninjured controls from Day 14 onward in all groups. Additional data for a follow-up time point are needed to confirm the existence of myofibroblasts after injection.

The treatment did result in a density variation of collagen type I in the LP. At Day 28, the collagen type I density was significantly greater in both HA-bulk and HA-Ge groups compared to the saline group. The significance of the data is unclear because an appropriate amount of collagen type I is necessary to replace the tissue lost after surgery. Yet excessive collagen deposition increases the risk of fibrosis. Decreased collagen type I densities were observed in both groups over the time period investigated, despite the fact that collagen levels in the HA-Ge group were greater than in the saline controls. It is thus possible that HA-Ge could have accelerated the replenishment of collagen type I in the short term and controlled excessive collagen deposition in the long term. No capsule formation was observed in our study. Thick collagen I formation shortly after injury near the biomaterial

surface is a common problem in engineered tissues (Jones, 2008). Hormones and growth factors resulting from the long-term cell–biomaterial interactions, however, should further regulate fibrosis through degradation of the collagens (Greenspan, 2005). Mechanical forces are also suggested to enhance scaffold remodeling and tissue assimilation (Petersen, Joly, Bergmann, Korus, & Duda, 2012). Because rats phonate at ultrasonic level, it is unclear if the ensued phonatory forces are relevant to fibroblast-induced scaffold remodeling. Thus, it is possible that HA scaffold could accelerate the replenishment of collagen type I in the short term. But phonation-related forces may be needed to facilitate scaffold remodeling and reconstruct the tissue in its native state in the longer term.

An optimal ratio of collagen types III to I may be essential for proper vocal fold mechanics (Gilbert et al., 2009). Simultaneous analysis of both collagen types sought further indications of the long-term impact of HA injections on the dynamics of collagen remodeling by hierarchically structured hydrogels. Only minimal collagen type III density, around 1%–2%, was detected in both the injured and uninjured samples. In contrast to a previous study (Tateya et al., 2005), our findings suggest that the concentration of collagen I may be greater than that of collagen III in uninjured (normal) rat vocal fold LP tissue. The ratio of collagen type III and I was not computed because the concentrations of collagen III were deemed excessively low. This could perhaps be because the enzymatic staining methods or analysis procedures may have underestimated the volume fraction of collagen III relative to other fluorescence-based IHC methods. Although the trends over time are likely to be correct, the comparisons between collagen III and elastin or collagen I may thus be misleading.

Elastin fibers are mainly found in the intermediate and deep layers of the vocal fold LP (Hahn, Kobler, Starcher, Zeitels, & Langer, 2006; Hahn, Kobler, Zeitels, & Langer, 2006). Rousseau et al. (2003) observed a decrease in elastin in canine vocal fold scars 2 months and 6 months postsurgery. Elastin fibers were found to be characteristically tangled and disorganized. A rabbit model of vocal fold scarring revealed fragmented and disorganized elastin fibers, mostly in the deep layer (Rousseau et al., 2004). Although elastin levels were statistically insignificant across groups except the saline control, the HA groups qualitatively showed higher elastin levels at Day 28 than at Day 3. Elastin was reduced in vocal fold scar tissue compared with controls. Aging vocal folds are also known to lose elastin and thus proper vibratory functions. Lower amounts of elastin and disorganized elastin fibers have been assumed to decrease tissue elasticity (Thibeault et al., 2002). Our results suggest that some restoration of elastin might result from HA injection. The stimulation of elastin secretion promoted by HA gels would promote the restoration of biomechanical tissue characteristics. A 2-month postoperative time frame for chronic scarring in a rat model was proposed in a previous study (Tateya et al., 2005). Thus, the upcoming 8-week results from the current study are needed to corroborate the observed trends. Data collection for the 8-week group is presently under way, and the results will be reported later.

The residence of the injectable biomaterial was not evaluated in the current study. In vivo degradation studies were reported on the half-life of disulfide cross-linked thiol modified HA-Ge hydrogels with moderate cross-linker density, used in this study. Those hydrogels were shown to maintain 30% of their initial weight 42 days posttransplantation in vivo in

mice (Shu et al., 2003). Hence, microgels inside the hierarchically structured HA-Ge gels were expected to reside in the tissue at least for 2 months after injection, due to their heavily cross-linked networks. The residence time of the external network of the composite system and the HA-bulk gel in tissue was expected to be more than 1 month.

The rat animal model was selected because the ECM components in rat and human vocal fold LP have been reported to be similar. In rat vocal folds, collagen III is reportedly present through the whole LP, whereas collagen I is sparse (Tateya et al., 2005). In human vocal folds, collagen III reportedly forms wavy organized fibers throughout the whole LP, whereas collagen type I is relatively thin and limited to the superficial layer around the basement membrane zone (Tateya et al., 2006). In both rat and human vocal folds, HA and elastin are reportedly found in the intermediate layer (Hammond, Zhou, Hammond, Pawlak, & Gray, 1997; Tateya et al., 2005), and fibronectin is found in the basement membrane zone (Courey, Scott, Shohet, & Ossoff, 1996; Tateya et al., 2005). The process of scarring occurs relatively quickly in the rat model. Tateya et al. (2005) found that ECM components reach a stable level by 8 to 12 weeks. A commensurate time period is therefore deemed adequate for scar maturity and the investigation of treatment approaches for scar formation in the rat model. Short treatment periods are advantageous because of reduced animal maintenance costs.

The chosen end points of this study represent the progression of tissue remodeling in acute vocal fold wound healing based on the same rat model (Tateya et al., 2005, 2006). The first time point (3 days after injection) corresponds to the time window of the transitions from proliferative to remodeling phases. The last two time points (2 weeks and 4 weeks after injection) represent the active phase of tissue remodeling in injured rat vocal folds.

Although rat vocal folds oscillate in the ultrasonic range (Knutson, Burgdorf, & Panksepp, 2002) and do not vibrate in the frequency range of human phonation, this model allows valid investigations of the chemical interactions between the injectable biomaterial and the tissue. Also, rat vocal folds demonstrate structural similarities to those of humans and therefore they are an excellent animal model for eventual translation to the human model. The human alternative is an unethical one because it requires creating an experimental injury. Human data could not supply information of the vocal fold histology and mechanics following the injections because no noninvasive biopsy method is available at the present time. The time period for vocal fold repair after injury is substantially shorter in rats than in human (Dorsett-Martin, 2004). Of course, the translation of results of any animal to human must be done with caution. Small rodents appear to exhibit a faster healing process than larger mammals following vocal fold stripping (Rousseau et al., 2004; Tateya et al., 2005, 2006). Therefore changes in cells and proteins might occur over a larger timespan in humans than in rats. Note that quantitative results on ECM proteins could not be compared to viscoelastic or mechanical properties because the small size of the rat vocal folds precludes standard rheology measurements or mechanical tests with readily available techniques. For this reason, an indentation test using an atomic force microscope has been developed (Heris, Miri, Tripathy, Bartherlat, & Mongeau, 2013; Kazemirad, Heris, & Mongeau, 2013), which could potentially be applied in the future for the characterization of the elastic properties of small LP specimens, such as human LP biopsy and rat vocal folds.

In this study, the surgical injury consisted of the complete stripping of the vocal fold LP until the underlying thyroarytenoid muscle was exposed. Such comprehensive injury is a reliable surgical model to induce a vigorous wound healing response and a consistent scarring outcome required for comparing the results across treatment groups. This surgical model, however, precluded the possibility of injecting the biomaterial into the LP at the time of surgery or during the early acute phase of wound healing. One might inject the biomaterial into the vocalis muscle instead of the LP. The focus of this study, however, was to evaluate whether the injectable biomaterial facilitated wound healing in the LP specifically. Preliminary trials indicated that the earliest time allowing a successful injection of the biomaterial into injured mucosa was 5 days after surgery. This time point falls within the overlapping phases of proliferation and remodeling in rat vocal folds using the same surgical model (Tateya et al., 2006). Soft collagenous LP was formed and provided a provisional matrix to host the injectable biomaterial without spilling over to the lumen. We hypothesized that the injectable biomaterial would control scar formation through regulating the subacute proliferative and tissue-remodeling response to surgical injury. The results indicated, however, that biomaterial injection during the proliferation phase did not significantly alter the scar formation during the 4-week postinjection period.

Last, in contrast to the microflap technique, mucosal stripping is no longer widely used in phonosurgery. In rats, ECM components are produced early in vocal fold injury. It has been suggested that the postoperative Day 3–5 time period is the plausible window in therapeutic research aimed at preventing scarring after vocal fold injury (Tateya et al., 2006). However, with the stripping method, only a small residual portion of the LP was left after the procedures. A few days of recovery were needed to create a space to inject the hydrogel. A period of 5 days was thus chosen in the current study despite the fact that it might be more advantageous to inject immediately after injury to facilitate the creation of a scaffold for cells involved in ECM component production. Because of the important role of HA in wound healing processes, HA injection at the time of injury might be more effective. The injection site should be within the LP for targeted and local healing effects. A microflap vocal fold surgical model has recently been reported in rabbits (Suehiro, Bock, Hall, Garrett, & Rousseau, 2012), which will allow the injection of biomaterial into the LP at the time of surgery. Future work will verify the location of the biomaterial injection using alcian blue staining with hyaluronidase digestion technique. Despite these limitations, the data obtained in the present study contribute to our understanding of scarring and tissue remodeling following injection with HA hydrogels.

Conclusions

The effects of two different HA hydrogel injections on tissue remodeling after acute injury were investigated using a rat animal model. The aim was to assess the biocompatibility of a hierarchically structured HA-Ge hydrogel and its potential for tissue reconstruction. Results revealed that the hierarchical HA-Ge was biocompatible and did not induce significant inflammatory response. Differentiated wound healing patterns were observed between the HA hydrogel groups and the controls. A more complete interpretation of the long-term effects of hydrogel injection will be possible after including the 8-week data.

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Figure 1.

Hematoxylin and eosin-stained sections of vocal folds (20 $\times$). Granulation tissue was observed 3 days postinjection (black arrows). The cross-sectional area decreased after injury, and the lamina propria (LP) did not remodel to its original size. An irregular epithelial surface was observed mainly 2 and 4 weeks postinjury. Scale bar = 200 μ m.

Figure 2.

ECM protein levels and cell counts normalized to the area of vocal fold lamina propria in rats following vocal fold surgical injury. Panel A: Collagen type I. Panel B: Collagen type III. Panel C: Elastin. Panel D: Macrophages. Panel E: Myofibroblasts. Metaphor software was used to digitize and quantify the staining images as described in the Method section. The bars and the error bars represent the means and the standard errors of the data ($n = 108$), respectively. Asterisks denote that the data at that time point are statistically significant compared to the saline controls ($p < .05$).

Figure 3.

Macrophages were stained for CD68 (red). Representative CD68-stained sections at each time point are shown (40 $\times$). Most macrophages were found 3 days postinjection, and the number decreased over time. A majority of the macrophages was found in the superficial and intermediate layers of the vocal fold LP. At 4 weeks, the macrophage level was still slightly elevated. Scale bar = 100 μ m.

Figure 4.

Myofibroblasts were stained with α -SMA (red, at arrow heads), which also stains the blood-vessel walls (black arrows) and myoepithelial cells in glands (yellow arrows). Representative α -SMA-stained sections at each time point are shown (40 $\times$). Very few myofibroblasts were found, with no significant difference between groups.

Figure 5.

Representative sections of vocal folds (40×) stained for elastin. Elastin appears red and nuclei appear blue. Elastin levels were elevated in all treatment groups at each time point, but no significant difference was found between the groups. Elastin was mainly found in the superficial and intermediate layers.

Figure 6.

Representative sections of vocal folds (40 $\times$) stained for collagen type I. Collagen I appears green and nuclei appear blue. For all treatments, postinjury, a thick layer of unorganized collagen I was formed immediately under the epithelium. The bundles in the intermediate and deep layers were observed to be bigger (red arrows).

Figure 7. Representative sections of vocal folds (40×) stained for collagen type III. Collagen III appears brown and nuclei appear purple. For all treatments and uninjured tissue, collagen III was sparsely distributed.

Table 1

Differences of extracellular matrix (ECM) and cell measures between right (injured) and left (uninjured) vocal folds.

Measure		Day 3	Week 2	Week 4
		<i>M (SD)</i>	<i>M (SD)</i>	<i>M (SD)</i>
Collagen type I	Saline	15.3 (10.0) *	8.0 (8.2)	6.7 (10.0)
	HA-bulk	13.5 (11.7)	14.1 (8.2)	14.9 (7.1) *
	HA-gelatin	18.0 (13.2) *	14.4 (10.3)	11.8 (8.8)
Collagen type III	Saline	0.3 (1.2)	0.1 (0.3)	1.4 (2.0)
	HA-bulk	0.0 (0.9)	-0.2 (0.4)	1.0 (1.8)
	HA-gelatin	0.0 (0.8)	0.3 (0.5)	0.3 (0.9)
Elastin	Saline	12.2 (8.8) *	9.7 (9.5)	5.5 (12.0)
	HA-bulk	6.5 (5.7)	6.8 (12.5)	13.9 (6.2) *
	HA-gelatin	6.6 (10.4)	5.9 (8.4)	10.1 (12.3)
Macrophages	Saline	8.9 (4.0) *	3.0 (3.1)	2.3 (0.9) *
	HA-bulk	12.2 (7.0) *	4.8 (1.8)	2.8 (2.0) *
	HA-gelatin	8.3 (3.8) *	4.0 (2.1)	1.6 (1.0) *
Myofibroblasts	Saline	0.8 (2.8)	0.8 (1.6)	-0.4 (1.4)
	HA-bulk	0.8 (1.6)	-0.1 (1.7)	-0.2 (3.3)
	HA-gelatin	0.3 (2.4)	0.3 (1.7)	0.8 (1.8)

Note. The difference between right and left vocal folds for each treatment group were compared at each time point using Wilcoxon matched-pairs signed rank test. Bonferroni correction was applied for each measure due to multiple comparisons ($\alpha = .05 / 9$).

* $p < .005$.