Functional outcomes of injectable substances on surgically injured rabbit vocal folds: a comparative study

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January 2019

A thesis submitted to McGill University in fulfillment of the requirements for the degree of

Master of Science in Otolaryngology

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Abstract

Objectives: The objective of this study was to evaluate the phonatory function of rabbit larynges 42 days after surgical injury of the vocal folds and immediate injection treatment of dexamethasone, hyaluronic acid/gelatin hydrogel, glycol-chitosan solution or saline as a control.

Methods: A modified microflap procedure was performed on the left vocal fold of 12 rabbits to induce an acute injury. Animals were randomized into one of four treatment groups with 0.1 mL injections of dexamethasone, hyaluronic acid/gelatin hydrogel, glycol-chitosan or saline as control. The injection was performed at the level of the lamina propria of the left mid vocal folds, immediately following the injury. Right vocal folds served as uninjured controls. Larynges were harvested at Day 42 after injection, and were subjected to airflow-bench evaluation. Acoustic, aerodynamic and laryngeal high-speed videoendoscopy (HSV) analyses were performed. In addition, HSV segments of the vibrating vocal folds were rated by three expert laryngologists on six parameters related to vocal fold vibratory characteristics on a Likert scale.

Results: The fundamental frequency, one possible surrogate of vocal fold stiffness and scarring, appeared to be lower in all treatment groups compared to that of the saline control (411.52 ± 11.63 Hz). The lowest fundamental frequency value was observed in the dexamethasone group (348.79 ± 14.99 Hz). Expert visual ratings of the HSV segments indicated an overall positive outcome in the Dexamethasone treatment group.

Conclusion: Dexamethasone injections might be a useful intraoperative adjunct to guide the vocal fold healing process after surgical injury. An increased sample size and a histological correlate would be helpful to confirm this finding.

Résumé

Objectif: L'objectif de la présente étude était d'évaluer la fonction vocale de larynx de lapins excisés 42 jours suivant une chirurgie laryngée et une injection de Dexamethasone, d'Hydrogel d'acide hyaluronique/gélatine, de solution de Glycol-chitosan ou de solution Saline en contrôle.

Méthodes: Une microchirurgie a été effectuée sur les cordes vocales gauches de 12 lapins afin de provoquer une inflammation aigue. Les animaux furent assignés à l'un des 4 groupes de traitement de façon aléatoire. Des injections de 0.1 mL de dexamethasone, d'hydrogel d'acide hyaluronique/gélatine, de solution de glycol-chitosane ou de solution saline en contrôle ont été faites. Le matériel fut injecté au niveau de la lamina propria du milieu de la corde vocale, immédiatement après l'incision. Les cordes vocales droites ont été préservées comme contrôle. Les larynx furent recueillis 42 jours après l'injection, et furent soumis à des évaluations fonctionnelles avec phonation induite par un ventilateur externe. Des analyses acoustiques, aérodynamiques et d'images à haute définition ont été faites. Les segments vidéos ont été évalués par trois experts laryngologistes examinant six paramètres de la vibration des cordes vocales sur une échelle de Likert.

Résultats: La fréquence fondamentale, présumément représentative de la raideur et de la présence d'une cicatrice, semble plus basse dans tous les groupes de traitements que celle dans le groupe de contrôle salin (411.52 ± 11.63 Hz). La valeur la plus basse est dans le groupe dexamethasone (348.79 ± 14.99 Hz). Les évaluations d'experts basés sur la revue des segments vidéos semblent aussi indiquer un effet d'adoucissement de la cicatrice dans le groupe dexamethasone.

Conclusion: Les injections de dexamethasone pourraient avoir un effet bénéfique positif sur la guérison des cordes vocales après chirurgie. Des études de plus grande envergure ainsi qu'une analyse histologique sont nécessaires pour confirmer ce résultat.

List of Abbreviations

AGS: Anterior Glottic Stenosis

BVFL: Benign Vocal Fold Lesion

CAD: Computer Aided Design

CT: Computed Tomography

cDNA: complementary DeoxyriboNucleic Acid

DPI: Days Post Injection

ECM: Extra Cellular Matrix

FACC: Facility Animal Care Committee

FNA: Fine Needle Aspiration

GE: Gelatin

HA: Hyaluronic Acid

HK: House Keeping

HSV: High-Speed Videoendoscopy

rmANOVA: Repeated Measure Analysis of Variance

RNA: Ribonucleic Acid

TA: Thyro-Arytenoid

Acknowledgements

I would like to extend my sincere appreciation and gratitude to my supervisors, Drs. Nicole Li-Jessen, Luc Mongeau and Karen Kost. They all contributed in an immense way to this work and none of it could have been done without their patience, advice and experience. Their doors were always opened and they were all always one phone call away when I had any questions or concerns. They are exceptionally talented and thorough researchers. I look up to them for their desire to always improve and reach for excellence. I am also thankful to Dr Bernard Segal for chairing my thesis committee meetings.

A special thank you to Neda Latifi and Annie Douillette from the Mongeau lab and to all the amazing students and researchers who helped carry out this project.

I would also like to thank our expert laryngologists raters, Drs Karen Kost, Jonathan Young and Christopher J. Hartnick for their help with the visual rating component of the project.

Preface

Contribution of Authors

Dr. Sarah Bouhabel is responsible for the literature review and Introduction in their entirety. Dr. Bouhabel was responsible for the concept, design, data collection and drafting of the manuscript. Scott Park, Eng. was responsible for data collection and partial drafting of the manuscript. Aurelien Masson and Annie Douillette provided help with the data collection. Drs Nicole Li-Jessen, Luc Mongeau and Karen Kost contributed to the concept and design of the study. They also provided supervision, critical review and editing. Dr Neda Latifi provided help with critical review and editing. Zhengdong Lei provided help with data analysis. Eric Boucher provided partial drafting of the overall discussion.

Chapter 1: Introduction

1.1 Thesis Rationale

Surgical resection is the mainstay of treatment for patients with benign vocal fold lesions (BVFL) who do not respond to conservative management options [1, 2] . Excision can be performed using various surgical methods. For example, a cold steel technique or an office-based laser can be used [1]. Phonomicrosurgery allows immediate removal of the lesions. However, the procedure itself often inevitably disrupts the vocal fold microstructure, namely the extracellular matrix (ECM), and induces a cascade of inflammation and repair responses [3]. During the healing process, fibroblasts are attracted to the injured area. They proliferate and generate collagen deposition and remodeling. If the process is not controlled in a timely manner, a chronic scar can result. A scar can also result when the severity of the trauma surpasses the repair capacity of the local macrophages and myofibroblasts [4]. Iatrogenic vocal fold scars increase their stiffness and impair their vibratory amplitude and symmetry, leading to recalcitrant hoarseness and dysphonia [3, 5, 6]. The occurrence of unpredictable vocal fold scarring after surgery remains a difficult clinical challenge to be addressed [5, 7-9].

At present, no effective measures are available to either prevent or treat postoperative vocal fold scars [5, 7-9]. When a glottal gap develops as a consequence of scarring, laryngologists can perform injection medialization laryngoplasty procedures to alleviate the patient's symptoms. This procedure is done by injecting a resorbable material in the medial aspect of the thyroarytenoid (TA) muscle, at the midmembranous and posterior portions of the vocal fold [10]. As a result, the injectable material reapproximates the vocal folds towards the midline and decreases the glottal

gap. Laryngoplasty itself however does not remediate the disrupted mucosal waves and the scarred tissues [7].

1.2 Thesis Objectives and Outline

When addressing iatrogenic vocal fold scarring, one effective approach might be to prevent the formation of a post-operative scar by using injectable biomaterials. The concept of a preventive measure in this context is to rationally utilize a substance that preemptively guides the wound healing process towards a regenerative instead of a fibrotic outcome. The overall aim of this project is to investigate three promising injectable materials regarding their possible preventive effects for iatrogenic vocal fold scarring, specifically: 1) hyaluronic acid-based hydrogels; 2) corticosteroids; and 3) chitosan. The effects of hyaluronic acid (HA) and dexamethasone on the vocal folds biological response have been studied [11, 12]. The effects of these materials on phonation function, however, have not yet been assessed. Acoustic and vibration phonation characteristics are indicative of the elasticity, toughness, and vibration modality of the repaired tissue.

The specific aim of this project was to evaluate the phonatory function of rabbit vocal folds after immediate injection treatment of dexamethasone, hyaluronic acid/gelatin hydrogel, glycol-chitosan or saline controls 42 days after surgical injury. An objective evaluation of the vocal fold function was done through the study of acoustic and aerodynamic data obtained from laryngeal high-speed videoendoscopy (HSV). A subjective expert rating of the HSV segments was also performed. The research hypothesis is that at least one of the evaluated injectable materials will soften the vocal fold scar, and this will be evidenced by phonatory functional parameters that are similar to those of uninjured controls. If one of the tested substances shows promising effects on the scar (as defined by positive functional analysis and visual rating findings), this study could

serve as a pilot for a larger scale animal study. Eventually, this research might translate into clinical human trials which could confirm positive wound healing post-operatively. This research might also improve the quality of life of patients suffering from iatrogenic hoarseness.

The organization of this thesis is as follows. The thesis has five chapters: chapter 2 presents a general literature review, chapter 3 presents a manuscript ("Functional outcomes of injectable substances on surgically injured rabbit vocal folds: a comparative study" by myself, S. Park, A. Masson, A. Douillette, N. Latifi, K. Kost, N. Li-Jessen, and L. Mongeau) that evaluates the phonatory function of rabbit vocal folds after injection of: (a) dexamethasone; (b) hyaluronic acid/gelatin; (c) glycol-chitosan; or (d) saline 42 days after surgical injury. Chapter 4 presents an overall discussion. Chapter 5 is an overall conclusion. Chapter 6 lists claims of originality, and Chapter 7 lists the references.

Chapter 2: Literature review

Multiple agents have been studied in the literature as preventive measures to restore surgically injured vocal folds, for both *in vivo* and *in vitro* models [8, 13]. For example, vocal fold scar prevention through the use of vitamin A has been demonstrated in a rabbit animal study [8]. Mitomycin C has also been investigated with satisfactory results. However, concerns about toxicity and potential carcinogenicity has hampered its use in clinical practice [8, 14-17]. There has been growing interest over recent years in hyaluronic acid (HA), corticosteroids and chitosan for the prevention of post-operative vocal fold scars.

2.1 Hyaluronic acid

Hyaluronic acid (HA), a sulfated glycosaminoglycan, is one of the main components of the ECM of the vocal folds [2]. It is biocompatible, bioactive and non-immunogenic [4], which explains why it is currently widely used in clinical practice as an injectable material for injection medialization laryngoplasty procedures [7, 13]. The chemical structure of HA consists of a long chain of a disaccharide repeating unit containing D-glucuronic acid and N-acetyl D-glucuronic acid [11]. The viscoelastic properties of HA serve to dissipate and modulate the fluctuating mechanical stresses acting on the vocal folds during phonation [4]. Therefore HA represents an important determinant in vocal fold biomechanics and cellular mechanisms.

From a biomechanical standpoint, the concentration of HA in the superficial vocal folds significantly influences the vocal fold viscoelastic properties [2, 13]. More specifically, HA injections in a rabbit vocal fold model have been shown to decrease the elastic shear modulus as well as the viscous modulus when compared to saline controls six months after injection [11, 18,

19]. From a histological standpoint, hyaluronic acid has been found to yield favorable wound healing effects on injured vocal folds [6, 11, 20]. The residence time of HA in the body before elimination, however, is relatively short. It has been reported to be 0.5 and 4 days in its unaltered form [6, 11, 13, 20]. When used with other materials to form composite scaffolds, HA has been shown to decrease fibrosis, type I collagen deposition and to contribute to a better organization of the elastin in rabbit vocal folds' models at time points between one day and six months [4, 18].

2.2 Corticosteroids

Corticosteroids have been extensively used in the field of otolaryngology, specifically in laryngology [21]. Their anti-inflammatory properties have rendered them very useful in the treatment of laryngeal pathologies involving local or systemic reactions [12, 22-24]. Their effect stems from the modulation of multiple cell types including macrophages, mast cells, fibroblasts and epithelial cells [24].

For instance, corticosteroids regulate the synthesis and maturation of collagen fibers and they inhibit fibroblasts [8]. Different steroid compounds have been tested for vocal fold scar treatment or prevention. Triamcinolone was found ineffective in decreasing scarring of surgically injured vocal folds in rabbit and canine models [12, 23, 24]. Dexamethasone is a more potent steroid compound with significantly greater anti-inflammatory activity than triamcinolone [25]. In a rabbit study, a volume of 0.1 mL of dexamethasone was injected in the vocal folds immediately after surgical resection [12, 23]. Collagen deposition was significantly lower in the treated vocal folds compared to the control vocal folds at 3 and 7 days after injury. A previous study in rabbits suggested the potential use of dexamethasone to prevent iatrogenic vocal folds scars [12, 23].

Recently, the investigation of steroids for post-operative scar prevention in laryngology has extended to human trials with positive vocal outcomes [9, 12, 24, 26, 27].

2.3 Chitosan

Chitosan is a natural injectable biomaterial that presumably has the potential for scar prevention. It is a derivative of chitin, the second most abundant natural polymer and the main component of arthropod shells [28]. Chitosan has been used in the development of biomaterials for controlled delivery and tissue engineering, owing to its biocompatibility, biodegradability and good residence time with a half-life up to 84 days [29].

In terms of histological effect, chitosan was shown to decrease the mature collagen deposition in a rat *in vivo* subcutaneous injury model [30]. The effective modulation of collagen deposition and remodeling is mainly related to its impact on the migration and proliferation of fibroblast cells [31]. More specifically, chitosan was previously shown to decrease the number of fibroblasts in subcutaneous rat skin wounds at 8 and 14 days after injury [30]. Similar trends were observed in animal and human fibroblast cultures [31, 32].

Chitosan was shown to prevent anterior glottic stenosis (AGS) following a CO2 laser-assisted cordectomy procedure in a laryngeal canine model [16, 33]. More recently, chitosan was reported to decrease post-surgical adhesion in patients undergoing sinus surgery [34, 35]. The chitosan gel was shown to decrease stenosis of the ostium at 12 weeks following sinus surgery in human patients operated for chronic rhinosinusitis [34, 35].

Based on this review of the literature, hyaluronic acid, dexamethasone and chitosan may potentially restore surgically injured vocal folds. It is still currently unclear, however, whether they

should be used as surgical adjuncts. More specifically, we don't know which one of these agents would be better than the other in reducing scarring after iatrogenic injuries in terms of phonatory outcomes. This motivated the present study described in the subsequent chapters.

2.4 Rabbit model of laryngeal injury

A rabbit injury model was selected. From an anatomical point of view, rabbit larynges have common features with human larynges. More specifically, the structure of their lamina propria is comparable [36, 37]. From a practical point of view, the rabbit model is relatively easy to work with in terms of animal size and surgical manipulations. There are abundant *in and ex vivo* experiments using the rabbit model reported in the literature [13, 37, 38]. The size of the rat or mouse model makes functional analysis on a flow bench challenging, although feasible. Larger animals such as pigs or dogs may constitute a better option, but also a much more expensive one. The rabbit larynx composes the smallest animal model that can easily be phonated in laboratory settings.

Chapter 3: Functional outcomes of injectable substances on surgically injured rabbit vocal folds: a comparative study

Manuscript in preparation for submission in the *Journal of Voice*

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Keywords:

Vocal fold scarring; wound healing; tissue engineering; functional analysis; excised animal larynx; injectable hydrogels

3.1 Introduction

Surgical resection is the mainstay of treatment for patients with benign vocal fold lesions (BVFL) who do not respond to conservative management options [1, 2] . Excision can be performed using various surgical methods. For example, a cold steel technique or an office-based laser can be used [1]. Phonomicrosurgery allows immediate removal of the lesions. However, the procedure itself often inevitably disrupts the vocal fold microstructure, namely the extracellular matrix (ECM), and induces a cascade of inflammation and repair response [3]. During the healing process, fibroblasts are attracted to the injured area and become proliferative and secretive in collagen deposition and remodeling. If the process is not controlled in a timely manner, a chronic scar can result. Vocal fold scars increase their stiffness and impair their vibratory amplitude and symmetry, which can lead to recalcitrant hoarseness and dysphonia [3, 5, 6]. The occurrence of unpredictable vocal fold scarring after surgery remains a difficult clinical challenge to be addressed [5, 7-9].

At present, no effective measures are available to either prevent or treat postoperative vocal fold scars [5, 7-9]. When a glottal gap develops as a consequence of scarring, laryngologists can perform injection medialization laryngoplasty procedures to alleviate the patient's symptoms[10]. The injectable material reapproximates the vocal folds towards the midline and decreases the glottal gap. This procedure however does not remediate to the disruption of the mucosal waves and to the scarred tissues [7].

A more effective approach to address this problem might be to prevent the formation of a post-operative scar by using injectable biomaterials. Multiple agents have been suggested as preventive measures in both *in vivo* and *in vitro* models [8, 13]. These could have the potential to restore

surgically injured vocal folds with minimal scarring. In light of a careful review of the literature, we have chosen three different biomaterials with potential for scar prevention:

3.1.1 Hyaluronic acid

Hyaluronic acid (HA) is a sulfated glycosaminoglycan and one of the main components of the ECM of the vocal folds [2]. HA represents an important determinant in vocal fold biomechanics and cellular interactions. The concentration of HA in the superficial vocal fold significantly influences vocal fold viscoelastic properties [2, 13]. HA injections in a rabbit vocal fold model have been shown to decrease the elastic shear and the viscous modulus when compared to saline controls 6 months after injection [11, 18, 19]. HA has shown favorable wound healing effects on injured vocal folds [6, 11, 20] as it decreases fibrosis and type I collagen deposition[4, 18]. HA is biocompatible, bioactive and non immunogenic[4], which also explains why it is currently widely used in clinical practice as an injectable material for injection medialization laryngoplasty procedures [7, 13].

3.1.2 Corticosteroids

Corticosteroids' anti-inflammatory properties have rendered them very useful in the treatment of laryngeal pathologies involving local or systemic reactions [12, 22-24]. Their effect stems from the modulation of multiple cell types including macrophages, mast cells, fibroblasts and epithelial cells [24]. Corticosteroids regulate the synthesis and maturation of collagen fibers and inhibit fibroblasts [8]. Different steroid compounds have been tested as vocal fold scar treatment or prevention. Triamcinolone was found ineffective in decreasing scarring of surgically injured vocal folds in rabbit and canine models [12, 23, 24]. Dexamethasone is a potent steroid compound with

anti-inflammatory activity six times higher than triamcinolone [25]. In a rabbit study, 0.1 cc of dexamethasone was injected in the vocal folds immediately after surgical resection[12, 23]. Collagen deposition was significantly lower in the treated vocal folds compared to the control vocal folds at 3 and 7 days after injury[12, 23]. Results from this study suggested the potential use of dexamethasone to prevent iatrogenic vocal folds scars. Recently, the investigation of steroids for post operative scar prevention in laryngology has extended to human trials with positive vocal outcomes [9, 12, 24, 26, 27].

3.1.3 Chitosan

Chitosan is another injectable biomaterial that demonstrates favorable potential for scar prevention. It is a derivative of chitin, the second most abundant natural polymer and the main component of arthropod shells [28]. Chitosan has been used in the development of biomaterials for controlled delivery and tissue engineering, owing to its biocompatibility, biodegradability and good residence time with a half-life up to 84 days [29]. The effective modulation of collagen deposition and remodeling by chitosan is mainly related to its impact on migration and proliferation of fibroblast cells [31]. Chitosan was previously shown to decrease the number of fibroblasts in subcutaneous rat skin wounds at 8 and 14 days after injury[30]. Similar effects on fibroblast numbers were also shown in animal and human fibroblast cultures [31, 32]. Chitosan was shown to prevent anterior glottic stenosis (AGS) following a CO2 laser-assisted cordectomy procedure in canines [16, 33]. Chitosan gel was also shown to decrease post surgical scarring at the level of the ostium 12 weeks following sinus surgery in human patients operated for chronic rhinosinusitis[34, 35].

3.1.4 Purpose of this Study

Hyaluronic acid-based hydrogels, corticosteroids and chitosan are three potential injectable biomaterials that can be used as preventative measures for vocal fold scarring. These substances have been studied histologically and more specifically in the vocal folds for HA and dexamethasone. These materials, however, have not been assessed regarding their functional effects on voice physiology and acoustics. The primary goal of this study was to evaluate the phonatory function of rabbit vocal folds after immediate injection treatment of Dexamethasone, Hyaluronic acid/gelatin, Glycol-chitosan or Saline controls at 42 days after surgical injury. Vocal fold function will be evaluated objectively through the analysis of acoustic and aerodynamic data using laryngeal high-speed videoendoscopy (HSV). Subjective expert ratings of the HSV segments were also conducted to evaluate vocal fold vibratory characteristics. The working hypothesis was that at least one of the tested injectable materials would have a positive effect on the vocal outcome, defined as an improved functional outcome when compared to the Saline control group.

3.2 Methods

3.2.1. Animal selection and handling

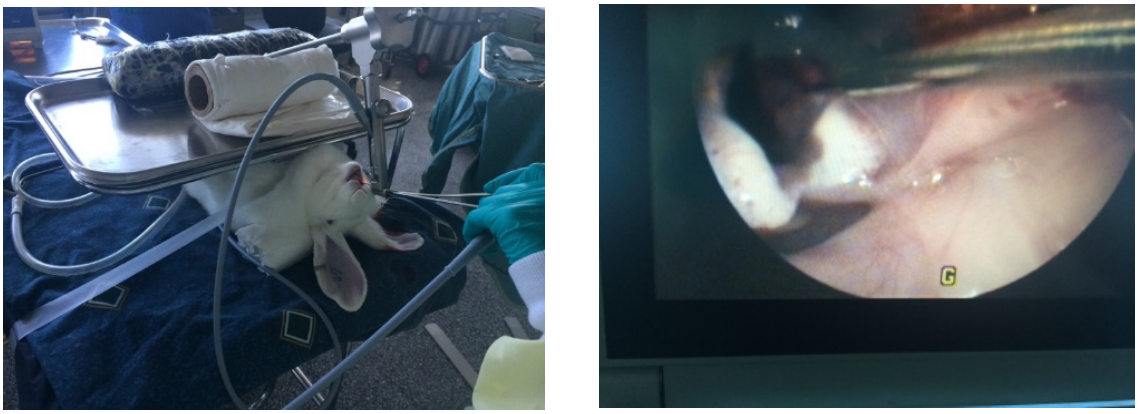
Twelve (12) New Zealand white breeder healthy female rabbits weighing between 2.8 and 3.4 kg were used for this study. This weight range is similar to that reported in a previous study describing the microflap injury model, which was adopted with slight modifications for the purposes of the present study [39]. All experimental and animal handling procedures were done in accordance with the National Institute of Health (NIH) guidelines for care and use of laboratory animals. Animals were housed in pens in groups of up to 8 per pen, and they were kept under a 12-hour-to-12-hour light cycle. There was a 1-week acclimation period before proceeding with the surgeries. The animal use protocol was approved by the Facility Animal Care Committee (FACC) at McGill University (Protocol number: 7556).

3.2.2. Surgical procedures

3.2.2.1 Anesthesia and Laryngeal exposure

Rabbits were kept fasting over a period of at least four (4) hours prior to surgery. Animals were anaesthetized using intramuscular (IM) injections of ketamine (35 mg/kg), xylazine (5 mg/kg) and acepromazine (0,75 mg/kg). Careful monitoring of vital signs (heart rate, oxygen saturation, and body temperature) was carried out by a veterinarian assistant throughout the procedure to ensure proper anesthesia. When needed, additional injections of anesthetics were administered to the animals to reach an adequate level of sedation (ketamine 17.5 mg/kg and acepromazine 0.375mg/kg)[38, 39]. The operating room set up and surgical technique described by Suehiro et al. 2012 [39] were replicated in order to create a microflap injury (Figure 3.1A). Exposure of the vocal folds was obtained using a pediatric anterior commissure side-slotted laryngoscope (Karl

Storz Endoscopy – America, Inc., El Segundo, CA, USA). Visualization of the surgical field was obtained via a 30 degrees, 2.7mm rigid endoscope (Karl Storz Endoscopy – America, Inc., El Segundo, CA, USA) coupled to a Telecam C camera (Karl Storz Endoscopy – America, Inc., El Segundo, CA, USA) [38, 39].



A **B**

Figure 3.1 Experimental methods. A) Operative setting for the procedure featuring rabbit under anesthesia. A bimanual technique was employed to adequately expose the larynx and perform the surgical interventions and injection procedures. B) Illustration of vocal fold exposure. A sickle knife was used to create the microflap injury after the larynx was properly exposed.

3.2.2.2 Acute laryngeal injury model

Microflap procedures were used to create the vocal fold injuries in this study with an intent to closely replicate microphonosurgery for BVFL [40] and [3, 15, 16, 41, 42]. A 5 mm long incision was made in the left vocal fold through the epithelium and the mucosal cover, following

the protocol described in a published surgical injury technique on a rabbit model [39] (Figure 3.1B). A fine microlaryngeal angled spatula was then introduced in the incision and an antero-posterior motion was repeated several times to exacerbate inflammation and scarring, without which the treatments could not be evaluated. The surgical area was injected with the treatment substance. Each animal was randomly assigned to one of the four treatment groups. A volume of 0.1 mL of each substance was injected using a straight 27-gauge collagen injection needle (L70.904 - Instrumentarium, Terrebonne, QC, Canada). The four treatment groups considered were: (1) glycol-chitosan solution 2% (group #1), (2) HA/Gelatin (Ge) hydrogel with 0.5% HA, 0.1% gelatin and 0.5% polyethylene glycol diacrylate (group #2) [43], (3) dexamethasone (group #3), and (4) saline (group #4) as the control group.

3.2.2.3 Laryngeal harvesting

The animals were humanely euthanized 42 days after injury. The larynges were harvested immediately post mortem. A 15-blade was used to create a vertical midline neck incision extending from the sub mental area to the sternum [44]. The midline strap muscles were separated and the thyroid notch was exposed down to the trachea. Horizontal cuts were made to release the larynx: one above the thyroid cartilage and one below the cricoid, including about 1cm of tracheal rings [44]. Larynges were kept at -80 C in a deep freezer until the functional analysis experiments.

3.2.3. Functional analysis

3.2.3.1 Setup for Flow-Bench Experiments

An experimental apparatus was designed to perform functional analysis on the excised rabbit larynges (Figure 3.2). In order to achieve better visualization of the true vocal folds, the

supraglottic structures of the larynx were removed. Each larynx was mounted on a 3D printed custom-designed support by fastening the trachea to the lateral port of the support using a zip tie. Adduction of the vocal folds was achieved by applying pressure on top of the arytenoids with two slender rods. A suture was placed through the thyroid cartilage and connected to a rotational knob that controlled the tension of the suture. At the start of each trial for each larynx sample, the tension of the suture was slowly increased until the initiation of phonation, and was kept constant throughout the rest of the experiment. The process was repeated between 3 and 10 times for each larynx sample. Only the best tokens were kept for analysis, i.e. the ones where phonation was continuous and stable. Average values of the phonation parameters were calculated using data from the three best tokens, and were used for analysis.

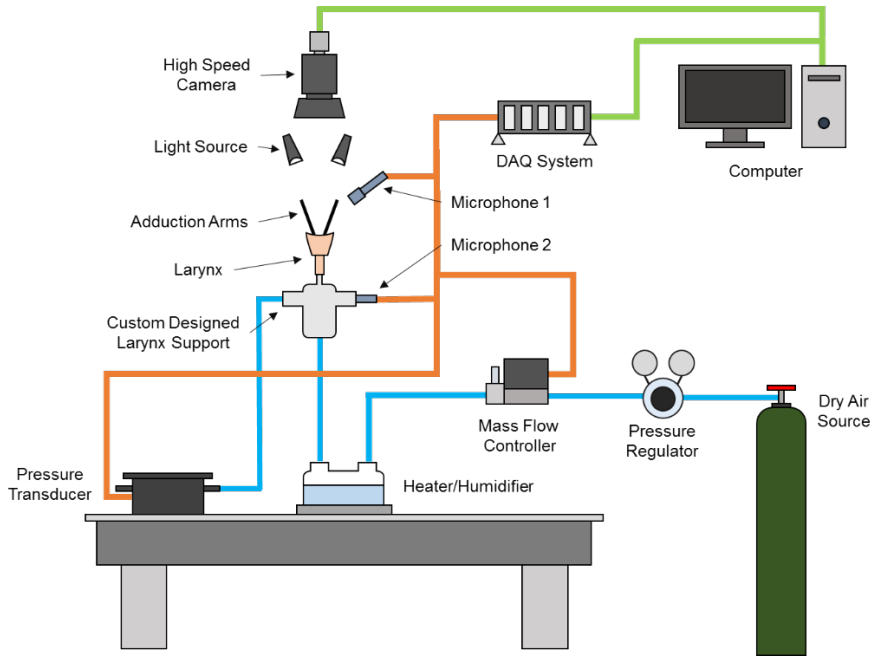


Figure 3.2 Schematic of the experimental apparatus to perform functional analysis on the harvested larynges. A high speed camera was held on a support above the customized excised laryngeal set up.

An illustration of the computer-aided design (CAD) model of the support is shown in Figure 3.3. The lateral ports of the support were connected to a differential pressure transducer (Baratron 220D; MKS, Andover, U.S.) and to a microphone (130D20; PCB Piezotronics, Depew, U.S.) for the measurement of the subglottal static and dynamic pressures, respectively. The ports were designed such that the connecting hoses fit tightly. Additional hose clamps were installed to prevent air leaks. Another microphone was fixed 10 cm above the larynx at an angle of 45 degrees for the measurement of the supraglottal dynamic pressure. The inlet port of the support was connected to the air source. Air flow from a regulated compressed air bottle (medical grade) was fed through a mass flow controller (Type 1500; MKS, Andover, USA) and a heater/humidifier system (HC150; Fisher&Paykel, East Tamaki, New Zealand) to obtain a 37 degrees Celsius and 100% humidified inflow. During the experiment trials, the larynx was sprayed with saline solutions to keep the vocal fold tissues hydrated. All of the sensors, mounts, and the sample were securely attached to a support made using T-slot extruded aluminum frames (80/20 Inc., Columbia City, U.S.). The entire apparatus was housed within panels made of leaded foam to reduce reverberation and extraneous noise.

3.2.3.2 Laryngeal high speed videoendoscopy, acoustic, and aerodynamic data acquisition

A high speed camera (FASTCAM MC2; Photron, Tokyo, Japan), equipped with a micro lens (AF 60mm F/2.8D; Nikon, Tokyo, Japan), was positioned directly above the larynx. Sufficient lighting was provided by halogen goose neck fiber optic lamps, positioned near the larynges at angles that minimized glare. The images were recorded at 6000 frames per second with a resolution

of 512 X 128 pixels. Each high-speed imaging data sample consisted of 1500 images, corresponding roughly to 100 glottal cycles. The larynx was installed on a 3D printed larynx support (Figure 3.4). A total of twelve video sequences, 3 for each treatment groups, were recorded and analyzed out of all tokens, as previously mentioned.

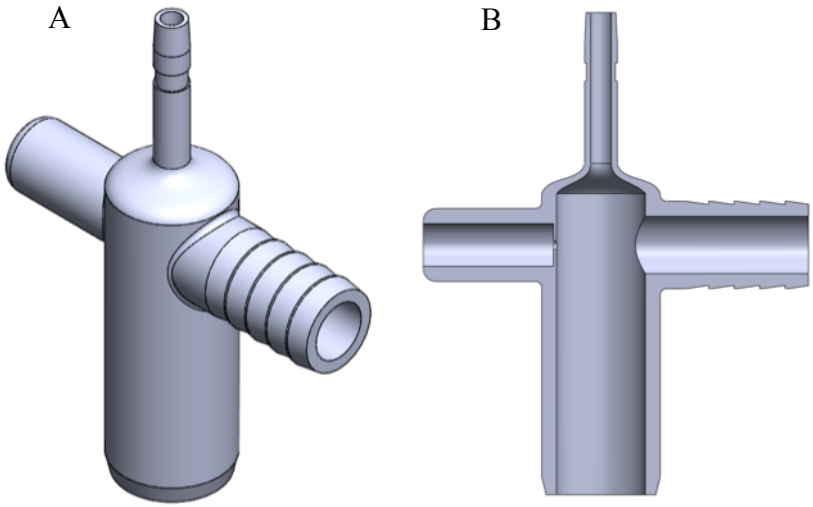


Figure 3.4 A computer model of the 3D printed larynx support. The top port is connected to the larynx and a zip-tie is taut around the groove to securely mount the excised larynx. The left port is connected to the microphone for subglottal dynamic pressure measurement. The right port is connected to the pressure transducer for subglottal static pressure measurement. A. Isometric view. B. Cross section view.

An in-house data acquisition (DAQ) system was created using LabVIEW (National Instruments, Austin, U.S.) and a DAQ board (National Instruments). The system was used to simultaneously record data from the microphones, pressure transducer, and mass flow controller. Airflow was regulated using the mass flow controller fed by a sinusoidal ramp voltage function input; the amplitude and period of the input were varied to control the rate of airflow to the larynx.

Each recording was 20 seconds, which was sufficient to create a gradual change in the air flowrate to capture both phonation onset and offset.

3.2.4. HSV Data Analysis

The high speed imaging data was analyzed using Glottis Analysis Tools (Department of Phoniatrics and Pediatric Audiology, University of Erlangen, Germany). Around 100 representative vibratory cycles were selected for analysis per video data samples. Seed points were then added to selected frames and gray thresholds were adjusted for each frame in order to refine the software's detection of edges during glottal opening and closure. During this process, the glottal midline was automatically detected as well. The vibratory amplitudes and areas were measured in pixels for the generation of glottal area waveforms (GAW).

In order to quantify the symmetry of the vocal folds dynamics, the dynamic range symmetry (DRS) was used, defined as

$$\text{Dynamic Range Symmetry (DRS)} = \frac{A_i^L}{A_i^R} \tag{1}$$

where A_i is the glottal area dynamic range for cycle *i,* and *L* and *R* represent the left and right portions of the glottal surface area, respectively. Thus, a DRS value smaller than unity represents a greater dynamic range of motion for the right vocal fold. Other variables, such as closing quotient and speed quotient were calculated as well.

The units of acoustic and aerodynamic data were converted from voltage to engineering units using the Lab VIEW program. The exported data was processed using custom MATLAB subroutines (2014b; The MathWorks, Inc., Natick, U.S.). The noise was first filtered using a Butterworth filter then the fundamental frequency was obtained using a fast Fourier transform

(FFT). Additional variables, such as jitter, shimmer, and signal-to-noise ratio were also calculated. Key quantitative data were selected amongst a comprehensive set of 309 parameters based on the most frequently used parameters in human laryngological and stroboscopic assessments [45]. Furthermore, the onset and offset threshold pressure and flow rate values were extracted from the synchronous sound pressure, flow rate and static pressure data.

3.2.5 Expert evaluation of the laryngeal high-speed videoendoscopy videos

3.2.5.1 The scoring system for visual expert rating

The scoring system used in this study was based on the HSV assessment form for human patients developed by Yamauchi et al. [46]. Revisions were made to adapt the tool for our *ex vivo* laryngeal samples. For instance, the presence of secretions or supraglottal edema was removed from the original form. Also, a parameter characterizing vocal fold stiffness was added to the form. The scoring tool contained six rating parameters including symmetry, periodicity, glottal closure, mucosal wave, amplitude and stiffness (Table 3.1).

	0	**1**	**2**	**3**
Symmetry	Symmetric	Slight asymmetry	Moderate asymmetry	Severe asymmetry
Periodicity	Periodic	Aperiodic	-	-
Glottal closure	Complete	Incomplete	-	-
Mucosal wave	Normal	Decreased	Absent	-
Amplitude	Normal	Decreased	Absent	-
Stiffness	None	Mildly stiff	Very stiff	-

Table 3.1 Rating scales for the subjective evaluation of HSV data.

The scoring template for the six different parameters is shown in Table 3.1. The Likert scale varied from 0 to 3 for the case of symmetry. It varied from 0 to 1 in the case of periodicity and glottal closure. It varied from 0 to 2 for mucosal wave, stiffness and glottal closure. The minimum possible overall score was 0, and the maximum possible score was 11 for each laryngeal sample. A higher score presumably indicates poorer tissue mechanics.

3.2.5.2 Definition of rating parameters

Symmetry compares the mucosal displacement amplitudes of the injured (right) and uninjured (left) sides [47]. It was evaluated on a scale from 0 to 3. Periodicity is a measure of regularity of the vibratory cycle [47]. It was scored as being present or absent. Glottal closure refers to the pattern of mucosal closure [47]. Raters were asked to assess if the closure between the vocal folds was either complete or incomplete. The mucosal wave refers to the presence of a bending wave on the superior and medial surfaces of the vocal folds [48]. It was rated on a scale from 0 to 2. Amplitude characterizes the extent of the vocal fold lateral displacement [48]. It was also rated on a scale from 0 to 2. Finally, raters were asked to evaluate the stiffness of the vocal folds. This parameter was added as it constitutes a byproduct of scarring. It was rated on a scale from 0 to 2.

3.2.5.3 Raters selection and training

Three laryngologists with respectively 7, 18 and 33 years of clinical experience in laryngology were recruited as expert raters for the HSV video segments. Before the evaluation, raters were provided with instructions and explanations of the rating template. Raters were blind to the research group.

3.2.5.4 Evaluation of the samples and data analysis

Twelve HSV videos (3 animals x 4 treatment groups) were chosen from the segments used for the glottal analysis tool and segmented for visual rating with the removal of the acoustic track. Each video was shown in the same sequential order to each rater. Each video sequence had variable durations but all were less than one minute long. Raters were allowed to repeat the recordings as needed to complete their evaluation. Means and standard deviations of the scoring data were calculated for each group.

3.3 Results

3.3.1. Functional Analysis

Phonation was achieved in all groups. The onset/offset and steady state phonation measurements were analyzed. The HSV results and acoustic/aerodynamic results for different treatment groups are listed in Table 3.2 and Table 3.3, respectively.

Table 3.2. Laryngeal high speed videoendoscopy (HSV) and acoustic results of the phonatory parameters in rabbit larynges (n = 3 per treatment group).

	Saline controls		*Glycol-chitosan solution 2%*		*HA/Ge hydrogel*		*Dexamethasone*	
Parameters	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Fundamental Frequency (Hz)	411.52	11.63	385.78	12.70	342.96	7.94	348.79	14.99
Jitter (%)	3.90	1.41	3.89	1.55	2.73	1.47	2.54	1.28
Jitter (ms)	0.10	0.04	0.10	0.05	0.08	0.05	0.11	0.05
Shimmer (%)	0.23	0.07	0.51	0.34	0.35	0.16	0.34	0.24
Shimmer [30]	0.16	0.05	0.35	0.23	0.23	0.11	0.23	0.17
Signal-to-noise Ratio	5.06	1.19	3.45	0.73	5.24	0.50	5.38	1.02
Closing Quotient	0.39	0.03	0.23	0.03	0.28	0.02	0.27	0.04
Speed Quotient	1.44	0.21	1.52	0.35	1.05	0.20	2.66	0.58
Dynamic Range Symmetry	0.77	0.09	0.88	0.09	0.74	0.08	0.87	0.07

Table 3.3. Aerodynamic results of the phonatory parameters in rabbit larynges (n = 3 per treatment group).

	Saline Controls		*Glycol-chitosan solution 2%*		*HA/Ge Hydrogel*		*Dexamethasone*	
Parameter	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Onset Pressure (cmH2O)	9.24	1.17	16.02	2.21	12.79	1.75	11.69	1.72
Offset Pressure (cmH2O)	6.43	1.07	7.09	0.65	7.52	0.69	6.05	1.42
Onset Flow Rate (L/min)	0.90	0.25	1.62	0.46	1.84	0.31	1.84	0.79
Offset Flow Rate (L/min)	1.08	0.15	1.16	0.10	1.71	0.21	1.42	0.62

3.3.2. Expert evaluation

Table 3.4 shows mean scores and standard deviations obtained for each visual rating parameter as a function of the treatment group. Regarding the overall scores (Figure 3.4), the dexamethasone group had the lowest values with an average of 2.78±1.98 among the groups. The Glycol-chitosan group had the highest overall score of 4.00±1.15. The Saline control group values were similar to those for the Glycol-chitosan group, with an overall score of 3.56±1.10. The HA/Ge group had an overall score of 3.00±1.66.

When considering individual parameters, a noteworthy difference was observed in the mucosal wave characterization for the Glycol-chitosan group. The mean scores for the mucosal wave and the stiffness were the highest for the Glycol-chitosan group (0.78±0.19). The Dexamethasone group had a slightly smaller average score for stiffness (0.67±0.58) whereas the Glycol-chitosan solution 2% (1.00±0.00) and the Saline group showed worse result patterns as indicated by their total score values.

Table 3.4. Means and standard deviations of expert visual rating results.

Parameter	Treatment group							
	Glycol-chitosan Solution 2%		*Dexamethasone*		*HA/Ge Hydrogel*		*Saline*	
	Mean value	SD	Mean value	SD	Mean value	SD	Mean value	SD
Symmetry	1.11	0.19	0.78	0.51	0.89	0.51	0.78	0.39
Periodicity	0.11	0.19	0.11	0.19	0.22	0.39	0.22	0.39
Glottal closure	0.22	0.38	0.22	0.39	0.11	0.19	0.33	0
Mucosal wave	0.78	0.19	0.56	0.51	0.56	0.39	0.67	0.33
Amplitude	0.78	0.19	0.44	0.39	0.44	0.19	0.67	0
Stiffness	1.00	0	0.67	0.58	0.78	0.69	0.89	0.39
Total score	4.00	1.15	2.78	1.98	3.00	1.66	3.56	1.10

3.3.3. Bifurcation

During the beginning phase of the onset, the vocal folds vibrated at a low amplitude and after reaching a specific flow rate threshold, the vibration amplitude rapidly increased with the subglottal static pressure. This bifurcation was observed throughout the experiments trials along with a change in frequency. It appears that the vocal folds' vibration and their cycle was more efficient moments after the bifurcation phenomenon happened, during the second mode of phonation. This phenomenon is shown in Figure 3.5, where the change in glottal amplitudes are visible in both the HSV and the acoustic measurements.

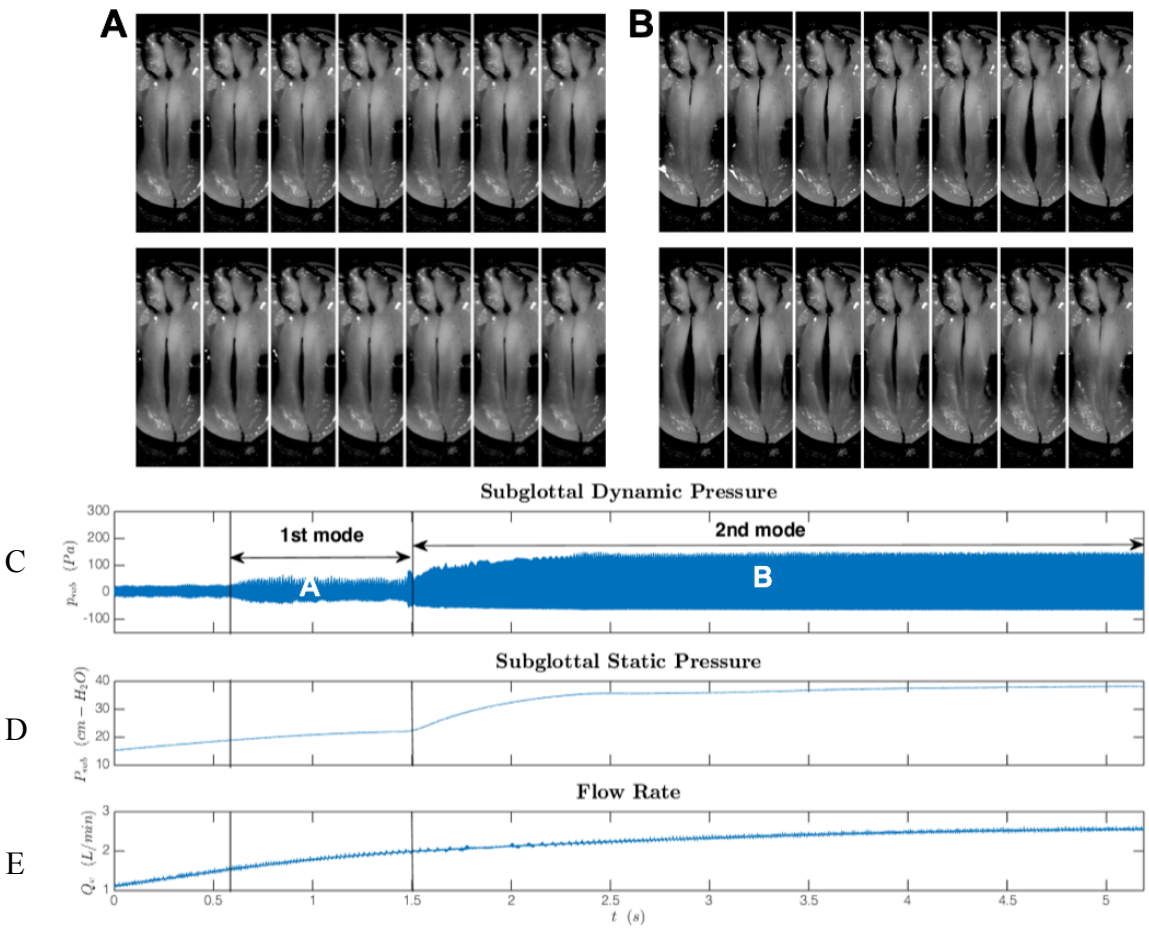


Figure 3.5. HSV, aerodynamic, and acoustic results showing bifurcation

A. First mode of phonation, showing smaller glottal amplitude with an almost immobile left vocal fold. Low efficiency vibratory pattern with no closed phase. **B.** Second mode of phonation with higher glottal amplitude, periodic vocal folds abutting and efficient vibratory pattern. **C.** Subglottal dynamic pressure in Pa. **D.** Subglottal static pressure in cm - H_2O. **E.** Flow rate in Liters/min by time (seconds).

A closer observation of the bifurcation phenomenon revealed that the oscillatory mode also changed at the bifurcation point. In the first mode of phonation, the oscillation is aperiodic and

unstable. As the flow rate increases, the phonation becomes more stable and switches to a second mode, where the oscillation is nearly harmonic, as shown in Figure 3.6.

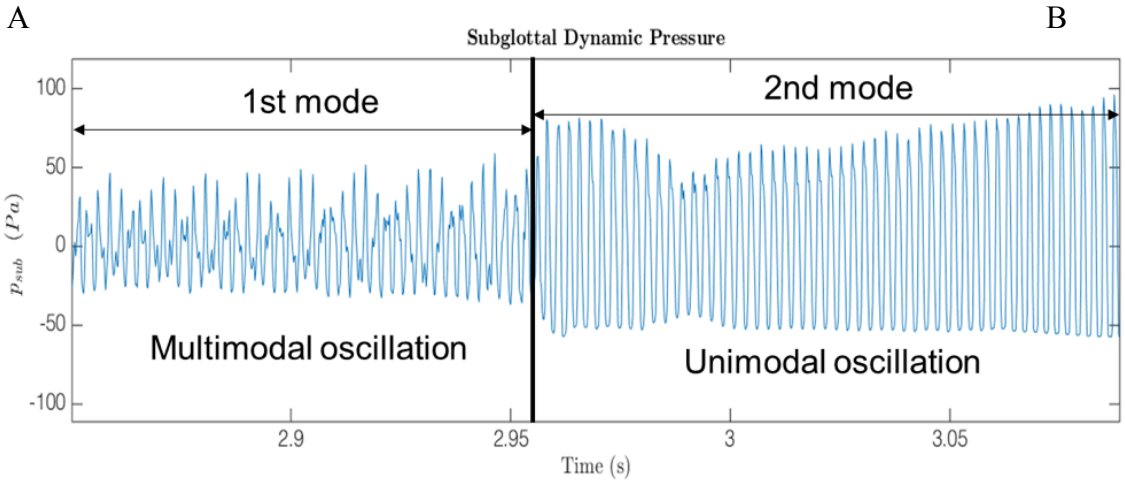


Figure 3.6. Close-up view of the subglottal dynamic pressure. Two different modes of oscillation can be observed. **A.** 1st mode: multimodal oscillation. **B.** 2nd mode: unimodal oscillation.

3.4 Discussion

There is currently a paucity of data regarding the correlation between HSV parameters and acoustic and aerodynamic data pertinent to vocal fold scarring. The combination of objective and subjective expert analysis of the HSV samples conducted in this study offers a complementary evaluation of the phonatory function of the vocal folds [49]. In fact, objective measurements from the HSV (e.g., fundamental frequency) were used to assist the interpretation of the visual ratings of vocal fold vibration features (e.g., mucosal wave visual appearance).

3.4.1. Functional analysis

The observed phonatory properties generally agree with the values reported in a previous excised rabbit larynx study [44]. HA/Ge hydrogel group showed the lowest fundamental frequency value at around 320 Hz and the saline control group showed above 400 Hz. It is currently believed that vocal fundamental frequency is in part determined by the vocal fold stiffness, with higher stiffness producing voice with higher fundamental frequency [2, 50, 51]. Other parameters, such as vocal fold mass and aerodynamics may contribute to variations in the fundamental frequency [50, 51]. Thus, the lower fundamental frequencies seen in the 3 treatment groups compared to the untreated Saline group may suggest that all substances had a softening effect on the vocal fold tissues. More specifically, HA/Ge hydrogel and dexamethasone might be the most effective substances in reducing the stiffness of the vocal folds.

From the HSV results, the dynamic range symmetry (DRS) was used to provide another perspective on the effects of the tested substances. With increased stiffness in the vocal folds, the resulting decrease in vocal fold compliance decreases the vocal fold glottal dynamic area. In other

words, using Equation 1, a stiffer left vocal fold in comparison to the right fold should yield a DRS value less than unity. A DRS value close to unity indicates that the vocal fold vibration is nearly symmetric. The calculated mean DRS values from the study are all below 1, implying that the right fold was stiffer compared to the left fold. Table 3.2 shows that glycol-chitosan and dexamethasone were the most effective substances for restoring vocal fold symmetry.

It should be noted that due to the intrinsic anatomical vocal fold asymmetry, the DRS alone is not a sufficient measure of evaluating the effectiveness of the biomaterials. However, when used relative to the saline control group, along with the relative values of the fundamental frequency values, the functional study can be validly used to determine the relative effectiveness of a specific group compared to others. Combining the fundamental frequency data and the DRS results, dexamethasone was suggested as a potential anti-fibrotic substance.

The bifurcation phenomenon has been previously documented in other excised larynx studies [52, 53]. These studies have explored the effect of asymmetry on the nonlinear behavior of excised larynx phonation. Thus, the asymmetry caused by the vocal fold scarring could have caused bifurcation in some samples. In a clinical setting, bifurcations change in oscillatory modes were reported from a patient with muscle tension dysphonia (MTD) [54]. Although the sudden increase in amplitude was similarly observed, a unimodal oscillation was exhibited in the first mode and a bimodal oscillation was measured in the second mode. A study on human vocal fry, however, reveals a similar vibratory pattern as the first mode shown in Figure 3.5[46, 55]. Although vocal fry can result from vocal fold damage or habitual voice use, another parameter that can induce vocal fry is the amount of mechanical stress applied to the vocal folds.

During the experiment, vocal fold adduction was achieved using two arms pushing the arytenoids together and also by applying tension perpendicular to the axial direction. This combination

induced the necessary in-plane stress on the vocal folds for phonation. As mentioned in the Methods section, the tension was increased for all samples until phonation onset and remained constant throughout the rest of the experiment. However, it should be noted that the tension could be increased to achieve a higher fundamental frequency. A greater tension often induced asymmetry resulting in different types of phonation. Although every effort was made to avoid to unintentional changes in the adduction, due to the nonlinear nature of the vocal fold phonation, the initial posture of the larynges could have contributed to the observed bifurcation in both acoustic and HSV measurements.

3.4.2. Expert visual rating evaluation

When comparing the four groups, the total score of the expert ratings was the lowest in the dexamethasone group (2.78±1.98), suggesting better visual rating results overall. The dexamethasone group also demonstrated lower stiffness and amplitude results. When considering both the functional analysis results and the perceptual data, dexamethasone seemed to potentially improve the healing process and contribute to decreased vocal fold scarring. In fact, our data seemed to corroborate previously published data on the positive effects of dexamethasone injections on vocal fold scarring [12, 23]. While previous studies showed a reduced collagen deposition in the acute phase at day 3 and 7 [12, 23], our results suggest that the steroid effect might be longer lasting as our rabbits were sacrificed at Day 42. This time point was chosen as the healing process of the rabbit vocal folds has been studied and the acute phase is generally resolved by day 21 after surgery[12, 56].

The worst total score was attributed to the glycol-chitosan treatment group, closely followed by the Saline control group. It should be noted that the glycol-chitosan treatment group refers

to the rabbits injected with a solution of glycol-chitosan 2% in deionized water, not a glycol-chitosan hydrogel. This may result in fast degradation of the glycol-chitosan solution *in vivo*, which further might yield a similar response to the saline controls. This may explain the similarity in the obtained results for the glycol-chitosan group and the saline controls, as both also showed poor mucosal wave ratings. In human subjects, vocal fold scarring presents with impaired glottal closure, and decreased vocal fold vibration associated with an alteration of the mucosal wave [57]. Therefore, the mucosal wave impairments as well as the increased stiffness detected in the glycol-chitosan and saline treatment groups are two parameters that could be correlated with a more significant scarring process.

3.5 Study limitations

The first limitation is related to the inherent nature of an *ex vivo* setting. *Ex vivo* models, although helpful for a better understanding of the vocal physiology and biomechanics, have their limitations. Tissue degeneration and the required artificial creation of a vocal fold tension and adduction are examples of the caveats of this approach [58]. Future work may involve functional analysis *in vivo* via a tracheostomy insertion. This invasive approach would allow a more representative substrate for analysis as the larynx is kept in its natural location. Technical challenges with this method would be maintaining the animals under general anesthesia and inducing phonation via the introduction of air through the tracheostomy. Tracheostomized animals also require supplemental extra care and handling.

A second limitation is that the vocal fold injury model could have been better validated prior to proceeding to the study. In fact, it has been shown that rabbit vocal folds recover vibration amplitude and lateral phase difference by 3 to 7 days following a microflap

injury[59]. It is for this reason that the model used in this study was a modified version of the microflap procedure in which a laryngeal spatula was briskly introduced in the wound to amplify the inflammatory response. However, no histological confirmation of scarring was obtained to properly confirm that this modified microflap technique induced significant ECM disruption.

Inter-specimen inconsistencies also represented a challenge in this study especially considering the small sample size. Each rabbit larynx has its own variability in anatomy. The amount of tension applied to the sutures and the arytenoids also had to be variable in order to ensure proper phonation in each sample. Despite the fact that the experimental rabbits fitted into specific inclusion criteria, the intrinsic diversity of subjects needs to be taken into account when interpreting these results [46]. This may explain the relatively important standard deviation values seen amongst the different treatment groups. The small sample size used as well as the different variables in this study does not allow clear statistically significant conclusions, but rather shows trends to be validated in a larger scale animal work.

In terms of the HSV expert rating, three different raters scored the videos. Unfortunately, the dataset was too small to reliably estimate the inter-rater variability. This would have been an interesting additional data to obtain as it could have contributed to explain the overlap and the range of values reported. Likewise, it would have been interesting to have the raters rate more video segments to increase the data set. Finally, the customized scoring template for visual rating analysis was not standardized and externally validated for excised rabbit larynges. The use of such a tool, had it been available, would have been beneficial in order to generalize and to compare our results with future subsequent studies in rabbits.

3.6 Conclusions

Our protocol demonstrated the feasibility, efficiency and effectiveness of an excised rabbit larynx model for injectable evaluation. The Dexamethasone treatment group showed a trend towards better functional results, possibly suggesting less fibrotic wound healing, with less scar and less aperiodic areas seen on visual expert ratings. Functional analysis was performed to quantify the effectiveness of injectable biomaterials as preventive measures for surgically injured vocal folds in rabbits. The HSV technique was shown to be useful not only as a tool to measure the symmetry of vocal folds during post-processing, but also as a qualitative measurement tool in clinical settings. The acoustic data were used to verify each sample's phonatory parameters. Results suggested a promising use of HSV and acoustic analysis techniques to identify and monitor post-surgical vocal fold repair and scarring.

3.7 Acknowledgments

This study was funded by the NIH grant DC005788 (Principal investigator: Luc Mongeau). We also would like to acknowledge Mme Karen Hope Stone, animal health technician from the McGill University – Comparative Medicine Resources Centre for her assistance during the surgical portion of this study.

Chapter 4: Overall discussion

The previous manuscript reported an investigation of functional outcomes of surgically injured rabbit vocal folds subjected to three injectable materials. The design was a multi-armed study in which three chosen injectable biomaterials were compared to a saline control group to study their potential scar preventing effect. A rabbit model of an acute laryngeal injury was created and the vocal folds were injected with different biomaterials (dexamethasone, HA/Ge hydrogel, glycol-chitosan or saline). Forty-two days after injury, the rabbits were sacrificed and the larynges were mounted on an experimental apparatus to mimic normal physiology and to obtain phonation. A functional objective data analysis and a subjective expert ratings analysis of the obtained images were done. The dexamethasone treatment group showed a trend towards better functional results, possibly suggesting less fibrotic wound healing, with less scar and aperiodic areas seen on visual expert ratings. This confirmed our hypothesis that at least one of the tested injectable biomaterials would show trends towards a positive functional outcome.

The sample size used for this work was relatively small to draw strong conclusions regarding the superiority of one injectable material versus the others in regards to the possible scar prevention effect. This project constituted the first milestone as a pilot study to demonstrate the feasibility, efficiency and effectiveness of an excised rabbit larynx injury model.

The original aim of the thesis was to pair the results of the functional analysis (HSV data and Visual expert ratings) to a histological analysis as well as a gene expression analysis. This multi-parameter analysis would have led to a more comprehensive evaluation of the scarring process and thus to a better assessment of the tissue rearrangements.

In fact, a separate experiment was started to examine preserved vocal folds from the different treatment groups. The analysis was to be done using a new technology, called non-linear

laser scanning microscopy (NLSM). This type of imaging allows visualization of microstructural details such as collagen and elastin, two important ECM determinants of vocal fold scarring. The excised rabbit larynges were embedded in OCT, frozen and sectioned into 6 μm slices. Following this, the slides were fixed in paraformaldehyde and mounted using phosphate buffered saline [60]. Conventional hematoxylin and eosin (H&E) staining was used to locate what appeared to be the mid vocal fold for each animal. It was realized later that what was believed to be vocal folds on the H&E staining surprisingly turned out to be of cartilaginous origin. This was discovered as another member of the group investigated the correlation between high resolution computed tomography (CT) scanning images and NLSM data. This new information will be the described in a publication currently in preparation.

Due to the surprising observations outlined above, the most anterior cuts of the rabbit larynges (which probably contained the true vocal folds) were unfortunately discarded. It is also possible that the buffers used during the rabbit sample preparation could have contributed to the distortion of the specimen, rendering the true vocal folds extremely small and harder to process. As a result, very important histological data was lost. However, this experience contributed in a positive way to the future animal rabbit studies: This study has strongly suggested that rabbit vocal fold tissue is located more anterior than is commonly thought.

Gene analysis also represents an additional option to obtain data on tissue rearrangements and wound healing. It was previously shown that fine needle aspiration (FNA) is a feasible method to obtain tissue from the vocal folds of animal larynges [61]. The use of FNA was also initially chosen during the design of the experiment to reduce the number of required rabbits. It also allowed for the use of a very attractive repeated-measure analysis of variance (rMANOVA) statistical model.

Thus, an additional separate experiment was set up in order to obtain FNA samples at 3, 10 and 38 days after injury (or days post injection (DPI)) and trend inflammatory markers and determinants of scarring. The FNA samples were processed for total ribonucleic acid (RNA) extraction, which were later used to prepare complementary deoxyribonucleic acid (cDNA). Real-time quantitative polymerase chain reaction (PCR) was done to assess the expression of genes associated with extra-cellular matrix deposition (Collagen type 1 alpha 2; Collagen 3 alpha 1; Elastin), the inflammatory response, (TGFβ1; Interleukin-6, IL-6), or with house keeping (HK) function (SDHA, SBPTN1, GAPDH).

Unfortunately, variation in sample size, sampling quality (blood contamination), cellularity, and sampling sites between left and right vocal folds, rabbits, as well as DPI rendered several FNA samples unusable on the basis of low RNA, PCR signal quality, or HK gene expression. Repeated sampling of the wound-site also seemed to have an overall negative effect the overall quality of the FNA samples and most of the 38 DPI samples were similarly determined to be unusable. These issues prevented taking full advantage of the MANOVA approach, and making a definite pronouncement as to the efficacy of HA/Ge hydrogel, glycol-chitosan, or dexamethasone as it relates to vocal fold wound healing.

However, valuable data was obtained regarding the overall effect of these treatments at 3 DPI and 10 DPI. Indeed, it was possible to observe a marked decrease (≥15-fold) of collagen 3 expression at 3 and 10 DPI in rabbits treated with the Ha/Ge hydrogel and dexamethasone, compared to the saline control treated vocal folds. Rabbits treated with glycol-chitosan lead to a non-statistically significant decrease of collagen 3 expression compared to the saline control group (<5-fold) at 3 DPI and to a non-statistically significant increase (>5-fold) at 10 DPI. Expression data for IL-6 yielded similar and compatible data, as dexamethasone was the only treatment able

to significantly decrease IL-6 expression at wound site compared to saline controls at both 3 DPI (7.5-fold) and 10 DPI (20-fold). For its part, glycol-chitosan injections lead to an expected marked increase in IL-6 expression at both 3 DPI (≈10-fold) and 10 DPI (50-fold). Conversely, the experimental HA/Ge hydrogel compound did not lead to significant changes in IL-6 expression compared to the saline treated controls at 3 or 10 DPI.

This portion of the experiment also contributed to knowledge in a positive way as it suggested ways to better plan future research studies. The low cellularity of vocal fold tissue was at the source of some of the difficulties encountered in the quantitative PCR studies.

Moving forward, future studies (a) should pool at least 2 vocal folds of the same experimental group prior to RNA extraction, and (b) should move to a multiplex qPCR approach with pre-amplification of the RNA targets.

Chapter 5: Overall conclusion

This work demonstrated the feasibility, efficiency and effectiveness of an excised rabbit larynx model for injectable evaluation. The primary aim was to test three different injectable materials in the vocal folds and compare them to Saline controls in order to assess their potential scar preventing effect. The Dexamethasone treatment group showed a trend towards better functional results, possibly suggesting less fibrotic wound healing, with less scar and less aperiodic areas seen on visual expert ratings. Functional analysis was performed to quantify the effectiveness of injectable biomaterials as preventive measures for surgically injured vocal folds in rabbits. The HSV technique was shown to be useful not only as a tool for measuring the symmetry of vocal folds during post-processing, but also as a qualitative measurement tool in clinical settings. The acoustic data was used to verify each sample's phonatory parameters. In particular, the acoustic measurements clearly revealed nonlinear behaviours of vocal folds during phonation. Results suggested a promising use of HSV and acoustic analysis techniques to identify and monitor post-surgical vocal fold repair and scarring.

Chapter 6: Claims of originality

(1) This is the first study comparing multiple injectable biomaterials regarding their efficacy as potential scar preventive measure in an excised rabbit vocal fold model. (2) This is the first study combining the results of a functional objective analysis and a subjective expert rating. (3) A novel scoring template for expert rating was created to score the HSV images for an excised rabbit laryngeal model.

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