VISCOELASTICITY IN SCARRED RABBIT VOCAL FOLDS AFTER HYALURONAN INJECTION – SHORT TERM RESULTS

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Short title: Viscoelasticity in scarred rabbit vocal folds after hyaluronan
Keywords: vocal fold scarring, viscoelasticity, hyaluronan.

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Abstract

Vocal fold scarring is accompanied by stiffness of the lamina propria and results in severe voice problems. Hyaluronan has been shown to improve the viscoelastic properties after injections in normal rabbit vocal folds and in patients with unilateral paresis and vocal fold atrophy.

Objectives: The main aim of the study was to analyze the short term viscoelastic properties after injection of hyaluronan in scarred rabbit vocal folds. Another aim was to examine the degree of scarring achieved by the experimental model.

Material and Methods

Vocal folds of 15 New Zealand rabbits were scarred by a localized resection. After 8 weeks one group received injections with a cross-linked hyaluronan and another group was injected with saline. After 11 more weeks both groups and a third group of control animals with normal vocal folds were sacrificed. The larynges were dissected out, 15 vocal folds were frozen for viscoelastic measurements, whereas 14 vocal folds were prepared and stained for histology. The histological analysis included measurements of the lamina propria thickness and of the relative content of connective tissue. Two methods were used for the viscoelastic measurements: 1. analyses were made on intact vocal folds with a linear skin rheometer (LSR) adapted to laryngeal measurements. 2. the vocal folds were dissected and analyzed in a parallel-plate rheometer.

Results

Measurements on the digitized slides showed a thickened lamina propria and a higher content of connective tissue in the scarred samples as compared to the normal vocal folds ($p<0.05$). The viscoelastic LSR analysis on intact vocal folds showed a tendency to stiffening of the scarred vocal folds as compared to the normal group ($p=0.05$). The parallel plate rheometry on the same samples after dissection showed a decreased dynamic viscocity and lower elastic modulus in the scarred samples injected with hyaluronan as compared to the normals and to the untreated scarred group ($p<0.01$)

Conclusions

The experimental model for vocal fold scarring resulted in deviation of the normal lamina propria structure with increased connective tissue content. Injection of scarred rabbit vocal folds with hyaluronan rendered improved viscoelastic parameters in short term.

Background

Vocal fold scarring may have different etiology, such as trauma, surgical defects of the vocal folds, post radiotherapy, or inflammation (1). This results in tissue defects and/or disturbance of the vocal fold lamina propria viscoelasticity. Voice is often breathy or aphonic and the phonation threshold pressure which corresponds to "easiness of phonation" (2) is elevated. The treatment is usually difficult, and may include voice therapy by a speech and language therapist and injection augmentation. Many substances have been tried. Bovine or autologous human collagen has been used for superficial injections into the vocal fold ligament (3, 4). Autologous fat implantation into the lamina propria has also been tried in selected cases (5). Drawbacks with collagen and fat is the need for allergy testing (for bovine collagen) and the unpredictable degree of resorption over time (for both) (6).

Due to different drawbacks with all existing materials for augmentation**,** there is an ongoing search for new materials (7). The ideal substance has to fulfil various criteria, e.g. to be non-toxic, non-allergic, can be precisely injected or implanted superficially into the vocal fold lamina propria**,** and persists for a long time.

Hyaluronan (HYA) is a glycosaminoglycan identical for all vertebrae species. It is present at high concentrations in the extracellular matrix of many tissues in the body (8) and has also been found in the vocal fold lamina propria (9, 10). HYA functions as a space filler, lubricates, is a shock-absorbing substance and has important biological functions in, e.g., wound healing (8). The viscoelastic properties of native HYA showed a similar dynamic viscosity as that of normal vocal fold mucosa (11). Its rheological properties, however, vary substantially with concentration, molecular weight and degree of molecular cross-linking (12, 13).

Pure cross-linked hyaluronan in the gel form (hylan b gel) was found to be persistent in rabbit vocal folds for up to at least one year after injection**,** with no inflammatory reaction or granuloma formation (14). Our previous results using hyaluronan in patients with glottal insufficiency due to unilateral vocal fold paresis and atrophy showed no side effects, improved voice and glottal closure up to at least 2 years. Parameters related to improved viscoelasticity, such as amplitude of vocal fold vibrations and phonation threshold sound pressure were also improved. No vocal fold stiffening was found after injection in the superficial lamina propria with hylan b gel (15, 16). The drawbacks are related to some resorbtion with need for reinjections. The effects of hylan b gel in treatment of vocal fold scarring has so far only been studied in a few patients but in the substance seemed to have the potential for improving vocal fold function (15) .

The *aim* of this experiment was to investigate the short-term viscoelastic properties of scarred rabbit vocal folds after injections of cross-linked HYA as compared to scarred vocal folds injected with saline. Vocal fold mucosa from non-injected rabbit larynges served as controls. A second aim of the experiment was to study the degree of scarring in the lamina propria achieved in the experimental procedure.

Material and Experimental procedures

Fifteen New Zealand white rabbits (bw 2.9-3.5 kg) were used in the experiment. The American principles of laboratory animal care and the Swedish National law on animal care ethics were followed. The experiment was approved by the local ethic committee of Karolinska Institute (S-149-01, 2001-10-15).

Vocal fold scarring. After premedication with glycopyrrolate (0.1mg/kg s.c.) and fluanizonum (10mg/ml fentanyl 0.3/mg/ml, 0.3ml/kg diazepam, 0.3ml/kg i.m.) the animals were anaesthetized with diazepam 1-2mg/kg i.v. The laryngeal structures and mobility were found normal at examination by means of a modified 4.0 mm pediatric laryngoscope (model 8576E,

Karl Storz Endoscope, Tuttlingen, Germany) and a Storz-Hopkins 0° 2.7 mm rigid endoscope, (model 7218A). The scarring procedure was performed with a 2 mm microcup forceps and microscissor (MicroFrance). A localized excision of the mucosa and superficial thyroarytenoid muscle was made under direct vision through an otomicroscope (Figure 1). The procedure yielded 22 excised (scarred) vocal folds and 8 normal vocal folds without scarring. All animals survived the procedure.

Vocal fold injections. After 8 weeks the animals were again examined with direct laryngoscopy under general anaesthesia. Injections were made under vision through a microscope into the lamina propria and/or to the superficial part of the thyroarytenoid muscle of the vocal fold using a Medtronic Xomed laryngeal injector with a 27 gauge needle. Systematic injections in either of the structures mentioned above was not possible due to the narrow space and the equipment available at the time of the experiment. Eleven out of the 22 scarred vocal folds were injected with 0.1ml saline each. Six vocal folds were injected with 0.1 ml Hylaform®, hylan b gel, a cross-linked pure HYA at 5.5 mg/ml concentration (Genzyme Biosurgery, Ridgefield, MA, USA), and 6 vocal folds were injected with Restylane®, a non-animal stabilized HYA from bacterial fermentation at 20mg/ml concentration (Q-Med Inc. Uppsala, Sweden). The 8 non-scarred vocal folds were not injected. No animal suffered from breathing problems or bleeding after the injections.

Dissection

Eleven weeks after the injections the animals were killed by an i.v. overdose of sodium pentobarbital. The larynges were dissected out and each larynx was divided in the posterior midline. Sixteen of the hemilarynges were immediately fresh frozen at –20°C until viscoelastic analysis (5 non-injected, 5 scarred vocal folds injected with saline, 3 scarred vocal folds injected with Hylaform® and 3 scarred samples injected with Restylane®).

Fourteen of the hemilarynges were placed in 10% formaldehyde for later preparation and histological analysis (4 non-injected, 5 scarred folds injected with saline, 2 scarred folds injected with Hylaform® and 3 folds injected with Restylane®).

Histological measurements

Fourteen vocal folds removed from the hemilarynges were further processed, dryed in microwave owen at 630W, paraffin-embedded and. cut into 5µm thick sections (17). These were stained with hematoxyline eosine and van Gieson for histological analysis. Image analysis on the stains at 20x magnification were made after digitization of the microscopic images (…….). The thickness of the lamina propria (LP) was measured with the software Image Pro Plus® (version 3.0 Media Cybernetics). The relative content of connective tissue in LP was measured from the digitized stains after a colour filtering and normalization process with Photoshop (version 8.0) and a custom made software (written by Hans Larsson at Karolinska Institute, Stockholm, Dept of Logopedics and Phoniatrics), Figure 2.

Viscoelastic measurements

1. Linear skin rheometry (LSR)

Analyses were made on intact vocal folds with a linear skin rheometer (LSR) adapted to laryngeal measurements. This device was originally developed for measurements of skin viscoelasticity (18, 19). A lightweight tipped probe with a cross section surface of 1mm^2 is driven to produce a sinusoidal compression over a distance of 1-2mm at 0.3Hz. The resulting relative Youngs´s modulus (ΔY) (20) parameter is derived from analysis of stress/strain curves. These parameters are related to tissue stiffnes. An advantage with the method is that the measurements can be made without dissecting the tissue samples. The hemilarynges were thawed at room temperature, kept moist with saline and fixed with needles at a plate during the measurements. Measurements were made at the vocal fold edge on midmembranous position during compression of the vocal folds (4 untreated samples, 5 scarred folds injected with saline, 3 scarred folds injected with Hylaform® and 3 with Restylane®). One vocal fold

was used to test the experimental set-up and the results for this were not further analyzed in the experiment. The measurements of each vocal fold lasted 15-20 minutes. After the measurements the hemilarynges were again frozen at –20°C until the parallel plate rheometry.

2. Parallel-plate rheometry

The linear viscoelastic shear properties of vocal fold tissue has been studied by several researchers (11, 21-23). A parallel-plate rheometer produces sinusoidal shear small amplitude oscillations at increasing frequency (from 0.01-15Hz). We used an AR 2000 Rheometer (TA Instrument) with a stationary lower plate (15mm diameter) separated by about 0.5mm from a rotating upper plate. Tissue samples from the same fifteen vocal folds as in the LSR experiments were dissected and analyzed at 37°C in the parallel plate rheometer (4 untreated, 5 scarred fold injected with saline, 3 scarred folds injected with Hylaform® and 3 with Restylane®). The samples included vocal fold lamina propria and the superficial part of the thyroarytenoid musce. The tissue were kept most with saline during the measurements. All rheometric measurements were performed in the linear region with constant stress level transferred from the sample to the upper plate where it is measured with a linear variable displacement transducer. The dynamic viscocity (η', Pas) and elastic modulus (G', Pa) were derived as a function of frequency. Dynamic viscocity is a measure of a material´s resistance to shear flow. The elastic modulus (G') represents a measure of a materials stiffness in shear. In this experiment the gap between the plates was not completely filled with tissue. Thus the absolute level of η' and G' may not be accurate. However, the same dissection procedure and amount of tissue was used for all samples which allows for comparison between the different treatment groups.

Statistics

Non-parametric comparisons between the groups were made (Statview program, SAS Institute Inc., version 5.0). The two types of HYA treatments were analyzed as one group due to the small number of samples. Due to the exploratory nature of the study a significance levels with $p<0.05$ are reported.

Results

Histological analysis

In 4 out of the 5 scarred vocal folds treated with HYA (Hylaform® or Restylane®) showed remaining substance. HYA was identified either in smaller well localized islands in the lamina propria or deep in the thyroarytenoid muscle. These aggregates were surrounded by a thin capsule of connective tissue. The measurements of the vocal fold lamina propria (LP) showed that both the scarring groups (scarring+saline and scarring+HYA) had significantly thicker LP than the non-scarred folds ($p<0.05$). There was no difference in LP thickness between the scarred folds who were treated with HYA or injected with saline. Analysis of the relative content of connective tissue in LP also showed that both scarring groups had higher relative connective tissue content in LP as compared to the untreated vocal folds, $p<0.05$ (scar+saline versus scar+HYA ns).

Viscoelastic analyses

1. LSR analysis

As shown in Figure 3 the relative Young´s modulus (ΔY) was lowest for the normal vocal folds and higher for both the scarred groups. This indicates a stiffening for the scarred groups. The difference between the normal vocal folds and the scarred folds injected with saline was close to significant ($p=0.05$). (normal versus scar+HYA: ns, scar+saline versus scar+HYA: ns).

2. Parallel plate rheometry

Figure 4 shows that the dynamic viscocity was lower for the scarred vocal folds treated with HYA as compared to both the untreated samples and to the scarred samples injected with saline ($p<0.05$). There was no significant difference between the untreated samples and the

scarred samples injected with saline). Figure 4 also shows that the elastic modulus was significantly lower for the scarred vocal folds treated with HYA as compared to both the untreated and to the scarred samples who were injected with saline, $p<0.01$ (untreated versus scar+saline: ns).

Discussion

Many attemps have been made to find a treatment for vocal fold scarring. In order to restore the vibratory capacity of scarred vocal folds the characteristics of a bio-implant used for injection or implantation should match the viscoelastic properties of normal vocal fold mucosa. Previous studies in normal rabbit vocal folds after injection of hylan b gel (Hylaform®) showed similar dynamic visocity after injection as for native rabbit vocal folds (22, 23). The results after treating a few patients with vocal fold scarring were also promising (15). The in vitro characteristics of Restylane® are similar as Hylaform® and both substances are easy to handle and inject. The rabbit vocal folds are similar to human in structure although the lamina propria is less well developed. We used rabbits vocal folds in a similar experimental model for vocal fold scarring as other researchers (21, 24). The total observation time was about five months after the scarring procedure. Hirano et al. recommended an observation time in the "chronic" scar model close to six months in order to obtain a realistic deposition of collagen in the vocal fold which probably correlates to a stiffening of scarred folds. The results of the colour analysis and measurements of the lamina propria thickening in the present study also indicates that significant scarring was achieved.

The difficulties to inject or augment with precision in scarred vocal folds are well known. This may explain the results of the histologic analysis showing that the HYA injected was found at various locations in the vocal folds. No inflammatory changes or granuloma were observed and the HYA was surrounded by a thin capsule. This is similar to the findings by Hallén et al. (14). Remaining HYA was found at histological analysis in 4 out of the 5 injected samples. The observation time after injection was 11 weeks. Thus for one animal either we did not succeed to inject into the the scarred tissue or there was resorbtion of the material.

We performed two types of viscoelastic analyses. The LSR analysis has the advantage that it can be made on intact vocal folds, but on the other hand the vocal fold tissue is compressed at only a single slow rate (not corresponding to phonation). The LSR analysis showed an elevated relative Young´s modulus for the scarred vocal folds as compared to the normal folds. This corresponds to stiffening probably due to scarring. There was no significant difference between the scarred folds injected with saline or with HYA (Hylaform® or Restylane®). The parallel plate reometry is performed after dissecting the vocal folds, but the method permits measurements at different frequencies (0-10 Hz in this experiment). The results of this analysis showed a lowered dynamic viscocity and elastic modulus for the HYA treated samples as compared to the scarred folds treated with saline. This indicates less stiffening which corroborates the results noted for some patients treated for vocal fold scarring (15). Titze, Gray and Chan performed parallel plate reometry analysis on normal human vocal folds before and after removal of the natural hyaluronan (11). Their results showed a higher viscoelasticity without hyaluronan. It may be that HYA alters the viscoelasticity directly in a favourable way in vocal fold scarring Our results must, however, be interpreted with caution. The HYA samples had lower viscocity than the normal controls. We have no clear explanation for this, but possibly there may be some degenerative changes due to the repetitive analysis which affected the normal samples more than the sacrred folds.

Conclusions

The results showed that the experimental model used resulted in vocal fold scarring both at histological analysis and indicated from the LSR viscoelastic analysis. The parallel plate

reometry showed improved biomechanical properties for the scarred vocal folds treated with HYA. This results must however be confirmed in order to permit definite conclusions.

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Figures Hertegård et al Viscoelasticity in scarred rabbit vocal folds

Figure 1

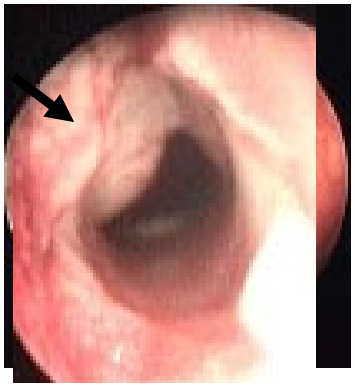

Figure 1. To the left: image of a rabbit larynx efter a resection (arrow) in the left vocal fold. To the right: image of a larynx of another animal after injection of hyaluronan into the left vocal fold.

Figure 2

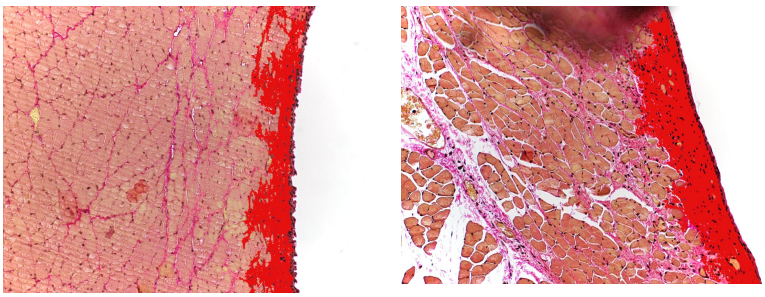

Figure 2. Hematoxyline-eosine stained sections (at 20x) after digital color filtering of connective tissue of the lamina propria (red color). left: a normal vocal fold. right: a scarred vocal fold

Figure 3.

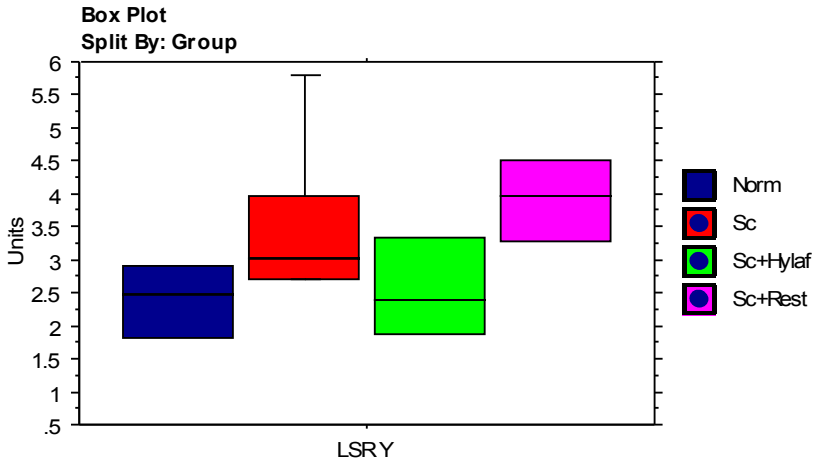

Figure 3. Box plots showing the relative Young´s elastic modulus (LSR Y) from the Linear skin rheometer (LSR) analysis for the normal vocal folds, scarred vocal folds and scarred folds treated with HYA (Hylaform and Restylane).

Figure 4

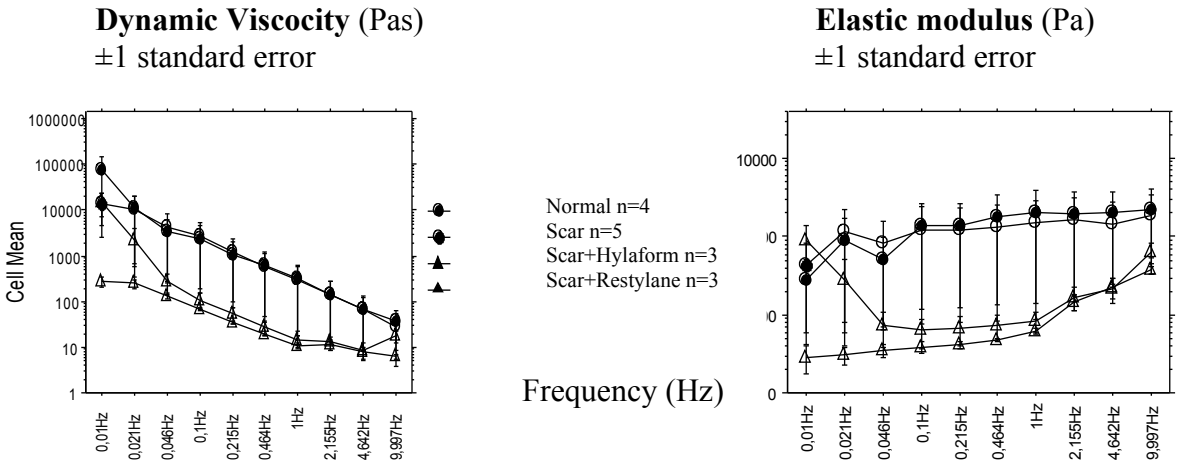

Figure 4. Graphs of the dynamic viscocity (left) and elastic modulus (right) as a function of oscillatory frequency of the parallel plate reometer for the controls, scarred fold injected with saline, and scarred folds injected with HYA (Hylaform and Restylane)