An In Vivo Study of a Novel Composite Hyaluronic Acid and Gelatin Hydrogel to Improve Healing of Vocal Fold Scars in a Rat Model

By

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Dedication

To my loving mother and to my precious wife.

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List of Abbreviations
HA: Hyaluronic Acid

Ge: Gelatin

DXN: doubly cross-linked network

HA-Ge: Hyaluronic Acid – Gelatin hydrogel

LP: lamina propria

PCA: posterior cricoarytenoid

LCA: lateral cricoarytenoid

CT: cricothyroid

ECM: Extracellular matrix

HGF: Hepatocye Growth Factor

ROI: Region of interest

ANOVA: analysis of variance

Abstract

Objectives/Hypothesis:

The primary objective of this study was to investigate the healing potential of a novel hierarchically micro-structured Hyaluronic Acid (HA)-gelatin (Ge) hydrogel in the treatment of acute vocal fold injury using a rat model. A secondary objective was to evaluate the feasibility of the rat vocal fold for scar and injection studies.

Study Design: Experimental Randomized Prospective Study

Methods:

Vocal fold injury was performed unilaterally in 36 rats. The animals were stratified into three groups. Each group had 25 µl of either saline, HA bulk or HA-gelatin hydrogel injected into the lamina propria five days after injury. Vocal folds were then harvested at 56 days after injection and were analyzed using immunohistochemistry. Immunofluoresence staining was performed on collagen type I, collagen type III and elastin

Results:

No major reaction to the injectable material was observed. When comparing protein densities between the right injured and left uninjured vocal fold; Type I collagen densities was higher in the saline and HA-Ge groups relative to the uninjured samples (p=0.31 and 0.917 respectively). Collagen type III densities, on the other hand, were greater than in the uninjured controls in both HA-bulk and HA-GE groups (p=0.012 and 0.028 respectively). The density of elastin was higher in the HA-bulk and HA-GE groups when compared to the uninjured vocal folds but statistically significant in only the HA-GE group (P=0.128 and 0.036 respectively).

On the other hand, when comparing protein densities on the right vocal fold between the treatment groups; we found The relative densities of elastin and collagen III were greater in the HA-bulk group than in the saline group (p = 0.032 and 0.07, respectively). Likewise, elastin and collagen type III were of greater densities in the HA-GE group than in the saline group (p =0.014 and 0.004, respectively).

Conclusions:

Local HA-gelatin injection shows some potential tissue remodeling and did not cause any inflammatory response during the course of this study. The rat vocal fold is an excellent model for laryngeal studies.

RÉSUMÉ
Objectifs:

L'objectif primaire de l'étude est d'évaluer le potentiel guérisseur de l'injection d'un nouvel hydrogel d'acide hyaluronic-gelatiné (AH-Ge) dans le traitement d'une blessure aiguë sur une corde vocale de rat. Un objectif secondaire est d'évaluer l'utilité du rat en tant que modèle pour l'étude des cicatrices et des injections sur les cordes vocales.

Conception de l'Étude: Étude Expérimentale Prospective Randomisée

Méthodes:

Une lésion a été faite sur les cordes vocales droites de 36 rats. Les rats ont ensuite été séparés en trois groupes. Vingt-cinq microlitre de solution saline, d'acide hyaluronic (AH) ou d'hydrogel AH-Ge ont été injectés dans la lamina propria cinq jour après le trauma initial. Les cordes vocales ont été retirées 56 jours après l'injection afin d'être analysées par immunohistochimie. La coloration par immunofluorescence a été réalisée sur du collagène de type I, du collagène de type III et l'élastine

Résultats:

Aucunes réactions majeures à la matière injectée n'ont été observées. Lorsque l'on compare la densité des protéines entre les cordes vocales blessées (droite) et les cordes vocales intactes (gauche), la densité de collagène type I est plus élevée dans les spécimens lésés ayant reçu une injection saline ou d'AH-Ge par rapport aux spécimens intacts (p=0.31 et 0.917 respectivement). D'autre part, la densité de collagène type III est plus élevée dans les spécimens lésés ayant reçu une injection d'AH et d'AH-Ge (p=0,012 et 0,028 respectivement). La densité d'élastine est aussi plus élevée dans le groupe ayant reçu une injection d'AH et celui ayant reçu l'AH-Ge. La différence était toutefois seulement significative dans le groupe d'AH-Ge (p=0,128 et 0,036, respectivement).

En comparant les densités des protéines parmi les divers traitements, on observe que les densités d'élastine et de collagène type II sont plus élevées dans le groupe ayant reçu de l'AH que dans le groupe ayant reçu une solution saline (p = 0,032 et 0,07, respectivement). De plus, l'élastine et le collagène type III sont en plus grandes densités dans le groupe ayant reçu de l'AH-Ge que dans le groupe ayant reçu une solution saline (p = 0,014 et 0,004, respectivement).

Conclusion:

Dans cette étude, l'injection locale d'AH-Ge semble avoir provoqué des changements au niveau tissulaire sans causer de réponse inflammatoire. Aussi, les cordes vocales de rats sont un excellent modèle afin d'effectuer des études sur le larynx.

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Chapter 1: Introduction

Scarring of the vocal folds is a common problem. Its true incidence is under-reported and there is lacking of literature about its prevalence. It may be secondary to trauma, smoke inhalation, acid reflux, allergies, inflammation and cancer. Scarred vocal folds are significantly stiffer and more viscous than normal vocal folds, owing to the fact that normal vocal fold tissue is replaced by scarred fibrotic tissue. This produces a voice disorder that may manifest as hoarseness and glottic incompetence. Treatment of scarred vocal folds is often required, as it is known that voice disorders in general have a significant impact on quality of life (1, 2). Surgical management of scarred vocal folds, although beneficial, may worsen the problem by creating more scars.

Different modalities aiming to treat vocal fold scarring have been proposed, include tissue replacement with flaps and grafts (3, 4) . Other injection materials, either permanent such as Teflon, which became obsolete in the last decade, or temporary such as fat (5) and cross-linked collagen, have also been utilized (6) . One major problem with these biomaterials is that they degrade rapidly over time and require frequent re-injection to maintain glottic competency (3). Also, all the above-mentioned materials do not restore the injured lamina propria to its normal shape; thus, viscoelasticity remains suboptimal.

To date, a satisfactory treatment modality for scarred vocal fold remains to be identified. Tissue engineering is a promising new field with the potential to produce an approach that would restore the disrupted lamina propria after injury. Three elements can be manipulated with the use of tissue engineering: the scaffold, cells and regulatory

factors (4). The development of scaffolds is aimed at facilitating the cross-linking between local host tissue and transplanted tissue for cellular regeneration and, eventually, the restoration and regaining of normal function. Regulatory constituents such as growth factors are essential elements to control cells in order to successfully achieve tissue formation (7).

One of the most popular and frequently used materials for vocal fold scarring is hyaluronic acid hydrogel, which usually promotes fibroblast migration (8-10). Gelatin has been successfully used for a range of applications including burn dressings, cardiovascular surgery and scaffolding for tissue engineering of skin (11). Combining gelatin with HA hydrogels improved cell attachment and the migration of fibroblasts (12) and also yielded improvements in viscoelastic properties during tissue repair (11). A doubly cross-linked network, or DXN, allows for easy modulation of their viscoelasticity, decreased degradation, which obviates the need for re-injection due to increased residence time in situ (13). Previous research has validated the success in the cross-linking of gelatin to HA for wound dressing and healing.

During the in vitro phase of the present study, gelatin was incorporated into DXNs (Figure 1), with the goal of combining favorable mechanical properties with a bioactive facilitator in tissue repair and engineering (14).

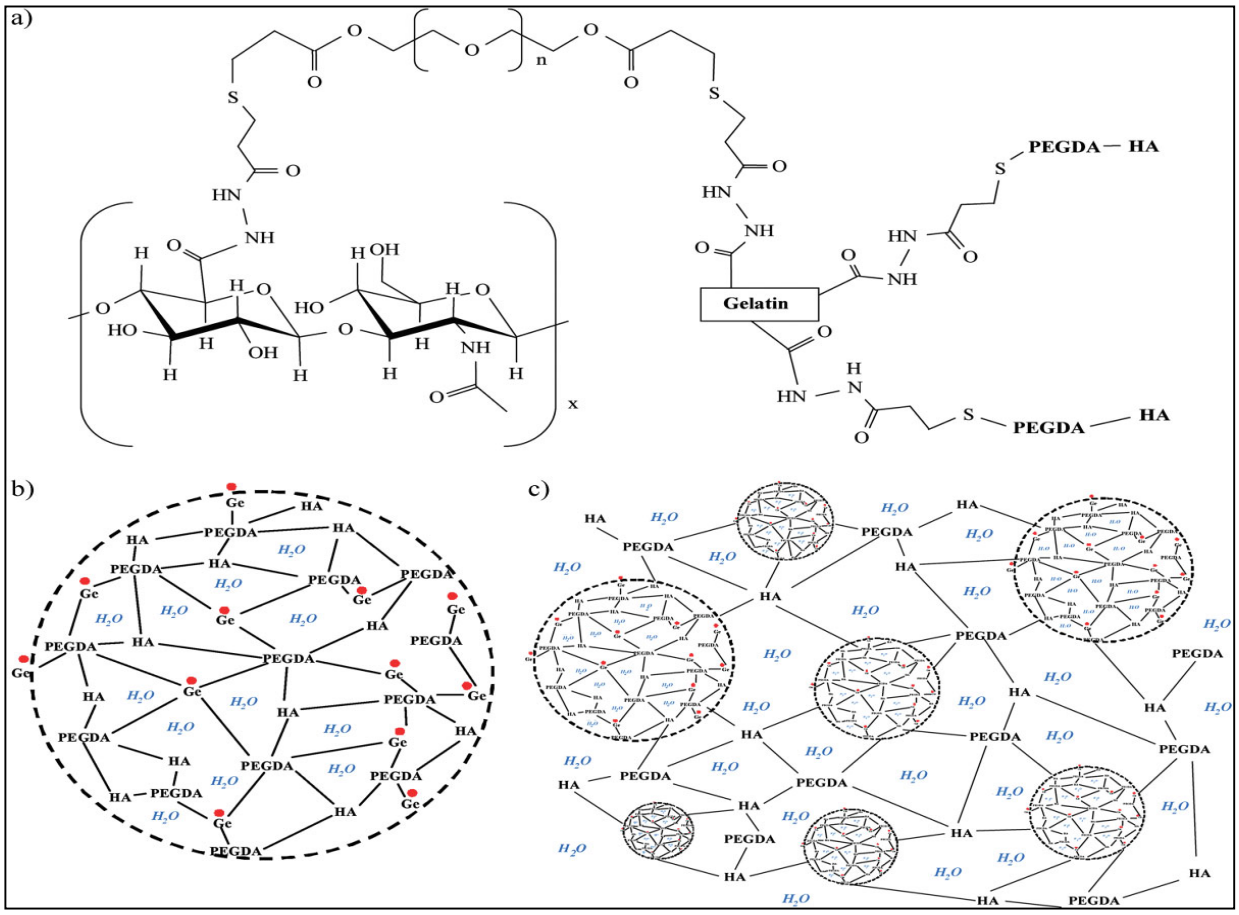

Figure 1: a)Schematic of thiol-modified HA and Ge crosslinked by Polyethylene (glycol) Diacrylate (PEGDA).b) Microparticle of crosslinked HA and Ge. c) Hierarchical network of HA–Ge microgels with HA network in water.(14)

To date, promising results in vitro have supported the effectiveness of newly created HA- Ge hierarchical hydrogels as a scaffolding material for vocal fold regeneration (14).

The results of the above in-vitro study (14) suggested that usage of HA-GE would improve vocal fold function after injury. Attempting to validate this suggestion was the rationale of the current study. To confirm these results, an in vivo study is needed to test

the hypothesis of tissue-regenerating capabilities. In this study, a rat model of acute vocal fold injury was used to assess the biocompatibility of the hierarchically structured HA- gelatin hydrogel and its potential for tissue reconstruction.

We hypothesize that the HA-Ge biomaterial will not cause inflammatory response, will provide a scaffold to facilitate vocal fold cell migration, attachment and proliferation and will provide the injected tissue with the structural integrity needed for enhanced wound healing. The primary aim of the current study is to test this hypothesis. A secondary aim is to assess the rats' larynges as a suitable model for vocal fold injury and injection studies. We compared the effects of our novel Ha-Ge composite with those of control materials on the healing of surgically induced vocal-fold injury in a Sprague-Dawley rat animal model. The specific methodology and our findings will be discussed in the coming chapters in detail.

Chapter 2: Literature Review

2.1 The vocal fold

2.1.1 Anatomy

The human vocal folds are a pair of laminated structures composed of five different layers: the epithelium, the lamina propria (LP), which is further subdivided into superficial, intermediate and deep layers, and the vocalis muscle (Figure: 2). The epithelium and superficial LP are jointly called the mucosa, while the intermediate and deep LP make up the so-called vocal fold ligament. The body of the vocal fold is the vocalis muscle. The muscle is attached to the thyroid cartilage anteriorly and extends posteriorly to the vocal processes of the arytenoid cartilages on both side

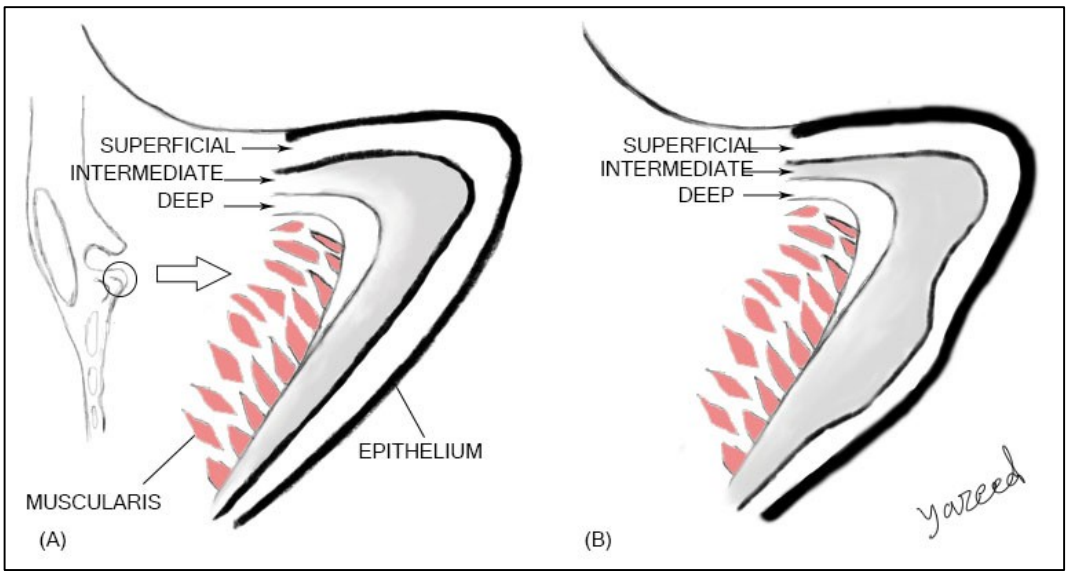

Figure 2: Sketch of the layered structure of the vocal folds. A) Coronal section of the vocal fold; and B) deep layers of the vocal fold.

The vocal folds show variations in size in both genders. Adult males typically have larger, longer folds due to the gender dimorphic laryngeal prominence, resulting in a lower-pitched voice. This gender-related dimorphism manifests after puberty and is believed to be secondary to increased testosterone levels, which promotes laryngeal cartilage growth (15).

Several muscles control the tension and the motion of the vocal fold. The main function of the vocalis muscle (thyroarytenoid muscle) is to maintain vocal fold tone. It adducts, lowers, shortens and eventually thickens the vocal fold. The posterior cricoarytenoid (PCA) muscle is the only muscle that abducts the vocal fold; it also elongates and thins the vocal fold. The lateral cricoarytenoid (LCA) muscle is another adductor, which by its action medialize and stritch the vocal fold. The inter-arytenoid muscle is the only unpaired muscle that contributes to vocal fold adduction with little effect on stiffness. The cricothyroid (CT) muscle rotates the cricoid cartilage with respect to the thyroid cartilage, resulting in elongation of the vocal fold (2). All internal laryngeal muscles receive innervation from recurrent laryngeal nerve except the CT muscle, which is innervated by the superior laryngeal nerve (Figure: 3).

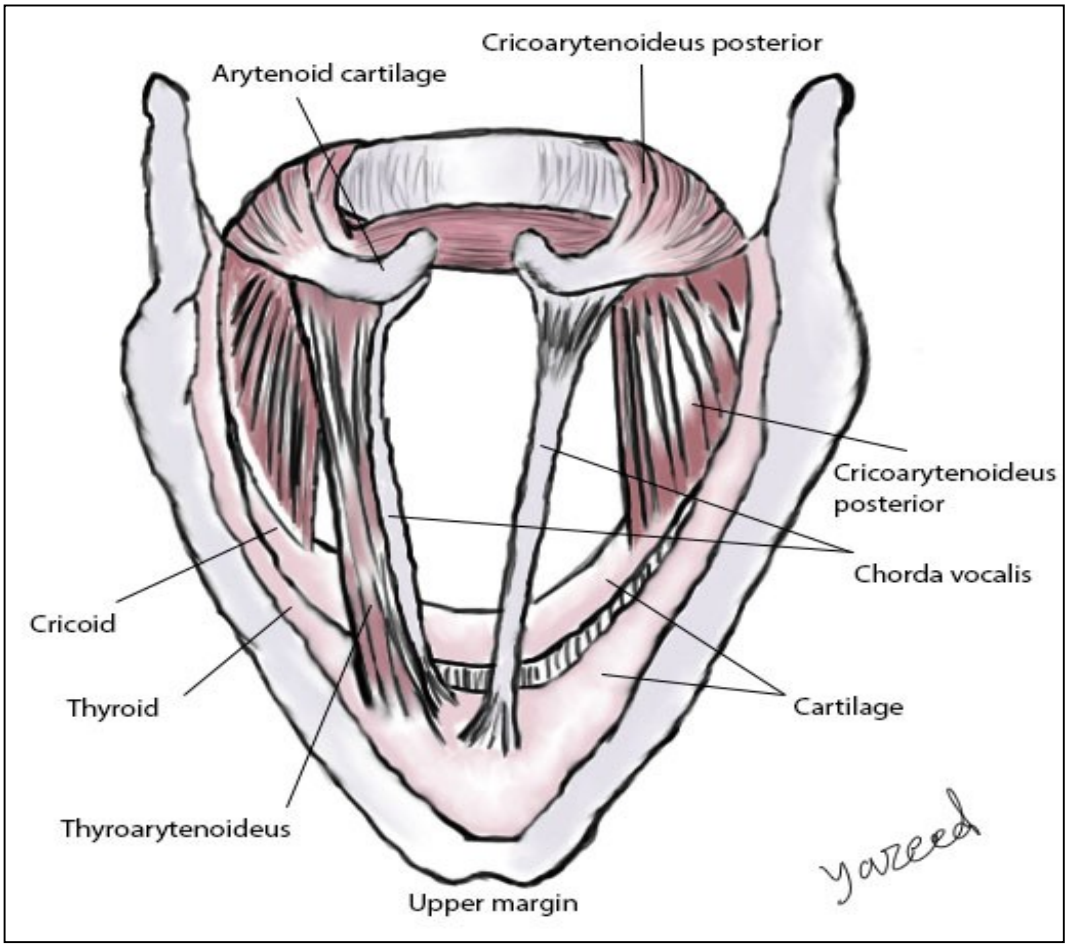

Figure 3: Illustration of the intrinsic muscles of the larynx, superior view.

2.1.2 Histology

The subdivisions of the lamina propria (superficial, intermediate and deep layers) differ in the distribution of fibrous components, specifically elastin and collagen. Vocal fold tissues have been shown to be a viscoelastic organ that demonstrates both viscous and elastic properties (9). Extracellular matrix constituents are collagen, procollagen, elastin and hyaluronic acid. Collagen (types I & III) is considered the main component of scar tissue. The main role of collagen in vocal fold healing is not well

understood, but it has been shown that collagen III acts as a scaffold for the granulation tissue, while collagen I adds strength to an incipient fragile wound (16). Increase in collagen type I is believed to be the basis of fibrosis and scar strength (17). Tensile elasticity of the vocal fold is usually maintained by elastin. Elastin and collagen play important roles in phonation and vocal fold vibration (18, 19).

In the last two decades, numerous research studies have been performed in order to evaluate the histological changes in vocal fold scars. Thibeault et al. have shown that collagen plays a role in scar tissue, and other ECM components could also participate in this mechanism (9). They used a rabbit as the animal model; a unilateral vocal fold injury was made, and the animal was euthanized two months after the injury to facilitate the development of a chronic scar. They found an increase in fibronectin and procollagen and decreased decorin and fibromodulin. The most striking observation in that study is increased tissue stiffness regardless of the total content of collagen.

Hyaluronic acid (HA) is a glycosaminoglycan that is widely distributed in the superficial lamina propria and is believed to be an important contributor to the viscoelasticity of the vocal fold (18). HA has been shown to decrease the collagen level in injured human skin tissue (20). In fetal wounds, increased HA levels are believed to prevent scarring in some wounds (21).

2.1.3 Vocal fold scar

Injury to the vocal fold lamina propria, usually in the form of repeated phonotrauma or inflammation, often leads to structural changes and scarring, which has been reported in human (22) and in animal models (16, 23-25). It causes a marked diminished vibration of the vocal fold mucosa, resulting in a hoarse, breathy and

sometimes weak voice (22). Once scar tissue forms in the lamina propria of the vocal fold, the viscosity and shear strength of the vocal fold changes; this eventually disrupts the normal mucosal wave during phonation (26). Scarred tissue is characterized by an increase in organized thick-bundled collagen matrix with a defragmented, disorganized elastin fiber network (23, 24). The changes in the density and distribution of protein following lamina propria scarring affect the biomechanical properties of the vocal fold tissue and, consequently, voice quality.

Singers, teachers and other professionals who use their voices extensively are more prone to vocal fold scars. Vocal fold scarring may also affects patients who undergo endoscopic laser surgery for any glottic lesions mainly neoplasm (22). Despite the recent advances in laser surgery techniques, postoperative scarring is still an inevitable complication.

2.2 Animal model

2.2.1 Overview

Animal models have been used in vocal fold research because they show similar characteristics to human vocal fold. Rabbits and dogs have been used extensively in the past to study vocal fold anatomy and scarring. Rats have also been reported to be an excellent model for vocal fold scarring due to the similarities of the vocal fold LP between rats and humans (25).

2.2.2 Canines

The canine larynx is the most commonly used model for scar and other laryngeal studies. Its size and overall gross structure are similar to the human larynx; it also phonates in a laboratory setting, and produces vocal fold vibration similar to that observed in humans. Despite these similarities, it is difficult to extrapolate their results to humans due to the differences in the histological structures of the vocal fold (27). Furthermore, dogs have a poorly defined vocal fold ligament, which makes them a challenging research model. For these reasons, other animal models, such as rabbits and rats, are utilized more often nowadays for vocal fold research.

2.2.3 Rabbits

Rabbits are commonly considered in studies of vocal fold scars due to the similarities of their lamina propria to human vocal folds (9). Rabbit vocal folds basically have the same three-layer structure as humans. However, there are some differences in vocal structures between rabbits and humans. For example, the vocal ligament is better developed in humans (10); see Table 1 for a comparison.

	Rabbits	Humans	Canines
Superficial Lamina propria	Loose ground substance	Loose ground substance	Sheets of collagen and elastins
			Loose ground substance
Intermediate lamina propria	Ground substance and collagen	Dense ground substance and elastin	Ground substance and collagen
Deep lamina propria	Collagen	Collagen	Collagen

Table 1 A schematic diagram showing the vocal fold tissue layers in rabbit, human, and canine larynges.(27)

2.2.4 Rats

The rat model has several advantages for studies of vocal fold scars. It has a tri-layered structure as for human vocal fold (28). Also, the scarring behavior in rats is somewhat similar to that in humans. It has been reported that chronic scars form within two months, while it takes as long as six months in canines and rabbits (23, 24). Scar healing in rats is expected to be fast due to their short lifespan, making them suitable for this type of study. Petersen et al. extensively studied the genome of rat vocal folds and reported important data relating to their biological and genetics constituents (29). The deep layer contains more collagen fibers than the superficial layer, which is similar to what is found in human lamina propria (25). Additionally, rats are cost-effective when compared to rabbits and canine models. However, one of the drawbacks of rats is that they do not phonate, which makes them unsuitable for rheological studies. Another limitation is the difficulty in enforcing voice rest as a form of therapy following an experimentally induced injury as in human vocal fold; this limitation is common to all animal models.

2.3 Vocal Fold scar Management

2.3.1 Introduction

Vocal fold scar is a challenging pathology and require multidisciplinary treatment including voice therapy, medical treatment and perhaps surgery. Regardless of the causative factor of the scar, injured vocal folds display a disruption in the mucosal wave often associated with glottic incompetency, which leads to clinically discernible voice problems and possible aspiration. Patients with scarred vocal folds usually complain of hoarseness, voice fatigue, strain and breathiness. Mucosal stiffness prevents the epithelium from traveling medially, which leads to a glottic gap and breathiness. Voice fatigue is caused by the hyperfunctional supraglottic region, a compensatory mechanism to correct for diminished glottic closure (30). Video-stroboscopy is the best examination modality for vocal fold scars. Findings suggestive of vocal fold scars include the presence of motionless segment, asymmetrical vibration, incomplete closure, absent or loss of mucosal wave and/or change in the amplitude of vibration (31).

2.3.2 Nonsurgical Rehabilitation

Medical management and voice therapy is considered the first line of treatment of vocal fold scars. Surgical intervention has no role in the immediate management of scarred vocal folds, especially without having tried voice rehabilitation and therapy (12). Several parameters can be used to improve the quality of voice such as voice hygiene, resonant voice therapy and vocal flexibility exercises (12). Direct voice therapy has

been described as the mainstay modality; this aims at avoiding and eliminating the compensatory mechanism that results in the reduction of breathing effort and fatigue (3). However, speech therapy has a number of limitations such as failure to address the disrupted histology of the scarred vocal fold. Medical treatment such as antireflux, steroids and antibiotics can be used as a sole treatment or coupled with another modality. The limitations of these modalities include the requirement for prolonged treatment duration and unpredictable outcomes.

2.3.3 Surgical Treatment

Surgical management is utilized when the non-surgical treatment modalities fail. A standard surgical modality for the treatment and prevention of vocal fold scars is yet to be developed. Many surgical approaches have, however, been described. These include injection (32), framework surgery, implants and tissue engineering. The goal of any surgery is to ensure sufficient glottic closure and to restore a mucosal wave. Surgical intervention is usually reserved for the recalcitrant cases that do not respond to medical and voice therapy.

Injection laryngoplasty is the most commonly used treatment for vocal fold scars. Different approaches (transoral and percutaneous) have been used to medialize scarred vocal fold tissue in order to improve voice and prevent aspiration (32). In 1992, Ford and colleagues revolutionized the concept of injection laryngoplasty with the introduction of a temporary injectable collagen to soften the scar tissue and restore glottic competency (6). Many injectable materials have been developed subsequently

such as collagen, hyaluronic acid and autologous fat (33). Most of these injectable chemicals have been tested in both animals and humans and are considered to be safe

Ideally, injectable materials should be bio-compatible and cause minimal or no inflammation and reaction. They should have a slow absorption rate to decrease and prolong the period of reinjection if needed. Fat, which is usually harvested from the lower abdomen of patients, is an autologous material used for vocal fold augmentation (5). The main problem with fat injection is its rapid absorption, which was confirmed in a canine study in a three-month follow-up period (34). To date, none of these injectable materials have shown the ability to restore the disrupted lamina propria.

Medialization laryngoplasty (framework surgery or thyroplasty) describes a different modality of medializing a paralyzed or scarred vocal fold. Isshiki described a procedure with successful medialization of a vocal fold through a small window created in the thyroid cartilage and the insertion of a silastic implant into the subperichondrium lateral to the vocalis muscle (35). This surgery is usually done under sedation in the operating room in order to evaluate the voice during insertion (Figure 4). Again, this procedure does not address the mucosal wave of the scarred vocal fold.

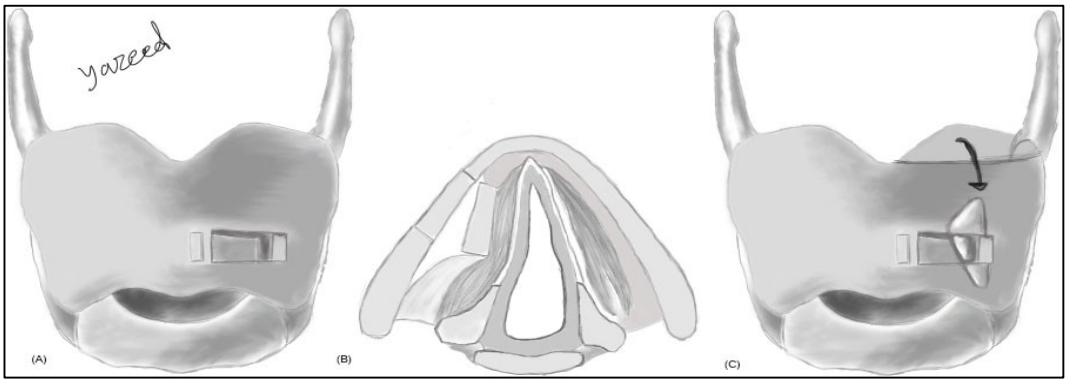

Figure 4: Medialization thyroplasty

2.3.4 New approaches for vocal fold scar management

The absence of an existing optimal approach to the management and prevention of scarred vocal fold tissue makes it imperative to find new approaches that will provide an adequate solution. The relatively newer approaches that have been described include the use of tissue engineering. Implantable biomaterial can be divided into two groups according to the site of implantation. The first group has the implant injected outside the mucosa, which is in, or lateral to, the thyroaryenoid muscle; this is called a non-mucosal implant. This technique facilitates the medialization of the vocal fold as needed in cases of paralysis or paresis. The second group was subjected to direct injection into the mucosa to soften the scar tissue or treat special cases such as sulcus vocalis (36).

It is important to quantify the mechanical viscoelastic of the vocal folds and implantable biomaterials; particularly the Young's modulus, the shear modulus and the poisson ratio. Young's modulus (elastic modulus) is used to measure the stiffness and elastic properties of the vocal fold, it is calculated as the ratio of the stress divided by the strain (37). Shear modulus is another quantity for measuring the stiffness of the vocal fold; specifically it describes the vocal fold response to shear stress(38). The presence of implantable material within the vocal folds can change these properties, with subsequent effects on the mechanics of phonation (36). Therefore, the viscoelastic and shear properties of the implantable material should be tailored to the native tissue properties for the optimal treatment of any vocal fold mucosal defect.

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The challenge of restoring a scarred vocal fold is the presence of multilayer structure lamina propria (39). To date, there has been no successful trial to rebuild the lamina propria to its native status. So, it is imperative to find a scaffold that possesses the ability to do so. An ideal scaffold should have chemical, structural and mechanical properties that are similar to the lamina propria (40).

Hyaluronic acid is widely distributed in the superficial lamina propria and is believed to be an important contributor to the viscoelasticity of the vocal fold, it is one of the ECM molecules investigated largely for the treatment of vocal fold scar. Hyaluronic acid is the major constituent and acts like a shock absorber during phonation (41) and has similar viscoelastic properties to the human vocal fold mucosa (42). HA has been shown to decrease the collagen level in injured human skin tissue(20). A study that compared HA with collagen implant in a rabbit model showed that the viscoelastic properties of HA-injected animal vocal folds were similar to those of the normal vocal fold (43). Hyaluronic acid is a popular and frequently used injectable material for managing vocal fold scarring. In addition to its similarities to normal vocal folds, it also promotes fibroblast migration and decreases the inflammatory process (8-10).

Lately, research has focused on combining HA with other materials. For example HA-dextran has been used and exhibited a promising results, it matches the mechanical properties of vocal fold tissue. The degradation of HA-dextran hydrogels was seen to be consistent with the degree of crosslinking in the hydrogel matrices, but it elicited a foreign body reaction (44). Hyaluronic acid together with fibronectin have been used as vocal fold implants (36). An important function of fibronectin is the promotion of adhesion between cells and ECM. It offers a binding site for several macromolecular

components of ECM, for example fibroblast, collagen and HA. However, studies have shown that fibronectin does not contribute significantly to the viscoelastic and shear properties of HA (36).

Engineered, injectable, modified HA with gelatin was developed by for the purpose of treating vocal fold scars (45). With HA-Ge injected into one side of injured vocal folds and saline into the other side as a control, the HA-Ge-treated vocal folds showed less fibrosis and better viscoelastic and shear properties at six months post-treatment when compared with the saline-treated vocal folds.

Hepatocye Growth Factor (HGF) has also been used to treat vocal fold scarring due its strong activity against fibroblast. Hepatocye Growth Factor suppresses collagen I and promotes HA production (46). Hirrano and colleagues injected HGF into stripped rabbit vocal folds immediately after injury and animals were harvested after 6 months (46). They assessed the ability of HGF to prevent scar formation. The HGF-treated vocal folds demonstrated less collagen and better vibratory function compared to the non-injected vocal folds (47).

Stem cells have also been suggested as a therapy for managing vocal fold scarring. It is a regenerative medical approach that has been attracting a lot of attention in the literature recently. Stem cell is un-differentiate cell that can divide and become differentiated and more specialized. They are known to divide to two main group of cell, Pluripotent (embryonic) that can differentiate to every cell of the body and multipotent (adult) that can differentiate to multiple cells but not all cell linage (48). Although there is inadequate data available in the field of stem cells in laryngology, success in any organ

could be extrapolated to the larynx, since stem cells have the ability to differentiate into any type of mature cell. Vocal fold regenerative medicine aims can be divided into two main goals: the first is to inhibit scarring and fibrosis, and the second is to rebuild the native ECM (39). Kanemaru et al. used mesenchymal stem cells for the treatment of vocal fold injury in a canine. They reported improvement in wound healing in the stem-cell-treated vocal folds (49). The underlying mechanism behind wound healing improvement is, however, poorly understood, as there is a scarcity of published evidence regarding stem cell treatment of scarred vocal folds.

2.4 Thesis Rationale

In view of the above review, there is a need to find a better way for tissue reconstruction and scar treatment after vocal fold injury. The next section will describe the methods of an experimental study on rats that will have the following objectives; to investigate the healing potential of a novel hierarchically micro-structured Hyaluronic Acid (HA)-gelatin (Ge) hydrogel in the treatment of acute vocal fold injury using a rat model. A secondary objective was to evaluate the feasibility of the rat vocal fold for scar and injection studies.

Chapter 3: Materials and Methods

3.1 Study Design

In order to compare utility of HA and HA-GE in injured vocal fold, 36 adult male Sprague-Dawley rats (4-6 months old), each with body weight of around 400-500 g, were used for this study. At the start of the study, all rats were injured in the right vocal fold (as described in Section 3.3). Then 5 days after injury, they were randomly assigned to one of the three treatment groups (i.e., n=12 for each group): 1) saline controls; 2) HA bulk hydrogel; and 3) hierarchically structured HA-Ge hydrogel. All animals were injected in the right injured vocal fold. . The injected volume was 25ul for all animals. This permitted comparisons with the uninjured left vocal fold for each animal, in addition to comparisons between the two treatment methods and the control group (saline injection). Rats from all groups were sacrificed on day 56 after injection.

3.2 Institutional Animal Care

All rats were housed at the Montreal Children's Hospital Research Institute's animal care facility. A period of 7-14 days for acclimatization was allowed. During this period, the animals were weighed twice weekly and cared for in a dedicated room at the animal facility of the Research Institute. Physical examination was conducted on each animal to identify physical anomalies. The animals were housed in clean, quiet and uncluttered rooms, with adequate lighting and a diurnal light cycle (12 hour light/12 hour dark). The room was kept well-ventilated and at a temperature between 68 and 74 ^{0}F. All experimental procedures were approved by The Facility Animal Care Committee of

the Research Institute McGill University Health Centre (RI MUHC), in accordance with the NIH guidelines for care and use of laboratory animals.

Each animal was identified with coded ear punches, and additional identification was provided by the use of cage cards. Approximately two rats were housed in a cage to give adequate space for free movement. The bedding material in each cage was soft, clean and dry, and was changed whenever visibly wet.

The rats had food and water ad libitum. Proper documentation was made on the weight and health status of all animals.

3.3 Surgical Procedure

Anesthesia was induced with inhalational anesthesia using 2-3% isofluorane (Abbott laboratories, Montreal, Canada), maintained in a ketamine/xylazine mixture; ketamine (90mg/kg)(Bioniche, Ontario, Canada) and xylazine (0.4- 0.6mg/kg)(Bayer Healthcare, Ontario,Canada) were administered in a dose of 2ml/kg, given intraperitoneally. To minimize saliva secretion and maintain the dryness of the larynx, diluted atropine sulfate (Alveda Pharma, Toronto, Canada) was also given in a dose of 1ml/kg, intraperitoneally. Carprofen 4mg/kg was given to reduce post-op pain. Assessment of depth of anesthesia was made by checking for loss of pedal withdrawal reflex. When deeply asleep, the animal was mounted on an operating platform, which allowed the animal to be positioned in a near-vertical supine position with the head up (Figure 5). The mouth was maintained opened using a fabricated mouth gag. The oral cavity, base of the tongue and vocal folds were then further anaesthetized with topical 1% Lidocaine (Astra Zeneca, Ontario, Canada).

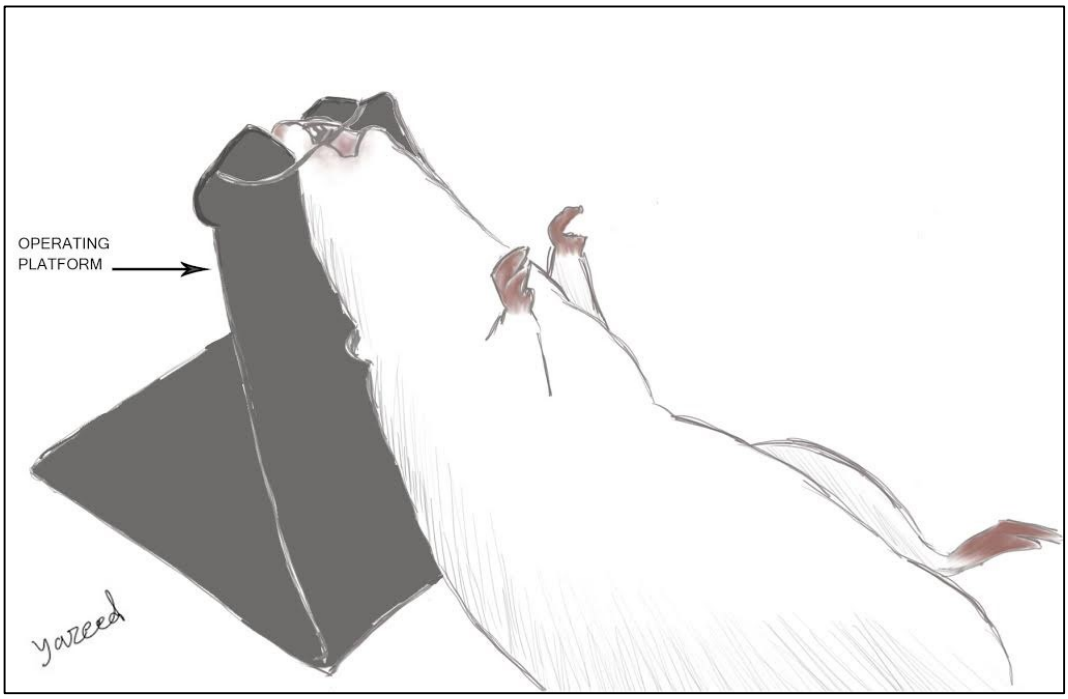


Figure 5: The surgical set-up for rat vocal fold experiments.

With the aid of a custom- fabricated 1.9-mm diameter, 25-degree endoscope connected to an external light source, the vocal folds were exposed and visualized on a monitor (Figure 6,7). The stripping injury was performed using a size 25 gauge needle slightly angulated at the tip, to expose the thyroarytenoid muscle. Small cotton pledges were used to maintain homeostasis when needed.

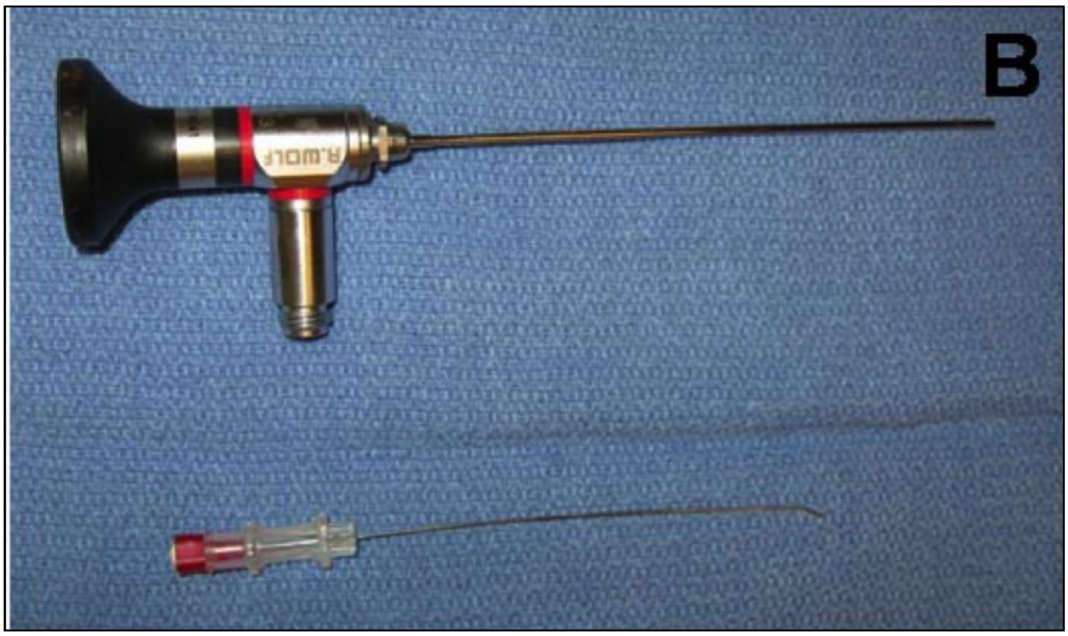


Figure 6: A 1.9-mm diameter 25° endoscope for vocal fold visualization and a 25-gauge needle for vocal fold injury.

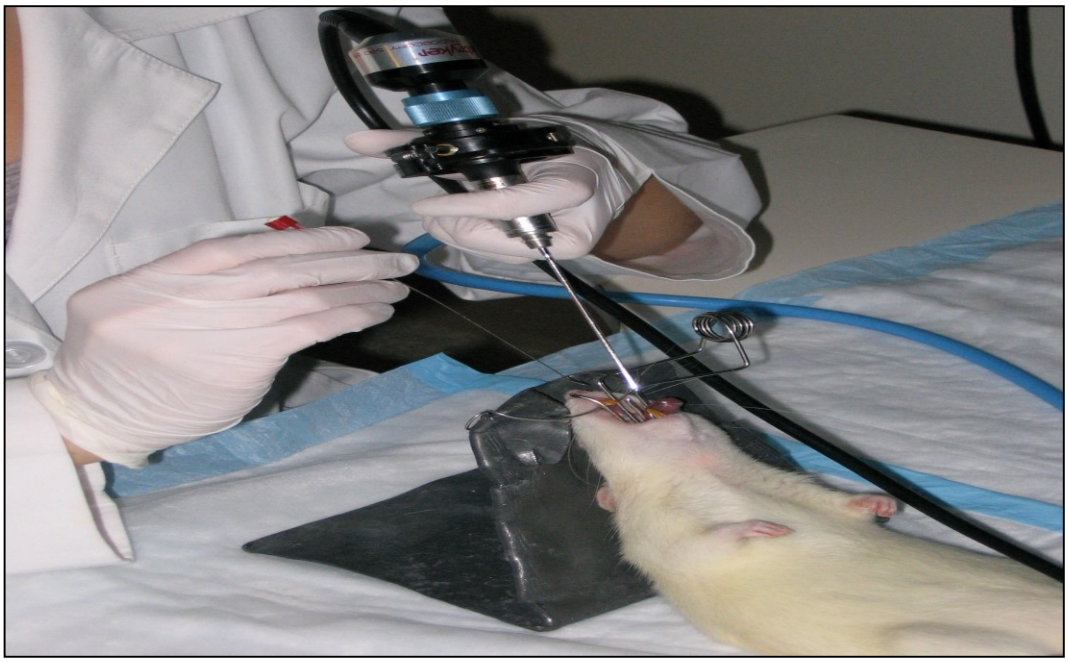


Figure 7: endoscope connected to the camera and light source.

The right folds were injured in all animals, and the left was left un-injured, thus serving as controls. Following a satisfactory injury, the animal was allowed to recover from anesthesia while being kept warm and comfortable with a heating lamp and monitored every 10 minutes post- operation until recovery was complete. Recovery was assessed based on a complete return to normal recumbence and posture. Five days after vocal fold injury when the rate of collagen synthesis starts to increase rapidly, the animals were anesthetized following the same procedure. The animals were re-scoped, this time to assess the injury and also to inject one of the three materials (with 25µl saline, HA bulk gel or hierarchically structured HA- gelatin gel) using a 50µl syringe with a 27 gauge needle (Figure 8,9) depending on which group the animal belonged to into the vocal fold. The material to be injected was freshly prepared beforehand as mentioned below and injected into the right vocal fold lamina propria in each animal.

Figure 8: Special 50µl syringe with a 27 gauge needle used for injection.

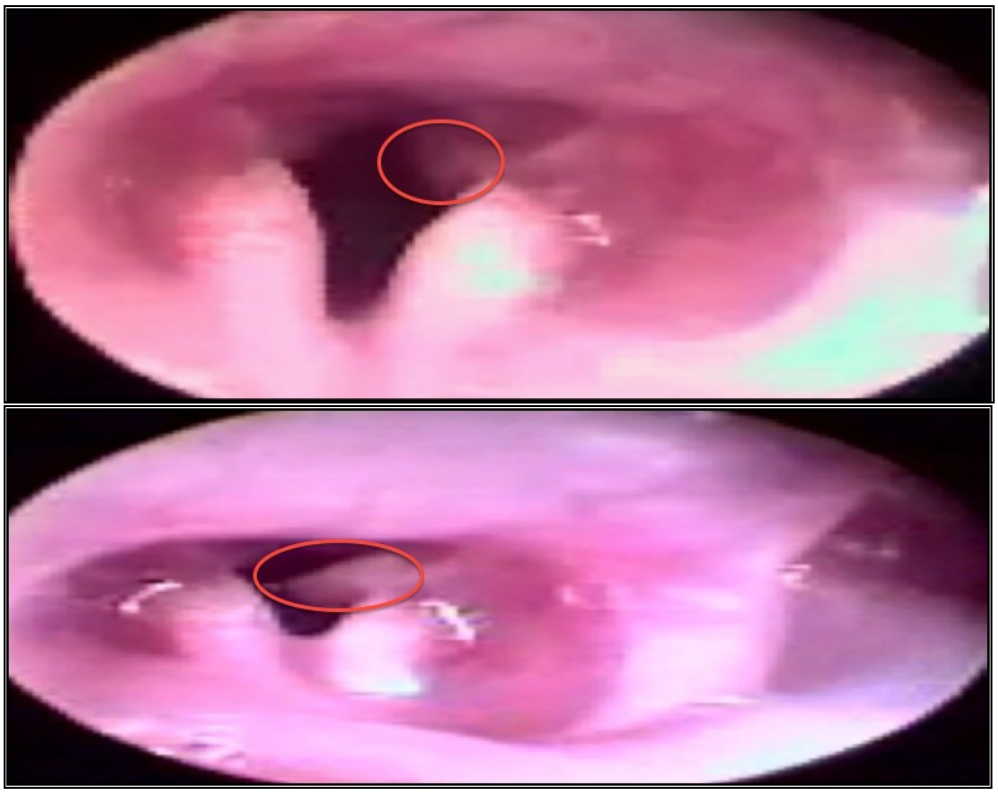


Figure 9 upper picture illustrate the vocal fold before injury. Lower one shows the injured right fold.

3.4 Hierarchical Hyaluronic Acid -Gelatin composite preparation

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 mixture was dispersed by sonication of the solution for 5 minutes. A total of 50μL of HA-Ge particles in thiolated HA solution and 50ul of 1% PEGDA cross-linker were loaded into a syringe. The syringe was placed in a sonicator for one minute to mix the solution. A total of 25ul of HA-Ge was injected into the vocal fold of each animal

within 4-6 minutes of preparation. The detailed preparation procedures for the HA-Ge biomaterial can be found in Heris et al. (14).

3.5 Hyaluronidase digestion – Alcian Blue staining:

To examine the HA hydrogel, Alcian Blue was used to detect the hydrogel.

3.6 Immunohistochemistry (IHC):

Immunofluoresence staining was performed on collagen type I, collagen type III and elastin. Skin tissue was used as a positive control for collagen type I, collagen type III and elastin. Phosphate-buffered saline (PBS) was used as a negative control. The immunohistochemistry preparation was done in a 2 days protocol. First day, we Deparaffinize the sections and rehydrate it through a graded ETOH series to dH20. Antigen retrieval was done by Immersing slides into a buffer solution-containing dish and incubated for 2 hours. Once the antigen retrieval done, a primary antibody (negative control only) added to the tissue and incubated over night. On the second day, a secondary antibody applied and incubated in a dark room for 35 minutes.

(refer to appendix II for more details).

3.7 Image analysis

Fluorescence digital images were captured using a Zeiss Axioskop microscope. Fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filters were used to image collagen subtypes and elastin, respectively.

MetaMorph was used to quantify the protein levels within the lamina propria as follows: 1) Green, red and blue channels were separated for each image. 2) The desired image was kept for further analysis and converted into a 16-bit image. 3) Background correction was performed. 4) The region of interest (ROI) was identified. 5) The minimum threshold was determined as the maximum intensity value in the lamina propria of the negative controls. 6) The area of the entire ROI and also the bright objects within the ROI were obtained. 7) The relative density of the protein of interest was calculated as the ratio of the area of the bright objects to that of the entire ROI.

3.8 Statistical analysis:

Differences observed in the lamina propria cross-sectional area; cell density and protein density for the three treatment groups 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. In addition, differences between the right (injured and treated) and left (uninjured) vocal folds of each treatment group were compared using a Wilcoxon matched-pairs signed rank test.

Bonferroni correction was applied for each test due to multiple comparisons**.** All statistical analyses were conducted using the SPSS 18.0.3 software (SPSS, Chicago, IL). Descriptive data are shown as mean and standard deviation. The overall **a**-level for these tests was set at .05 (refer to appendix I for more details).

Note: a manuscript based on this thesis is in preparation.

Chapter 4: Results

4.1 General observations

A total of 36 adult male Sprague-Dawley rats were used in this study. Five animals died after induction at different stages. Three animals died before undergoing any surgical procedure, one after the first procedure (injury), and one during the injection (the second procedure). Eight larynges were sectioned incorrectly, and we could not run stains and analysis on them. A total of 23 animals in total, 7 in the saline group, 8 each in the HA-bulk and HA-gelatin groups, were sectioned. However, due to some staining problems, the results presented for statistical analyses were for 7 animals in the saline group, 8 animals in the HA-Bulk group and 6 animals in the HA-gelatin group (see Table 2 for details).

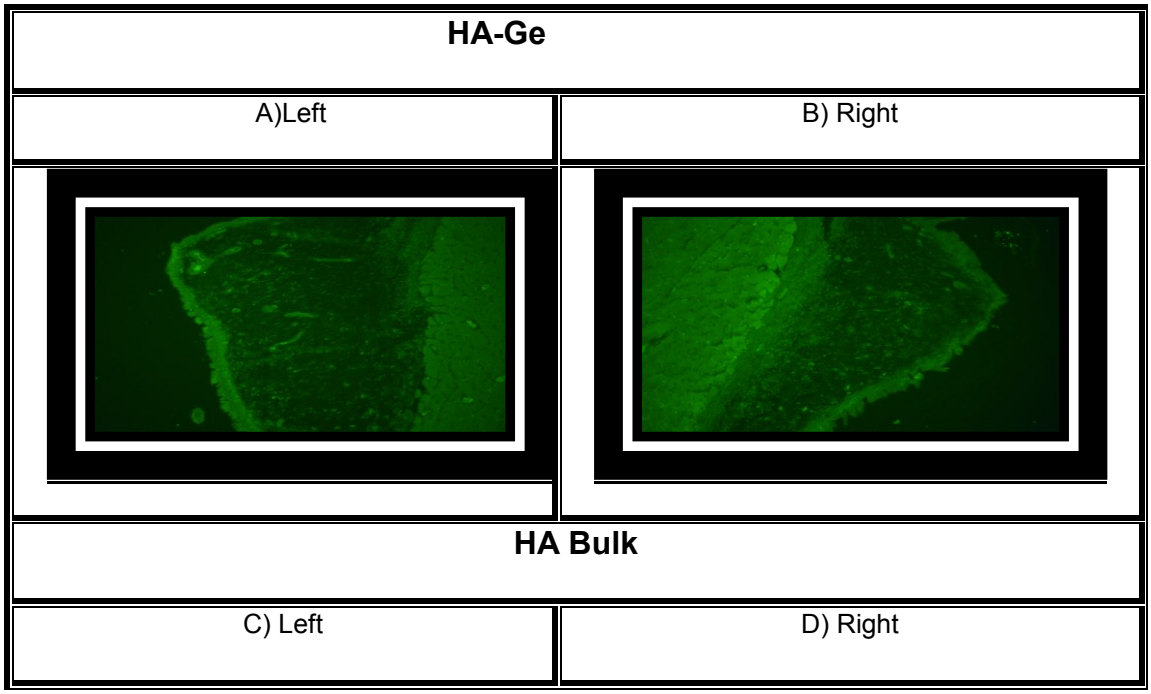
Group	No.	Died	Causes	Sectioned	Analyzed
Saline	12	2	1-reaction to anaesthesia 2-Laryngeal spasm	7 animals	7 Animals
HA Bulk	12	2	Both laryngeal spasm and edema	8 animals	8 animals
HA-Ge	12	1	Laryngeal spam	8 animals	6 animals

Table 2: General descriptive of the study groups

Laryngeal exposure was clear in all but two animals. They developed edema in the airway, so the procedure had to be aborted and repeated on another day. Minimal bleeding was encountered during the procedure, and we were able to control it with a cotton soaked in adrenaline. Injection was performed with the same needle (27 gauge needle) in all animals with minimum spillage outside the fold, and we were able to suction it before aspiration into the lung. No adverse reactions notified during or after the injections in all treatment groups. Vocal fold were completely covered with epithelium at the time of fold sectioning.

4.2 Matrix protein distribution

The relative densities of matrix proteins in the lamina propria were estimated. Figures 10, 11 and 12 depict examples of the relative collagen III, elastin and collagen I protein densities for the HA-Ge (A), HA bulk (B) and saline (C) groups, respectively.



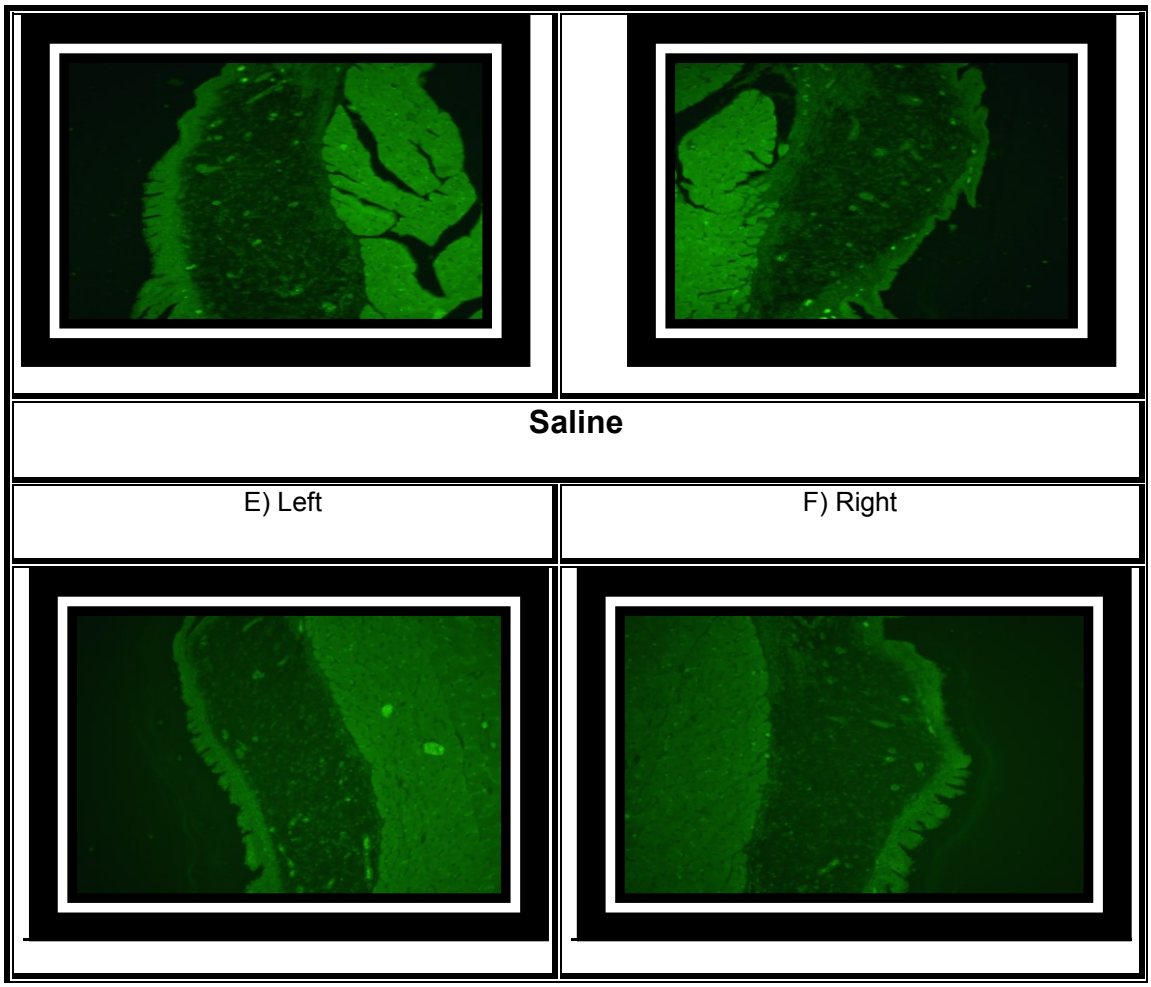
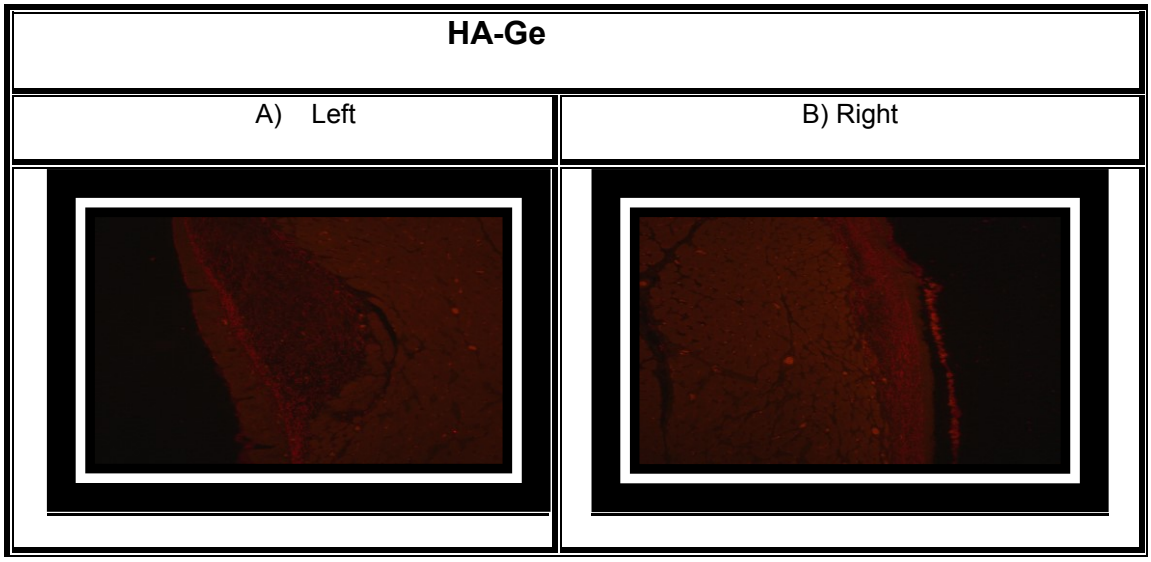


Figure 10: Examples of Collagen III distribution in both vocal folds in the three groups



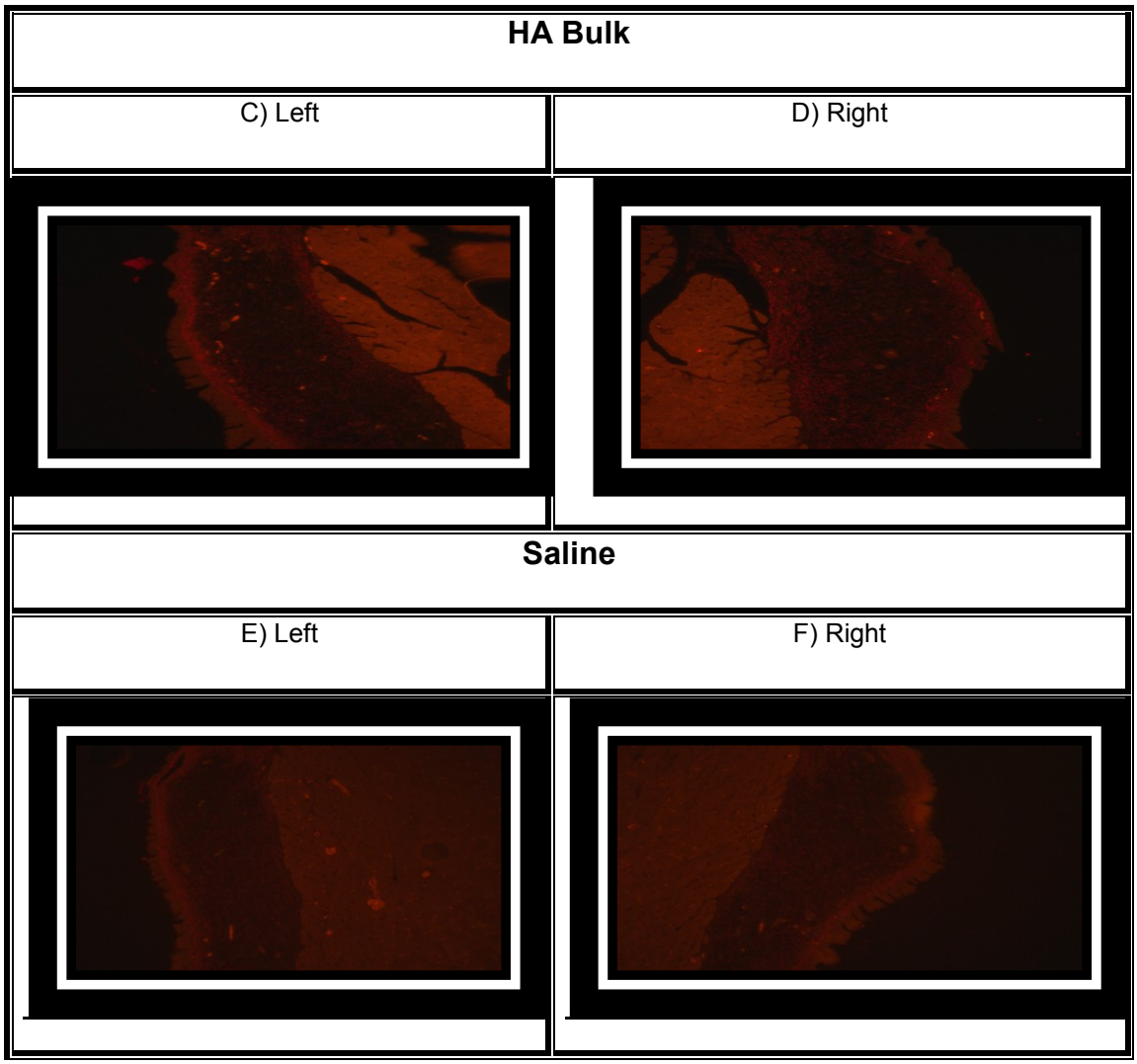
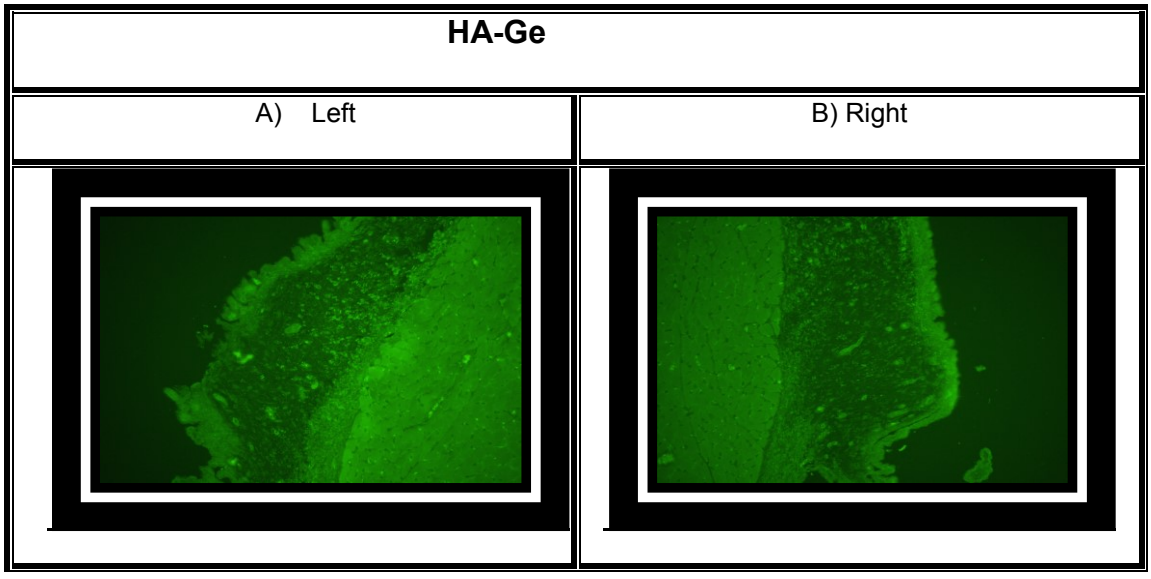


Figure 11: Examples of Elastin distribution in both vocal folds in the three groups



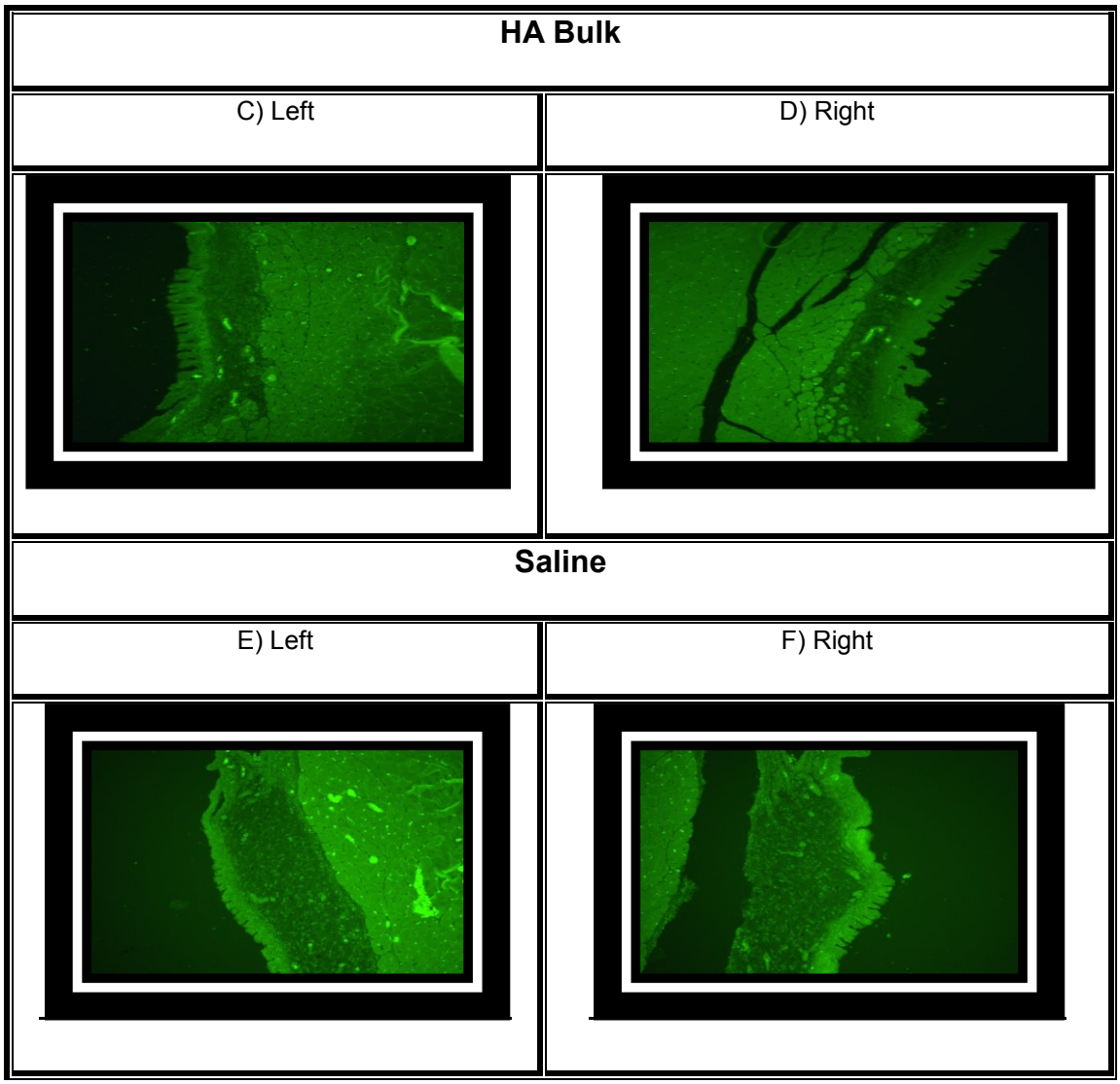


Figure 12: examples of Collagen I distribution in both vocal folds in the three groups

The densities of type 1 collagen in uninjured versus injured vocal folds were 35% and 51%, respectively; the densities of type III collagen in uninjured versus injured vocal folds were 12% and 9%, respectively; while for elastin in uninjured versus injured the densities were 22% and 16%, respectively. These were statistically significant for all groups (Figure 13).

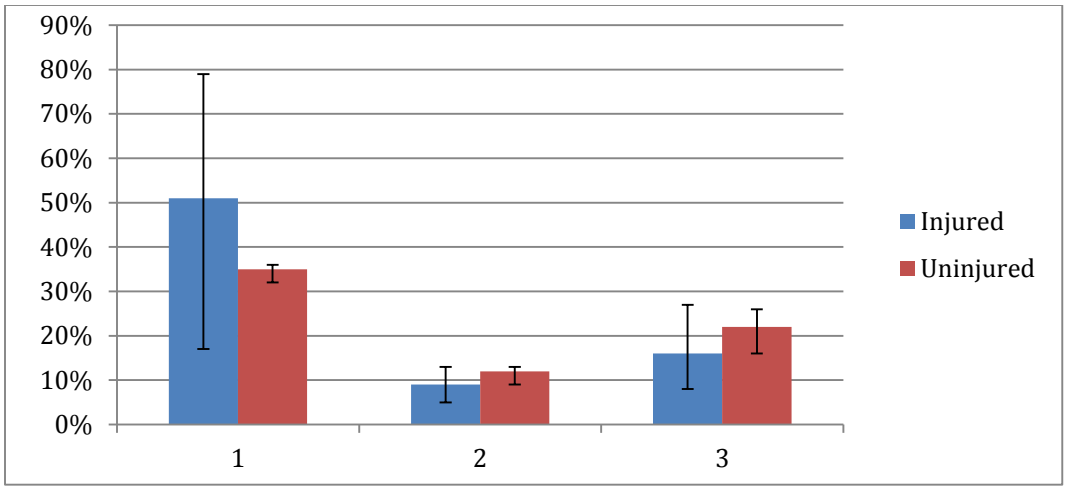


Figure 13: comparison between overall Protein Density in both vocal fold.

1-Collagen I. 2- collagen III. 3-Elastin.

Type I collagen was 35% in uninjured vocal folds, the density of collagen I was increased in all treatment groups (48%, 58% and 36% 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 (Figure 14). Collagen type III densities, on the other hand, were greater than in the uninjured controls (11%) in both HA-bulk and HA-GE groups (16% and 19%), whereas the density in the saline-treated was lower (8%) (Figure 15). The density of elastin was 22% in the uninjured vocal folds. This density was slightly higher in the HA-bulk and HA-GE groups (25% and 28%, respectively) and lower in the saline-treated group (16%) (Figure 16).

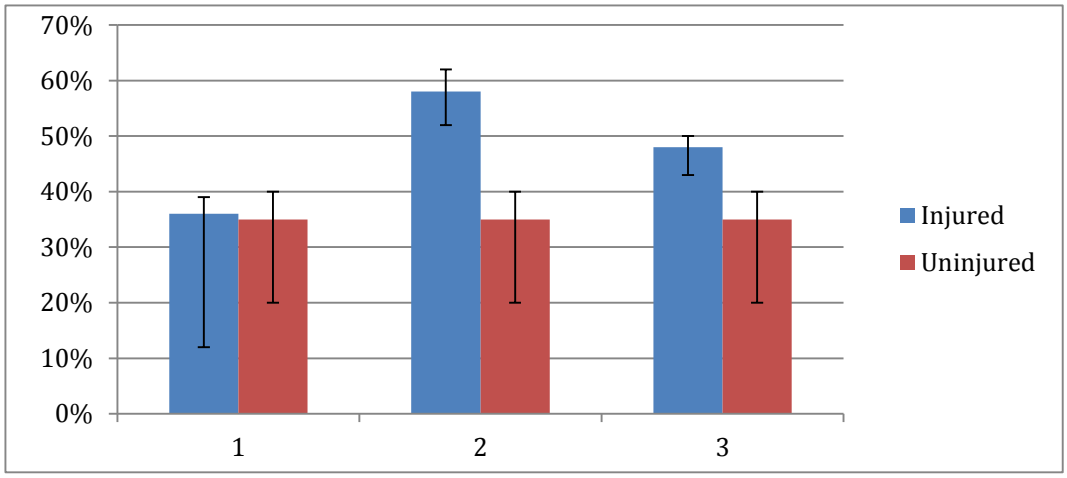


Figure 14: collagen type I density in the three treatment group.

1- HA-Ge 2- HA Bulk 3-Saline

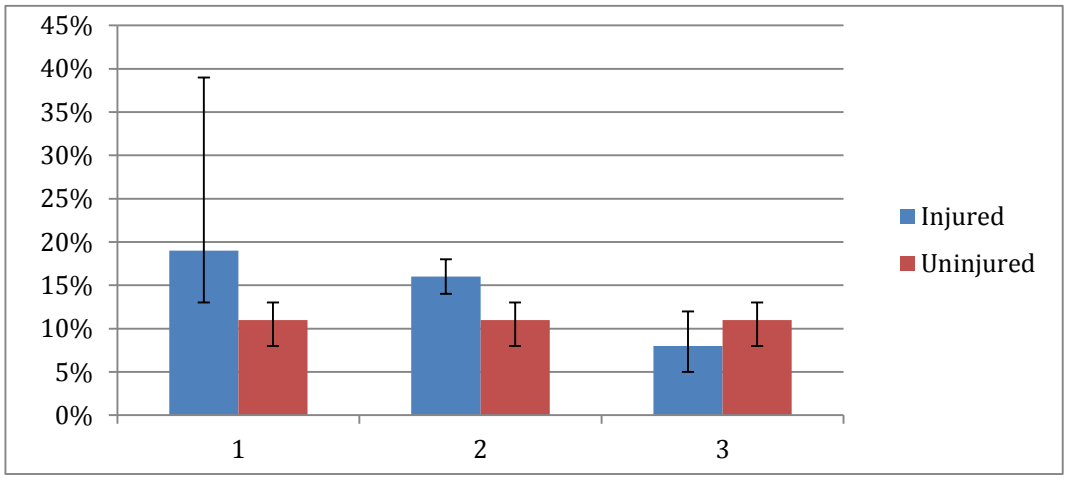


Figure 15 collagen type III density in the three treatment group

1- HA-Ge 2- HA Bulk 3-Saline

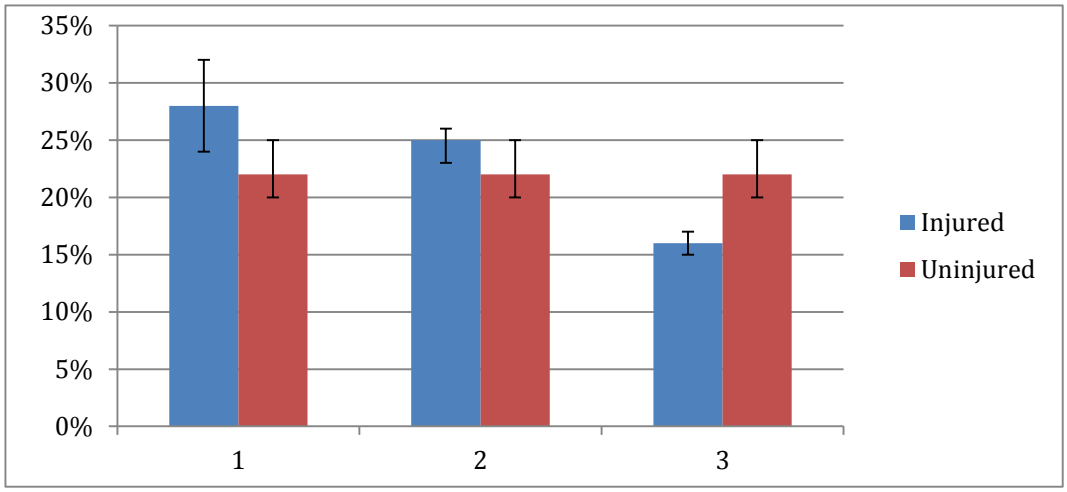


Figure 16 Elastin density in the three treatment group

1- HA-Ge 2- HA Bulk 3-Saline

The relative densities of elastin and collagen III were greater in the HA-bulk group than in the saline group ($p = 0.032$ and 0.07, respectively). Likewise, elastin and collagen type III were of greater densities in the HA-GE group than in the saline group ($p = 0.014$, 0.004, respectively). Although elastin and collagen type I appeared greater in the HA-GE group than in the HA-bulk group, these differences were not statistically significant (0.56 and 0.16, respectively).

Chapter 5: Discussion

5.1 Animal model:

The rat has been shown to be an excellent model for laryngeal studies. In the past, several other animal models have been used owing to the fact that their vocal folds are remarkably similar to those of humans. Previous studies have shown the rat to have similar vocal fold scarring properties to those of humans. (25). The rat lamina propria contains three layers, with more collagen fibers in the deep layer similar to the human vocal fold, while less collagen is found in the superficial layer (28, 50). In the current study, we demonstrate the ease of injuring and injecting the VF of rats, despite the small size of their larynges and vocal folds. Some technical difficulties encountered at the beginning of the trial, such as vocal fold spasms and edema, prevented further work on the animal. We believe this was due to the longer manipulation of the airway and less experience of the examiner at the beginning. We recommend the examiner practice on rat models and train further on the use of endoscopic procedures and try to minimize the time required for vocal fold manipulation.

5.2 HA and Inflammatory Response:

In this study, we showed that hyaluronic acid (both as unmodified HA- bulk hydrogels and HA- gelatin) was found to be biocompatible, with no severe adverse

reaction observed. No granuloma or granulation tissue had developed in our model after injection, and the lamina propria was completely covered with epithelium at harvest. These findings validate previous reports regarding the safety of this product (51).

HA plays an important role in the process of wound healing. In early wound healing, an increase in the levels of hyaluronic acid is associated with diminished inflammatory response, while a decrease in its level promotes a significant inflammatory response (52). However, HA hydrogel, by itself, is non-inflammatory, with little or no reaction to hyaluronic injection (53, 54). Previous results by Coppoolse, Van Kooten, Heris, Mongeau, et al also corroborate these facts; it was shown that HA- gelatin-injected vocal folds contain significantly fewer macrophages than the control- injected samples (51). This weakened the inflammatory response following injection, while HA-gelatin thereby facilitated repair by the host tissue (55).

5.3 Extracellular Matrix Protein Distribution:

Scarring of the vocal fold still remains a challenging pathology that mainly affects the outer layers of the vocal fold; specifically the mucosa and the superficial lamina propria. The outcome in terms of voice quality is dependent upon the distinctively layered ultrastructure of the vocal folds, which is defined mainly by its extracellular matrix. Pathological changes in the vocal-fold ECM alter voice quality because of the alteration of tissue viscosity that yields the loss of normal vibratory function of the vocal folds. Different aspects of the ECM are affected by the VF-scar; these include

myofibroblasts, collagen type I & III and elastin.

Myofibroblasts play a role in the synthesis of extracellular matrix components such as collagen types III and I. They also provide, to some degree, tensile strength to the wound. However, in some cases, accumulation of myofibroblasts may lead to excessive scarring, which would eventually affect tissue biomechanical properties (56). In our earlier study (51), we found no significant differences in myofibroblast cell counts between the HA-Ge and the control groups. This could be explained by the fact that the timing of the injection after injury was probably a bit late; earlier injections post-injury may be a useful strategy to ensure the presence of a scaffold for myofibroblasts.

The roles of collagen types I and III in cutaneous wound healing have been studied extensively in the literature. It is well-known that following an injury, collagen type III is rapidly synthesized, acting as an early scaffold for fibroblast migration and proliferation. Collagen type I then replaces type III during the remodeling phase, which acts to provide long-term tensile strength to the wound site (25).

This present study showed that the presence of collagen type I was sustained significantly longer in the HA-Ge group when compared with the control group at eight weeks post-injection. An earlier study showed that collagen I in the HA-Ge-injected group increased by 29% and 27% at 14 and 28 days post-injection, respectively; in our study, we showed an increase by 36% at 56 days post-injection. It seems possible that these results are due to the enduring residue of HA-Ge creating a suitable environment for collagen I production for a longer time.

On the other hand, collagen III was higher in both treatment groups when compared to the un-injured vocal fold and the control group. These findings are perhaps surprising. We were expecting a lower collagen III level at this point. This discrepancy could be a result of the fact that there is a prolonged need for a scaffold for myofibroblast proliferation. Other possible explanation could be the timing of injections. We think collagen and scar forms earlier than five days and injections should be done in the first three days.

Elastin is responsible for the tissue's ability to recoil after stretching in the lamina propria. It is found in a small amount in the superficial lamina propria with a higher concentration as you go deeper into the intermediate and deep lamina propria (57). Elastin usually decreases with aging, which contributes to vocal fold thinning and voice changes (58). Several animal studies have shown the decreased level of elastin in scarred vocal fold (9, 23). In our study, elastin levels were higher in both treatment groups when compared to the uninjured side. The difference was highly significant when we compared the HA-Ge to the control saline group with a p value of .014. This finding is consistent with the previous study, which showed sustained high levels of elastin in HA groups compared to the control group (51). This finding further supports the idea that HA hydrogel helps restore the elastin, which will eventually restore the biomechanical tissue characteristics. A rheological study is needed to further assess the changes in vocal fold vibration and voice outcomes.

5.4 Limitations:

This study has some limitations, some of which relate to the use of rats as a model for vocal-fold scarring and remodeling. The biomechanical environment of rat vocal folds is markedly different from that of human vocal folds, which somehow limit extrapolation of the results to human vocal fold studies. In addition, there are no known ways of ensuring post-surgical voice rest in the rat model; whereas in humans, strict adherence to therapeutic treatment including voice rest is required. Furthermore, our study is limited by the relatively small sample size. Despite these challenges, the observations and results presented here are valuable contributions to the current literature.

Chapter 6: Conclusions

This study has investigated the role of HA hydrogel in the treatment of vocal fold scars. The relationship between tissue stiffness in vocal fold scar and fibrous and interstitial proteins does not seem to be straightforward and requires further investigation. HA based hydrogels was biocompatible and showed no reaction. Collagen III have shown unexpected higher values in the HA based hydrogels groups in contrast to collagen I that showed optimum result. The present study provides additional evidence with respect to the effectiveness of HA based hydrogels in the treatment of vocal fold scar, but still provides limited information on structural changes only and not on biomechanical characteristics of the vocal folds. Improving the histological part of the Vocal fold scar will indeed improve the quality of voice, but further studies using voice handicap index (VHI) and voice related quality of life (VR-QOL) is warranted. Finally, rat vocal folds are similar in structure to those of humans, which makes it easy to extrapolate and extend the study to human vocal folds. Future studies on the current topic are therefore recommended to assess whether these changes observed will also be seen in the human vocal fold and if these changes improve the rheological properties of the Human scarred vocal fold.

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Appendix I:

Elastin Results:

Rat saline		LEFT un-injured			RIGHT injured	
Sample ID	Area (μm^2)	Threshold Area (μm^2)	%	Area (μm^2)	Threshold Area (μm^2)	%
1L2	291635.0	36408.0	12.5%	362722.0	26105.0	7.2%
3L18	162535.0	21679.0	13.3%	150459.0	19892.0	13.2%
2R4	214786.0	24157.0	11.2%			
1R7	527955.0	52253.0	9.9%	56838.0	4467.0	7.9%
1R10	139103.0	16804.0	12.1%	141306.0	15487.0	11.0%
1R4	94106.0	10905.0	11.6%	112537.0	9353.0	8.3%
3R4	296620.0	28278.0	9.5%	304519.0	16166.0	5.3%
Rat HA-bulk						
1L11	137725.0	15690.0	11.4%	39633.0	6970.0	17.6%
1R16	238716.0	24345.0	10.2%	175455.0	22209.0	12.7%
1R13	74011.0	8506.0	11.5%	183969.0	35823.0	19.5%
1L17	380104.0	48432.0	12.7%	398613.0	56614.0	14.2%
2L12	228104.0	22839.0	10.0%	35868.0	7591.0	21.2%
2L15	305718.0	30327.0	9.9%	293886.0	49038.0	16.7%
3R17	166861.0	15635.0	9.4%	204988.0	19686.0	9.6%
3L12	230151.0	27693.0	12.0%	166948.0	33304.0	19.9%
Rat HA-gelatin						
1R7						
2R7	195625.0	21578.0	11.0%	145500.0	32786.0	22.5%
2L9	263296.0	32966.0	12.5%	68030.0	12503.0	18.4%
3L6	570492.0	70873.0	12.4%	291340.0	48246.0	16.6%
3L9	265030.0	24707.0	9.3%	157548.0	34493.0	21.9%
2L6	79500.0	9503.0	12.0%	295016.0	47932.0	16.2%
1L5	406114.0	42853.0	11.1%	196840.0	39513.0	19.25%

Collagen I

Rat saline		LEFT			RIGHT	
Sample ID	Area (µm^2)	Threshold Area (µm^2)	%	Area (µm^2)	Threshold Area (µm^2)	%
1L2	192347	69349	36.05%	213097	49602	23.28%
1L2	190451	65613	34.45%	234159	55680	23.78%
1R1	401843	134125	33.38%	134848	95367	70.72%
1R1	302756	107024	35.35%	88939	41469	46.63%
3L18	166581	56826	34.11%	167793	129302	77.06%
2R4	304700	109272	35.86%	151249	42413	28.04%
2R4	324379	119451	36.82%	132937	55180	41.51%
01R7	360746	117502	32.57%	63895	63824	99.89%
1R10	134844	47825	35.47%	116615	27530	23.61%
1R4	94439	32657	34.58%	73173	31735	43.37%
Rat HA-bulk						
1L11	163697	56508	34.52%	43928	37771	85.98%
1L11	223594	77924	34.85%	189420	93932	49.59%
1R16	467363	175088	37.46%	102609	91240	88.92%
1R13	63216	24327	38.48%	84951	14209	16.73%
1L17	160107	50957	31.83%	317319	199157	62.76%
2L12	73349	16565	22.58%	259225	157647	60.81%
2L15	149953	53415	35.62%	258609	159125	61.53%
3R17	118757	43993	37.04%	93020	35298	37.95%
Rat HA-gelatin						
1R7	161146	56093	34.81%	20359	6171	30.31%
2R7	157510	55665	35.34%	87601	39554	45.15%
2R7	125839	48529	38.56%	111366	43211	38.80%
2R7	90837	30584	33.67%	49169	13588	27.64%
2L9	106477	38440	36.10%	77910	40210	51.61%
3L6	267850	99318	37.08%	330323	72286	21.88%
3R5	379098	144417	38.09%	211988	49433	23.32%
3R14	41756	14538	34.82%	60738	28140	46.33%

Collagen III

Rat saline		LEFT			RIGHT	
Sample ID	Area (µm^2)	Threshold Area (µm^2)	%	Area (µm^2)	Threshold Area (µm^2)	%
1L2	291635.0	36408.0	12.5%	362722.0	26105.0	7.2%
3L18	162535.0	21679.0	13.3%	150459.0	19892.0	13.2%
2R4	214786.0	24157.0	11.2%			
1R7	527955.0	52253.0	9.9%	56838.0	4467.0	7.9%
1R10	139103.0	16804.0	12.1%	141306.0	15487.0	11.0%
1R4	94106.0	10905.0	11.6%	112537.0	9353.0	8.3%
3R4	296620.0	28278.0	9.5%	304519.0	16166.0	5.3%
Rat HA-bulk						
1L11	137725.0	15690.0	11.4%	39633.0	6970.0	17.6%
1R16	238716.0	24345.0	10.2%	175455.0	22209.0	12.7%
1R13	74011.0	8506.0	11.5%	183969.0	35823.0	19.5%
1L17	380104.0	48432.0	12.7%	398613.0	56614.0	14.2%
2L12	228104.0	22839.0	10.0%	35868.0	7591.0	21.2%
2L15	305718.0	30327.0	9.9%	293886.0	49038.0	16.7%
3R17	166861.0	15635.0	9.4%	204988.0	19686.0	9.6%
3L12	230151.0	27693.0	12.0%	166948.0	33304.0	19.9%
Rat HA-gelatin						
1R7						
2R7	195625.0	21578.0	11.0%	145500.0	32786.0	22.5%
2L9	263296.0	32966.0	12.5%	68030.0	12503.0	18.4%
3L6	570492.0	70873.0	12.4%	291340.0	48246.0	16.6%
3L9	265030.0	24707.0	9.3%	157548.0	34493.0	21.9%
2L6	79500.0	9503.0	12.0%	295016.0	47932.0	16.2%
1L5	573757.0	55831.0	9.7%	259521.0	56789.0	21.9%
1L5	238471.0	29875.0	12.5%	134159.0	22237.0	16.6%

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Collagen_I_right	20	48.1078	23.85335	16.73	99.89
Elastin_right	21	23.92	9.007	8	43
Collagen_III_right	20	15.0837	5.44924	5.31	22.53

Kruskal-Wallis Test

	Condition	N	mean
Collagen_I_right	saline	7	10.71
	HAbulk	7	13.00
	HAgelatin	6	7.33
	Total	20	
Elastin_right	saline	6	5.33
	HAbulk	7	12.43
	HAgelatin	8	14.00
	Total	21	
Collagen_III_right	saline	6	4.00
	HAbulk	8	11.75
	HAgelatin	6	15.33
	Total	20	

Test Statistics

	Collagen_I_right	Elastin_right	Collagen_III_right
Chi-Square	2.978	7.246	11.605
df	2	2	2
Asymp. Sig.	.226	.027	.003

t test: Saline vs HA bulk

Condition	N	Mean Rank	Sum of Ranks
Elastin_right			
saline	6	4.50	27
HAbulk	7	9.14	64
Total	13		
Collagen_III_right			
saline	6	4	24
HAbulk	8	10.13	81
Total	14		

Test Statistics

	Elastin_right	Collagen_III_right
Mann-Whitney U	6	3
Wilcoxon W	27	24
Z	-2.143	-2.711
Asymp. Sig. (2-tailed)	.032	.007

t test: Saline vs HA-Ge

Condition	N	Mean Rank	Sum of Ranks
Elastin_right			
saline	6	4.33	26
HAgelatin	8	9.88	79
Total	14		
Collagen_III_right			
saline	6	3.50	21
HAbulk	6	9.50	57
Total	12		

Test Statistics

	Elastin_right	Collagen_III_right
Mann-Whitney U	5.000	.000
Wilcoxon W	26	21.00
Z	-2.453	-2.882
Asymp. Sig. (2-tailed)	.014	.002

t test: HA bulk vs HA Ge

Condition	N	Mean Rank	Sum of Ranks
Elastin_right			
HAbulk	7	7.29	51
HAgelatin	8	8.63	69
Total	15		
Collagen_III_right			
HAbulk	8	6.13	49
HAgelatin	6	9.33	56
Total	14		

Test Statistics

	Elastin_right	Collagen_III_right
Mann-Whitney U	23.000	13.000
Wilcoxon W	51.000	49.00
Z	-.579	-1.420
Asymp. Sig. (2-tailed)	.563	.156

HA bulk group; Lt vs Rt

	N	Mean	Std. Deviation	Minimum	Maximum
Collagen_I_right	7	56.6411	23.04938	16.73	88.92
Elastin_right	7	25.40	5.501	18	31
Collagen_III_right	8	16.4152	3.98634	9.60	21.16
Collagen_I_left	7	33.9571	5.46956	22.58	38.48
Elastin_left	7	20.4764	3.60995	16.21	25.25
Collagen_III_left	8	10.8938	1.18525	9.37	12.74

HA-Ge group; Lt vs Rt

	N	Mean	Std. Deviation	Minimum	Maximum
Collagen_I_right	**6**	**35.1084**	**12.16164**	**21.88**	**51.61**
Elastin_right	**8**	**28.70**	**8.793**	**14**	**43**
Collagen_III_right	**6**	**19.5825**	**2.86517**	**16.25**	**22.53**
Collagen_I_left	**6**	**36.1267**	**1.28657**	**34.81**	**38.09**
Elastin_left	**9**	**20.0542**	**1.74552**	**16.57**	**21.97**
Collagen_III_left	**6**	**11.1617**	**1.37903**	**9.32**	**12.52**

Appendix II:

Immunohistochemistry Protocol

Elastin / Collagen I – Day 1

Materials

Sodium Citrate buffer	
TBS + 0.05% Tween20	
10% BSA	Invitrogen – ready to use
1% BSA in TBS	
Primary antibody	Rabbit polyclonal anti collagen I – abcam
Pap pen	
Kim wipes	
Water bath + dish	
Humidified black box	

Deparaffinize sections and rehydrate through a graded ETOH series to dH20

Antigen retrieval

Set up the water bath and preheat the buffer solution (Sodium Citrate buffer) to 80 °C.
Immerse slides into the buffer solution containing dish and incubate for 2 hours..
Turn off water bath and remove the buffer dish to room temperature.
Cover the dish with and allow slides to cool down for 15 minutes.
Wash 2x 2 min dH_20.
Kimwipe slides carefully
Pap pan: Draw rectangles around the tissue. Allow liquid to dry around 15 sec.
Gently wash slides 2 x 5 min in TBS + 0.05% Tween20

Serum blocking (200uL BSA + 200uL Goat + 1600uL TBS 1X)

Kim wipe slides
Pipette ~ 50 ul 10% BSA in TBS on the section. Incubate 1 hour at room temperature.

Primary antibody

(Negative control – only PBS. Positive control – Skin tissue)
Tap off excess serum. Kim wipe around the sections.
Dilute primary antibody to (Collagen I 1: 50, Elastin 1:100) with 1% BSA in TBS
Thaw antibody and then centrifuge briefly. Vortex briefly.
Add ~20 ul diluted primary antibody. Make sure tissue is fully covered.
Incubate over night at 4°C (humidified black box)

Protocol Elastin / Collagen I – Day 2

Materials

TBS + 0.05% Tween20	
Secondary antibody	Goat anti-rabbit Alexa 448 – invitrogen
1% BSA in TBS	
Kim wipes	
DAPI	D3571 - invitrogen, 5mg/ml stock solution (10.9mM)
PBS	
Antifade reagent	P36930 – invitrogen, ready to use
Cover slip	

Rinse 3 x 5 min TBS + 0.05% Tween20.

Secondary antibody

Kim wipe slides carefully.
The next steps should be done in the dark to avoid photobleaching.
Dilute secondary antibody 1:1000 with 1% BSA in TBS (Collagen I: Goat anti-rabbit Alexa-488, Elastin: Goat anti-rabbit Alexa 594)
Apply diluted secondary fluorescence antibody. Incubate for 35 minutes at room temperature *in the dark*.
Wash 3 x 6min TBS + TBS + 0.05% Tween20

DAPI counterstain
Caution: DAPI is known as mutagen. Handle with care.
Dilute the DAPI stock solution in PBS (1:10.000)
Apply DAPI solution to tissue. Incubate for 1 minute.
Rinse 3 x 5min TBS + 0.05% Tween20
Kim wipe slides
Add a drop of Prolong Gold antifade reagent
Allow the reagent to warm up to room temperature before use / opening
Mount and store in the dark at 4C
At least 24h before microscopy

Results:
Collagen I……green
Elastin …… red
Nuclei……..blue