JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE **16** (2005) 553–557

Evaluation of injection augmentation treatment of hyaluronic acid based materials on rabbit vocal folds viscoelasticity

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The viscoelastic properties of vocal folds after injection of hyaluronic acid (hyaluronan, HA) based materials have been studied in an animal model (rabbit) six months after injection. The results indicate that the viscoelastic properties of the vocal folds injected with the HA based materials are similar to the healthy vocal folds (non-injected samples) used as control. Histological analysis has been also performed to investigate on the fate of the injected materials after six months from the implant. The HA based materials remain up to six months and they recruited fibroblasts that induce the ingrowth of new connective tissue resulting in an endogenous soft tissue augmentation. The HA based compounds are good candidate for further studies aimed at restoring/preserving the vibratory capacity of the vocal folds with injection treatment in glottal insufficiency

1. Introduction

Injection laryngoplasty is a common practice in phonosurgery to treat disphonia. Dysphonia is represented by glottal insufficiency generated by scarring, paralysis or inflammation of the vocal folds joint. During phonation, the vocal folds (Fig. 1) are brought together near the centre of the larynx by muscle attached to the vocal folds basis. As air is forced through the vocal folds, they vibrate and produce sound. When vocal folds does not move well enough to be close each other in the midline during speech, air leaks out too quickly thus causing the voice to sound breathy and weak. In these cases, injection or implantation of augmentation substances may be suited to achieve complete glottal closure. The ideal substance should be easy to inject and able to maintain the volume of the augmented vocal fold over time. It has to devoid of immunogenic and toxic effects and must not influence the viscoelastic properties of vocal folds and their vibratory capacity [1–4]. Indeed the vocal folds biomechanical functions such as vibratory capacity, ability to initiate and sustain phonation (phonation threshold pressure) are strictly related to viscoelasticity of the vocal folds tissues, such as lamina propria which depends on macromolecular components of its extracellular matrix (ECM). The ECM contains fibrous proteins, collagen and elastin, and interstitial constituents, proteoglycans and glycosaminoglycans, among which hyaluronic acid (HA) is one of the main components. HA is a natural occurring, non sulphonated glycosaminoglycan composed of repeating disaccharide units of β-D-glucuronic acid and N-acetyl-β-D-glucosamine residues linked at the 1, 4 and 1, 3 positions, respectively, with a molecular weight ranging from 100 to 4000 KDa and it is present in all extracellular matrices of human connective tissue. HA molecules, are characterized by a random coil configuration in aqueous solution, with a large excluded volume, which determines the viscoelastic properties of tissues [5–8].

In laryngeal augmentative surgery nowadays, the most commonly used materials are Teflon, collagen and fat. They all have different drawbacks, such as granuloma formation and migration from the injected site as reported for Teflon [9], absorption which leads to the need for multistage surgical procedures when collagen and fat are used [10–11]. To overcome the above mentioned problems and because of its biophysical properties, hyaluronic acid based materials have attracted research interest for their potential use in vocal folds augmentation surgery. It has been reported that some HA based compounds [1] give, from a biological point of view, good results when injected in the vocal folds; they provide, indeed, a durable augmentation without causing any serious inflammatory or adverse reaction [1, 12].

The aim of this work was to study the viscoelastic properties of vocal folds injected with HA-based compounds, in an animal model (rabbit) after 6 months from

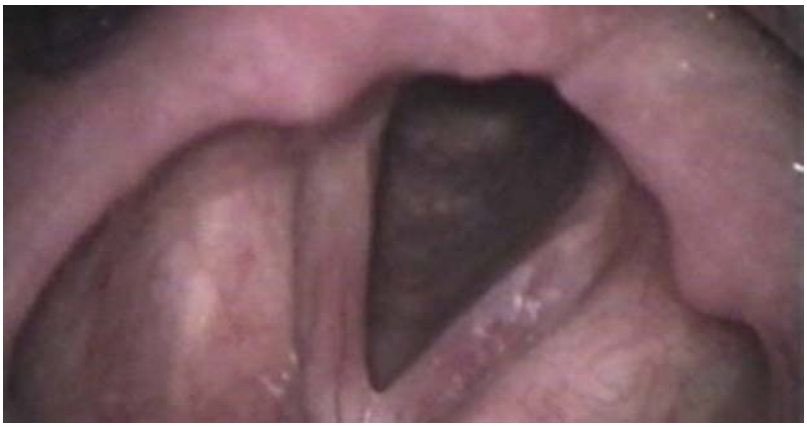

Figure 1 Human Vocal Folds.

the injection, to ascertain that those materials do not alter the vocal folds biomechanical properties related to their viscoelasticity. Histological analysis has been also performed to investigate on the fate of the injected materials after six months from the implant.

2. Materials

Restylan (Q-Med AB, Uppsala, Sweden) is a biodegradable, non-animal stabilized hyaluronic acid (NASHA(TM)) at a concentration of 20 mg/ml. It is produced biotechnologically by fermentation using a stabilization process.

Hylan B gel (Biomatrix/Genzyme Biosurgery, Ridgefield, NJ, USA) is a hydrogel derived from hyaluronan. Hylan B gel is results from cross-linking reaction of hyaluronan with divinylsulfone. Hylan B gel concentration was 5.5 mg/ml, in a hydration fluid of 0.15 M NaCl. The average size of particles in Hylan B gel slurry is 200–700 microns.

Deflux® (Q-Med, Uppsala, Sweden) is made of dextranomere microspheres and 1% hyaluronic acid solution. The dextranomere particles consist of a three dimensional network of a cross-linked dextran.

3. Methods

3.1. Injection procedure

Injections were performed into the left vocal folds of New Zealand white rabbits, the right one served as control. Rabbits were chosen as an animal model since their larynx has a relatively human-like complexity and anatomy [13].

The American principles of laboratory animal care and the Swedish National law on animal care ethics were followed.

The animals were pre-medicated with a tropine (2 mg/kg,s.c.) and diazepam (1 mg/kg, i.v.) and then anaesthetised with Hypnorm® ((fluanizonum, 10 mg/ml, fentanyl, 0.2 mg/ml) 0.3 ml/kg, i.m.). The internal larynx was visualised by means of a modified paediatric laryngoscope (Parson, 8576E, Karl Storz Endoscope, Tüttlingen, Germany) and an otomicroscope. The injections of the augmentation materials, used as received, were performed with a High Pressure Handle (27200, Karl Storz Endoscope, Tüttlingen, Germany) coupled to a needle (0.5 mm outer diameter) into the lamina propria and/or the medial partofthethyroarytenoid muscle in one vocal fold. Groups of four animal each were formed. In each one 0.07–0.08 ml of the following substances were injected: Restylan, Hylan B gel, Deflux®.

To study the long term effect of the augmentation materials, after the injection, the animals were kept alive for six months.

3.2. Histological analysis

The fixation and staining procedures for hystological analysis were done according to well estabilished procedures [7] and briefly reported as follows.

After animal sacrifice, the larynges were transferred to a solution of 2% formalin and 0.5% glutaraldehyde in a 0.1-M phosphate-buffered saline (PBS), pH 7.35. Each specimen was fixed by irradiation in a microwave oven at a setting of 450 °C for 5 min, 630 W. After dehydration the specimens were embedded in paraffin wax. Serial sections (3 to 5 μm) of the glottic region were cut in the horizontal plane, and the sections were mounted on glass slides. Sections from the right vocal folds served as a control.

After deparaffinization in xylene, routine hematoxylin and eosin and Gieson's staining were performed. Alternate sections from the specimens, resected after 6 months, were stained for histochemical localization of hyaluronan. The sections were examined in a light microscope and photographed in a Zeiss Axiophot microscope.

3.3. Rheological measurements

A Bohlin VOR Rheometer (Bohlin Reologi A B, Lund, Sweden), strain controlled, was used to evaluate the dynamic viscosity of the samples as a function of frequency during small amplitude oscillatory shear tests. Frequencies from 0.01 to 10 Hz were tested. The geometry of the measuring system was plate and plate.

The size of the samples (about 4 mm in length, 2 mm thick and 3 mm deep) proved to be optimal to fit into the rheometer and the results were also consistent during repetitive measurements. Anyway, statistical analysis was performed by means of one way ANOVA test, where P-values <0.05 were considered significant.

The tests were performed at temperature controlled by a thermostatic bath (37 ± 0.1).

The strain sweep tests were performed to evaluate the region of deformation in which is valid the linear viscoelasticity [3].

In dynamic experiment the material is subjected to a sinusoidal shear strain:

$$\gamma = \gamma_0 \sin(\omega t) \tag{1}$$

where γ_0 is the shear strain amplitude, ω is the oscillation frequency (which can be also expressed as $2\,\pi f$ where f is the frequency in Hz) and t the time. The mechanical response, expressed as shear stress τ of viscoelastic materials, is intermediate between an ideal pure elastic solid (obeying to the Hooke's law) and an ideal pure viscous fluid (obeying to the Newton's law) and therefore is out of phase respect to the imposed deformation as expressed by:

$$\tau = G'(\omega)\gamma_0 \sin(\omega t) + G''(\omega)\gamma_0 \cos(\omega t) \tag{2}$$

where $G'(\omega)$ is the shear storage modulus and $G''(\omega)$ is the shear loss modulus. G' gives information about the elasticity or the energy stored in the material during deformation, whereas G'' describes the viscous character or the energy dissipated as heat. The dynamic viscosity is a measure of the gel resistance to shear flow which is always associated with internal dissipation, generally, as heat. The dynamic viscosity η' is related to the loss modulus G'' by:

$$\eta' = \frac{G''}{\omega} \tag{3}$$

4. Results and discussion

Small amplitude oscillatory shear experiments were performed to evaluate the frequency dependence of dynamic viscosity of rabbit non-injected vocal folds (controls) and those injected with different materials.

In Fig. 2 the dynamic viscosity as a function of frequency of the vocal folds injected with Deflux and Hylan b gel and of the non-injected vocal folds used as

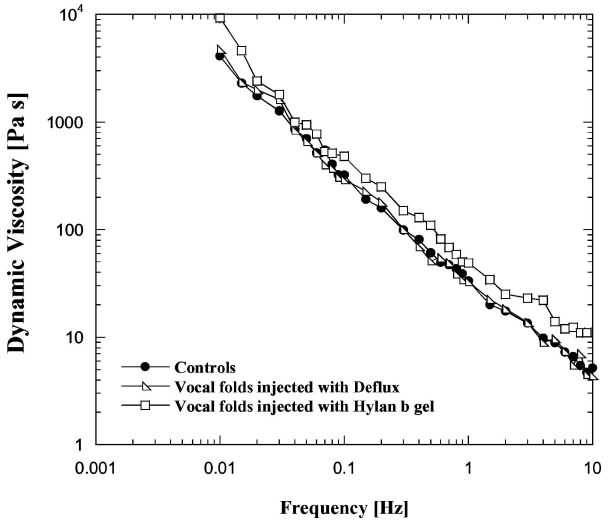

Figure 2 Dynamic Viscosity at 37 °C of control samples and of rabbit vocal folds injected with Hylan b gel and Deflux after 6 months from the injection.

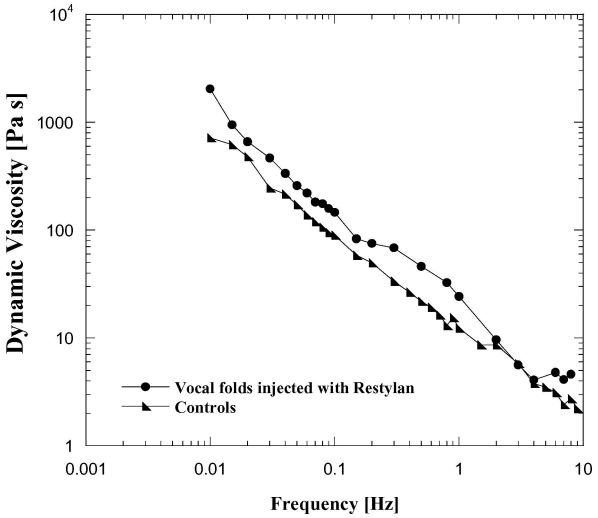

Figure 3 Dynamic Viscosity at 37 °C of control samples and of rabbit vocal folds injected with Restylan after 6 months from the injection.

control is reported. In Fig. 3 dynamic viscosity of vocal folds injected with Restylan and of the respective control is shown. The dynamic viscosity values of the controls used for Restylan are lower than those of the controls used for Delux and Hylan b gel. This could be ascribable to variability in the animals characteristics.

All the vocal folds tested showed a dynamic viscosity monotonically decreasing with frequency. This is the typical rheological behaviour of vocal fold mucosa and, more specifically, of all soft tissue made up of ECM. In particular, among vocal folds proteoglycans, hyaluronic acid plays important roles in determining their biomechanical properties [6]. Indeed, this bio-macromolecule can deform and align its flexible chains in the streamlines of shear flow thus leading to a decrease of tissue viscosity.

Generally, the viscosity of a material is a measure of its resistance to flow and is always associated to dissipation of internal energy as heat and also in the case of the phonation, the energy involved in the process is associated to the viscosity. In particular, the energy involved during the phonation process can be divided into two types: the energy for initiate phonation, known as phonation threshold pressure (P_{TP}), and the energy to sustain phonation (E_p) and both energies strongly depend on viscosity of vocal folds tissue. In detail, Titze described two quantitative relationship, equations 4 and 5, which correlate those quantities to vocal folds tissue characteristics:

$$P_{TP} = \frac{\mathrm{VDW}}{T} \tag{4}$$

where V is the mucosal wave velocity, D is the tissue-damping coefficient (which is proportional to tissue viscosity), W is the prephonatory glottal width, and T is the thickness of vocal folds. Moreover:

$$E_p = \left(\frac{LT}{D}\right)\eta'\omega^2\xi^2 \tag{5}$$

where L, T and D are the length, thickness and depth of the vocal folds, respectively, η' is the tissue viscosity,

TABLE I Dynamic viscosity values at 100 Hz, of the vocal folds injected with the different materials, extrapolated from the experimental data by least-squares regression (η(100 Hz)). Value of the ratio between the dynamic viscosity values at 100 Hz of the vocal folds injected with the different materials and the dynamic viscosity values of the corresponding healthy vocal folds ($\eta_{\text{injected vocal folds}}/\eta_{\text{control}}$)

Sample	η(100 Hz) (Pa*s)	$\eta_{\text{injected vocal folds}}/\eta_{\text{control}}$
Control samples (Restylan)	0.26	–
Vocal folds injected with Restylan	0.39	1.5
Control samples (Deflux and Hyaln b gel)	0.48	–
Vocal folds injected with Deflux	0.45	0.94
Vocal folds injected with Hylan B gel	0.86	1.8

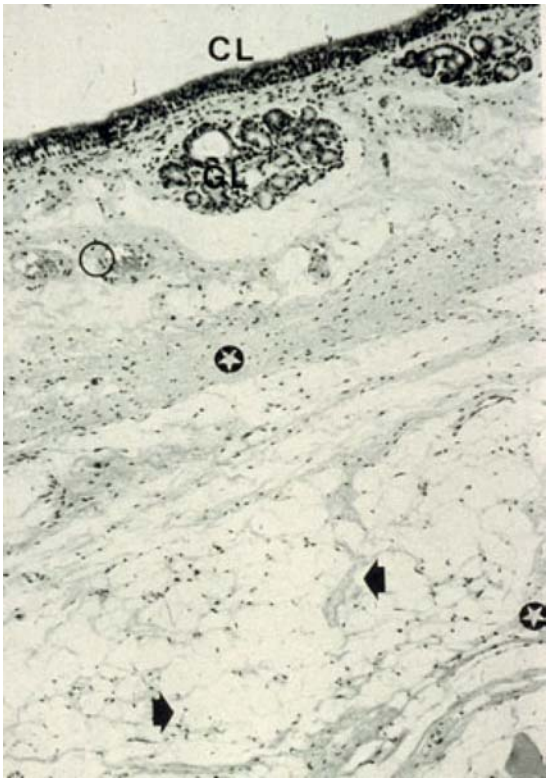

Figure 4 Micrograph of a section of vocal folds injected with Deflux after six months from the injection. Microspheres of Deflux remain (arrows). Persisting collagen (stars) is evident.

ω is the angular frequency of oscillation and ξ is the vibrational amplitude [14, 15].

The typical male vocal folds vibrating frequency is about 100 Hz, value not accessible by the conventional testing apparatus. The dynamic viscosity values of the vocal folds injected with the different materials at 100 Hz were extrapolated by least-squares regression and normalised to those of the corresponding healthy vocal folds. The results are reported in Table I. According to Equation 5, from the knowledge of the normalised values it is possible to estimate the energy increase required to sustain phonation in case of augmented vocal folds.

The results indicate that the use of HA based-compounds as augmentative materials implies a little increase of energy to sustain phonation, being less than twice the energy involved in the physiological phonation process.

Moreover, histological analysis have been performed to investigate on the fate of the injected materials after six months from the implant. From the micrograph (Fig. 4) of a section of a vocal fold injected with Deflux it is possible to notice that after six months the microspheres are still present. The dextranomeres recruited fibroblasts that generated new collagen resulting in an endogenous soft tissue augmentation. Only a minimal inflammatory reaction was noted.

The histological study performed on Hylan b gel (Fig. 5) has shown that, when injected into vocal folds, this materials can remain up to six months, it promotes the ingrowth of newly formed connective tissue thus giving a durable augmentation of vocal fold. The new soft connective tissue contains collagen, HA and fibroblasts. Looking at the micrograph of the vocal fold injected with Restylan (Fig. 6), it is possible to notice that also this HA based material stays up to six months stimulating the formation of new connective tissue. In conclusion from a biological point of view the HA based materials gel attracted fibroblasts, which generated new collagen and ECM macromolecules, resulting in endogenous soft tissue augmentation, without causing any serious inflammatory or other adverse reactions.

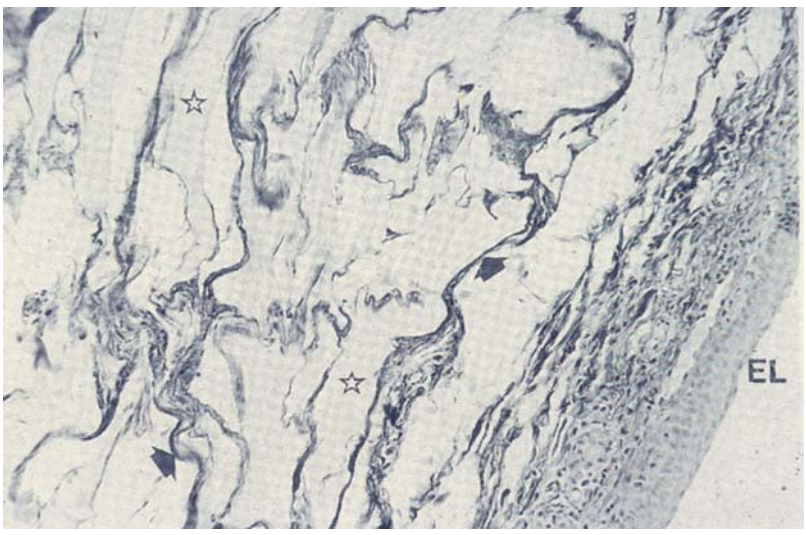

Figure 5 Micrograph of a section of vocal folds injected with Hylan b gel after six months from the injection. It is shown collagen formation (arrows) within the Hylan b gel materials (stars).

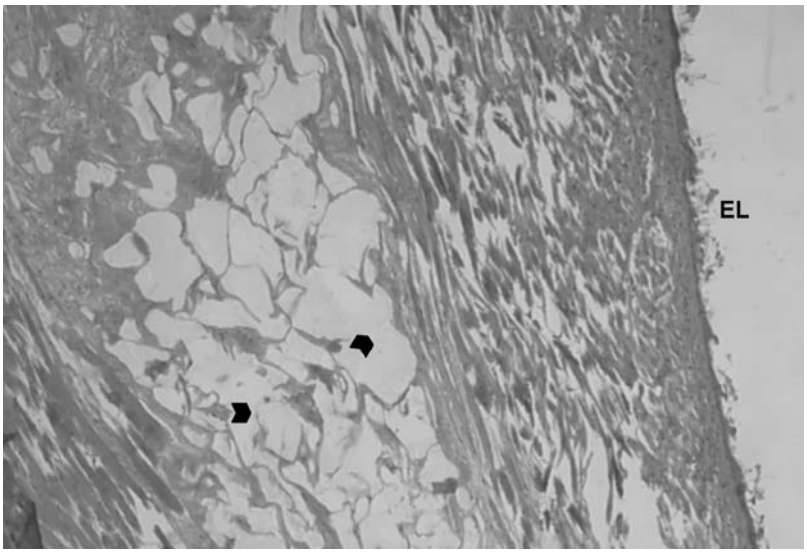

Figure 6 Micrograph of a section of vocal folds injected with Restylan after six months from the injection. Restylan is still present after six months (arrows).

5. Conclusions

In laryngology, as in other surgical specialities dealing with augmentative surgery, there has been an intensive search in recent years for alternative biocompatible materials with optimal performances, as compared with those previously or currently in use. In this field when voice problems connected with glottal insufficiency occur, the injections of augmentation substances in the vocal folds are often used to achieve complete glottal closure. These substances must not influence the viscoelastic properties of vocal folds and their vibratory capacity

In this work, we investigated the viscoelastic properties of vocal folds in an animal model, after injection of different hyaluronic acid based materials six months after the injection of implantation. The results from this animal experiment indicated that the HA based materials , Restylan, Deflux®, and Hylan B gel, do not alter the viscoelastic properties of the vocal folds. Moreover, the HA based materials gel attracted fibroblasts, which generated new collagen and ECM macromolecules, resulting in endogenous soft tissue augmentation. The novel connective tissue, originated from the host itself, gives a durable vocal fold augmentation without causing any serious inflammatory or other adverse reactions.

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Received 15 August
and accepted 15 October 2004