# VISCOELASTIC MEASUREMENTS AFTER VOCAL FOLD SCARRING IN RABBITS
## SHORT TERM RESULTS AFTER HYALURONAN INJECTION

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VISCOELASTIC MEASUREMENTS AFTER VOCAL FOLD SCARRING IN RABBITS– SHORT TERM RESULTS AFTER HYALURONAN INJECTION

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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 results in stiffness of the lamina propria and severe voice problems.

Objectives: To examine the degree of scarring achieved in the experiment and to measure the viscoelastic properties after injection of hyaluronan in rabbit vocal folds.

Material and Methods

Twenty-two vocal folds from 15 New Zealand rabbits were scarred, 8 vocal folds were controls. After 8 weeks 12 of the scarred vocal folds received injections with two types of cross-linked hyaluronan products and 10 scarred folds were injected with saline. After 11 more weeks the animals were sacrificed. After dissection, 15 vocal folds were frozen for viscoelastic measurements, whereas 14 vocal folds were prepared and stained. Measurements were made of the lamina propria thickness. Viscoelasticity was measured on intact vocal folds with a linear skin rheometer (LSR) adapted to laryngeal measurements.

Results

Measurements on the digitized slides showed a thickened lamina propria in the scarred samples as compared to the normal vocal folds (p<0.05). The viscoelastic analysis showed a tendency to stiffening of the scarred vocal folds as compared to the normal controls (p=0.05). There was large variation in stiffness between the two injected hyaluronan products.

Conclusions

The scarring model resulted in significant damage and elevated viscoelasticity of the lamina propria. Hyaluron preparations may alter viscoelasticity in scarred rabbit vocal folds.
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Background

Vocal fold scarring may have different etiology; such as trauma, scar healing after vocal fold surgery, 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. Effective treatment is lacking. Many injection 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 are 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 fulfill various criteria, e.g. to be non-toxic, non-allergic, and to allow precise injection and superficial implantation into the vocal fold lamina propria. It should persist for a long time.

Hyaluronan (HYA) is a glycosaminoglycan identical for all vertebrate 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 is a major constituent of extracellular soft connective tissue matrix where it exerts lubrication, shock absorption, filtering, exclusion of particles and many biological functions in, e.g., wound healing (8, 11). The viscoelastic properties of native exogenous HYA showed a similar dynamic viscosity as that of normal vocal fold mucosa (12). Its rheological properties, however, vary substantially with concentration, molecular weight and degree of molecular cross-linking (13, 14).

Pure cross-linked hyaluronan in a gel-like form (hylan b gel, Hylaform®) 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 (15). Our previous results using hyaluronan in
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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 (16, 17). Parameters related to improved viscoelasticity, such as amplitude of vocal fold vibrations and phonation threshold sound pressure were also improved. No vocal fold stiffening or signs of scarring was found after injection in the superficial lamina propria with hylan b gel. The drawbacks are related to some resorption 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 (16).

The first aim of the experiment was to study the degree of scarring in the lamina propria achieved in an experimental model for vocal fold scarring. The second 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.

**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.** Before surgery premedication was administered consisting of glycopyrron, (Robinul®, 0.1mg/kg s.c.) and Hypnorm (a mixture of fluanizonum 10mg/ml and fentanyl 0.3/mg/ml), 0.3ml/kg i.m.. The animals were then anaesthetized with diazepam 1-2mg/kg i.v. The laryngeal structures were found normal at examination by means of a modified 4.0 mm pediatric laryngoscope (model 8576E, Karl Storz Endoscope, Tuttingen, Germany) and a Storz-Hopkins 0° 2.7 mm rigid endoscope, (model 7218A). The scarring
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procedure was performed with a 2 mm microcup forceps and microscissor (MicroFrance) (18, 19). A 2-3 mm biopsy was taken from the midmembranous mucosa and superficial thyroarytenoid muscle under direct vision through an otomicroscope (Figure 1). Seven animals were operated on both vocal folds and 8 animals were operated on one side only. Thus the total of 22 vocal folds were operated (scarred) and 8 normal vocal folds were kept as control group. 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 (Figure 1). 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. Ten out of the 22 operated (scarred) vocal folds were injected with 0.1ml saline each. Six vocal folds out of the 22 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 out of the 22 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. This included 5 non-injected (normal), 5 operated (scarred) vocal folds injected with saline, 3 operated (scarred) vocal folds injected with Hylaform® and 3 operated (scarred) samples injected with Restylane®.
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Fourteen of the hemilarynges were placed in 10% formaldehyde for later preparation and histological analysis (3 non-injected (normal), 5 operated (scarred) folds injected with saline, 3 operated (scarred) folds injected with Hylaform® and 3 operated folds injected with Restylane®).

Analysis

Histological measurements

Fourteen vocal folds removed from the hemilarynges were further processed in 2% formaldehyde and 0.5% glutaraldehyde in 0.1 M PBS, fixed in microwave oven at 630W, paraffin-embedded, dried, and cut into 5µm thick sections (20). 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 (using a Nikon Digital Camera, DXM 1200 attached to a Nikon Ecclipse, E600 microscope). The thickness of the lamina propria (LP) was measured with the software Image Pro Plus® (version 3.0 Media Cybernetics). The thickness measurements of LP were enhanced by 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 Phoniatics), Figure 2.

Viscoelastic measurements

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 (21, 22). A lightweight tipped probe with a cross section surface of 1mm² is driven to produce a sinusoidal compression over a distance of 1-2mm at 0.3Hz. The resulting relative Youngs’s modulus (ΔY) (23) parameter is derived from analysis of stress/strain.
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curves. These parameters are related to tissue stiffness. 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 normal 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.

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 6 scarred vocal folds treated with HYA (Hylaform® or Restylane®) there was remaining HYA substance, either in smaller well localized islands in the lamina propria or deep in the thyroarytenoid muscle. No inflammatory changes or granuloma were observed. 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.

Viscoelastic analyses (LSR analysis)
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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 variation was large within the scar group treated with different HYA substances, the Hylaform® samples had the lowest stiffness. 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).

Discussion

Many attempts have been made to find a treatment for vocal fold scarring. The main aim of the study was to examine the degree of scarring achieved in a rabbit animal model. The rabbit vocal folds are similar to human in structure although the lamina propria is less well developed (20). We operated rabbit vocal folds in a similar surgical scarring procedure as other researchers (18, 19). The total observation time was close to five months after the scarring procedure.

The main histological finding was a significant thickening of the lamina propria. Previous research with scarring models in rabbits showed ultrastructural changes of the lamina propria with increased pro-collagen and fibronectin and a decrease in elastin at 2 months after surgery (24). Studies on scarred canine vocal folds showed similar findings as for the rabbits 2 months after surgery and a co-deposition of collagen in the lamina propria developing 6 months after scarification (19, 25). Levels of fibronectin were also elevated both 2 and 6 months after surgery. Although we did not analyze the mechanisms of the LP thickening this finding indicates that a significant damage was achieved by our scarring procedure.

The viscoelastic testing with the LSR method showed an elevated relative Young’s modulus for the scarred vocal folds injected with saline as compared to the normal folds. This corresponds to tissue stiffening. Reological analyses in other studies both in scarred rabbits and dogs larynges have shown increased stiffness and viscosity (25, 26). Thus both the
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histological and reological findings indicate that a significant degree of scarring was achieved by the vocal fold surgery in this experiment. The injection in itself might also have contributed to some scarring (no animals in the experiment were operated but not injected). However, no scarring has been found in a previous study of rabbits vocal folds were injected with HYA substances without any surgical scarring procedure (27). Furthermore no signs of scarring has been noted in our studies in our previous injection studies on patients with glottal insufficiency caused by vocal fold paresis or atrophy (16).

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 viscosity after injection as for native rabbit vocal folds (27, 28). Furthermore it was demonstrated that the level of hyaluronan was lowered in pigs rabbit and vocal folds within 15 days after a scarification procedure with a simultaneous increase in tissue stiffness (29, 30). The results after treating a few patients with vocal fold scarring were also promising (16). Both HYA substances used in this study (Restylane® and Hylaform®) are easy to handle and inject. 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, and the HYA was surrounded by a thin capsule. This is similar to the findings by Hallén et al. (15).

Remaining HYA was found at histological analysis in 4 out of the 6 injected samples. The observation time after injection was 11 weeks. Thus for two animal s either we did not succeed to inject into the scarred tissue or there was resorbtion of the material.

The reological testing with the LSR method showed no significant difference between the scarred folds injected with saline or with HYA (Hylaform® or Restylane®). The variation was large for the HYA treated samples, but Hylaform® had a lower relative Young´s modulus.
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close to the normal controls. This indicates less stiffening which corroborates the results noted for some patients treated for vocal fold scarring (16). Titze, Gray and Chan performed parallel plate reometry analysis on normal human vocal folds before and after removal of the natural hyaluronan (12). Their results showed a higher viscoelasticity without hyaluronan. Hansen and Thiebeault injected a HYA scaffold hydrogel in ewly scarred rabbit vocal folds. Viscoelasticity was improved after 3 weeks with well organized collagen fibrils (31). It was hypothesized that HYA accelerated wound repair and altered the viscoelasticity directly in a favourable way in vocal fold scarring.

Conclusions

The results showed that the experimental model used resulted in thickening of vocal fold lamina propria. The LSR viscoelastic analysis showed elevated elasticity for the scarred vocal folds injected with saline as compared to the normal controls. These findings indicate that significant damage to the lamina propria was achieved by the surgical procedure. The reometry also showed improved biomechanical properties in some of the scarred vocal folds treated with HYA. This results must however be confirmed in order to permit definite conclusions.

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References


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Figures Viscoelasticity in scarred rabbit vocal folds

Figure 1

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

Figure 2

*Figure 2*. Hematoxyline-eosine stained sections (at 20x) after digital color filtering of the lamina propria (red color). left: a normal vocal fold. right: a scarred vocal fold
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 (Norm, n=4), scarred vocal folds (Sc, n=5) and scarred folds treated with HYA (Hylaform® Sc+Hylaf, n=3, and Restylane® Sc+Rest, n=3).

Figure 3.