Does botulinum toxin type A alter the consequences of recurrent laryngeal nerve transection in the rat model?

by

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Abstract

Introduction: Laryngeal paralysis (LP) is a disorder that impacts significantly the health and the quality of life of patients. It causes a dysfunction of vocal folds (VF) movement as a result of affection of the laryngeal neural supply. While several theories have been proposed to explain the pathophysiology, synkinesis or misdirected re-innervation of laryngeal muscles is the currently accepted mechanism. Several experimental treatments to restore VF functional mobility have been proposed. However, most of them were unsuccessful due to the persistence of synkinesis. Injection of neurotoxins into laryngeal muscles was used to overcome this problem, but unfortunately did not restore functional mobility, and was associated with significant side effects. Only one animal experiment previously tested botulinum toxin type A (BTX-A) injections into laryngeal muscles, and resulted in widening the laryngeal inlet, although recovery of function was not assessed. In a study on children with LP, BTX-A was injected into external laryngeal muscles and resulted in recovery of function in nearly all of them. This study and others suggested that toxin's action at the neuromuscular junction, may be not be the only one at play, and that other promising sites of action (e.g. central) can reverse the synkinetic phenomenon.

Objective: To investigate whether an injection of BTX-A into external laryngeal muscles enhances the recovery of laryngeal function, in laryngeal paralysis rat model.

Methods: A single blind randomized controlled animal study (Sprague Dawley rats) with surgically induced LP was conducted. After transection of the right recurrent laryngeal nerve (RLN), a BTX-A or normal saline was injected into the intervention or the control group's cricothyroid, sternothyroid and sternohyoid muscles. Under general intravenous anesthesia, laryngoscopy and LEMG recordings from thyroarytenoid (TA), cricothyroid (CT) and posterior cricoarytenoid (PCA) muscles were performed before, immediately and four to six weeks after transection of RLN. LEMG activity of each muscle was graded (0-4), according to the amplitude and relation to the phase of respiration. VF movement on laryngoscopy was graded as normal, partially mobile, or immobile.

Main Outcome Measures: Primary: Difference between median LEMG grades of the two groups. Secondary: 1- difference between the proportions of recovered and persistently paralyzed VF in the two groups, 2- Difference between the medians of burst amplitude and duration on LEMG in the two groups.

Results: Twenty-four Sprague Dawley rats were randomized. Only nineteen animals were available for the final evaluation. LEMG analysis showed a statistically significant difference between the median LEMG grades of right PCA (p= 0.02, 95% CI 0.017-0.023). The median grade for the intervention group (n=10) was 4 (25^{th}%=2.75, 75^{th}%=4), and for the control group (n=4) was 1 (25^{th}%=0.25, 75^{th}%=3.25). No difference was found in the TA median grades. Recovery of movement was observed in four out of nine animals, and four out of ten animals of the intervention group (p 1.00). No statistically significant difference was found between the amplitude and the burst durations between the two groups.

Conclusions: BTX-A appears to enhance the phasic activity of the laryngeal abductor muscle in the rat model at the short-term. However, this was not translated into functional VF movement. Further work will be necessary to clarify the impact on clinically significant mobility.

Preface

This thesis is an original work by Mohammed A. Jomah. The research project, on which this thesis is based, received research ethical approval from the Animal Care and Use Committee for Health Sciences in University of Alberta, Project name "Does Botulinum toxin alter the consequences of recurrent laryngeal nerve section in a rat model?", No. 712/02/14/D, February 11, 2013.

Dedication

To my dearest parents Khairia Bin-Nouh & Abdulkader Jomah, with love.

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List of abbreviations

25th%	25th percentile
75th %	75th percentile
ACh	Acetylcholine
ACSS	Anterior cervical spine surgery
BTX	Botulinum toxin
BTX-A	Botulinum toxin type A
CA	Cricoarytenoid joint
Ca^{++}	Calcium
CI	Confidence interval
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CT	Cricothyroid muscle
CTX	Cardiotoxin factor
EJV	External jugular vein
EMG	Electromyography
FDA	Food and drug administration (United states of America)
FFL	Flexible fiber-optic laryngoscope
HR	Heart rate
Hz	Hertz
IA	Interarytenoid muscle
IGF-1	Insulin like growth factor type 1
IV	Intravenous
K^{+}	Potassium
L	Liter
LCA	Lateral cricoarytenoid muscle
LEMG	Laryngeal electromyography
LMN	Lower motor neuron
LP	Laryngeal paralysis

Lt.	Left side
MRI	Magnetic resonance imaging
MSC	Muscle stem cells
MU	Motor unit
MUP	Motor unit potential
Na^+	Sodium
NMJ	Neuromuscular junction
NS	Normal saline
O_2	Oxygen
PCA	Posterior cricoarytenoid muscle-
RLN	Recurrent laryngeal nerve
RR	Respiratory rate
Rt.	Right side
SD rats	Sprague Dawley rats
SH	Sternohyoid muscle
SLN	Superior laryngeal nerve
SM	Sternomastoid muscle
SNAP-25	synaptosomal-associated proteins of 25kDa
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
ST	Sternothyroid muscle
TA	Thyroarytenoid muscle
TEF	Tracheo-seophageal fistula
TIVA	Total intravenous anesthesia
VF	Vocal fold

Chapter 1: General introduction

1 Anatomy of the larynx

1.1 Objectives

- Overview of the anatomy of the larynx.
- Overview of the neuroanatomy of the larynx, its complexity and variability, with emphasis on relevance to laryngeal paralysis

1.2 Introduction

The larynx is a musculo-cartilaginous organ that has an anatomically complex structure. It is a part of the respiratory system, and is located at the inlet of the lower airway. In males, it lies opposite the third to sixth cervical vertebrae. In females and children it is slightly higher. In children there is a little difference in the size of the larynx between boys and girls. However, after puberty the anterior-posterior diameter increases to almost the double in males. The laryngeal cavity extends from the laryngeal inlet, at the lower end of pharynx, to the beginning of the trachea [1]. It can be divided into three regions: supraglottic, glottic and subglottic regions. The supraglottic region includes the epiglottis and the aryepiglottic folds. The glottic region includes vocal folds, anterior and posterior commissures. The subglottic region extends from 5-10 mm below vocal folds until the end of cricoid cartilage [2].

The essential function of this organ is to provide protection for the lower airway from the entry of foreign materials. In addition to this function, the larynx is the main organ responsible for voice production. It also acts as a sphincter, that when closed helps in building up the intra-thoracic pressure, which is important in cough reflex and in aiding performing muscular effort [1].

1.3 Laryngeal skeleton framework

The laryngeal framework, or skeleton, is composed of the hyoid bone and a group of cartilages; namely the thyroid, the cricoid, the epiglottis, the paired arytenoid cartilages, the corniculate and the cuneiform cartilages (Figure 1-1 to 1-4). These are connected by soft tissue attachment (i.e. ligaments and membranes), which permit changes in their relative positions. This flexible connection allows alteration in the shape and tension of the structures attached to them as a result of action of the intrinsic and extrinsic laryngeal muscles [1].

The hyoid bone lies at the superior aspect of the skeleton. It is a U shaped bone with its curvature facing posteriorly. On each side it has inferiorly located lesser cornua and superiorly located greater cornua. It is held in position by its muscular and soft tissue attachments to the skull base and the mandible. It gives attachment to mylohyoid, geniohyoid and hypoglossus muscles superiorly and is connected to thyroid cartilages by thyrohyoid membrane inferiorly [1].

The thyroid cartilage is a shield-like cartilage and is the biggest in the framework. It is composed of two laminae that fuse anteriorly in the midline forming an angle. This angle measures about 90 degree in males and 120 degree in females [3]. Posteriorly each lamina extends superiorly and inferiorly forming the superior and

inferior cornua. The superior cornu gives attachment to the lateral thyrohyoid ligament, which attach this cartilage to the hyoid bone. The inferior cornu articulates with cricoid cartilage by synovial joint. This joint has a rotatory movement and is affected by the contraction of cricothyroid (CT) muscles. On the external surface of each lamina an oblique line runs downward and forward from superior thyroid tubercle, located inferiorly to superior cornu root, to the inferior thyroid tubercle, located at the lower border of the lamina. This line gives attachment to thyrohyoid, sternothyroid and inferior laryngeal constrictor muscles. The inner surface is lined with loosely attached mucous membrane. The thyroepiglottic ligament is attached to the inner surface just below the thyroid notch. Inferior to it, and on each side of the midline, the vestibular and the vocal ligaments and the thyroarytenoid (TA), thyroepiglottic and vocalis muscles are attached [1].

The cricoid cartilage is the only complete circular cartilage in the airway. It is shaped like signet ring, with broad posterior lamina and narrow anterior arches. Each lateral surface articulates with the inferior cornu of the thyroid cartilage at a point close to the junction of lamina and arch. The upper border of the lamina articulates with the arytenoid cartilages forming two synovial cricoarytenoid joints. Fixation of this joint affects the vocal fold mobility and can be confused with laryngeal paralysis [1].

The arytenoid cartilages are paired pyramid-shaped cartilages that rest on top of the cricoid cartilage lamina. Each cartilage has a vocal process anteriorly that gives attachment to the vocal ligament, and a lateral muscular process that gives

attachment to the posterior (PCA) and lateral cricoarytenoid (LCA) muscles. The interarytenoid (IA) muscle extends from the posterior surface of one cartilage to the posterior surface of the contralateral one [1].

The corniculate and cuneiform cartilages are two paired small cartilages that are located in the aryepiglottic fold and may help to improve its rigidity [1].

The epiglottic cartilage is a thin big leaf-like structure with its narrow stalk attached to the anterior inner angle of the thyroid cartilage by the thyroepiglottic ligament. The upper broad part projects upward and backward behind the hyoid bone and the tongue. It is also attached to the hyoid bone by the hyoepiglottic ligament, and its free edges are attached to the arytenoid cartilages by the aryepiglottic folds [1].

1.4 Laryngeal muscles

Laryngeal muscles can be divided into two groups: extrinsic and intrinsic laryngeal muscles. The extrinsic muscles attach the larynx to the adjacent structures and control the position of the larynx in the neck. They exert minimal effect on vocal fold shape and movement indirectly. The intrinsic muscles are responsible for controlling the dynamics and the morphology of the vocal folds [1].

1.4.1 Extrinsic muscles

Extrinsic muscles can be divided according to their relation to the hyoid bone into two groups: suprahyoid and infrahyoid. The suprahyoid muscles include the mylohyoid, the geniohyiod, the stylohyoid, the digastric, the stylopharyngeous, the palatopharyngeous and the salpingopharyngeous muscles. The infrahyoid muscles include the thyrohyoid, the sternohyoid, the sternothyroid and the omohyoid. The

extrinsic muscles aside from linking the larynx to the adjacent structures and stabilizing it, they function to control the position of the larynx in the neck. During the various aero-digestive physiological functions they elevate or depress the larynx or move it anteriorly or posteriorly. These movements are important for airway protection, during swallowing, and also in controlling the vocal tone during phonation [1]. Extrinsic muscles can play a role in the breathing and phonation functions of the larynx by affecting the vocal folds movement and tension indirectly, however this effect is minimal [4-6]

1.4.2 Intrinsic muscles

These muscles are the main muscles responsible for controlling the vocal folds movement, position, mechanical properties and shape (Figure 1-2, 1-3, 1-4). They abduct, adduct or change the length and the tension of the vocal folds. All these muscles are paired except for the IA muscle, which is a midline single muscle [1] (Figure 1-5, 1-6, 1-7).

The PCA is the only muscle that abducts the vocal folds, and is the second biggest intrinsic muscle. It extends from outer posterior surface of cricoid lamina to the back of the muscular process of the arytenoid cartilage. The upper fibers of this muscle are horizontal, while the lateral fibers are vertical. Contraction of the PCA muscle rotates the arytenoid outward and downward by two synchronous actions. The horizontal action rotates the arytenoid cartilage causing the muscular process to move towards the midline and the vocal processes to move laterally, and thus the glottis widens. The vertical action pulls the arytenoid cartilage down thus separating the arytenoid cartilages from each other. The net result of this combined

action is abduction, elevation, elongation and thinning of the vocal folds. All the layers of the vocal folds become stiff and the edge becomes rounded [1].

The LCA muscle extends from the superior lateral aspect of cricoid arch, and inserts into the anterior lateral surface of the muscular process of the arytenoid cartilage. Its contraction results in inward rotation of the arytenoid cartilage. The vocal folds become adducted, lowered, elongated and thinned [1].

The IA muscle (A.K.A. arytenoid or arytenoidus) is the only unpaired muscle of the intrinsic muscles. It consists of transverse and oblique fibers. The transverse fibers extend from posterior aspect of muscular process of one arytenoid to the contralateral arytenoid. The oblique fibers extend from the posterior aspect of muscular process of one arytenoid "superficial to transverse fibers" to the apex of contralateral arytenoid cartilage. Some fibers pass around the arytenoid apex to enter the aryepiglottic fold and form the aryepiglottic muscle, which act as a sphincter for the laryngeal inlet. The IA muscle functions chiefly to close the posterior glottis by adducting the cartilaginous portion of vocal folds [1].

The TA muscle originates from the inner surface of the thyroid cartilage, and inserts into the vocal process and the anterolateral surface of the body of arytenoid cartilage. This muscle is a broad sheet of muscle fibers, the lower medial part of it is thicker and form a distinct muscle bundle called vocalis, which is the deepest layer of vocal fold. Some of the fibers pass to enter the aryepiglottic fold and run along the margin of the epiglottis as a thyroepiglottic muscle, which function to widen the laryngeal opening [1]. The TA muscle can be divided into two portions; the medial portion of the muscle has high percentage of slow-twitched muscle fibers, while the

lateral portion is composed of fast-twitched muscle fibers predominantly [7,8]. Contraction of the TA muscle results in adduction of the vocal folds, especially the musculo-membranous portion, and stiffens the muscular layer while the superficial layer becomes passively loose. The folds also become thicker with a rounded edge, shorter and lower in position [1].

The CT muscle is the largest laryngeal muscle, the only muscle supplied by the superior laryngeal nerve, and the only one lying completely outside the cartilaginous skeleton. It is a fan shaped muscle that arises from the lateral surface of the anterior cricoid arch. The fibers diverge and form two bellies; the oblique fibers (rectus belly) run postero-laterally and insert into the inferior aspect of the inferior cornu of the thyroid cartilage. The anterior straight fibers (oblique belly) insert into the posterior surface of the lower border of the thyroid lamina [1]. A recent study on human laryngeal cadavers documented the existence of a third belly (horizontal belly) [9]. This belly is located medial to the upper part of the oblique belly. It arises from the top surface of the posterior cricoid arch and runs posterio-superiorly to insert into the posterior third of the medial surface of the inferior edge of thyroid lamina and the inferior horn [9]. The CT muscle acts to rotate the cricoid cartilage along the horizontal line passing through the cricothyroid joints, thus approximating it to the thyroid cartilage and increasing the distance between the arytenoids and the thyroid cartilage. The net result of its contraction is stretching, elongation, thinning, and lowering of the vocal folds. All the layers of the folds become stiff and the edge sharp [1].

The intrinsic laryngeal muscles are believed to be composed of functionally separate compartments with separate nerve supply [8-11]. The finding of different concentrations and different types of muscle fiber inside each compartment supports this theory [7,12,13]. For example, TA muscle is found to be composed of two compartments: the medial compartment (vocalis muscle) contains slow twitch muscle fibers, which make it appropriate for phonation, and the lateral compartment contain fast twitch muscle fibers that make it suitable for adduction function during reflex mechanism for airway protection [7,8].

1.5 Laryngeal mucosa

The mucosal covering of the larynx is continuous with the mucosa of the pharynx superiorly and with mucosa of the trachea inferiorly. It is mainly composed of stratified ciliated columnar epithelium (respiratory epithelium), except for the area of the vocal folds, the posterior surface of the epiglottis, the upper part of aryepiglottic folds and the posterior commissure, which are covered with stratified squamous epithelium. The difference in the mucosal lining of these areas allows them to tolerate the trauma during phonation where these structures come into contact. Mucous glands are distributed along the laryngeal epithelium to provide lubrication to the vocal folds. However, the vocal folds themselves contain no glands[1].

The vibratory edges of the vocal folds have complex structure. Each fold is composed of five layers. The first layer is the epithelial layer, which is thin and devoted of mucous gland. Deeper to the epithelial layer is the lamina propria, which is divided into three layers: superficial, intermediate and deep lamina propria. The

superficial lamina propria (also known as Reinke's space) consists of loose fibers and matrix with the lowest concentration of collagen and elastin among the three layers. The intermediate lamina propria consists of more concentrated collagen and some elastin. The deep lamina propria is a dense fibrous layer and contains the highest concentration of collagen fibers. The intermediate and deep lamina propria layers comprise the vocal ligament. The last layer is the muscular layer, which is formed by the vocalis muscle [1].

The movement and the shape of the vocal folds are controlled by the intrinsic laryngeal muscles and to a lesser extent by the extrinsic laryngeal muscles [4].

1.6 Laryngeal neuroanatomy

The vagus nerve (Cranial nerve X) provides sensory and motor neural supply to the larynx through two branches: superior laryngeal nerve (SLN) and recurrent laryngeal nerve (RLN) (Figure 1-8). The vagus nerve leaves the skull through the jugular foramen accompanied by the spinal accessory nerve, the glossopharyngeal nerve and the jugular vein. It travels in the neck enclosed within carotid sheath, between the carotid artery and internal jugular vein. The vagus nerve has two ganglia: the superior ganglion (jugular ganglion), which is situated in the jugular foramen, and the inferior ganglion (nodose ganglion), which is located in the neck slightly below the jugular foramen. The branches of right and left vagus nerve supply the corresponding ipsilateral parts of the hemilarynx. Sensory afferent fibers from the larynx travel through the RLN and SLN to the inferior ganglion and then ascend to the tractus solitarius nucleus in the medulla oblongata. The motor neural supply to the laryngeal muscles originates from the cell bodies in the motor cortex.

The neural fibers descend through the internal capsule to synapse with the second order neurons in the nucleus ambiguous. The motor neuron cells supplying CT muscle are situated rostrally in the nucleus ambiguous. The motor neurons for TA, PCA, LCA and IA are found in overlapping pools and situated more rostrally in the nucleus ambiguous. The motor fibers originating from the nucleus ambiguous travel through the vagus nerve, and synapse with the third order neuron in the inferior ganglion of the vagus nerve. These fibers travel to the target laryngeal muscles through SLN (CT muscle fibers) and RLN (TA, PCA, LCA and IA muscles fibers) [1,14]. Some animal studies showed that single motor neuron could give two branches with one branch travelling through SLN and the other through RLN [15]. This simultaneous innervation is believed to play a role in specific coordinated movement of the laryngeal muscles such as swallowing and respiration [16]

The external laryngeal muscles receive their motor neural supply from the trigeminal, the hypoglossal, the facial and the cervical spinal nerves (C1- C3) [1].

1.6.1 Superior laryngeal nerve

The superior laryngeal nerve leaves the vagus nerve at the inferior ganglion of the vagus nerve. It travels medial to internal and external carotid artery lateral to the pharynx, and split into internal and external branches at the level of hyoid bone. The internal branch carries afferent fibers from supraglottis and lower part of the pharynx. It also carries secretomotor parasympathetic fibers that supply the mucosal glands above vocal folds. The external branch travel inferiorly accompanied by superior thyroid artery and vein. It provides motor supply to the cricothyroid muscle [1,14]. One study found a presence of an extension branch from the external

branch of SLN that provide motor and sensory innervation to the vocal folds. This communicating branch exit the medial surface of the CT muscle, and enter the lateral surface of the TA muscle [17].

1.6.2 Recurrent laryngeal nerve

The RLNs have different courses on the right and left side. The right RLN arises from right vagus nerve, anterior to subclavian artery and loops under it, and then it ascends in the tracheoesophagel groove to enter the larynx. On the left side the nerve arises from vagus nerve as it crosses the aortic arch, then it loops under ligamentum arteriosum and ascends in tracheoesophageal groove towards the larynx. Both nerves enter the larynx behind the cricothyroid joint [1,14].

There are some anatomical variations of the RLN, which make this nerve vulnerable to injury during neck surgery [18]. These variations include: a non-RLN, an extra-laryngeal branch of RLN, a distorted RLN, and intertwining between RLN branches and inferior thyroid artery [19,20]. Non-RLN is a rare variation found in 0.3-1.6% of individuals and occurs mostly on the right side [20]. The extra-laryngeal branch of RLN is a common variation with multiple types that differ in the number of the branches and their branching pattern [21-23]. Distortion of RLN can result from any local disease that shifts the nerve from its normal position, such as large goiter or substernal extension of the thyroid gland [20]. The RLN lies in a close relation to the inferior thyroid artery, and this relation is inconsistent. In some cases the inferior thyroid artery divides into many branches, and RLN intertwines with these branches [20]. These variations predispose the nerve to injury due to failure of identification during surgery [20,24].

The nerve provides motor neural supply to all the intrinsic laryngeal muscles, except the CT muscle, which is supplied by the internal branch of SLN. Once the RLN enters the larynx it gives many branches. The branching pattern is complex and variable [10]. Some studies have proposed that RLN has two separate abductor and adductor divisions [25]. However, other studies challenged this assumption [26,27], and found that the neural supply of adductor muscles and the abductor muscle arose from a common trunk. The RLN also carries afferent sensory fibers of the laryngeal mucosa from glottis and subglottis. It also gives sensory and motor branches during its ascending course to the esophagus and trachea and a motor branch to the cricopharyngeus muscle [1].

2 Physiology of the larynx

2.1 Objectives

- Overview of the physiological mechanisms responsible for the various laryngeal functions
- Emphasis on the mechanisms of failure of laryngeal function

2.2 Introduction

The basic and the most important function of the larynx is airway protection, which is aided by the valvular, or the sphincteric, structural configuration of the laryngeal inlet. This function is achieved through involuntary reflex. The integrity of this reflexive mechanism prevents the entry of any foreign material into the lower airway. The larynx also plays an essential role in respiration. It allows for air passage from and to the lower airway through a coordinated reflexive movement of the laryngeal muscles. In humans, the larynx is more sophisticated than in other animals, and is capable of producing voice. It is the main organ involved in phonation, which requires a complex interaction of many structures and body systems to produce a comprehensive speech [28]. In this chapter the main focus will be on the protective and respiratory role of the larynx; the phonatory function is discussed briefly as the full description of this function is beyond the scope of this project.

2.3 Airway protection

Airway protective function is achieved through a polysynaptic reflexive mechanism, the glottic closure reflex, which is evoked by stimulation of the sensory receptors in the upper respiratory tract [28]. The greatest number of mechanical sensory receptors is located at the inlet of the larynx [29]. The supraglottic area also contains chemical and thermal receptors [30-32]. The stimulatory signals are carried by the afferent limb, internal branch of the superior laryngeal nerve (SLN) to the ipsilateral nucleus tractus solitarius and then to the ipsilateral nucleus ambiguous. The efferent limb starts from the nucleus ambiguous, which sends signals through the motor branches of the recurrent laryngeal nerve (RLN) to the target ipsilateral laryngeal muscles that result in forceful glottic closure (Figure 1-9) [33]. This reflex has been investigated in experimental studies in cats, dogs and pigs, as well as in clinical studies in anesthetized humans and it was found to have a latency period of 10 to 18 seconds [32,34]. Another reflex (with a similar latency period) that involves simultaneous contraction of the ipsi- and contralateral adductor muscles known as the crossed adductor reflex was found in some experimental animal studies (Figure 1-10) [33,34]. It was consistently elicited in anesthetized cats, but less frequently in anesthetized dogs and seldom in anesthetized humans. A third reflex with longer latency period of 66 to 70 milliseconds that involves bilateral adductor muscles contraction was demonstrated in awake humans [35]. Later studies showed that the crossed reflexes are affected by anesthesia in a reverse relation, which might suggest that these reflexive mechanisms are under control of central facilitation mechanism [33,36].

During swallowing, airway protection is achieved by closure of the laryngeal inlet combined with cessation of breathing and laryngeal elevation. Stimulation of the internal branch of SLN bilaterally results in closure of the laryngeal inlet at three levels. The first is at the level of aryepiglottic folds, which when contracted cover the anterior gap of the superior laryngeal inlet. The posterior gap is closed by the bulk of the arytenoid cartilages. The second level of protection is achieved by contraction of the false vocal folds, and the third and the most important level is provided by the true vocal folds [37]. Laryngeal elevation is mediated by the action of the extrinsic laryngeal muscles, which act to elevate and tilt the larynx anteriorly under the tongue, thus providing extra protection of the airway and in the same time widening the hypopharyngeal space [38]. Temporary and simultaneous cessation of respiration prevents airflow into larynx thus providing additional protection against aspiration[38].

2.4 Respiration

Vocal folds abduction is essential for providing a patent tract for airflow during respiration. Negus found that the glottis opens millisecond before the beginning of airflow into the lower respiratory airway, which results from a diaphragmatic descent [37]. Glottic opening during each respiratory cycle occurs as a result of rhythmic discharge in the RLN and SLN, and is mediated by the respiratory center in the medulla. It is decreased by hyperventilation and hypocapnia, and increased by hypoventilation and hypercapnia [39]. The posterior cricoarytenoid muscle (PCA) actively contracts to open the airway with each respiratory cycle [39,40]. Its contraction results in widening the glottic aperture by increasing the horizontal distance

between the vocal folds as a result of its abductor effect [41]. The activity of PCA depends on the presence of ventilatory resistance in the airway during respiration. This activity is controlled centrally through a reflex mechanism with the vagus nerve probably acting as the afferent limb [42]. This dependence of the abductor activity on ventilatory-resistance is supported by the observation that PCA muscle contraction disappears after tracheostomy [40]. Moreover, vagotomy was found to result in eliminating this reflex mechanism [42]. PCA activity was found to increase with hypercapnea [39] and hyperpnea [43]. It also was shown to has expiratory activity in some studies, and this activity is augmented by hypercapnea [44].

The respiratory role of the cricothyroid (CT) muscle is not clear. Some studies found this muscle to be active during inspiration, expiration or to have biphasic activity [45-50]. The discrepancy might have arisen as a result of the different methodology, such as the anesthesia level, the animal model used, and the different endpoint utilized in these studies. In general, most of the studies agreed on the role of this muscle during inspiration. Its contraction results in lengthening of the vocal folds, therefore increasing the anterio-posterior diameter of the glottic opening. The synchronous activities of the CT and the PCA muscles result in an increase of the total cross-sectional area of the glottis [51]. In addition, this synchronous activity was found to reduce the laryngeal resistance [51]. The activity of the CT muscle was found to be dependent on the respiratory condition and the level of consciousness [47,52]. During quiet spontaneous breathing in awake humans the activity was found to be very weak, and increased with deeper breathing and upper airway occlusion accompanied by appearance of expiratory activity [50]. In quiet spontaneous

breathing of anesthetized individual, the inspiratory activity was again weak during light anesthesia, and it was abolished with deeper level of anesthesia [45,53]. The activity increased with the addition of respiratory stimulus that increases the respiratory effort such as hypercapnea, hyperpnea and upper airway occlusion [50,54], and was accompanied by expiratory activity as well [50]. On the other hand, some studies found the expiratory activity of the CT muscle to be the main role of this muscle in the respiratory cycle [46,55]. This activity was thought to be produced through a peripherally evoked mechanism, rather than being a spontaneous activity generated in the medulla as in the case of the inspiratory activity [46]. The trigger for this mechanism is the elevation of the subglottic pressure influenced by carbon dioxide level, and the reflex is mediated centrally with the vagus nerve acting as the afferent limb. Hypercapnea was found to increase the activity of CT muscle in both respiratory phases [46,47], and the expiratory activity was found to relate inversely with the anesthesia level [47,52]. The afore mentioned information on the activity of CT muscle made many authors describe it as an accessory muscle of respiration, as its role is limited to conditions where stronger respiration is required [45,50].

2.5 Phonation

Phonation is the most complex function among the three functions of the larynx, and is the least understood. This is because this function is well developed in humans [37], thus experimental animal studies cannot provide a representative model. For phonation to occur, respiratory support to provide airflow, in addition to the adduction of normally covered vocal folds and control of vocal folds' length and tension should be present [4].

The larynx is responsible for the production of voice through passive vibration of the vocal folds created during the passage of air between them, producing the mucosal wave [4]. The tongue, palate, oral cavity and lips are responsible for resonance, and articulation of the sounds. The mucosal wave cycle begins with an increase in the subglottic pressure, which leads to opening of the glottis. At the maximum opening of the glottis the upper lip of the edge of the vocal folds remain lateral and the lower lip move medially as a result of the recoiling force, drop in subglottic pressure and the physical effect of the negative pressure (the so called Bernoulli effect). This negative pressure pulls the vocal folds toward each other thus approximating the upper edges. This cycle is repeated time and again [4].

The passive vibratory mechanism of phonation is based on aerodynamic principles, and is supported by the observation that a completely denervated cadaveric larynx can produce sound in an experimental setting [37]. This phenomenon, in addition to the fact that the vibration disappears after tracheostomy insertion [4] challenged the neurochronaxic theory that proposed that vibration results from active phasic contraction of TA muscle under the control of RLN [4].

However, phonation is not an entirely passive mechanism as the glottic closure and the control of vocal fold length and tension are under active neurological control. Active contraction of the intrinsic laryngeal muscle is essential for glottic closure as well for changing the tension and position of the vocal folds. Controlling the frequency is associated with the speed of the mucosal wave which is affected by laryngeal muscle contraction, length of the vocal folds, airflow rate and subglottic pressure [56,57]. Extrinsic laryngeal muscles affect the pitch by changing the spatial

configuration of laryngeal cartilages [6]. The activities of the sternothyroid and sternohyoid muscles result in elevation of the subglottic pressure, the fundamental frequency and the vocal intensity. They also cause a decrease of the distance between the cricoid and thyroid cartilages, and an increase in the length of the vocal folds. In contrast, the thyrohyoid muscle decreases the subglottic pressure, the vocal intensity, the fundamental frequency and the vocal fold length [6].

3 Laryngeal Paralysis

3.1 Objectives

- Review of the condition, its epidemiology and the impact on the patient's quality of life.
- Review the current management options.
- Present the controversies on the pathophysiological basis of the disease.

3.2 Introduction

Laryngeal paralysis (LP), (A.K.A. vocal fold paralysis), is defined as the absence of normal movement of one or both vocal folds (VF) as a result of affection of the neural supply to the larynx [58]. It should not be confused with VF fixation, which results from mechanical fixation of the VF, due to cricoarytenoid (CA) joint pathology. These two diseases share some clinical features and are usually referred to as VFs immobility, however their management approach is entirely different. Interruption of the neural supply from the brainstem nuclei to the targeted laryngeal muscle fibers at any level can result in a variable degree of altered mobility. The pathway might be affected by iatrogenic insults (such as surgical injury, drug neurotoxicity, radiation, etc.), accidental trauma to the nerve, inherently neurological conditions (such as tumors, vascular diseases, central nervous system (CNS) diseases, neuromuscular disorders, infections, etc.), or by an unexplained mechanism (i.e. idiopathic).

The recurrent laryngeal nerve (RLN) is especially vulnerable to mechanical trauma due to its anatomical relations and its length. It can be by stretched, crushed, or transected during any surgical procedure within the vicinity of its course. It might also be injured as a result of compression by an adjacent mass. Malignant tumors in the neck or the chest regions can invade the nerve or exert pressure on it, which result in progressive demyelination or devascularization leading to dysfunction. Radiation therapy directed at the regions where the nerve run can cause fibrosis in or around the nerve and impair its function.

The extent of the nerve dysfunction depends on the degree of the damage. Minor injury, such as stretching or mild pressure, may result in a limited segmental demyelination, which impairs the axonal transport mechanism and results in a conduction block. Spontaneous re-myelination results usually in restoration of the nerve conduction. More severe injury, such as crushing, may interrupt the neural axons with preservation of the nerve conduit (axonotmesis). This usually results in Wallerian degeneration of the nerve segment distal to the injury site. Recovery of the nerve function after such an injury requires the presence of neural channel for axonal regeneration. If the injury is in a form of complete nerve transection, neurotemesis takes place and the chances of functional recovery depend on: the percentage of the axons that find their way to re-innervate their original target, and the degree of the aberrant synkinetic re-innervation of the remaining axons [14].

Neurological or neuromuscular diseases can cause neural dysfunction at any level of the laryngeal neural pathway. The recovery of functional mobility of VF usually follows the effective treatment of the underlying cause in some diseases [58,59].

Neurotoxic drugs, such as Vincristine, can cause peripheral neuropathy and cause a reversible dysfunction of the laryngeal nerves. Discontinuation of these drugs usually results in recovery of functional VF mobility [60,61]

A large number of patients have no identifiable cause for the paralysis, even after extensive investigative workup [58,59,62-68]. The reported recovery rate for this group of patients is variable among the published reports. Some authors suggested the presence of infectious or inflammatory processes in this category of patients [69-72], however no casual relationship has been established, and many cases have no clinical evidence that support such a notion.

3.3 History of the disease

Historically, the information about laryngeal function and structure was obtained from cadaveric dissections and animal experiments. Galen, the father of Roman medicine, in the second century AD, was the first to describe the anatomy of the intrinsic laryngeal muscles [28]. He distinguished the three laryngeal cartilages, and six pairs of muscles, which he classified into adductors and abductors muscles [28,73]. He also reported the loss of phonation as a sequel of sectioning the RLNs in dogs [28]. The knowledge postulated by Galen's was unchallengeable, and dominated for many centuries to follow because of the prohibition of cadaveric dissection by the church in Europe. After the Renaissance, Vesalius (1543) described the anatomy of the larynx in his book *De Humani Corporis Fabrica* [28]. He performed many experiments on animals and used tracheostomy for respiratory support during thoracic procedures [28]. Later, in 1602, Nicolas Habicot described the laryngeal movement during swallowing, and in 1790 Andresch was the first to demonstrate the

innervation pattern of the larynx before his work was elaborated upon and published by Swan in 1830 [73].

For many centuries, the focus on laryngeal diseases was limited to the infectious categories, such as diphtheria and croup. These diseases were prevalent and incurable, frequently leading to airway obstruction and death, which led many clinicians to devote their efforts to find effective treatments. However, it was not until the identification of the infectious agents and the discovery of the antibiotics in the nineteenth century by Pasteur and Koch, when these diseases became curable [28]. Although many diseases were attributed to infectious processes, neurological etiology was postulated in some cases [74], however without a clear understanding of the exact mechanism.

The field of laryngology truly started to develop after the development of the tools and the skills that enabled routine examination of the larynx. However, most of the early inventions were not practical and did not gain popularity [73]. It was Manuel Garcia, a singing teacher, who first used the laryngeal mirror to visualize his own larynx in 1854 [73]. After that, many clinicians employed it for routine clinical examination, and started to describe different laryngeal pathologies and correlate them to the clinical manifestations. This allowed a better understanding of the laryngeal diseases and improved the treatment strategies.

The feasibility of laryngeal examination allowed the laryngologist to observe the behavior of the VFs, and helped in the evolution of the neurolayngology field [74]. After four years of Garcia's invention, Turk described LP and showed images of the paralyzed larynx in his atlas [18]. Ever since, the variations in the clinical presentation

of the patients with LP, and the pathogenesis behind it, stirred the interest of many laryngologists. In 1881 Felix Semon, one of the earliest pioneers in the world of laryngology, performed extensive investigations on the functional innervation of the larynx, and the laryngeal behavior after injury to the RLN. Based on his work, he proposed a theory to explain the variation of the VF's position after RLN injury (known as Semon's law), which states that the nerve fibers supplying the abductor muscles, posterior cricoarytenoid (PCA), are sensitive to injury more than the fibers supplying the adductor muscles, and that is why the paralyzed VFs attain a median position [75,76]. This law had some limitations and has been challenged later by multiple studies.

In 1897, Wagner and Grossman proposed a different theory (Wagner-Grossman hypothesis), which claimed that the cricothyroid (CT) muscle (the only muscle supplied by superior laryngeal nerve) was responsible for the medial position of paralyzed VFs [77]. However, subsequent studies that attempted to manipulate the CT muscle, and study its effect on the airway challenged this theory [78-82]. Moreover, with the advent of electromyography (EMG), multiple studies reported the presence of electrical activity in the laryngeal muscles of the paralyzed larynx, which suggested that the CT muscle was not the only muscle controlling the position of the VFs. Blitzer and Koufman challenged the Wagner-Grossman theory in their studies using EMG and found that the CT muscle doesn't influence the position of VF in LP, and that other factors were responsible for the VF position [77,82]. Siribodhi proposed that synkinesis "misdirected the re-innervation by the native or foreign neural fibers" is responsible for the position of the VFs [83].

The synkinetic innervation theory negates the previously common belief that the laryngeal muscles are totally denervated in LP [84]. However, not all studies are supportive of this view. Damrose et al. reported no evidence of synkinetic innervation, upon using evoked laryngeal electromyography (LEMG) in fourteen out of fifteen patients undergoing medialization thyroplasty [85]. However, there are concerns about the methods they used for obtaining the LEMG activity, and their interpretation of the data [14].

Additional evidence in support of the synkinetic theory came from several animal studies, which showed that the RLN regenerate to re-innervate the laryngeal muscles even after segmental excision with demonstrable LEMG, despite the absence of useful functional recovery [86-88].

To date, the management of LP aims primarily on symptomatic relief to compensate, for the lost physiological function and to improve the quality of life. Many surgical techniques have been described, however each of them requires a sacrifice of one or more of laryngeal structures and/ or functions temporarily if not permanently. Strategies aiming at re-innervation to restore the functional movement to the paralyzed VFs have been described, and electrical stimulation devices for pacing the larynx has met limited success in animal experimentation, but neither has been translated successfully into clinical practice.

3.4 Epidemiology

LP is reportedly the second most common congenital laryngeal condition after laryngomalacia, comprising 10% of all congenital laryngeal lesions [89]. The exact incidence and prevalence of LP are however not known [90,91]. Murty et al. estimated

an incidence rate of 0.75 cases per million per year for congenital bilateral LP [92]. It appears that the number of the pediatric cases diagnosed with LP has increased over the recent years, which could be attributed to the advances in the diagnostic techniques, such as the advent of the flexible fiber-optic laryngoscope, and to the successful salvage of more newborns with multiple congenital anomalies [65]. Thirty to sixty two percent of the pediatric LP cases are bilateral [93]. In the adult literature, there is a huge variability of the incidence rate reported in the literature. In Japan Yumoto et al. reported an incidence rate of 37.56 per 10 million [94], whereas Ahmad et al. reported an incidence rate of 42 per 10 thousand in India [95]. This variability between different papers could be attributed to the difference in the demographic and epidemiologic characteristics of the study's population, as well as to the difference of the level and the reporting practice of the institutions [14].

3.5 Etiology

The etiological factors reported in the literature can be generally categorized into: iatrogenic, neurologic, neoplastic, idiopathic, and genetic causes. These etiological categories are different proportionately between the pediatric and the adult populations, and between the unilateral and the bilateral affection. Moreover, even within these categories the reported rates are inconsistent in the literature.

3.5.1 Iatrogenic

3.5.1.1 Surgical trauma

Iatrogenic surgical injury of the RLN is a well-known cause of LP. Many surgical procedures in the neck, upper thoracic or skull base regions carry a potential risk of

injury to the RLNs. Usually the injury involves the nerve on one side, resulting in unilateral LP, but bilateral injury can also happen. The mode of the injury may be from partial or complete transection, cauterization, crushing, stretching, compression, devascularization or even minor manipulation of the nerve. The type and severity of the injury determines the prognosis.

In children, the proportion of LP resulting from surgical injury to RLN ranges widely from 6% - 47% of the total cases number in several case-series, and most of them are unilateral [58,59,63-67]. Cardiothoracic procedures are the most common culprits, especially ligation of a patent ductus arteriosus (PDA) [58,63,65,66]. The close relation of the left RLN to the ductus makes it very vulnerable to injury, and the injury can happen regardless of the surgical method used. The reported rate varies widely ranging from 0.7% to 52% [96,97]. However, most of the reports identified extremely low birth weight (ELBW) to be a significant risk factor [97-99].

Repair of tracheo-esophageal fistula (TEF) or esophageal atresia is another procedure associated with LP [58,64,100-104]. The reported rates range from 4% to 29%[103,105]. Other surgical procedures with lower frequencies include: thyroidectomy and excision of congenital branchial anomalies [58,100,106].

In adults, iatrogenic surgical injury is one of the commonest causes of unilateral and bilateral LP [107-110]. Several surgical procedures have been implicated, and thyroid surgery is reported to be the commonest in several reports [107,108]. However, this proportion has relatively decreased as injury from other surgical procedures increased [14]. Thyroid surgery carries a major risk to the RLN due to the close proximity of the nerve to the thyroid gland, the variability of its course and its

branching pattern around it [18]. A recent systematic review found a rate of 2.3% (0 – 18.6%) and 9.8% (1.4 -38.4%) for permanent paralysis and transient paralysis, respectively [111]. The authors attributed the variability of the reported figures to the different diagnostic methods used for assessment [111]. Most reports identified a higher frequency after revision surgeries [112-116]. That could be attributed to difficulties in identifying the nerve due to the anatomic distortion and the scarring resulting from the primary procedure. Several intraoperative nerve monitoring techniques have been used to aid in identifying the nerve, however their use did not significantly decrease the rate of injury [117]. Surgeries involving malignant pathologies, and Graves disease were reportedly associated with a higher rate of injury as well [112-114,116].

Anterior cervical spine surgery (ACSS) is another surgical procedure reported to commonly result in LP. Merati et al. reported ACSS as the commonest surgical procedure to result in RLN injury [110], occurring usually, on the side of the surgical approach, although bilateral affection is not unknown [118]. Most studies reported rates between 2% and 7% [118-124], although the frequencies varied drastically from 1.1 to 24.2% [118-126]. The injury is assumed to be secondary to stretching the nerve during dissection of the laryngotracheal complex from the lateral neck structures[127]. This can be supported by the high rate of recovery observed in most of the patients[118,125,126], in addition to the higher incidence of LP when right sided approach is used [118,119,125]. The right RLN is known to have a shorter course and wide angle of entry into the larynx, and that makes it more vulnerable to stretch injury from the applied retraction than the left nerve [128]. Another theory was

advocated by some authors, which attributed the paralysis to the pressure applied on the intralaryngeal RLN terminals by the cuffed endotracheal tube, [120,121,123]. They suggested an adjustment of the cuff pressure to decrease the risk of nerve injury based on their result [120,121,123]. However, when Audu et al. performed a prospective study on hundred patients undergoing ACCS comparing cuff manipulation with no intervention, they found no significant difference between the two groups [124].

Carotid endarterectomy is a procedure that can be associated with injury to the vagus, superior laryngeal or RLN. The injury might occur during dissection of the carotid artery from the surrounding tissues, or from stretching by retraction, crushing from vascular clamps, or electro-cauterization for hemostasis. The reported rates range from 0.6 - 7.6% [129-138].

During the course of skull base procedures, injury to the vagus nerve may take place. It could happen as an isolated mono-neuropathy, or as a part of multiple cranial neuropathies. Injury to the vagus nerve produces both motor and sensory deficits on the affected side, thus placing the patient at a greater risk of aspiration. Rosenthal et al reported on skull base surgery as the underlying etiology in 2% of the unilateral LP surgical category [107]. Whereas others reported several folds higher rates in their patients (7% and 9%, respectively) [109,110].

Thoracic surgeries including lung, mediastinal, esophageal, and cardiovascular procedures may be complicated by LP. During perihilar dissection, for left pneumonectomy or lobectomy, injury to the RLN can occur. The reported rates again vary from 6.7 – 28% [139-141]. Whereas following cardiovascular procedures, such as coronary artery bypass surgery and valve repair, LP reported rates range

from 0.67 to 1.9% [142], aortic and aortic arch surgeries had higher rates, ranging from 8.6- 26.7% [143-145]. RLN injury during valve or coronary artery surgeries can occur directly (i.e. direct trauma to the nerve), or indirectly from the excessive retraction applied during the median sternotomy while harvesting the internal thoracic artery [142]. Esophageal surgeries also carry a considerable risk to RLNs, and might result in bilateral injury. Two meta-analyses reported rates of 3.5% and 9.5%, and 5.6% and 10.9%, after transthoracic and transhiatal esophagectomy, respectively [146,147].

3.5.1.2 Drug toxicity

Several drugs and chemical agents have been known to induce LP and include vinca alkaloid (vincristine/ vinblastine), cisplatin, local anesthetics, and organophosphorous compounds.

Vincristine is a chemotherapeutic agent used for treatment of multiple hematologic malignancies. LP can result as a part of peripheral poly-neuropathies following Vincristine use, secondary to its neurotoxic effect that causes structural damage in the peripheral nerves [148]. Several cases of LP have been reported after the use of vincristine, most of which recovered after withdrawal of the drug [60,61] Similarly, cispaltin induced LP resolves after cessation of therapy [149].

The use of local infiltration anesthesia in carotid and tonsillar surgery was reported to transiently affect the RLN but the effect resolved after few hours [150,151].

Organophosphorous poisoning can result in LP as part of its neurotoxic effect, which again fully resolves after supportive medical therapy (such as anticholinergic drugs, and respiratory support) in few days [152,153].

3.5.1.3 Radiation therapy

Radiation therapy can cause fibrosis in the tissues exposed. The laryngeal neural supply can be affected ostensibly due to fibrosis, stretching or compression, or impaired blood supply to the nerve. Radiation therapies of the nasopharyngeal, oral, laryngeal, pharyngeal, mediastinal, and breast cancers have all been implicated [154-158]. The paralysis usually happens after a latent period of several months or even years [154-156]. Radioactive iodine I-131, a drug used for treatment of thyrotoxicosis and thyroid cancer, was reported to result in LP [159], allegedly from nerve stretching caused by the edema of the surrounding tissue [159].

3.5.1.4 Birth trauma

Birth trauma has been reported in the literature as a cause of LP, however the exact mechanism is unknown. Some authors suggested that the injury is a product of stretching of the RLN during difficult or forceps delivery, drawing a similarity to brachial plexus injury [62,160], but there is no evidence to support this theory especially in the absence of any associated other cranial or peripheral nerve deficits. The reported proportions of this category in several large case-series range from 1.3% to 49%, of these 20% to 81% are unilateral and 19% to 100% are bilateral[58,59,62,64-67,100].

3.5.1.5 Intubation

Endotracheal intubation has been reported as an etiology for unilateral and bilateral LP. Reported rates range from 4% to 14.3% of the all LP patients [107,109,110]. Compression of the anterior intralaryngeal branch of RLN between the inflated tube

cuff and the laryngeal cartilages is believed to be the responsible mechanism of the injury [161]. Reduction of the tube cuff pressure in patients undergoing ACSS resulted in lowering the rate of LP [120,121,123]. However, this notion is debatable [124]. Some cases of LP were reported after laryngeal mask airway use during general anesthesia [162]. Again from the postulated mechanisms is compression of the SLN or RLN from the incremental increase of the cuff pressure [162-165]. Traumatic CA joint dysfunction cannot be ruled out as the responsible cause following intubation or laryngeal mask placement. The diagnosis in most of these case series/ reports were based on symptomatic presentation and flexible fiber-optic laryngoscopy (FFL) only, without proper examination of the CA joint mobility, or neurophysiological evidence to document nerve dysfunction [166,167]. Paulsen et al. found that intubation can result in different form of CA joint dysfunction other than subluxation, although they did not fully exclude the possibility of nerve injury [168]. The reference standard diagnostic method to rule out CA joint pathology is by joint palpation during direct laryngoscopy [169], although LEMG could be useful in distinguishing between articular and neural pathologies [170].

3.5.2 Neurological

CNS pathologies can result in LP especially where the brainstem is involved. In children, neurological diseases are the second most common cause of bilateral LP[58,171]. Arnold-Chiari malformations (ACM) with the associated myelomeningiocele and hydrocephalus top the list [171]. Herniation of the cerebellum and brainstem stretches and compresses the vagi. Treatment by decompression can result in full recovery [58,59]. Other neurological diseases implicated include hydrocephalus,

meningomyelocele, cerebral agenesis, neuromuscular disorders, Charcot-Marie-Tooth disease and myasthenia gravis [160].

In adults, most of the neurological cases are secondary to cerebrovascular accident[109]. A prospective study of the newly diagnosed cerebrovascular accident patients, documented that 20.5% had LP, most of whom had a lesion in the lateral medullary region [172]. Other neurological disorders including multiple sclerosis, myasthenia gravis, Parkinson's disease and amyotrophic lateral sclerosis, have been cited [173].

3.5.3 Neoplastic

Malignant tumors are a common cause of LP in adults. They may invade or by exert pressure on the nerve leading to devascularization, demyelination, or even disruption. Neoplasms were reported as the etiological factor in 5% to 53% of LP patients; the majority metastatic mediastinal tumors [14]. Lung, thyroid, esophageal and laryngeal tumors were also implicated, but at lower frequency [14].

Non-malignant tumors can also affect RLN and result in LP. Mediastinal lymphadenopathy associated with sarcoidosis and tuberculosis were reported, however the reported cases are very few [14].

3.5.4 Idiopathic

LP is labeled as idiopathic after an extensive investigative work-up had been undertaken ruling out any plausible etiology. The work-up ideally should include computed tomography and/or magnetic resonance imaging of the head and neck, and X-ray of the chest to rule out any compressing mass. In children, it is the largest

category with bilateral affection [58,62,64], and the reported proportions range from 23% to 44% in the large case-series [58,59,62-67]. In adults, the idiopathic etiology is not uncommon, comprising 12% to 42% of unilateral LP [68]. Many authors tried to explain the underlying mechanism of the idiopathic cases. They proposed a link to viral infections such as herpes simplex virus, Varicella Zoster, Epstein-Barr virus and influenza-A virus [69-72,174,175]. However, there is no evidence to support these assumptions.

3.5.5 Genetic

Genetic source has been suggested to play a role in individual case reports of congenital idiopathic bilateral LP [176]. Different inheritance modes were proposed including: autosomal recessive, sex-linked [177,178] and autosomal dominant modes[179,180]. The autosomal dominant form of familial bilateral LP has been linked to specific gene located on chromosome 6q16 [181].

3.6 Presentation

The most common presenting symptom in children with unilateral and bilateral LP is stridor [58,62,65,171]. Gentile et al in a series of 10 children, reported it in 70% of the unilateral and in all bilateral cases [65], and Daya et al. reported similar rates (77% and 90%, respectively) [58]. Dysphonia or an abnormal cry, is also commonly noted in unilateral LP patients (51%) [58], followed by feeding difficulties with aspiration in 23% [58]. Cyanosis with chest retraction and apnea are commonly encountered in bilateral LP, particularly in patients with cardiac or neurological abnormalities [58]. In

older children and adolescents, the presentation is said to be similar to that of adults[182].

In adults, most of the patients with unilateral LP present mainly with dysphonia [183]. Other symptoms include cough, vocal fatigue, dysphagia, chocking, dyspnea and gastro-esophageal reflux disease symptoms [183]. Patients with bilateral LP commonly exhibit dyspnea and stridor, and less frequently voice change, dysphagia or aspiration [173].

LP has a negative impact on the general health of the affected individuals, and greatly affects the quality of life of most of the patients [184-186]. Unilateral LP may place the individual at risk of recurrent attacks of aspiration pneumonia, because of the inability to protect the airway during swallowing. It might also cause psychological or emotional problems that lead to social isolation due to the poor voice quality, and the restriction to speak in clear comprehensible voice. Patients with dyspnea might experience limitation of physical activity. In children, this might affect normal development and behavior, and result in psychological abnormalities.

3.7 Pathophysiology

Understanding the pathogenesis of LP requires a thorough study of the functional anatomy of the laryngeal muscles. The conventional view of the laryngeal neuroanatomy has been questioned, as many reports had found evidence for more complex anatomical configuration of the laryngeal muscles and their innervation pattern [7-12,187]

The anatomical dissection studies of the larynx suggested laryngeal muscles are compartmentalized, for specific functions. Some of these studies demonstrated the

presence of fascial barriers between the compartments, where muscle fibers differ in direction and attachment sites [8-11]. Other studies found differences in the biochemical properties of the muscle fibers in each compartment, which might reflect the functional role of these compartments [7,12,13]. For example, the horizontal compartment of the PCA and the vocalis muscle (the superior medial compartment of TA muscle) are rich in slow-twitched type I muscle fibers [7,13]. The contraction of this type of muscle fibers is prolonged, stable, and fatigue-resistant, a function needed for vocalization or quiet respiration [7]. In contrast, the vertical compartment of the PCA muscle is rich in fast-twitched type II muscle fibers, which adapt it to function during rapid breathing [13]. The same type of muscle fibers is found in the lateral compartments of the TA muscle, making it suited for airway protection [7]. Moreover, the compartmentalization theory is further supported by the histological studies of laryngeal muscles' innervation, which found that each of the compartments has a separate nerve branch that provides it with neural supply [8-10,187].

It was commonly assumed that LP results from complete denervation [85]. However, experimental and observational studies challenged this assumption. The impairment of VFs movement after RLN injury had been first claimed to be secondary to synkinetic reinnervation [188], by Siribodhi et al. [83]. Several animal and human studies seem to support this assumption [84,189-192].

In synkinesis, misdirected reinnervation occurs when the regenerating axons randomly innervate the laryngeal muscles in a chaotic manner. Synkinetic reinnervation can maintain the bulk and the tone of the muscle. However, because

the abductor and adductor nerve fibers are randomly re-distributed between and within the laryngeal muscles, the normal movement is not restored [193]. The net result is a paradoxical movement that is not consistent with the corresponding phase of respiration [77]. Paradoxical movement results from simultaneous contractions of the muscle while its antagonist is contracting causing either no or abnormal minimal movement [193]. Even when some neural axons reach their correct target muscle, the innervation is neither appropriate, nor enough to cause normal contraction [193]. Moreover, the compartmentalization of these muscles is another complicating factor, reinnervation may result in neural axon innervating the wrong muscle compartment (with different muscle fibers type), and result in early fatigue and a decrease of muscle force [8,9,194]. In addition to the random innervation pattern, animal studies showed evidence of re-organization of the motor neuron pool in the CNS [191,195].

Crumley has suggested a classification scheme for synkinesis, based on the symptoms and the vocal fold behavior and position [84]. Favorable, or type 1, synkinesis represents partial or complete impairment of the VF mobility, however with acceptable or normal voice quality. Unfavorable synkinesis, which has three subtypes (2-4), is characterized by abnormal VF' position or mobility, with compromised airway or decreased voice quality. Type 2 involves spastic, or jerky movement of the VF with acceptable to poor voice quality. In type 3, the VF tends to assume a hyper-adducted position, with normal or acceptable voice quality, and potential airway compromise. Lastly, type 4 is characterized by hyper-abducted VF, breathy voice quality, and patent airway [84]. This classification might be applicable to

the unilateral affection; however, it does not reflect the disease severity in bilateral LP, as it has not taken into account the airway protective function of the VFs.

3.8 Diagnosis

3.8.1 Laryngoscopy

Flexible fiberoptic laryncoscopy (FFL) provides important information about the VFs when used in a cooperative awake patient. It allows for evaluation of the dynamic function of the larynx [171]. During this examination, VFs are examined for their position at rest during quiet breathing, symmetry, bulk, movement pattern during phonation, glottic closure and any scarring or vocal process asymmetry [196]. Stroboscopy on the other hand is currently considered the reference standard for the demonstration of the mucosal wave and the glottal gaps, where the voice change is the main concern. But direct laryngoscopy with arytenoid palpation under general anesthesia is necessary to establish the diagnosis of LP [169]. In addition to providing clear visualization of the VFs movement during spontaneous breathing, it allows assessment of the airway for other anomalies such as subglottic stenosis, laryngeal scarring, arytenoid fixation or subluxation, and tracheal abnormalities.

3.8.2 Investigations

Investigations and workup are usually directed by the history and physical examination. If neurologic causes are suspected, magnetic resonance imaging of the brain is indicated. Computed tomography scan from skull base to the thoracic inlet covering the whole course of the RLN is helpful to rule out lesions invading or compressing the nerve.

Laboratory studies (e.g. serology, microbiology) should be only requested according to the suspected etiology based on the clinical picture.

3.8.3 Laryngeal electromyography

When Weddel et al. first introduced the LEMG they suggested that it might have diagnostic and prognostic values for LP [197]. In LEMG, fine electrodes are inserted into the laryngeal muscles to detect their electrical activity and assess the integrity of the neural pathway. It could be helpful in predicting the prognosis. However, the there is no strong evidence in support of its prognostic value [198].

LEMG is a commonly used tool for adult patients with laryngeal mobility problems. It can help differentiate between LP, and other causes of VF's immobility. Whereas it has a proven track record of safety and feasibility in the adult clinical practice, the scale of its utility is less in the pediatric age group [199].

A relatively recent evidence-based review recommended LEMG for guiding BTX-A injections into the laryngeal muscles in cases of spasmodic dysphonia. [198] The authors found, however, no robust evidence for the other uses (including its use as a prognostic adjunct), and recommended further studies. This notion was further supported by the recommendation of neurolaryngology study group on LEMG [200], which standardized its techniques and reporting practice. But Rickert et al. in a recent meta-analysis, found that LEMG was useful to predict poor prognosis [201].

3.9 Management

Management of LP is different for unilateral and bilateral disease. In general the management approach is either conservative or surgical. Clinical decision-making

depends on the stability of the airway, and the degree of affection of the other laryngeal functions, in addition to the general health status of the patient, and the presence of other co-morbidities.

In bilateral LP, if the airway is significantly compromised, it may dictate immediate intervention to secure the airway, usually by a tracheostomy. This will provide a temporary solution, while waiting for spontaneous recovery. However, tracheostomy has multiple potentially serious risks and results in a wide range of morbidities, therefore laryngeal expansion procedures are sought after as a permanent solution. These surgical procedures however, involve a structural change to the larynx, and affect the other laryngeal functions. If the airway is stable, the patient may be managed conservatively (e.g. alternate route of feeding) and frequent evaluations.

In unilateral LP, the airway patency is not affected in most of the cases, as the contralateral fold is functional. The main issues are the poor voice quality and the risk for aspiration, resulting from glottic insufficiency. In general, the management is conservative waiting for spontaneous recovery or compensation from the contralateral intact side. Definitive treatment requires employing one of the surgical medialization techniques.

All the existing surgical techniques for unilateral and bilateral LP aim to provide symptomatic relief for the affected patients, and do not address the core problem of the disease itself. These procedures do not restore the physiological VF mobility, but only provide static structural, or architectural, treatment by repositioning the paralyzed VF to the midline, relieving the obstruction, or providing re-innervation

to restore or preserve the tone of the laryngeal muscles [90,202,203]. Moreover, they usually result in varying degree of destruction of the laryngeal structure and function. There is neither consensus nor evidence based guidelines that direct the choice of surgical technique, and their optimal timing. This is partly due to gaps of knowledge about the natural history of the disease. The ideal treatment would involve restoration of the dynamic function of the larynx [204], however this is still not available.

3.9.1 Unilateral laryngeal paralysis

The management of unilateral LP depends mainly on the clinical presentation and the age of the patient. For children, it should be focused on airway protection and feeding, especially in infants. Tracheostomy is rarely needed, unless there are associated airway or neurological co-morbidities. Dietary modification by thickening oral liquids is usually effective to avoid aspiration, however an alternate route of feeding (gastric or nasogastric tube) may be required. In older children voice therapy can be used to augment the compensatory effect of the contralateral fold in closing the glottic gap [205], and sometimes there is no need for further surgical interventions. Medialization procedures are indicated if these conservative measures fail, but most authors recommended a waiting period of at least one year, to allow for spontaneous recovery, before performing any surgical intervention [58,64]. In adults, the management depends on the symptoms of the patient and the chances of spontaneous recovery. Conservative voice and swallowing strategies might benefit some patients and eliminate the need for further management [206]. Voice therapy involve multiple techniques and exercises, which aim to achieve better

glottic closure, enhance the activity of the laryngeal muscles and develop better abdominal breathing support [206]. Swallowing therapy utilizes different techniques in the form of exercises, behavioral techniques or dietary modifications, which are directed at improving the airway protection [207]. There is not enough evidence on the efficacy of voice therapy in comparison to surgical interventions [208]. Again, conservative measures are initially used but if spontaneous recovery did not take place, or where recovery is deemed unlikely, surgical medialization procedures are considered, especially for those with great risk of aspiration.

Several medialization techniques have been described including: injection laryngoplasty, laryngeal framework surgery, arytenoid adduction, and reinnervation surgery. However, there is no evidence or consensus for their optimal timing, or the favorable technique [93,208]. Most of the techniques have been developed and used for adults.

Injection laryngoplasty was first introduced by Breuning in 1911, where he used paraffin for unilateral LP [209]. However, because of the complications caused, the procedure was omitted and rarely used. With the discovery of the new injectable materials, the procedure regained popularity due to its minimal invasiveness and its lower morbidity rate [210]. The procedure can be performed in an office setting in the awake co-operative patients under local anesthesia. Either a trans-cutaneous or a trans-oral injection approach (guided by FFL) is used. Alternatively, it is performed in the operating room under general anesthesia using microlaryngoscopic guidance[211]. Several materials were developed and used for injection, and can be classified as xeno-, homo-, or auto-grafts and synthetic material [210]. However,

several adverse effects have been reported including airway narrowing, improper placement, migration, infection and foreign body reaction [208]. In addition, repeated injections are usually required, which can result in progressive scarring, and might lead to structural damage.

Payr (1915) was the first to describe medialization thyroplasty, which aims at reforming the thyroid cartilage to medialize the VF. Later on, this technique was modified by Isshiki, who used a thyroid cartilage flap inserted through a window in the thyroid cartilage at the level of VF to medialize the paralyzed VF. The technique was further modified, and synthetic materials were used for the medialization [212,213]. The procedure gained popularity as an effective method for long-term rehabilitation of voice, and several materials were used including silicone and titanium. However, it produces an irreversible destruction and alteration of the laryngeal structures [214]. Its complications include laryngeal edema, wound infection and extrusion of the implant [215]. Sometimes additional medialization procedures are performed simultaneously, such as arytenoid adduction, or arytenoidopexy. Arytenoid adduction involves pulling the muscular process of arytenoid forward by suturing it to the anterior thyroid cartilage, leading to medial fixation of the VF [216]. Arytenopexy involves medial displacement of the arytenoid cartilage after opening the CA joint, and suturing it to the cricoid cartilage in a more medial position, by thus repositioning also the VF [217].

Whereas all the aforementioned are static solutions, reinnervation surgery aims to restore neural supply to the laryngeal muscles. Although this procedure succeeded to restore mobility in some animal experiments, it was not translated successfully

into clinical setting [90,218,219]. This is probably secondary to synkinesis in addition to other factors related to chronic denervation [218,220]. The main advantage attributed is the preservation of muscle bulk and tone, and improvement of the voice quality [221]. The currently used techniques include: primary anastomosis of RLN ends, anastomosis of ansa cervicalis or the hypoglossal nerve to RLN, direct implantation of a ansa cervicalis to TA muscle, ansa cervicalis to TA neuromuscular pedicle, and CT muscle-nerve-muscle innervation [221]. The most commonly used technique is ansa cervicalis to RLN anastomosis, followed by primary repair of RLN injury [221]. Comparison of these techniques was not possible due to the heterogeneity of the end points in the published studies [221].

3.9.2 Bilateral laryngeal paralysis

Management of bilateral LP depends mainly on the status of airway patency. In children, the standard of care is to secure the airway by a tracheostomy until spontaneous recovery takes place or a glottic expansion procedure is resorted to. However, not all patients require tracheostomy, and the reported rate of its use ranges from 0 – 92% [58,63-66] For those who do not require a tracheostomy, close monitoring, family education and frequent follow up are required as the symptoms may deteriorate. In adults, tracheostomy has been used as an initial line of management in bilateral LP patients [222].

Tracheostomy is known to affect the quality of life of the patient. It requires special daily care with regular cleaning, suctioning and humidification of room air. In the case of pediatric patients, the effect extends to the caring family, because of the extra care required. An adult or an older child might experience social

embarrassment and isolation. It also has a wide range of medical complications, some of them are serious and life threatening. Early complications after the procedure include: pneumomediastinum, pneumothorax and wound complications[223]. The most common and serious life threatening problems are cannula obstruction and accidental decannulation [223]. Long-term complications include granulation tissue formation, laryngeal and/ or tracheal stenosis, tracheomalacia, trachoesophageal fistula and tracheal-innominate fistula [223]. Sometimes the situation requires performance of one of the surgical glottic expansion procedures. However, most of the previously published reports recommended a watchful waiting period of at least 12 months [58,64,66,93]. Several techniques and approaches had been described including arytenoidectomy, cordotomy, and laterofixation, which had been used either alone, or in combination to achieve better results. The aim is to lateralize one or both VFs and improve the airway patency.

Other experimental procedures such as selective reinnervation surgery, laryngeal pacing and botulinum toxin type A (BTX-A) injection have been described and showed variable degrees of success, mostly in animal experiments. Hengerer and Tucker were the first to describe selective reinnervation surgery for PCA muscle, to restore abduction [224]. They used a sternohyoid-ansa-hypoglossi nerve muscle pedicle to PCA muscle technique. The choices for the nerve graft are limited, as it should provide a phasic inspiratory activity. The phrenic nerve is thought to be the optimal choice, however, it is associated with the morbidity of denervated hemi-diaphragm. Some animal studies have achieved some success [225-229], and others

acheived variable results depending on the type of the nerve grafts and the technique used [230-234]. However, in clinical studies these results have not been replicated [218,235].

Laryngeal pacing is a procedure where an electrical stimulator is implanted to deliver impulses to the laryngeal muscles. It was first described by Zealear and Dedo[236]. It was used in animal experimental studies with only a few attempts in humans. Animal studies showed some success in restoring the abduction of the VFs[237-240] through stimulation of PCA muscle. In humans, the technical limitations of the device and the related complications associated with the electrode placement restricted its utility [194,241].

BTX-A injection has been used as a treatment of LP. Cohen et al. was the first to use BTX-A in LP in two animal experiments and suggested that it might have an important role in the management of LP [242,243]. The experiments were performed on Mongrel dogs with surgically induced LP, and BTX-A was injected into the CT muscle. It lateralized the VFs' position in most of the animals.

BTX-A injections were used in a few case series and showed a relief of airway obstruction. However, due to the temporary effect of this drug on the injected muscles, multiple injections were needed every 3-4 months [244-246]. El-Hakim used BTX-A in a small series of children with bilateral LP as a single injection into CT, sternothyroid and sternohyoid muscles bilaterally [247]. Complete functional recovery in six out of seven patients was documented.

4 Laryngeal Electromyography

4.1 Objectives

- Brief overview of the basic neurophysiology principles related to the electromyography
- Overview of the laryngeal electromyography recording and interpretation methods
- Review the clinical applications of laryngeal electromyography
- Overview of the effect of nerve injury and botulium toxin on electromyographic activity

4.2 Definition

Electromyography (EMG) is the recording of electrical activity of the muscle to evaluate its function [248], which can be used to aid in the diagnosis of neuromuscular disorders. It allows for assessment of the motor system integrity from the level of upper motor neuron in the cortex to the muscle fiber.

4.3 Basic neurophysiology

Muscle and nerve cells have specific physiological properties that make them suitable to conduct electrical signals. The interior of these cells contains a higher concentration of negatively charged ions in relative to the surrounding tissues, which contain a higher concentration of positive ions. The difference of ions concentration around the cell membrane creates an electrical potential called the resting membrane potential. Electrical activity in these cells results from a change in

the ions distribution around the cell membrane, which creates a local depolarization (positive ions influx) followed by repolarization (positive ions efflux) to restore the resting membrane potential. This change is a transient, fast and very short event that propagates rapidly along the axon, and is known as the action potential [249].

Usually, each LMN supplies multiple muscle fibers. The collection of the LMN and the muscle fibers supplied by it are collectively called the motor unit (MU) [250]. Once the LMN axon reaches the muscle, it divides into multiple small branches that supply different muscle fibers. The end of each branch expands to form a presynaptic button, the collection of which is called the endplate region. Each button contains cytosols containing Acetylcholine (ACh), and synapses with a junctional fold on the muscle fiber. The membranes of the presynaptic bouton and the junctional fold are separated by the neuromuscular junction [251].

The lower motor neuron receives excitatory input from other centers in the central nervous system [251]. The action potential is generated in the axon hillock (the area between the neuron cell body and the axon) of the LMN, and propagates along its axon to the neuromuscular junction on the target muscle fibers. Once the action potential reaches the synaptic bouton, it causes opening of the voltage gated calcium (Ca^{++}) channels. The influx of Ca^{++} into the presynaptic bouton induces a cascade of events that facilitate the fusion of the ACh cytosols to the cell membrane, and then exocytosis of ACh into the neuromuscular junction. Then, ACh molecules bind with ACh-gated receptors in the postsynaptic membrane of the endplate region [251], which contains channels that permit passage of sodium (Na^{+}) and potassium (K^{+}) ions located on its periphery. Influx of Na^{+} depolarizes the endplate region, and creates

the endplate potential (~70 mV), which in turn activates the surrounding voltage-gated Na^+ channels. Activation of these channels results in influx of Na^+, which causes depolarization of the surrounding area, and subsequently activation of more voltage-gated Na^+ channels in the muscle fiber. Following depolarization, repolarization of the muscle fibers takes place, which result in restoration of the resting membrane potential. This process spread very rapidly in the muscle fiber, and is known as the action potential [251].

4.4 Electromyography recording

Multiple action potentials generated by a group of muscle fibers in one MU summate to produce a motor unit potential (MUP), which can be recorded using the technique of EMG [250].

4.4.1 Recording process

MUPs are detected using an active electrode (needle or surface electrode applied to the target muscle), a reference electrode, and a ground electrode, which are connected to the amplifier. The recorded signal is the net difference between the electrical activities (i.e. potentials) detected by the active and the reference electrodes. The ground electrode is used to enhance the signal to noise ratio, and to protect the subject from the risk of electrical shock [252,253]. The amplifier functions to eliminate the artifact by acting as a differential amplifier, where it detects the potential difference from the electrodes and rejects the external interferences (external signals detected by both electrodes and referred to as "common mode" signals). The amplifier's ability to reject the common mode signals depends on its

"common mode rejection ratio", which is typically set to 10,000 (i.e. the potential difference is amplified 10,000). The amplifier has a band-pass filter that is usually set to detect signals between 10 and 10,000 Hz [252]. The amplified signals can be revealed graphically on oscilloscope, acoustically using a loudspeaker, or can be converted from analogue to digital signals by connecting the amplifier to a digitizer, which can be displayed and analyzed with a computer software [252,253].

4.4.2 Types of electrodes:

Different types of electrodes are available, and each type has special characteristics and uses. They can be classified as surface electrodes and inserted (needle or wire) electrodes. Surface electrodes have the advantages of being non-invasive, easily handled and conveniently used, as they are applied directly to the skin. The main limitation is that they can only detect activity of the superficial muscles. The wire electrode is composed of a fine insulated wire that is inserted into the muscle of interest using a hollow needle. Once the wire is deployed into the muscle, the needle is withdrawn, and the wire is anchored to the muscle by its hooked end, which make it able to stay in place for long period. The main limitation of the wire electrodes is the difficulty to readjust or reposition them once inserted into the muscle [253].
There are different types of needle electrodes. The main advantages of these electrodes are the small detection area, and the ability to reposition the needle to detect a better signal. The first type is the monopolar needle electrode, which is composed of an insulated needle with active detection surface at its tip. The detection area is the circular area of the muscle around the tip. Its reference

electrode is placed at a different area in the body. The second type is the bipolar needle electrode, which is composed of a hollow needle enclosing two insulated wires that are exposed at the end. Each wire is connected to a different channel in the amplifier, and the detection area is the area between the two wires. One of these wires acts as the active electrode, and the other acts as the reference electrode. The needle shaft acts as a ground electrode. The third type is the concentric needle electrode, which is formed of a hollow needle with a wire electrode running inside it. The wire is insulated except at the tip, which acts as the active detecting electrode. The detection area is restricted to the area facing the needle bevel, thus changing the direction of the needle results in detecting activity from a different area. The needle shaft acts as a local reference electrode. Other types of needles exist, but are not commonly used [253].

4.4.3 Laryngeal electromyography techniques

In clinical practice, LEMG can be performed in an office based setting on a conscious cooperative subject, or intra-operatively under general anesthesia. In the office setting, the electrode is inserted trans-cutaneously, or trans-orally under the guidance of a fiber-optic laryngoscope. A monopolar or concentric needle electrode can be used. If a monopolar needle is used, the ground electrode (either surface or needle electrode) is placed on a distant area (e.g. chest). The reference electrode, which also can be surface or needle electrode, is placed at the cheek or the forehead[252-254].

In the transcutaneous approach, the needle is inserted through the skin overlying the larynx, and the direction of needle insertion depends on the muscle being examined. For the cricothyroid (CT) muscle, the needle is inserted into the area overlying the cricothyroid membrane (slightly off the anterior midline of the neck), and then directed along the exterior surface of the cricoid cartilage upward and laterally until the electrical activity is detected. For the thyroarytenoid (TA) muscle the needle is passed through the cricothyroid membrane to enter the airway. Then the needle is directed upward and laterally to reach the TA muscle. For the posterior cricoarytenoid (PCA) muscle, two approaches can be used: a midline and a lateral approaches. The lateral approach requires rotation of the larynx, which is usually inconvenient to the patient, and is not commonly used. In the midline approach, the needle is inserted through the cricothyroid membrane. Then, the needle is advanced through the airway until it reaches the posterior lamina of cricoid cartilage. Once the lamina is felt the needle is passed through the cartilage while directed 15 degree horizontally from the midline aiming to the tested side [252-254].

The trans-oral electrode insertion can be done in a conscious patient under fiber-optic laryngoscope guidance, or in the operating room during direct laryngoscopy. In a conscious patient, a local anesthetic spray is used to decrease the discomfort. The electrode is inserted slightly lateral to the vocal fold edge, to detect the activity from TA muscle. For the PCA, the electrode is inserted about 10 mm posterior to the inter-arytenoid area. Recording the CT muscle activity is not possible using the trans-oral approach [252]. Specific maneuvers are used during LEMG recording in a

conscious subject to confirm the accuracy of needle insertion, such as vocalization, sniffing, or deep breathing [253,254].

In children and non-cooperative patients, the LEMG is done under general anesthesia during direct laryngoscopy. While the patient is lightly anesthetized and spontaneously breathing, the needle is inserted into laryngeal muscles (TA, and PCA) under direct visualization using the same trans-oral needle insertion technique described above [199]. The anesthetic agent can depress the muscle activity, and affect the LEMG recording [255]. Both inhalational and intravenous anesthesia were reported to decrease the MUP amplitude and increase the latency period, however, the effect is more pronounced with the inhalational agents [255]. Therefore, it is suggested to avoid inhalational agents, and to perform the recording after lightening the intravenous anesthesia.

In experimental animal studies, LEMG recording was mostly performed in anesthetized animals. Different insertion techniques were described including trans-oral, trans-cartilaginous, through cricothyroid membrane, and directly into the muscle through a laryngofissure. In the rat model, the trans-oral approach under direct laryngoscopy guidance was used in a few studies, where the needle was inserted into the adductor muscles complex and into the PCA muscle (Figure 1-11) [256-259]. Most of the studies used a trans-cartilaginous approach after surgical exposure of the larynx. The needle was inserted through a window created in the thyroid cartilage into the adductors muscle complex or the PCA muscle [260,261]. Few studies used a midline laryngofissure for direct needle insertion into the laryngeal muscles [262,262,263,263]. However, this approach is invasive and could depress the

activity of the laryngeal muscles, as it might eliminate the subglottic pressure that is important for the reflexive activity of these muscles [40,42].

4.5 Electromyography interpretation

The raw amplified EMG signals can be enhanced by applying additional filter, to decrease the background noise and remove the interfering artifacts that were not excluded initially by the amplifier. The EMG data can be analyzed qualitatively or quantitatively. In qualitative analysis, the EMG signals are evaluated during insertion, muscle relaxation, and with minimum and maximum voluntary contraction. During insertion, a brief (about 500 milliseconds) activity (insertional activity) is generated by the muscle. This is normally observed as the needle has some electrical energy, and once introduced into the muscle it creates a change of the electrical potential in the surrounding muscle fibers. Prolongation or shortening of this activity might imply muscular or neural injury. Normally, if the muscle is completely relaxed (non-contracting), no activity is observed. Presence of spontaneous activity might indicate an underlying muscle or nerve pathology [253,264]. During contraction, qualitative analysis includes the description of the MUP waveform morphology, and the recruitment pattern (firing pattern of MUP) in the raw or the processed EMG recordings. Waveform evaluation includes assessment of MUP amplitude, duration, and shape. These are usually described qualitatively without numerical description [264].

Quantitative analysis can be performed using different methods for measurement of the MUP parameters, and it can be performed manually or automatically using

computer software [265]. Several processing methods can be used prior to the quantitative analysis, in order to provide quantitative measurements that can be used for comparing different muscles or different individuals. Some of these methods include average of rectified signals, root mean square value, integrated rectified signal, turn analysis, and frequency analysis. These methods are usually done after rectifying the LEMG signals to avoid the problem of averaging the value of the EMG signals to zero (i.e. the amplitude in the EMG signal varies randomly above and below the zero line and the net result is equal to zero) [248]. Rectification can be performed by deleting the negative values (half-wave rectification), or by inverting the negative values (full wave rectification). The latter preserves the energy of the signal and is the preferred method [248]. After rectification, smoothing of the signals helps to decrease the random pattern of the EMG signal [248]. Among the processing methods, calculation of the integrated rectified signal was widely accepted as the preferred one [266], which involves mathematical calculation of the area under the curve of the rectified signal, over a specific period of time [248,266]. The average of rectified signal is calculated in a similar way, but the calculated value is divided by the time [266]. Both of these methods provide similar information, however they do not provide physical meaning [266]. On the other hand, the root mean square (mathematical calculation of the amplitude values in a specific period of time performed by squaring the amplitudes and taking the root mean square of the average value) provides more information about the power of the muscle, and is the preferred method of signal processing [248,266]. In the turn, or zero crossing analysis, the peak amplitudes or the times the signal crosses the zero line in a specific period

of time is counted. Although this method was used frequently to distinguish between the healthy and abnormal muscles, it is not recommended for measuring the force of the muscle (e.g. recruitment) [248].

4.5.1 Laryngeal electromyography interpretation

LEMG recording is evaluated in the same way as in other skeletal muscles, however with some differences. The differences are related to the nature of the muscle's functional and physical properties. First, while it is possible to assess a single skeletal muscle during active contraction, multiple laryngeal muscles usually contract simultaneously to perform a specific task. Therefore, during blind insertion of the needle (trans-cutaneously), the subject is asked to perform certain maneuvers (e.g. vocalizing certain vowels or taking deep breath) to ensure accurate needle insertion [252]. Secondly, laryngeal muscles are respiratory muscles, and have phasic activity during the respiratory cycle [252], and some of them have a continuous tonic discharge (i.e. repetitive bursts of activity to provide the muscle with a tone) [267,268]. This makes the evaluation of the pathologic spontaneous activity difficult [267,268]. Lastly, laryngeal muscles are small muscles, when compared with the other skeletal muscles, with smaller MU size. This structural characteristic results in lower amplitude and shorter MUP duration in comparison to the larger skeletal muscles[252].

The phasic activity of the laryngeal muscles is driven by central reflexive mechanisms. If the respiratory conditions are changed, such as during hypo- or hyperventilation and hypo- or hyper-capnea, the phasic activity of the laryngeal

muscles is altered [39,47,50,269]. Voluntary respiratory tasks, such as deep breathing or forced expiration, increase the activity of the adductor and abductor muscles [269]. Therefore, the LEMG recording might vary with the respiratory condition, and is better to be performed while the subject is spontaneously breathing.

4.5.1.1 The effect of nerve injury on laryngeal electromyography:

Following complete nerve injury, several stages of muscle activity have been reported. In the acute stage, LEMG recording shows electrical silence (i.e. absence of the phasic and the tonic activity patterns) [254]. After a period of time ranging from 1 to 3 weeks, pathological spontaneous activities, such as fibrillation potentials (i.e. spontaneous activity generated by the unstable muscle fiber) and prolongation of insertional activity are observed. These changes result from the instability of muscle fiber membrane [254]. Once re-innervation of laryngeal muscles from the original nerve or from the surrounding nerves takes place, the pathological potentials disappear, and abnormal MUPs with polyphasic morphology, low amplitude and prolonged duration are observed [254].

Synkinetic innervation can be evaluated by asking the patients to perform certain tasks (i.e. tasks produced primarily by the action of the muscle) to activate the muscle and then its antagonist during LEMG recording. The presence of activity in a muscle during the activation of its antagonist indicates synkinetic innervation. For example, in the TA muscle, synkinetic activity might be indicated by the presence of activity during forced deep inspiration. While in PCA muscle, the presence of activity during phonation or forced glottic closure (e.g. valsalva maneuver) might reflect

synkinesis [252]. During spontaneous breathing, synkinesis could also be indicated with the presence of activity in TA during inspiration, or in PCA during expiration.

4.5.1.2 The effect of botulinum toxin on laryngeal electromyography:

Botulinum toxin (BTX) is a powerful neurotoxin that blocks the release of ACh from the nerve ending at the neuromuscular junction. Intramuscular injection of BTX produces a paralytic effect on the injected muscles in a dose-dependent manner[270,271]. EMG assessment of the muscle following BTX injection shows a decrease or absence of the electrical activity [271], accompanied by appearance of denervation potentials (e.g. fibrillation) [272]. Multiple clinical studies on patients with various neuromuscular disorders found that BTX injections resulted in reduction of the different EMG parameters including MUP amplitude, interference pattern and the number of turns per second [272-277].

In laryngology, BTX was used for treatment of different conditions, and the most common use was for patients with spasmodic dysphonia. However, LEMG changes, following BTX injection, were rarely reported, instead most of the studies focused on symptomatic, aerodynamic and voice changes [278]. In experimental animal studies, very few studies investigated the LEMG changes after BTX injection. Inagi et al. reported the LEMG findings following BTX-A injection into the TA muscle of Sprague Dawley rats [279,280]. The authors found a reduction in the interference pattern, and the compound muscle action potential amplitude (summation of MUPs produced after stimulation of the RLN) [279,280], and the effect was more significant with higher doses [280]. The time until functional recovery of the EMG activity was

related to the dose of the injected toxin, ranging from eight to forty days with doses of 0.0007 and 0.07 units, respectively [279].

4.6 Clinical applications of laryngeal electromyography

EMG is widely used as a diagnostic tool for various neurological, muscular and neuromuscular disorders. In laryngology, LEMG was used for diagnosis, and predicting prognosis of various voice and neuromuscular disorders of the larynx. In addition, it was used for intraoperative RLN monitoring, and as an adjunct for guiding laryngeal muscle injections [198,200,254,281-283]. Despite the wide use of LEMG in laryngology, there are still variations in its methods, techniques, and results interpretation. This variability led to the lack of consensus about its validity as a tool for diagnosis or prognosis [200,252]. An evidence-based review performed by Sataloff et al. found evidence to support the use of LEMG for guiding botulinum toxin injection into TA muscle for patients with spasmodic dysphonia [198]. No evidence was found supporting its other uses in other laryngeal disorders [198]. This was further supported by the Neurolaryngology study group report, which also questioned many issues in the previously published studies [200]. The main concern of the authors was the variability among the methods used for recording and interpreting the results, especially the lack of quantitative LEMG analysis. The group made some recommendations to help standardize the methodology and reporting practice for LEMG, and the need for more prospective studies [200]. Recently, the European Laryngological Society published guidelines for LEMG recording and interpretation, and made similar recommendations [252].

4.6.1 The use of laryngeal electromyography for laryngeal paralysis

Many studies used LEMG as a diagnostic tool to differentiate between neurological and mechanical causes of vocal fold immobility, and to predict prognosis [198,252]. Although no evidence was found to support these uses [198], a recent systematic review reported results supporting the ability of LEMG in predicting the poor prognosis of LP [201]. The study found 91% of the patients with abnormal LEMG had no recovery in the follow-up [201]. However, this result might be misleading as the length of the follow up and the timing of the LEMG evaluation were variable among the included studies, and were not even specified in some. Moreover, most of these studies used qualitative ways to interpret the LEMG data.

Only a few studies utilized quantitative methods for LEMG analysis. Gavazzoni et al utilized quantitative and qualitative analytical methods of the LEMG data in a group of patients (n=40), with unilateral LP (idiopathic n=15, iatrogenic n=17, infectious n=6, and neurological n=2), in order to test the diagnostic accuracy of LEMG [284]. In the quantitative analysis, manual measurements of the MUP amplitude, and duration of the TA and CT muscles were performed. The authors reported higher MUP duration in the affected side but no difference in the MUP amplitude between the two sides. However, the study did not specify the methods used for making the measurement of the MUP amplitude and duration, or whether the signal was processed in a proper way to allow for comparison prior to analysis. Statham et al. used quantitative measurements of the maximum amplitude and turn counts of the TA and lateral cricoarytenoid (LTA) muscles, in healthy subjects (n=21), and a group of patient with unilateral LP (n=16)[285]. The authors reported higher turn counts and

maximum amplitude in the healthy control group, however the difference in the maximum amplitude was not statistically significant. Another study used turn analysis combined with qualitative assessment to test the ability of LEMG in predicting the prognosis of patients with unilateral LP (n= 23) [286]. The mean of the number of turns per second was not statistically significant between the group of patients who recovered movement (n=6) and those who did not over a follow up period of six months (n= 11). The authors reported that when qualitative analysis was combined with turn analysis, the accuracy of LEMG in predicting prognosis increased. Despite that, the study had many limitations such as variable durations between onset of paralysis and LEMG evaluation, inclusion of patients from different etiological categories without subgroup analysis, and the small sample of patients without a control group.

It seems that until now, there is no optimal methods for LEMG data quantification that can be used for assessment of the laryngeal muscles in LP. Most importantly, quantification of the synkinetic activity has not been accounted for. One study proposed a grading system [287] that might help in detecting the paradoxical activity of the synkinetically innervated muscles. However, it has not been validated yet. The system correlates the timing of the muscle activity with the respiratory phase, taking into account the degree of muscle activity. Although, quantification of the LEMG data was not described in this study, the proposed system appears to reflect the functional activity of the laryngeal muscles when combined with the quantitative analysis of LEMG.

5 Botulinum toxin

5.1 Objectives:

- Review the history and structure of botulinum toxin
- Overview of the mechanism of action of the toxin
- Overview of the clinical applications and its use in laryngeal paralysis (LP)

5.2 Introduction

Botulinum toxin (BTX) is a neurotoxin that has a powerful paralytic effect on the muscles. It exerts its effect by interfering with the release of the neurotransmitters (acetylcholine (ACh)) from the nerve ending at the neuromuscular junction (NMJ). The toxin was first discovered by Kerner in the early eighteenth century, when he described the clinical picture of botulism, and related it to a poison, which he called "sausage poison" [288]. At the end of the eighteenth century, Van Ermengem succeeded in isolating the bacteria producing the toxin, and in 1928 Tressmer Snipe and Sommer extracted the toxin [288,289]. When Krener discovered the toxin he suggested that it might have therapeutic applications. However, it was not until 1980 when the toxin was first used clinically by Scott et al. to treat strabismus [209,290].

The toxin is produced by Clostridium botulinum bacteria (gram-positive anaerobic bacteria), and some other species including Clostridium butyricum, Clostridium baratii, and Clostridium argentinense [270]. Seven serotypes of the toxin were identified and denoted by the letters A to G, where each has subtypes that differ in their immunological properties [270]. Only type A and B are used for therapeutic

purposes. Four subtypes of botulinum toxin type A (BTX-A) are available for clinical use [291], and all have the same effect but with different immunogenicity and potency[291]. OnabotulinumtoxinA (Botox®) has been the first to get FDA approval, and it has been approved for many clinical uses [292].

5.3 Mechanism of action of botulinum toxin type A

5.3.1 Paralytic local effect:

BTX-A is synthetized as a single polypeptide molecule, which is then proteolyzed into a heavy and a light chain linked by disulfide bond. The paralytic effect of BTX-A is produced as a result of the failure of the nerve terminal to secrete ACh. Normally, once the action potential reaches the lower motor nerve (LMN) terminal, ACh molecules are released from the cytosols in LMN terminal into the neuromuscular junction cleft, and then attach to their receptors in the muscle fiber membrane. The release of ACh from the cytosol requires the presence of a specific protein complex (soluble N- ethylmaleimide- sensitive factor attachment protein receptor (SNARE)), which facilitates the fusion of the cytosol to the cell membrane. BTX-A interferes with this exocytosis mechanism by targeting the SNARE complex. BTX-A enters the nerve terminal by endocytosis, mediated by the binding of the heavy chain part of the toxin with specific glycoprotein in nerve cell membrane [293]. After that, the light chain, which has a catalytic function, binds with and cleaves a specific protein in the SNARE chain called the synaptosomal-associated proteins of 25kDa (SNAP-25) [294], thus the ACh-containing-cytosol cannot bind with the cell membrane, and cannot be released from the LMN terminal into the synaptic cleft.

The described mechanism of the paralytic effect of BTX is the known and the most accepted. However, multiple experimental and clinical studies reported functional, biochemical and structural changes that raise the possibility of other effects occurring locally in the muscle, the NMJ and the LMN ending, or distally at the CNS level.

5.3.2 Other possible mechanism of action

5.3.2.1 Muscle level:

Multiple animal studies demonstrated morphological changes in the form of alteration of the proportion of the fast and the slow muscle fibers upon injection of the toxin [295,296]. BTX-A injections into the gastrocnemius and plantaris muscles of the rat resulted in an increase of the proportion of the slow muscle fibers, and a decrease of the fast fibers. The proportion of the fast type in the contralateral muscles was altered as well [295,296]. The increase of the slow fiber type weakens the muscles and explains the temporary beneficial effect for patients with spasticity and dystonia. However, in laryngeal muscles a different effect was found. Inagi et al. found an increase in the fast muscle fibers with no change in the slow type in the injected thyroarytenoid (TA) muscles [297]. The authors indicated that this change in the type of muscle fibers is different from what is seen after RLN transection. However, it is unclear if this change results from denervation or as a direct effect of the toxin [297], and whether it plays a role in the long term (e.g. ameliorating synkinesis).

At the cellular level, Inagi et al. studied the histological changes in the rat TA muscle after BTX-A injection. An increase in the mitotic activity was found, which might indicate that BTX-A induced a proliferative effect on the muscle [298]. One study investigated the proteomic changes in the rat laryngeal muscles injected with BTX-A. It demonstrated a change in the expression of many proteins associated with muscle energy metabolism, contractility, stress response, gene transcription, protein translocation and cell proliferation. Some of these changes persisted after the return of muscle function [299], which might be responsible for the effect observed in some injected muscles that persisted beyond the expected duration of action.

5.3.2.2 Neuromuscular junction level:

Several experimental animal studies found that blocking the ACh release from LMN triggers temporary synaptic remodeling and extensive sprouting of the LMN ending, to form new NMJs. However, this effect seems temporary and most of these new changes disappeared with the recovery of the original synapses from the toxin effect[300].

5.3.2.3 Lower motor neuron level:

One study found that BTX-A injection resulted in a alteration of the neurotransmitter gene expression in the injected gastrocnemius muscle of the cat[301]. This was observed as well after nerve injury in many studies, however in different pattern [301]. Some investigators demonstrated evidence of retrograde transportation and transcytosis of BTX-A and its product SNAP-25 [302,303]. These studies indicated that the intramuscularly applied BTX-A traveled along the axon of

the LMN and reached the CNS after transcytosis over the central synapses, in the rat model [302,303]. For example, Antonucci et al. found BTX-A-cleaved-SNAP-25 molecules in the facial motor nucleus after BTX-A injection into the rat whisker pad [302]. The authors also produced evidence that the toxin can be retrogradely transported in the visual system. In the same study, another group of animals were injected with BTX-A into the tectum. After the injection, BTX-A-cleaved-SNAP-25 molecules were found in the retinal cells, however, the molecules were not detected in retinal cells after severing the optic nerve, which indicates that the toxin was retrogradely transported along the nerve [302]. In another experiment, BTX-A-cleaved-SNAP-25 molecules were also detected in the spinal cord of the rat after being injected into the limb muscles, and Colchicine injection into the sciatic nerve prevented that confirming the retrograde transport process [304]. These findings suggest that the toxin can travel for a long distance from its injection site, and imply that BTX-A can exert direct effect on the CNS.

5.3.2.4 Central nervous system level

Several animal and clinical studies demonstrated neurophysiological evidence that supports the central action of BTX-A at the spinal, bulbar, and cortical levels.

5.3.2.5 Spinal cord level

In two clinical studies on patients with essential tremors and dystonia, investigators documented a reduced presynaptic inhibition between the flexor and the extensor muscle motor neurons [305,306]. Injecting BTX-A into the forearm muscles normalized the presynaptic inhibition level. This effect was attributed to the change in the

spindle afferent input level, which led to modulation of the central reflex mechanism.

5.3.2.6 Brainstem level

Animal studies showed that BTX-A block ACh release from the gamma motor neuron at the NMJ of the intrafusal muscle fibers [307]. This effect led to a change of the spindle afferent input, which might alter the brainstem reflex mechanisms [307]. Clinical studies on patients with spasmodic dysphonia showed improvement of the speech after BTX-A injection. Bielamowicz and Ludlow injected BTX-A unilaterally into the TA muscle. They found a reduction of the LEMG activity not only in the injected TA muscle but also the ipsi-lateral cricothyroid (CT), and the contra-lateral TA and CT muscles [278]. They concluded that the effect on the non-injected muscles could have been caused at the brainstem level, rather than by a local spread of the toxin from the injected muscle [278].

5.3.2.7 Cortical level

A clinical study on patients with writer's cramp found that the cortical representation of the hand muscles is different from normal. After BTX-A injection into the forearm muscles, the symptoms improved and the cortical map was reorganized. However, this effect was temporary and returned to the pre-injection state after the end of the duration of BTX-A effect [308].

5.4 Therapeutic uses

BTX has many clinical applications. It was used for different diseases affecting various body systems including neurologic, ophthalmologic, urologic, laryngeal,

gastrointestinal, painful, and hyper secretory disorders, as well as for cosmetic purposes. The FDA approved BTX-A for treatment of strabismus, blepharospasm, cervical dystonia, upper limb spasticity, neurogenic detrusor over-activity, glabellar lines, axillary hyperhidrosis, and as prophylaxis for chronic migraine [291]. Although BTX-A is one of the most powerful neurotoxins, it is considered as a safe drug [309]. One of the reported side effects is the spread of the toxin to the nearby or distant muscles, however this has been rarely reported [309]. Resistance to BTX-A was also reported after multiple injections, nevertheless these have been early experiences and were probably caused as by the preparations that contained a high amount of proteins that triggered antibody formation [309,310]. Other less frequently reported self-limited side effects include pain at the injection side, erythema, localized edema, headache, nausea, fatigue, flu-like symptoms, and rashes [309,310].

5.5 Uses in laryngeal diseases

In laryngology, BTX-A has been used widely for treatment of spasmodic dysphonia, which is characterized by involuntary hypermobility of the laryngeal muscles during phonation. BTX-A injection into the vocal fold of the affected patients results in improvement of the quality of voice. A systematic review found only one clinical trial where beneficial effects of its use were demonstrated, and concluded that firm evidence supporting its effectiveness is still limited [311].

BTX-A was used infrequently for treatment of other laryngeal movement disorders with variable results, such as stuttering, essential voice tremors, vocal tics, puberophonia, supraglottic spasm, cricoaryteniod joint dislocation, vocal fold granuloma, posterior glottic synechiae, and laryngeal paralysis (LP) [312].

5.5.1 Uses in laryngeal paralysis

There are only two experimental animal studies where BTX-A was used for LP. In both, Cohen et al. injected BTX-A into the CT muscle leading to a temporary lateralization of the vocal folds [242,243].

Few human publications reported the use of BTX-A in LP [244-247,313]. Rontal et al. reported a patient with unilateral LP caused by multiple sclerosis [314] where BTX-A was used. They first injected the toxin into the ipsilateral adductor muscles, however without any improvement. Six months later the PCA muscle was injected in order to medialize the vocal fold. One month after the second injection, full functional recovery was demonstrated, which the authors believed was a genuine effect of BTX-A rather than a remission of the multiple sclerosis (none over the 4-year follow up) [314]. However, given the natural history of multiple sclerosis, the possibility of natural remission cannot be excluded, especially that in the afore-mentioned case, none took place over the subsequent four years. In another study, Rontal et al used BTX-A injection into TA and lateral cricoarytenoid (LCA) along with gelfoam injection (as medialization laryngoplasty) in ten patients with unilateral LP [315]. The concept proposed was to re-balance the larynx by eliminating the action of the synkinetically innervated TA and LCA, and to leave the interarytenoid muscle to provide vocal folds adduction in the short term. An improvement of the voice quality was achieved, and persisted beyond the duration of the BTX-A effect and the resorption time of gelfoam [315].

BTX-A was also used for bilateral LP. Andrade Filho and Rosen reported a patient with bilateral LP that was sustained after surgical injury to the recurrent laryngeal

nerves [244]. The patient was repeatedly injected with BTX-A into TA and LCA muscles (unilateral), and subsequently his breathing improved with minimal temporary effect on the voice quality. Ekbom et al. used BTX-A injections into the adductor muscles of ten patients with bilateral LP [246] (Six with iatrogenic surgical injury, one neurological etiology, one post-intubation, and two idiopathic). Symptomatic improvement was reported in all patients, and six did not require a tracheostomy. The authors reported widened glottic gaps in all subjects with repeated BTX-A injections. Of the remaining four patients, two were decannualted after glottic expansion procedures, and two remained tracheostomized. In another study, BTX-A injections into the TA muscles were used as adjunct to the electrical pacing of the PCA muscle in a patient with bilateral LP [316]. There was no significant change in the voice parameters, however the authors reported improvement of airflow and the stimulated abduction after the injection. In another similar case report, improvement of airflow measurement was reported, allowing for decannualtion later on [313]. The authors of the latter two studies related the beneficial effect produced to the paralytic action of BTX-A in eliminating the action of the synkinetically re-innervated TA muscle, which allowed the PCA muscle to act unopposed during inspiration [313,316]. However, the effect was of short duration and repeated injections were required [313,316].

In children, BTX-A was used in two studies. Smith et al used bilateral BTX-A injection into TA muscles in a group of ten children, seven of which had bilateral LP, but several had other airway lesions [245]. Three out of the seven patients improved. They all underwent airway surgery with placement of tracheostomy, and were

decannulated prior to the injection. The toxin was deemed helpful in improvement of the stridor, and avoidance of tracheostomy reinsertion, but repeated injections were required. Later on, El-Hakim used BTX-A injections into CT, sternothyroid, and sternohyoid muscles in a series of seven children with bilateral LP [247]. Six of these patients had congenital idiopathic etiology and all of them recovered completely. The seventh patient had iatrogenic etiology and partially recovered on one side. None of the patients required further management following the injection. The author postulated that the short-term positive effect was secondary to the paralytic effect of the toxin, which relaxes and widens the glottic opening. The long-term effect (i.e. functional recovery) was related to a central mechanism that allowed proper re-innervation of the laryngeal muscles and elimination of synkinesis.

6 Experimental studies on laryngeal paralysis

6.1 Objectives

- Review the previously used animal models, and the modalities for treating laryngeal paralysis in experimental settings.
- Review the experimental literature on laryngeal paralysis models that tested botulinum toxin efficacy in particular.
- Review the end points utilized in the previous experimental studies with emphasis on laryngoscopy and laryngeal electromyography on the rat model.

6.2 Introduction

Most surgical experimental animal studies on laryngeal paralysis (LP) were performed in large animal models (e.g. dogs, horses, pigs, cats and rabbits) due to the advantage of their large, stable airway. In addition, changes of the laryngeal functions after the therapeutic intervention can be evaluated by assessing vocalization and exercise tolerance in these models [237-239]. Although rats were not commonly used probably because of the small size of their larynx, they were utilized more recently in studies investigating non-surgical treatment approaches, such as gene and stem cell therapy [203,262,263,317-322], and in some surgical re-innervation studies [260,323,324]. The rat model is a favorite choice for most researchers in different fields due to its low cost, commercial availability, and ease of handling, housing and care. As a laryngeal model, it has recently been found to be suitable for studying laryngeal function [258,259].

An animal LP model has mostly been created by inducing an injury (i.e. transection, crushing, clipping or ligation) to the recurrent laryngeal (RLN) [325,326] or the vagus nerve [263,327]. Although this model simulates mainly the clinical picture of iatrogenic surgical injury, it might be less representative of the pathophysiological picture of the other categories (e.g. idiopathic and neurological categories). However, since a representative model for the other categories cannot be obtained, this model appears to be the most suitable for LP research. Few studies employed other methods to simulate LP, such as chemical denervation of the laryngeal muscles [328], and application of local anesthetic around the RLN [239]. These methods were used to produce a short-term LP model, however, it might not be truly representative. Many of the studies investigating certain medialization procedures, induced a static picture of LP in an *ex-vivo* model (i.e. excised larynx) by creating a structural modification on the laryngeal framework or muscles [329]. This model has many limitations, although it might be acceptable as an approximate model to assess for the static effect of these procedures on the glottic dimensions.

Several end points were used in the LP experimental studies to assess for the change in the laryngeal muscle function after employing a certain intervention. Laryngoscopy was used commonly for assessment of the vocal folds (VF) mobility, and can be considered as the reference standard. Laryngeal electromyography (LEMG) is another commonly used tool that can provide an objective assessment of the laryngeal muscles function. Histology, on the other hand, was used to evaluate changes in the muscle bulk and the degree of innervation. Although it can provide an objective and quantitative assessment of the muscle structure, it cannot reflect the

functional status of the larynx or detect the synkinetic innervation pattern. Retrograde tracing studies though might be able to detect the changes at the central nervous system (CNS), and identify abnormal innervation patterns [191]. Other less frequently utilized end points include airflow measurement, swallowing assessment, voice assessment, symptomatic improvement and exercise tolerance[237,238,330,331]. Although these endpoints can reflect the physiological properties of the airway in the animal model, the results cannot be translated directly to humans, because of the difference in the laryngeal physiological properties and biomechanics between humans and animals [332].

6.3 Experimental treatment modalities

6.3.1 Glottic expansion and medialization procedures

Most of the previously published experimental studies on the management of LP investigated the efficacy of the static glottic expansion, and medialization surgical techniques. There are, in particular, many veterinarian clinical studies on the efficacy of these techniques for managing LP in dogs and horses [333,334].

The expansion procedures were performed either on an *ex vivo*, or an *in vivo* animal model with pre-existing or surgically induced LP. Many were applicable for the treatment of affected animals, and some were directed at humans [335-340]. Few studies proposed novel expansion techniques claimed to be less invasive than the existing options. One of those recently proposed involved placing a stent placed between the arytenoid cartilages [341,342]. However, this is unlikely to be applicable to

human, due to the expected high rate of morbidity associated with the placement (e.g. displacement, foreign body reaction, etc.) [342].

The medialization procedures were mostly studied utilizing *ex vivo* models, where aerodynamic end-points were employed. However, these models have several limitations, and might not reflect the functional status accurately. The *in vivo* experiments focused at the evaluation of various materials that are candidate for implantation, or injection laryngoplasty, with the aim of identifying less immunogenic and more stable choices. Materials used for injection included fascia, cartilage, fat, allograft dermal tissue, fibroblast growth factor, polycaprolactone, polymethylmetacrylate, hyaluronic acid, hydroxyl-apatite, calcium phosphate, calcium hydroxyl-apatite, and silicone [343]. Materials used for implantation included fascia, cartilage, Gore-Tex, titanium, sialistic, and adjustable balloon [344-349]. Most experiments assessed the histological changes evoked at different points in time, stability, and also functional outcomes such as voice quality.

Surgical expansion and medialization of the glottis can provide a solution to relieve the airway obstruction and the glottic incompetence, respectively. However, these procedures are mostly destructive in nature, and result in a compromise of the laryngeal functions. Most importantly, they do not restore normal VF mobility.

6.3.2 Laryngeal re-innervation

Re-innervation procedures aspire to restore the neural supply to the laryngeal muscles, either by using the native (RLN) or another donor nerves. The techniques employed included direct end-to-end anastomosis, nerve-muscle-pedicle, nerve into muscle, and muscle-nerve-muscle techniques [218,350]. Re-innervation can be classified

into selective and non-selective types. The canine model was the most commonly used for this procedure. Other less frequently used models include feline, equine, and rodent. The end-points utilized include laryngoscopy, LEMG, and histological changes.

6.3.2.1 Selective re-innervation:

The objective of selective re-innervation is to reestablish functional neural supply, by innervating the abductor and the abductor muscles separately [350]. Many animal studies tried different techniques to selectively innervate the posterior cricoarytenoid (PCA) muscle, aiming to restore the phasic abduction of the vocal folds (VF) during inspiration. The choice of the donor nerve is limited, as it should have an inspiratory phasic activity, and the optimal appears to be the phrenic nerve[351]. However, this leads to denervation of the hemi-diaphragm [218]. Using a split phrenic nerve was attempted (to avoid the hemi-diaphragm morbidity), however it had unfavorable results [351]. Other potential sources include superior laryngeal nerve (SLN), ansa-cervicalis nerve and the native RLN. Still, even upon using these nerves, the disparity in sizes between them and the abductor branch of the RLN poses a technical obstacle [218].

The phrenic nerve was used to re-innervate the PCA muscle, and successful results were reported in some studies based on laryngoscopy, LEMG, and histology [225-229], however, this was not universally reproduced [352,353]. Moreover, most of the more successful studies were uncontrolled and done on small number of animals, and did not assess the long-term functional status [225-228]. One study examined end-to-end or end-to-side anastomosis between the RLN and the phrenic nerves in thirty Sprague

Dawley (SD) rats. In the end-to-end group (n=10), partial recovery of movement was achieved in eight, and full recovery in one animal. No recovery was observed in the end-to-side group (n=10) [229].

The SLN was used in a few animal experiments, with less encouraging results [234]. Debnath et al. used muscle-nerve-muscle technique in four dogs, where the RLN on one side was severed, and the distal end was implanted into the ipsilateral cricothyroid (CT) muscle. The adductor branches of the RLN were transected, leaving only the abductor counterparts intact to innervation selectively the PCA muscle. No recovery of movement was detected in up to six months after the procedure, despite recovery of electrical activity in the PCA muscle. In this study, histological evidence showed preservation of the muscle fiber structure of the PCA muscle and atrophy of the thyroarytenoid (TA) muscle [234].

The ansa cervicalis nerve was used in different techniques to selectively re-innervate the PCA or TA muscle. Again, most of the studies were of a small sample size, not controlled, and the reported results were inconsistent [230-233].

6.3.2.2 Non-selective re-innervation:

The primary aim here is to restore tone and bulk to the laryngeal muscles [350]. The easiest and most commonly used technique is direct end-to-end RLN anastomosis, which usually did not result in functional movement [354,355]. Other donor nerves used to re-innervate the adductor muscles include the ansa cervicalis, hypoglossal nerves, and a nerve graft connected to contralateral laryngeal muscles. Non-selective re-innervation can be considered a static procedure to improve the symptoms of LP.

Although partial recovery was reported in some studies, this was in rare instances and was not consistently reproduced [356,357].

6.3.3 Laryngeal pacing

Laryngeal pacing, or functional electrical stimulation of the larynx, is achieved by implanting an electrical device that delivers electrical impulses to the nerve (intact RLN or vagus nerve, or graft nerve), or to the target muscle directly. The device is composed of a sensor, pacer, and efferent electrodes. The pacer generates electrical stimuli to the muscle synchronous with the respiratory phase. The afferent signals (i.e. those detected by the sensor) can be electrical, such as activity in the phrenic nerve, or mechanical, such as change of the intra-thoracic pressure [358]. Some types of pacers generate impulses at a pre-set adjustable rate, irrespective to the respiratory phase [237].

Laryngeal pacing was used in several animal experiments to restore functional mobility to the PCA muscle. Restoration of VF abduction was reported in few studies[237-240], however these were performed on very small number of animals. Zealear et al. investigated the effect of electrical stimulation in eight dogs after selective re-innervation of the PCA muscle using primary anastomosis of RLN. They reported that long term electrical stimulation enhanced selective re-innervation of the PCA muscle [193]. However, the end points used were neither validated nor standardized. Pacing of the TA muscle was also attempted for unilateral LP, and reportedly improved adduction and muscle tone and bulk [331,359].

Although pacing may produce phasic contraction in the stimulated muscle, so far full functional recovery has not been achieved. Unfortunately, the device had technical

limitations [240,358,360] that restricted its clinical application in humans [194]. It was also noted that despite enhancing mobility in most of the animal studies, the stimulation threshold of the device required readjustment over time [240,359], probably due to the newly re-innervating fibers reaching these muscles. This is a controversial point since synkinetic muscles were claimed to be better than the totally denervated ones for the pacing to work [194,358], since it provides neural conduit for the signal to travel through the muscle.

6.3.4 Non-surgical treatment

6.3.4.1 Gene therapy

Gene therapy is a new treatment modality for LP, which has been used in very few studies. It uses injection of genes that encode specific growth factors to either protect against neural degeneration, or promote nerve growth. These genes are carried in viral vectors, and can be expressed locally in the muscles, or in the CNS after retrograde transport through the RLN [203,262,263,319-322,361,362]. Most of the studies were done on the rat model, and mostly depended on histology end points.

Rubin et al. in a recent study of SD rats (n=10) injected an adenoviral vector carrying zinc finger transcription factor (used to stimulate production of insulin like growth factor type 1 (IGF-1)) into crushed RLNs [319]. After one week, the intervention group had better mobility of the VFs compared to the control group. Histological examination indicated better innervation, or less denervation of the laryngeal muscles in the experimental group [319]. Comparable results were found in a similar study, however the effect was only significantly different after one week, but

the difference disappeared after fourteen days [203]. Genes encoding other factors such as IGF-1 [322,361-363], and glial cell derived neurotropic factor (GDNF) [262,263,327] were used and showed comparable results.

The limitations of the gene therapy are the short duration of the effect observed and the inability to prevent synkinesis [202]. In addition, concerns about the safety profile, technical difficulties and ethical issues unfortunately pose challenges to its applicability in clinical practice [220].

6.3.4.2 Muscle stem cells

Muscle stem cells (MSC) injection into laryngeal muscles was used recently in two studies, as a novel treatment modality for LP [317,318]. The purpose of MSC therapy is similar to the gene therapy, since both of them can be used to deliver neurotrophic growth factors. In addition, MSC can provide protection against atrophy in the injected muscle [318]. Halum et al. injected the TA muscle of Wistar rats (n=16) with autologous MSCs after transection of the RLN [317]. The TA muscle was injected also with a neurotrophic factor IGF-1 (n=4), cardiotoxin (CTX) (n=4), ciliary neurotrophic factor (CNTF) (n=4), or normal saline (n=4). After one month all the animals exhibited high rates of survival of the MSCs. Muscle fiber diameter was particularly higher in the IGF-1 and CNTF groups. MSC injection might be useful as a material for injection laryngoplasty. However, it will probably be challenged by limitations similar to the case of gene therapy, most importantly, its inability to overcome the problem of synkinesis.

6.3.4.3 Pharmacological therapy

Pharmacological agents were used as experimental treatment for LP in few studies. Mori et al induced LP in SD rats using either RLN crush or vagus nerve avulsion [220]. One month later, T-588 (a neuroprotective drug used for Alzheimer disease) was administered orally. In the crush group nine out of twelve rats, and two of the nine control animals recovered VF movement after four weeks from the drug administration (2 months after nerve injury). This was assessed using direct visualization of the VF through a laryngofissure. Motor nerve conduction examination showed higher velocity in the crush group. Histological examination showed a higher number of the surviving neurons in the nucleus ambiguous of the vagal avulsion group (n=6), which was significantly different from the control group (n=5).

Nimodipine (a calcium channel blocker claimed to improve functional recovery of injured nerves and promote neural sprouting) was administered orally to SD rats for three days then a unilateral crush injury to RLN was applied. Sixty animals were divided into ten equal groups: one sham-operated control group, five intervention groups, and four control groups. Assessments were done at different time points (one to six weeks). Upon performing evoked LEMG, higher amplitudes of the compound muscle action potential in the nimodipine-treated animals than the untreated animals were recorded, which at six weeks were similar to the sham-control group. However, there was no difference in the latency time between the two groups. Retrograde labeling study at six weeks showed a higher number of motor neurons that re-innervated the PCA muscle in the intervention group.

Histological examination of PCA muscle did not demonstrate a difference in the number of neuromuscular junctions between the two groups [364]. Although the results of this study indicated some evidence of nerve regeneration in the nimodipine-treated group, the laryngeal functional status was not assessed (neither laryngoscopy nor spontaneous recording of LEMG was done). Moreover, retrograde labeling study was inconclusive about the arrangement of the motor neuron pool in the brain stem.

While these neurotrophic drugs have the advantage of easy administration and avoidance of the potential effects of gene and MSC therapy, their effect was limited in promoting functional neural regeneration. Moreover, as with the other pharmacological agents, these drugs might have systemic adverse effects.

6.4 Experimental studies on the use of neurotoxins

Very few experimental studies utilized neurotoxin injection into laryngeal muscle for management of LP. In one experimental study it was used to overcome the synkinetic re-innervation and enhance a selective re-innervation [204], and in another two for chemical denervation of CT muscles to achieve glottic expansion [242,243].

6.4.1 Vincristine and phenol

McRae et al. used vincristine and phenol to denervate the adductor muscles and enhance selective innervation of the PCA muscle [204]. They injected the TA muscle of SD rats with phenol (n=7), high dose vincristine (n=5), low dose vincristine (n=5), or normal saline (n=6) after three weeks from unilateral RLN transection. A month later, most animals in the intervention groups had immobile fixed VF in the lateral

position. This observation was made using a midline laryngofissure, which might have abolished the PCA activity. Despite the absence of mobility, LEMG of the PCA muscle was present in three animals of low vincristine group (two died during recording), and in all except one of the animals from the phenol group. In four animals from the low vincristine group, and in five from the phenol group there was no electrical activity in the adductor muscles. The high dose vincristine group was excluded from the analysis due to the high mortality following the administration of the toxin (three animals died). In the control group animals, electrical activity of the adductor muscles and the PCA were demonstrated in five out of six animals.

The authors concluded that low dose vincristine injection was effective in preventing synkinetic re-innervation. However, the method used for assessment of VF movement and detection of LEMG activity (i.e. midline laryngofissure) might have altered the muscle activity and the actual dynamic picture. Moreover, the high mortality resulting from toxicity raised concerns about the safety of administration.

6.4.2 Botulinum toxin

Only two studies investigated the effect of BTX-A on LP, and only one of them used a LP model. The two studies were performed by Cohen et al, where they injected BTX-A into the CT muscle of mongrel dogs to expand the glottic gap [242,243]. In the first study, twelve animals were divided into four groups, and only one group underwent a transection of the RLN to produce a LP model (n=2). In the first group (n=3), the animals received bilateral injection of normal saline. The second (n=3) and the fourth (n=4) groups received BTX-A injection unilaterally and bilaterally, respectively. Lastly, the third group, which represents the LP group, received BTX-A

injection in the same side of the RLN injury in one, and contra-laterally in the second. The end point was endoscopic assessment of the degree of VF lateralization (angle of the anterior commissure in resting, maximal abduction and maximal adduction) at four, and eight weeks after the intervention. In the animals that received the toxin, the VF was lateralized on the injected side in all states. For the third group, the angle was wider when the toxin was injected on the same side as the nerve transection. The authors observed that the effect of the toxin was dose-dependent, and more pronounced when injected bilaterally [242].

In a follow-up experiment, BTX-A was injected unilaterally into the CT muscle of ten normal animals (i.e. without induction of LP). Measurements of the anterior commissure angle were obtained during resting, maximal abduction and maximal adduction; pre-operatively, one, two, and three months post-operatively. The authors claimed that the degree of VF lateralization was more during abduction, followed by resting and lastly adduction, and that the effect of the toxin lasted for thirty days. There were no appreciable histological changes in the CT muscle [243]. The main criticism rests with the small sample size, the use of a non-standardized, or validated end point, and arguably difficult to reproduce.

The concept of eliminating the action of the CT muscle to improve the airway was investigated in multiple studies [45,80,82,242,243,365,366]. It was observed that the CT muscle has an inspiratory activity, which leads to widening the glottis upon contracting simultaneously with PCA muscle [51]. Upon eliminating the PCA muscle effect (as is the case in LP), the contraction of CT had the opposite effect [45]. Moreover, studies investigating the CT function under compromised respiratory

conditions, found a predominant inspiratory role [45,46,50,53,54,367]. Some authors reported that eliminating the CT muscle action in denervated larynx widened the glottis and decreased the laryngeal resistance [45,366] but this was not reproduced consistently in other studies [80,82,365]. It appears that this discrepancy is due to the difference in the experimental conditions. In acute airway obstruction, CT muscle removal or denervation might produce a relief of the respiratory obstruction and lateralize the VF [45,366]. However, after a period of time synkinetic re-innervation of the denervated muscles takes place, and different interactions with the other laryngeal muscles arise [80].

6.4.2.1 Botulinum toxin in the rat model

In the rat model, BTX-A was used on several occasions to investigate its effect on different disorders, such as spasticity, dystonia, chronic pain, and detrusor muscle over-activity [368-370]. In the laryngeal model, the experiments were mainly directed to test safety and effect on muscle structure and function [279,280,297-299,371]. However, in all these studies the toxin was injected into a normal larynx and the aim was to investigate its applicability in spasmodic dysphonia. Inagi et al. performed series of studies investigating the effect of BTX on laryngeal structure and function. In one experiment, they studied the short-term effect of BTX-A injected endoscopically into the TA muscle (unilateral) [280]. The toxin was injected in different concentrations and volumes. All the rats injected with more than 0.7 U died within the first 2 days. Three days after the injection, mobility of the injected VFs was decreased in the rest of the animals. The effect of the toxin appeared dose-dependent. On LEMG, a decrease in the interference pattern of activity in the injected muscles was

demonstrated, and this effect was more pronounced with higher concentrations. Histological examination showed higher degree of denervation in all laryngeal muscles (both ipsi- and contra-laterally), again, with higher concentrations. Functional outcome was assessed using measurements of the glottic dimensions during adduction and abduction. This method is again questionable as it assessed the static laryngeal photographs. The experiment was not controlled, nor was it performed on a LP model, which limits the conclusions that can be drawn.

6.5 Experimental studies utilizing laryngoscopy and LEMG as endpoints

6.5.1 Laryngoscopy

Several studies used laryngoscopy on LP models to assess the effect of therapeutic interventions (e.g. re-innervation, pacing, and non-surgical therapy) on the recovery of VF movement [80,203,229,234,237,238,242,260,319,354]. Different grading systems for the degree of VF mobility were used, however none of them was validated. Most studies used rigid telescope, or direct laryngoscopy with a mounted microscope for assessment, and few studies utilized flexible fiberoptic laryngoscope [229,324,372]. The assessment is ideally done while the animal is spontaneously breathing under light anesthesia to detect the dynamic behavior of the VFs during the respiratory cycle, which can be monitored visually, or by using chest wall EMG [53] or a respiratory belt[373]. However, some studies used methods that might not reflect the actual dynamic behavior of the VFs. As discussed earlier, some studies used photographs of the laryngoscopic views captured during particular phases, and made measurements of the glottic dimensions [242,243,280]. Again, this could be misleading,

and might not demonstrate the VF behavior. Other studies performed classical laryngoscopy while the animal was tracheostomized, or in a retro grade fashion (telescope through the stoma) [234]. However, there is considerable evidence that the activity of the PCA, the main abductor of VFs, depends on presence of ventilatory resistance [42], and presence of a tracheostomy abolishes its activity [40]. Therefore, assessment of the VF function using these methods might not accurately reflect mainstream clinical assessment. The same principle might apply to the studies that created a midline laryngofissure to observe the VFs, however no study had evaluated its effect [204,220,262,263,363].

6.5.1.1 Laryngoscopy in the rat model

Laryngoscopy was used in experiments performed on the rat model using direct laryngoscopy with a mounted microscopes, rigid telescopes, and flexible fiberoptic laryngoscopes [203,229,257,258,260,319]. Some studies used a midline laryngofissure to examine the VF mobility [204,220,262,263,363], which, as discussed in the previous section, might has limitations. Most of the studies used injectable anesthetic, such as ketamine with xylazine [220,257,258,260,260,262,319,374], and barbitone sodium [229], during laryngoscopy. One study used isoflurane inhalational anesthesia, and two mortalities during laryngoscopy were reported [203].

Trans-oral laryngoscopy in the rat model seems to be a safe and reproducible procedure, provided that an appropriate anesthetic technique is used. Although few studies reported mortality related to its use [203], under-reporting cannot be excluded given the caliber of the airway and the small size of the animal.

Laryngoscopy allows for assessment of the physiological function of the larynx, without creating artifacts that can affect the reflexive control mechanisms of the larynx. Moreover, it simulates the situation used in clinical setting, making it easier for prediction of the picture in humans, thus facilitating the clinical translation.

6.5.2 Laryngeal Electromyography

LEMG was used widely to assess for the innervation status and activity of the laryngeal muscles after interventions [234,260,260,261]. Some studies recorded the phasic LEMG activity in a spontaneously breathing animal, and others employed evoked LEMG, and nerve conduction velocity studies [260,261]. Evoked LEMG and nerve conduction studies might be useful to identify the supplying nerve after surgical re-innervation procedures, however it is not practical for clinical application. These tests require surgical exposure of the nerve, which is ethically unjustifiable in humans, as it can result in morbidity or inadvertent injury to many structures including the tested nerve. The recording has been done under different types of anesthesia, such as ketamine-xylazine [234,260]. Different methods for detection of the electrical signals and data analysis were used, which make it difficult to compare the results of these studies.

6.5.2.1 Laryngeal electromyography in the rat model

Few studies utilized LEMG in the rat model [257,258,260-263,375,376]. Different methods were used to insert the needle for signal detection. Some studies that investigated the re-innervation procedures employed evoked LEMG. The needle was inserted into the TA muscle through a window created in the thyroid cartilage. The reference

electrode was placed in the soft tissue around the larynx, and a stimulator was used for stimulation of the nerve graft. [260,261]. Other studies created a midline laryngofissre and inserted the needle under direct vision into the TA muscle, to test for nerve conduction velocity after RLN stimulation [262,263], or to record spontaneous activity [375]. Transoral insertion into TA and PCA muscles under telescopic guidance was also used successfully, albeit infrequently [256-259]. Direct needle insertion into the PCA muscle after rotation and separation of the larynx and trachea from the esophagus was described, however it was only done before sacrificing the animal[376], given its invasiveness.

The EMG signals were analyzed in different ways. With evoked LEMG, the ratio of action potential amplitude between the treated and untreated sides was calculated as a measure of the re-innervation [260]. LEMG recorded during spontaneous breathing was analyzed by evaluating the waveform morphology and the recruitment pattern [257,258,375]. Most of the previous studies used qualitative way for the analysis, and rarely correlated the muscle activity to the respiratory phase. Quantitative analysis of the EMG data was used in studies investigating a wide range of neural, neuromuscular, or muscular disorders. It gives a more objective assessment of the muscle activity, however it is limited with respect to detection of the pathological activities such as fibrillation potentials. The details of the EMG analytical methods are discussed in the laryngeal electromyography section.

7 Conclusion and formulation of the research question

The larynx is a vital organ that has a complex structure and performs highly sophisticated functions. These functions are controlled with high precision by the central nervous system through poorly understood mechanisms. Affection of the structural organization, or the central control mechanisms can result in disruption of the functional competence. Laryngeal paralysis results from a disruption of the neural supply to the larynx [58]. It is a challenging disease that severely impacts the affected individuals [184-186]. The lack of understanding of its pathophysiology, and its natural course leaves us with palliative treatment options, which do not restore function.

Several experimental studies investigated the treatment options of laryngeal paralysis. However, they were mostly unsuccessful in finding a reproducible and efficient modality that achieves recovery of vocal fold movement. A common denominator to the failure was their inability to resolve the synkinetic innervation pattern of the laryngeal muscles.

Since synkinesis was shown to be associated with central re-organization of the motor neuron pool [191,195], it might be possible to resolve it by a central mechanism. Botulinum toxin is an agent that was previously sought after because of its well-known neuromuscular junction effect. However, there is a body of work that suggests it posses a direct effect on the central nervous system [302,303]. The toxin, reportedly, improved function in various neuromuscular disorders by producing effects of duration beyond those anticipated from its temporary paralytic action[278,305-307]. Multiple clinical and experimental studies postulated that this effect

might be mediated by modulation of the central nervous control mechanisms, as well as by the normalization of the motor neuron pool organization [278,305,306].

The detection of synkinetic innervation requires a method that can assess the functional status of individual key laryngeal muscles. Although laryngoscopy is the reference standard for assessing overall dynamic laryngeal function, it cannot play that role. Laryngeal electromyography on the other hand has the potential to offer objective assessment of the laryngeal muscle activity, and when correlated with the respiratory phase, it may provide information about appropriate re-innervation.

The cricothyroid muscle is an external laryngeal muscle that has unique mode and changeable phase of action. Multiple observations indicated that under certain conditions, akin to LP, the muscle action could reduce the glottic aperture [45,45,46,50,53,54,367]. Some evidence also exists that similar effects can be exerted by the sternohyoid and sternothyroid muscles [377]. Given this information, one can imagine that neutralizing these actions may reduce the airway compromise in LP. When one considers that the neural supply to these muscles remains intact in most cases of LP, the retrograde transport of the toxin to the brainstem becomes a plausible route bypassing the interrupted RLN.

Based on these findings, we hypothesized that botulinum toxin injection into the external laryngeal muscles can enhance the consequences of recurrent laryngeal interruption in the rat model as assessed by laryngeal electromyography and laryngoscopy.

8 Figures

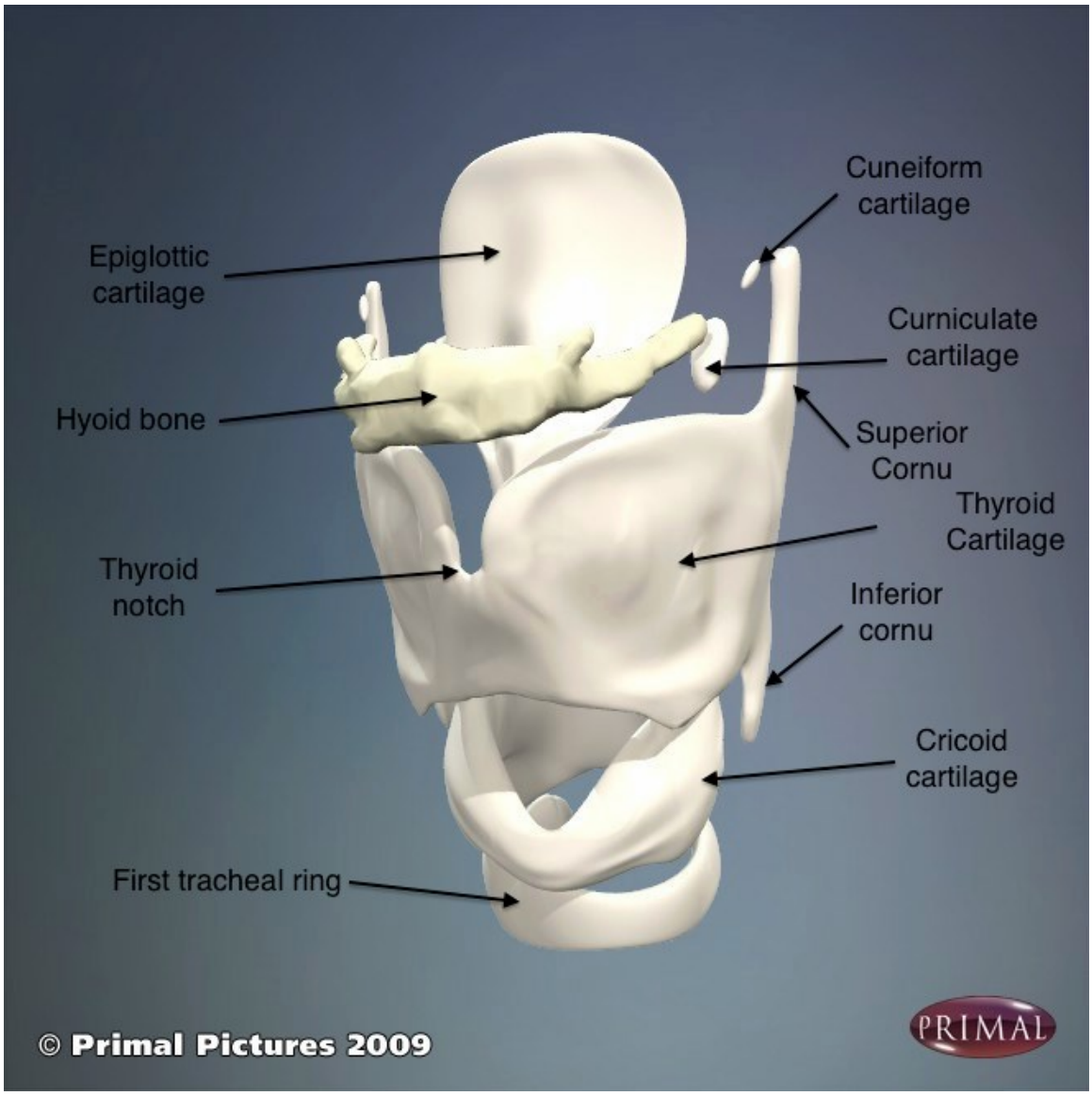

Figure 1-1 Laryngeal skeleton framework

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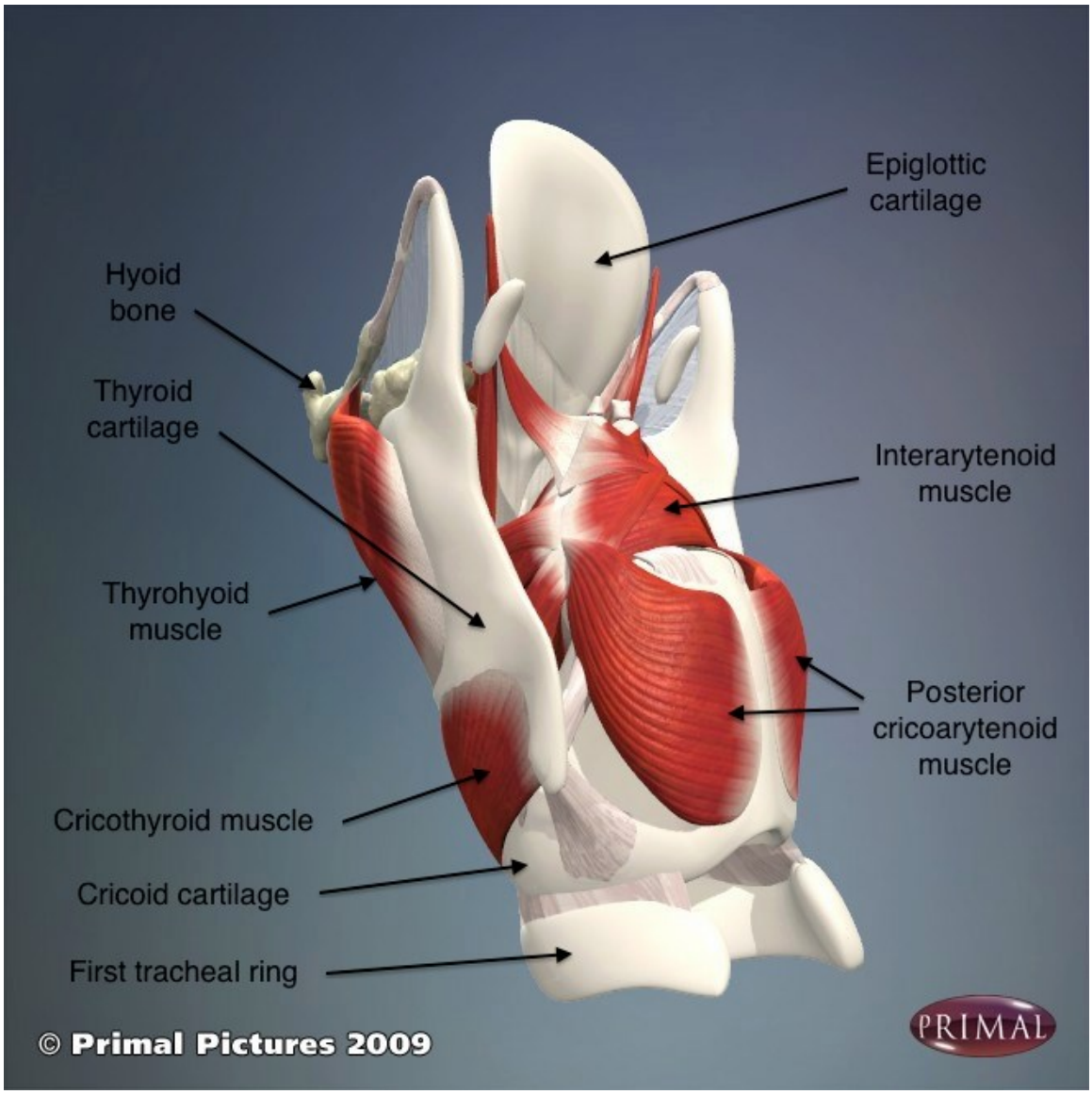

Figure 1-2 Posterolateral view of the larynx

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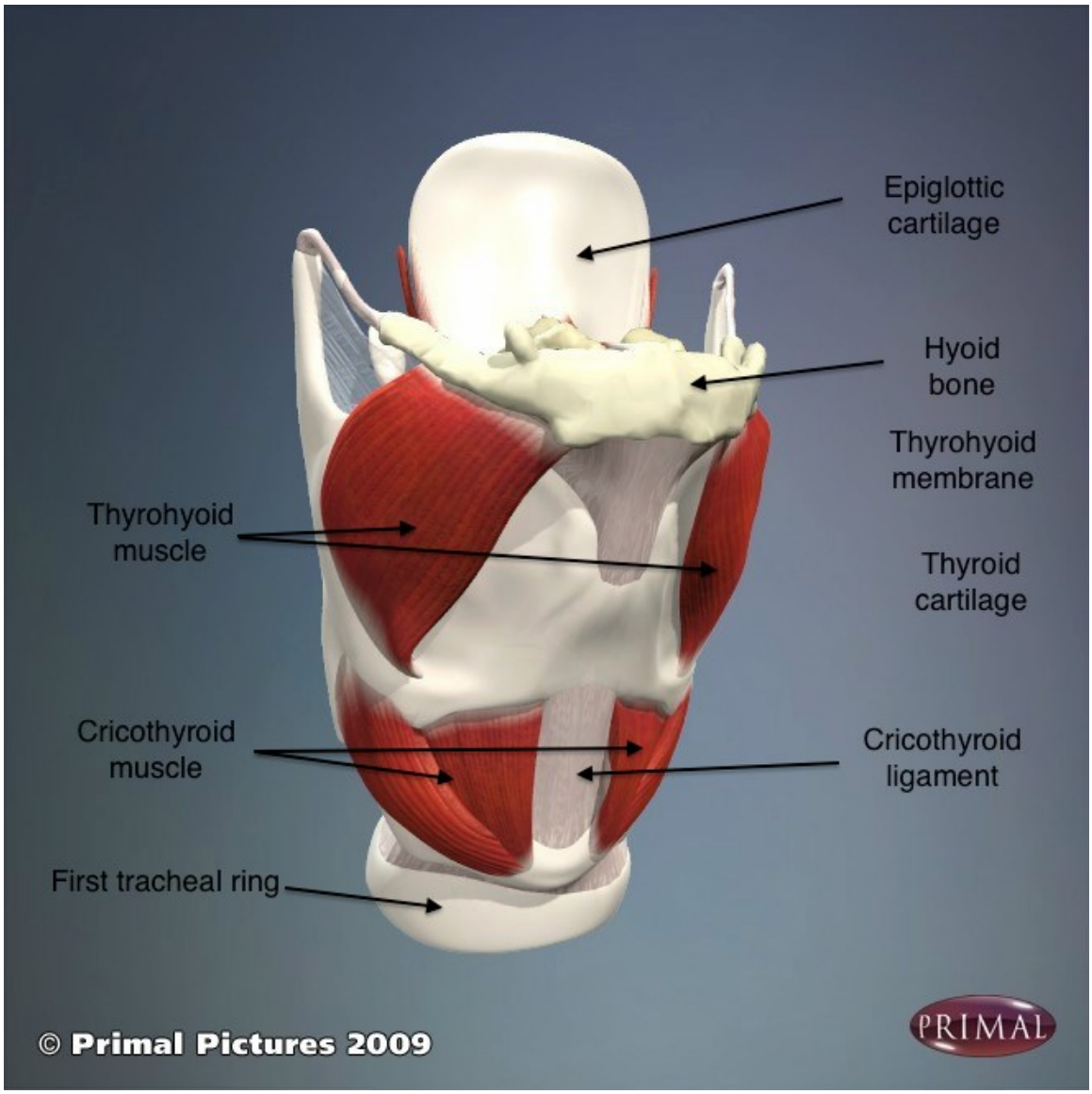

Figure 1-3 Anterior oblique view of the larynx

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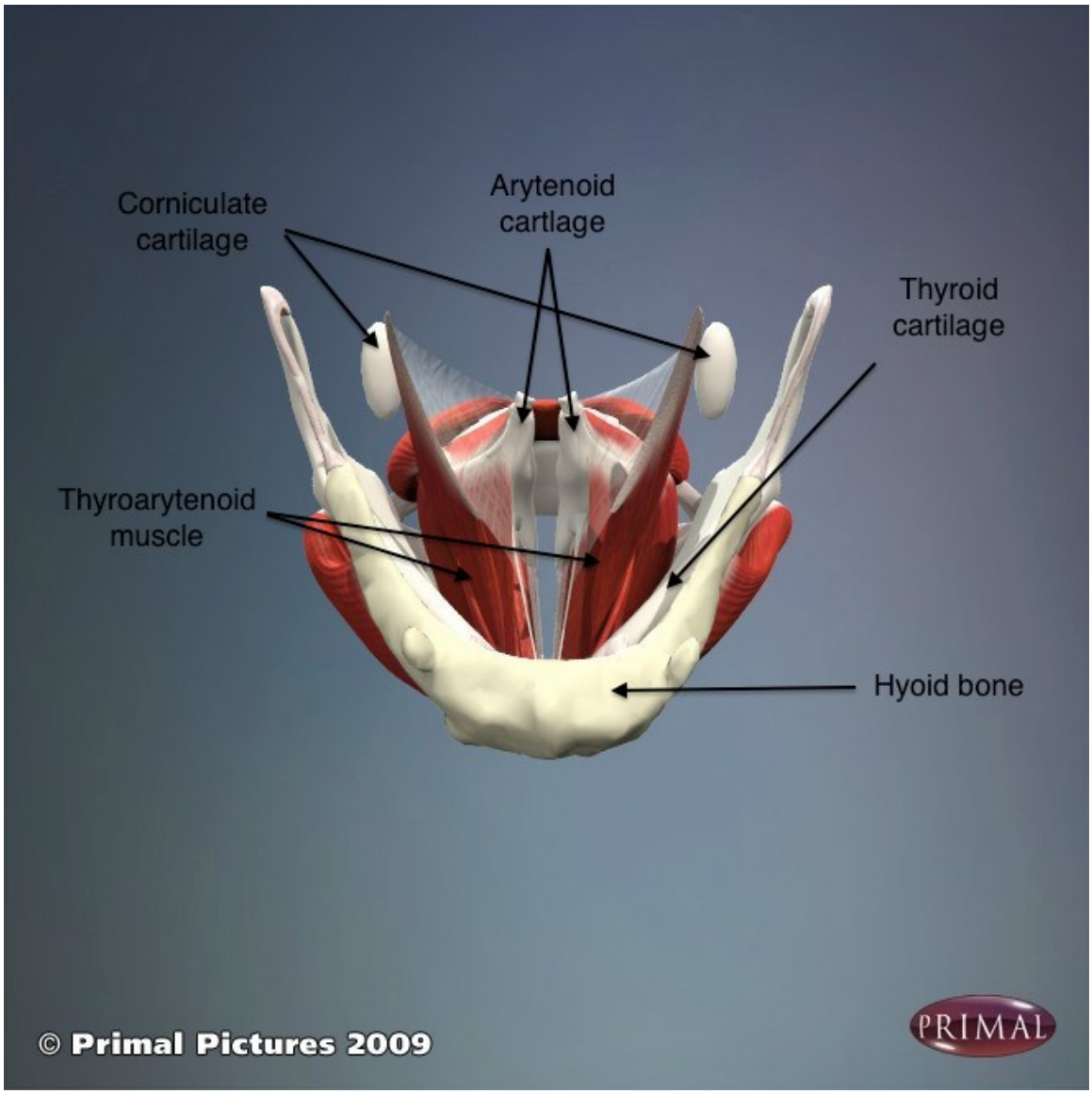

Figure 1-4 Superior view of the larynx

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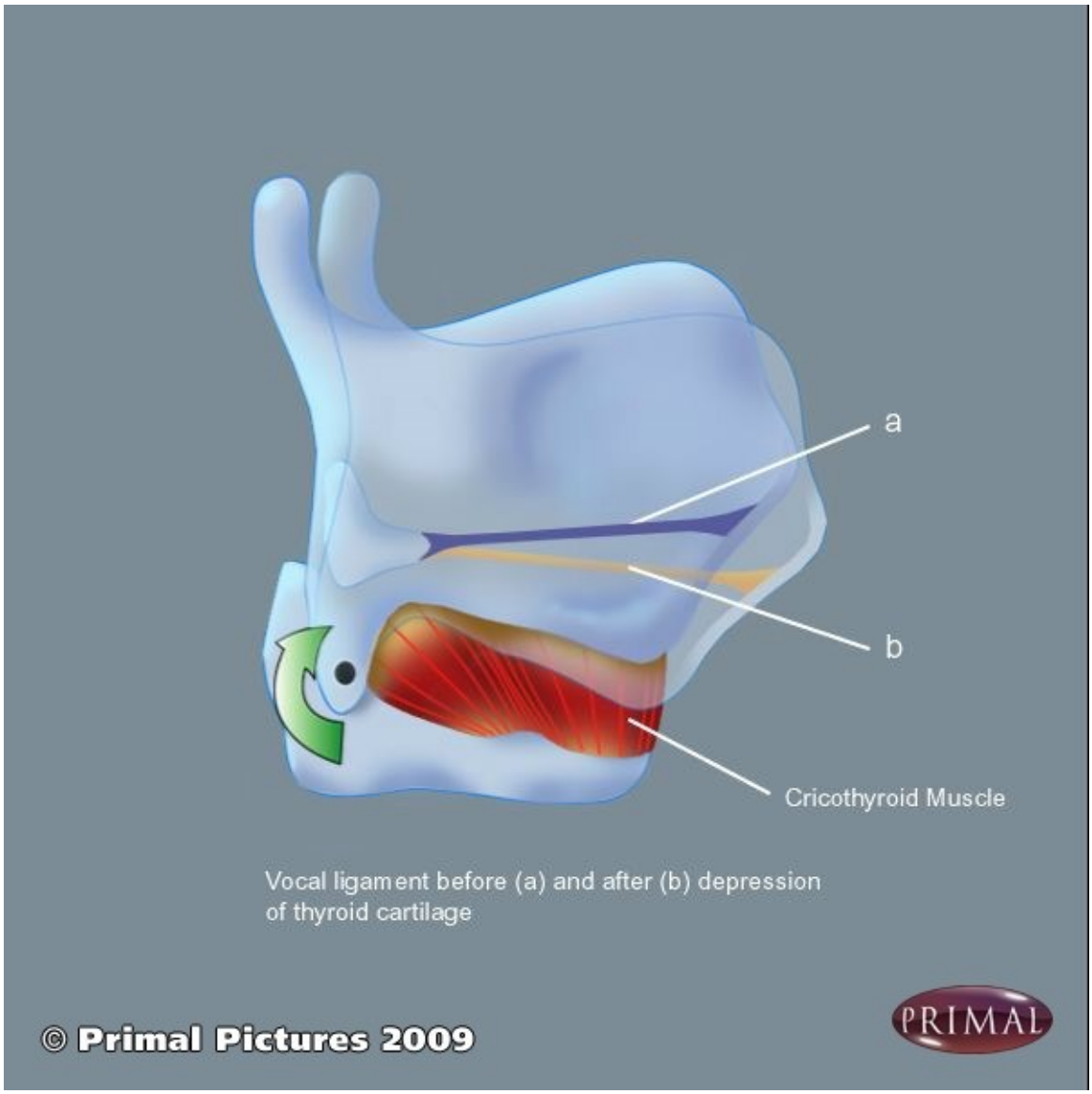

Figure 1-5 Illustration of the action of the cricothyroid muscle

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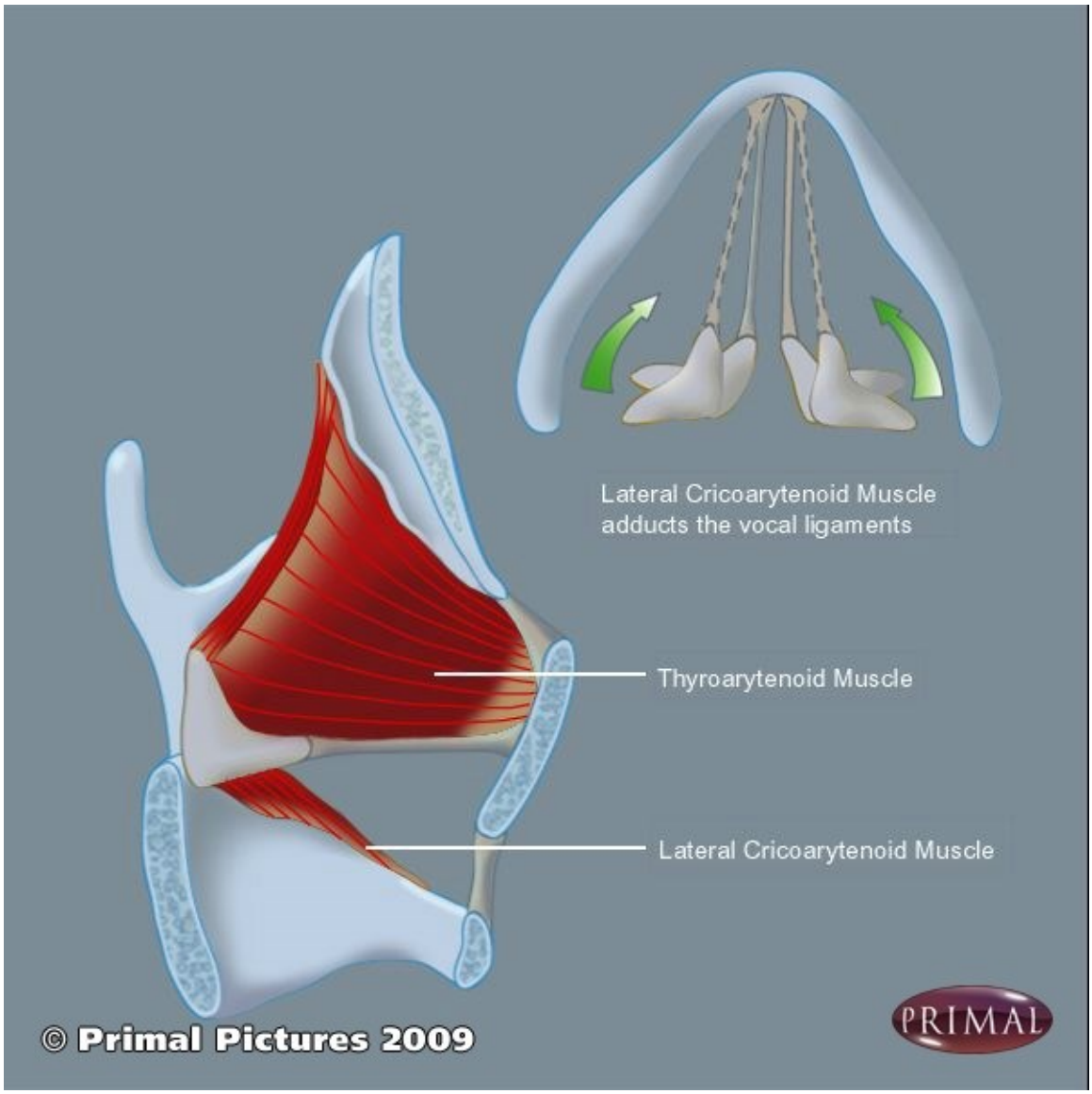

Figure 1-6 Illustration of the action of the lateral cricoarytenoid and thyroarytenoid muscles

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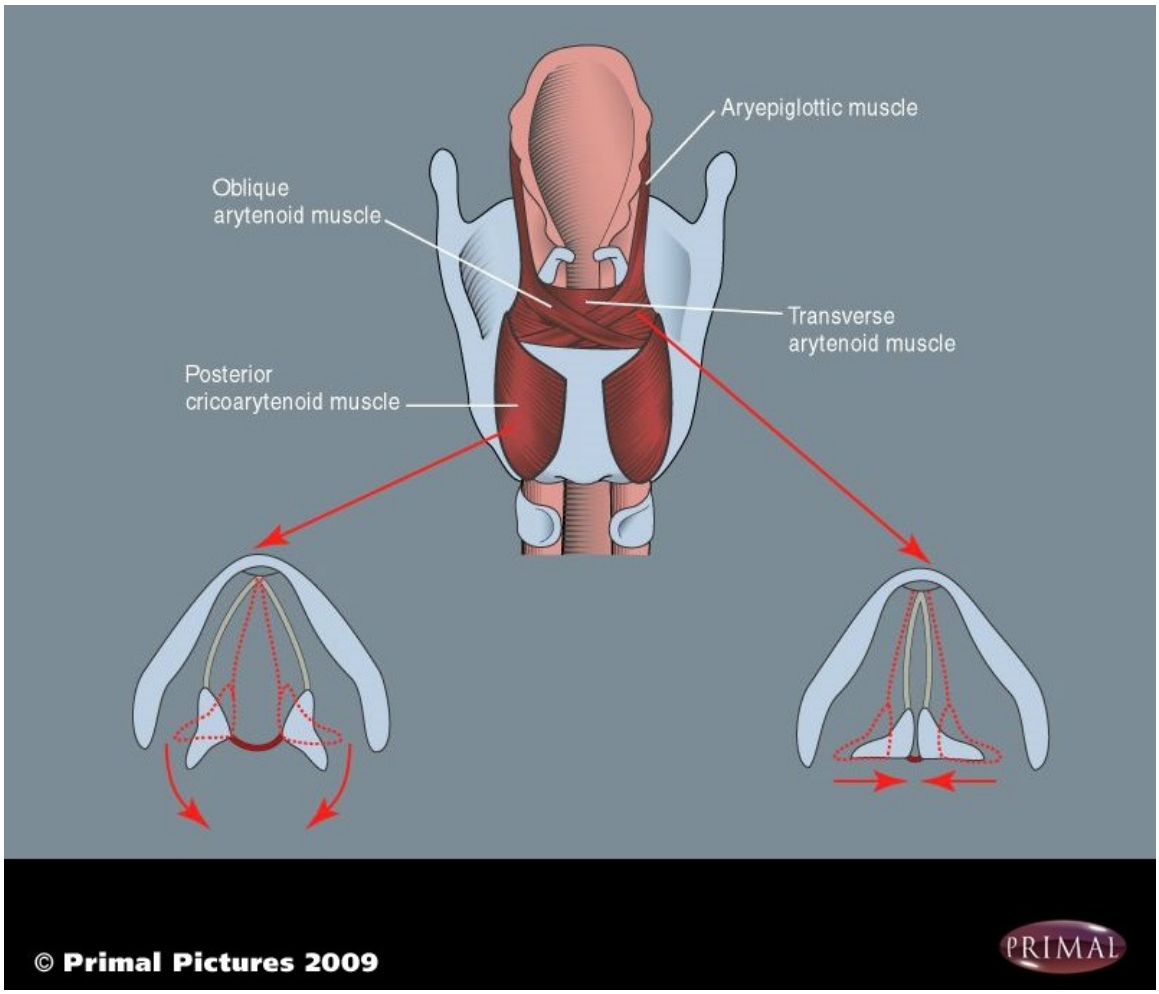

Figure 1-7 Illustration of the action of the posterior cricoarytenoid and arytenoid (i.e. interarytenoid) muscles

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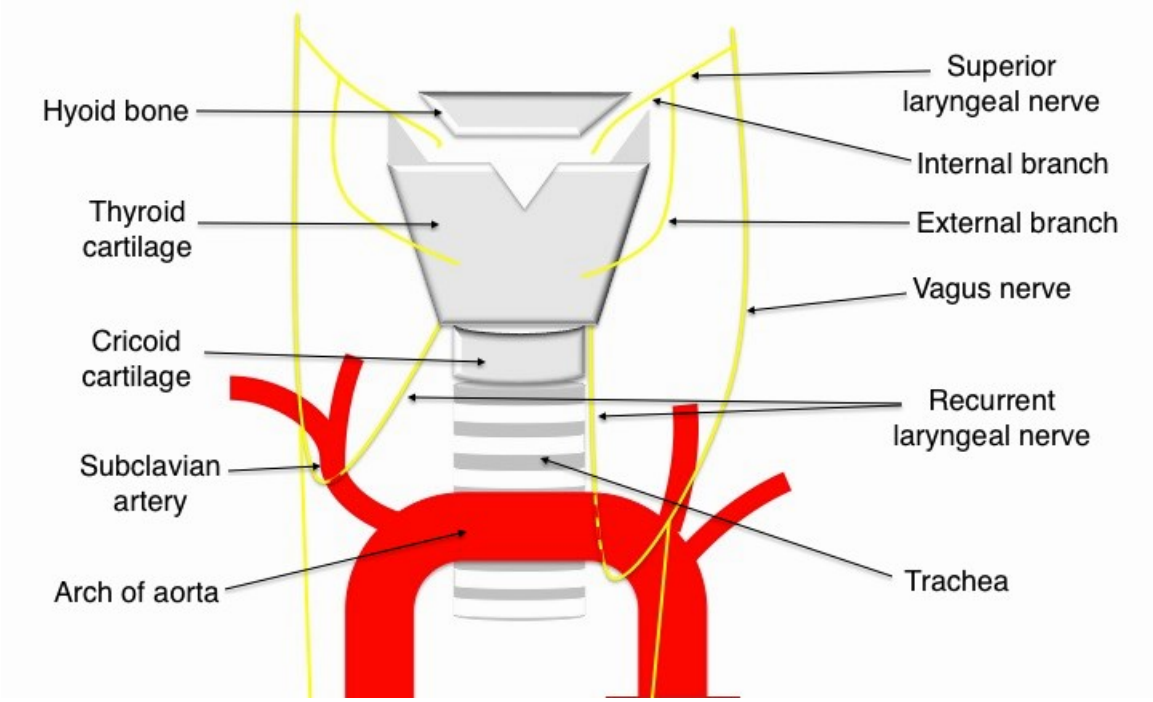

Figure 1-8 Neural supply of the larynx

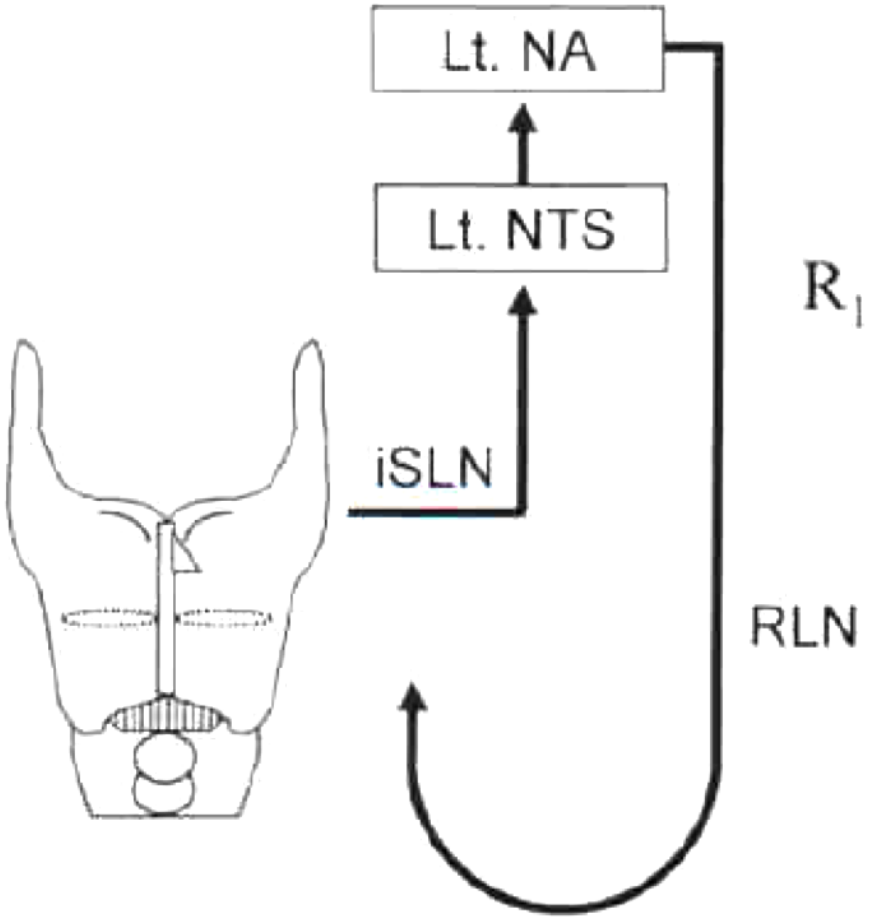

Figure 1-9 Ipsilateral adductor reflex.

R1: Ipsilateral reflex with latency period of 10-18 milliseconds, Lt.: left, NA: Nucleus Ambiguous, NTS; Nucleus Tractus Solitarius, iSLN: internal branch of Superior laryngeal nerve, RLN: Recurrent laryngeal nerve.

Taken with permission from (Sasaki CT et al. Central facilitation of the glottic closure reflex in human. Ann. Otol. Rhinol. Laryngol. 2003;112: 293-297). Annals Publishing Group©

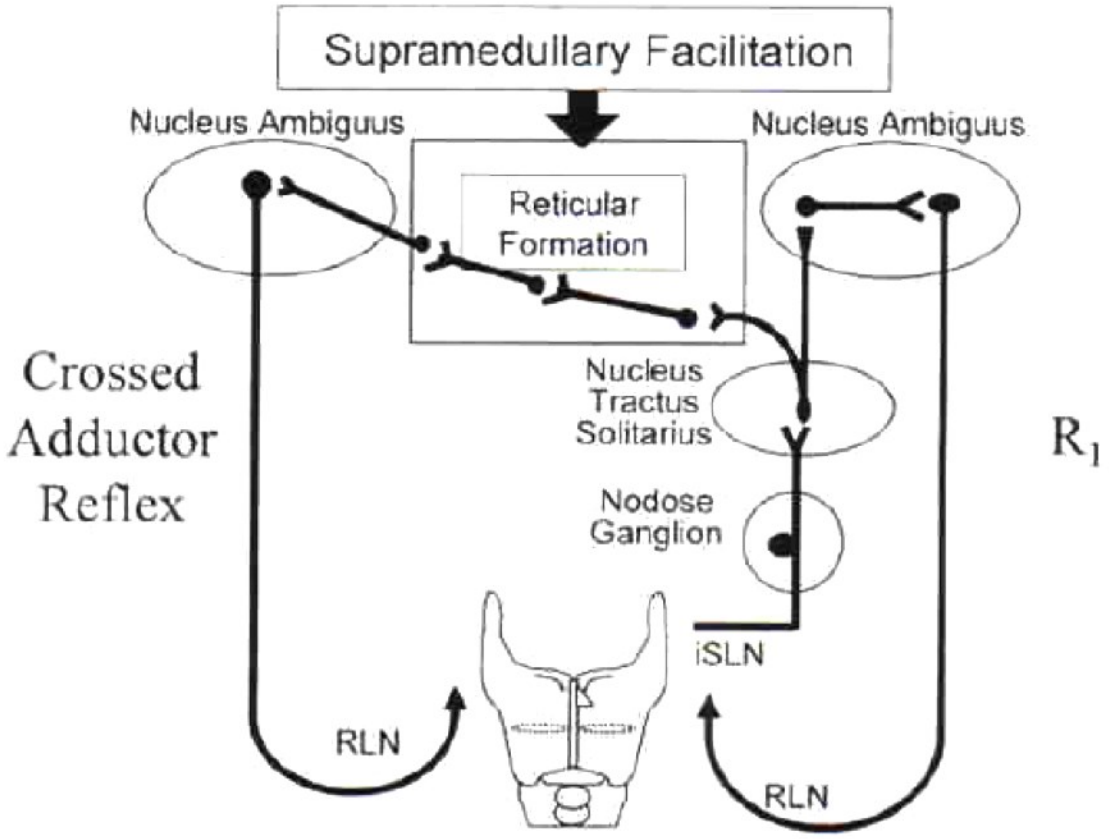

Figure 1-10 Crossed adductor reflex.

R1: Ipsilateral reflex with latency period of 10-18 milliseconds, iSLN: internal branch of Superior laryngeal nerve, RLN: Recurrent laryngeal nerve.
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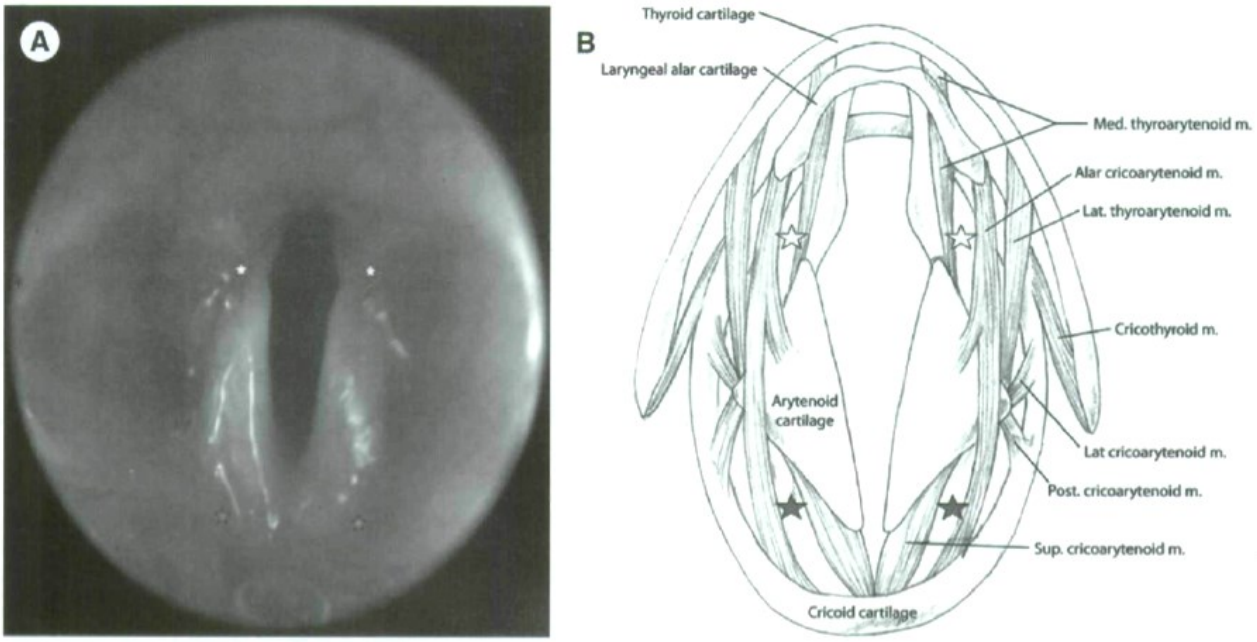

Figure 1-11 Endoscopic view of the rat larynx

A) Endoscopic view of the rat larynx showing the point of needle insertion, upper stars: TA muscle, lower stars: PCA muscle. B) Illustration of the intrinsic laryngeal muscles in the rat.

Taken with permission from (Tessema B et al. Evaluation of functional recovery of recurrent laryngeal nerve using transoral laryngeal bipolar electromyography: a rat model. Ann. Otol. Rhinol. Laryngol. 2008; 117(8): 604 608). Annals Publishing Group©

Chapter 2: Does Botulinum toxin type A alter the consequences of recurrent laryngeal nerve transection in the rat model?

1 Introduction

Laryngeal paralysis (LP) is not an uncommon disease that can affect all age groups, and severely impacts the patients' health and quality of life [184-186]. It is characterized by the absence of normal movement of one or both vocal folds (VF), due to an altered neural supply [58]. The severity of nerve injury is an important factor in determining the prognosis. Severe injury, such as complete transection of the nerve, is usually associated with poor prognosis [326,378].

Several animal studies demonstrated that after complete transection, the recurrent laryngeal nerve (RLN) regenerates to re-innervate the larynx, however, without necessarily regaining functional movement [86-88]. This is most likely attributed to synkinetic innervation, in which the nerve axons lose their way and reach a different muscle, or a different muscle compartment within its target [84].

The current surgical repertoire is directed to ameliorate the symptoms of LP by surgically modifying the caliber of the airway without restoring functional mobility. Whereas most of the published work focused on studying the efficacy of the surgical glottic expansion and medialization procedures, some studies searched for an alternative that would restore the functional mobility to the paralyzed VF. One option has been the re-innervation procedure, which unfortunately is as yet to prove consistently effective and reproducible [225-228,234,352,353]

Hence, neurotoxins were sought to deal with synkinesis. Vincristine and phenol, were injected locally into laryngeal muscles [204], but they were associated with significant mortality, and again limited success. Botulinum toxin type A (BTX-A) is another toxin that was utilized, initially, to achieve glottic expansion [242,243]. In two animal studies, it was associated with improvement of airway patency, and no significant morbidity or mortality were documented. However, the duration of effect was short and repeated injections were required [242,243]. Although the toxin succeeded in enlarging the airway according to the authors who used static end points, the functional status of the VFs was not assessed. In one clinical report, BTX-A was injected into the external laryngeal muscles that affect the articulation of the cricoid over the thyroid cartilage, thereby affecting the glottic aperture [247]. Most of the patients avoided a tracheostomy, and the effect lasted beyond the duration anticipated for the toxin, and in addition the patients recovered (nearly all) normal laryngeal mobility. the author postulated a neuromodulatory effect of BTX-A that negate synkinesis.

Our aim in the current study is to investigate whether BTX-A can alter the consequences of the LP in animal model, with specific focus on the functional recovery. We utilized quantitative LEMG as the main end point to assess the changes in the activity of the laryngeal muscles. The secondary end point was laryngoscopic evidence of recovery of VF movement.

2 Hypothesis

BTX-A injection into the external laryngeal and strap muscles, namely: Cricothyroid (CT), Sternothyroid (ST) and Sternohyoid (SH) muscles, enhances the recovery of laryngeal function after interruption of the RLN.

3 Material and methods

The present study was conducted in accordance with the Canadian Council of Animal Care (CCAC) guidelines and policies, and with ethical approval from the Animal Care and Use Committee for health sciences at the University of Alberta. The research was funded by Edmonton Civic Employee Fund, and the Saudi Cultural Bureau Student Research Fund.

3.1 Experimental procedure

3.1.1 Preparation and anesthesia

Pre-operatively the animal received water and food *ad libitum*. General anesthesia was inducted using 4-5% isoflurane while oxygen (O_2) was delivered via a nasal cone at a flow rate of 1.5-2 L/min. The animal's weight was noted, and a unique identification number was marked on the tail. During preparation, anesthesia was maintained using the induction agent. According to the animal's weight, a dose of atropine (0.05 mg/kg), ampicillin (20 mg/kg), meloxicam (1 mg/kg) and warmed normal saline (5 ml) were administered subcutaneously. The surgical area (~5mm below chin to ~5mm above sternal notch) was shaved then sterilized with iodine solution and alcohol, and an eye lubricant was applied to the corneal surfaces. The

animal was then transferred to the surgical table, and placed on a restraining board with an integrated circulating fluid heating pad (temperature 37^{0}C). A respiratory belt (Kent Scientific Co., USA), a rectal thermometer probe and a vital signs monitoring foot sensor (STARR Life Sciences® Mouse Ox® Plus) were attached. The animal was then covered with a transparent sterile plastic sheet, and vital signs and depth of anesthesia were assessed. The depth of anesthesia was determined by eliciting a toe pinch reflex, and observing the respiratory rate and pattern. Baseline readings of heart and respiratory rate, peripheral capillary oxygen saturation (SpO_2), temperature and mucous membrane color were all recorded. Once the surgical plane of anesthesia was confirmed (i.e. absent toe pinch reflex, slow rate and regular pattern of respiration) with stable vital signs, the surgical procedure was started. The anesthetic level and vital signs were monitored continuously and documented every five minutes.

3.1.2 Surgical procedure

All surgical procedures were performed under sterile aseptic technique. An operating microscope (S88 Carl-Zeiss, Germany) was used during dissection. The surgical incision site was infiltrated with 0.5-1 ml 2% lidocaine in 1:200,000 adrenaline. The marked midline incision was made using surgical Metzenbaum scissors. The subcutaneous fat and submandibular glands were dissected and retracted laterally, and the deep fascia was divided in the midline. The sternomastoid (SM) muscles were identified immediately at a deeper plane, extending from their sternal attachment towards their lateral insertion into the temporal bone. Between them and at deeper plane, the SH muscles were identified,

heading toward their insertion to the hyoid bone. With progressive dissection just lateral to the SM muscles, the external jugular vein (EJV) was identified. The vein was freed from the surrounding tissues, and two vascular clips were applied on it. A small incision was made between the clips, and an intravenous (IV) cannula was inserted into the lumen. The distal end was ligated and the clips were removed. The cannula was advanced into the proximal end and secured in place with ligature. At this point, total intravenous anesthesia (TIVA) was initiated. A continuous IV propofol infusion was started at the lower calculated dose (44-55 mg/kg/hr), and isoflurane was decreased to 0.5% maintaining a similar Fi O_2 flow rate. After approximately two minutes, the isoflurane was stopped. The surgical dissection to expose the larynx was continued after re-confirming the depth of the anesthesia. The SH muscles were separated in the midline and retracted laterally to expose the laryngotracheal complex. The laryngeal cartilages, thyroid gland and cricothyroid muscles were identified. Baseline spontaneous LEMG activities of CT, and thyroarytenoid (TA) on both sides were recorded. Then, the surgical field was covered with wet gauze and a baseline laryngoscopy, to document movement of vocal folds, was performed and captured digitally. After that, the wound was exposed and the laryngotracheal complex area on the right side was explored. The RLN was identified in the tracheo-esophageal groove where it runs on the lateral aspect of the trachea accompanied by blood vessels, then heads towards the crico-arytenoid joint to enter the larynx. The nerve was severed with microscissors at the level between the sixth and seventh tracheal ring excising approximately one millimeter. The LEMG recording was then repeated, along with laryngoscopy to

confirm the onset of the paralysis. The intervention drug (BTX-A [prepared by slowly adding 10 ml of normal saline into 100 units Botox® vial, to obtain a final concentration of 10 units/ml] or normal saline) was injected into CT, ST and SH muscles on the right side. A total volume of ~0.15-0.2 ml (~1.5-2 units of BTX-A), was injected. After that, the intravenous anesthesia was interrupted and the line was removed and the proximal end of EJV was ligated. General anesthesia was then delivered by isoflurane 1.25 % with O_2 flow rate of 1.5 L/min. The wound was closed in two layers. The subcutaneous tissue was closed using interrupted 5-0 Vicryl sutures, and the skin was closed using subcuticular continuous 3-0 Monocryl suture. The animal was then recovered from anesthesia and given a dose of butorphanol and normal saline subcutaneously. During the follow up (final evaluation) procedure (after four to six weeks), the same steps were repeated with the exception of the step of dividing the nerve. Additionally, LEMG was recorded from the posterior cricoarytenoid (PCA) muscle (in addition to the other muscles) endoscopically, and in some, via a laryngofissure approach.

3.1.3 Laryngeal electromyography

EMG recordings from CT, TA, and PCA muscles were obtained by inserting a monoplar needle electrode (29GA, 37mm) (Rochester Electro-Medical, USA) into the muscle of interest. The ground electrode (27G, 12mm) (Ambu® Neuroline Subdermal, Malaysia) was inserted into right thigh muscle and the reference electrode (27G, 12mm) (Ambu® Neuroline Subdermal, Malaysia) into the strap muscles. The electromyographic signals were amplified (1000x) (1902 CED, Cambridge UK), digitized (5 KHz) (1401 CED, Cambridge UK), and bandpass filtered

(4-5000 Hz). A Piezoelectric belt was used to detect the chest wall movements, which would allow us to correlate the EMG signal with the respiratory cycle. The belt was attached to the data acquisition unit to record the respiratory activity simultaneously with the EMG signals. EMG recordings were performed using Spike2 v. 6.0 software (CED, Cambridge UK). To obtain LEMG activity of the CT muscle, the monopolar needle was inserted into the belly of the muscle under microscopic guidance. For the TA muscle in each side, the needle was inserted in the midline through the cricothyroid membrane, directing the needle upward and laterally just off the midline (Figure 2-1). Recordings were obtained from both sides before and after transecting the right RLN. A minimum of five respiratory cycles were digitally recorded from each muscle for off-line analysis. At the final evaluation, recording from PCA muscle were obtained by inserting the needle trans-orally under telescopic guidance (Figure 2-2). In some animals, a mid-line laryngofissure was performed to record the LEMG activity of the PCA.

3.1.4 Laryngoscopy

Laryngoscopy was performed, while the animal was spontaneously breathing under TIVA. It was performed trans-orally with the toungue retracted using an artery forceps. The larynx was sprayed with ~0.3 ml 1% lidocaine. Then, lidocaine 1% nebulizer was attached to the O_2 source (flow rate 5-6 L/min), and administered through nasal cone for ~1 minute. A 0^0 2.7 mm rod lens telescope (KARL-STORZ®, Germany) attached to the light source and a high definition digital camera, which was connected to a high definition screen, was used to visualize vocal folds' movements. Video capture of the endoscopic view of five respiratory cycles of vocal

fold movement was recorded using Stryker® SDC HD digital capture (Stryker®, USA) for future assessment.

3.1.5 Recovery and postoperative care

Following the surgical procedure, the animal was placed in a clean cage to allow for recovery from anesthesia. Each animal was housed individually for the first twenty-four hour to assess the oral intake. The animal was monitored closely during the first hour (assessed every twenty minutes) or until the animal was fully awake, and then every eight hours for a total of forty-eight hours. Vital signs (respiratory rate and pattern, temperature and mucous membrane color), activity, appearance, behavior, feeding, and incision site were monitored and documented. Those animals that exhibited signs of uncontrolled pain or severe distress were humanely euthanized. Antibiotic (ampicillin sodium 20 mg/kg) was administered every twelve hours for five days. The standard analgesic protocol consisted of meloxicam (1 mg/kg once daily), and butorphanol (0.2 mg/kg every eight hours) were administered for the first forty-eight hours.

3.2 End points

3.2.1 LEMG

A blinded investigator performed the LEMG analysis, and the results were reviewed by a blinded neurophysiologist. For the CT and TA muscles, the raw LEMG signals were band-pass filtered (Butterworth, 100), fully rectified, smoothed and the root mean square amplitude was measured (time constant 0.002 seconds). For the PCA muscle, the high-pass filter (Butterworth, 70) was used, and the signals were fully

rectified and smoothed. For each muscle, five consecutive bursts were analyzed and the mean of maximum amplitude and burst duration were calculated. For each burst, measurement of the maximum amplitude, burst duration, and timing of the burst in relation to the respiratory cycle were noted. Finally a grade of zero to four according to a grading system previously published was assigned for each muscle[287]. The grading is based on the mean of the maximum amplitude measurements of the bursts, and the burst timing in relation to the respiratory cycle [287]. Grade four was assigned to the muscle when the bursts were of normal amplitude, and locked in time with the accurate respiratory phase more than 75% of the time. Grade three was given when the amplitude was around 50% of the normal, and the activity was in the accurate phase. Grade two denoted absence of the EMG activity, or an amplitude less than 25% of the normal. Grade one was for muscles with activity randomly distributed between the correct and the incorrect respiratory phases. Lastly, grade zero was allocated for muscles with normal amplitude, but with activity in the wrong respiratory phase.

3.2.2 Laryngoscopy

Video recordings of the laryngoscopic view of at least three respiratory cycles of vocal folds movements at three time points (baseline, post-RLN-transection, and final observation) were reviewed and graded by a senior blinded otolaryngologist. Vocal fold movement was graded as 3= normal, 2= minimal movement, or 1= absent movement. Grade three was assigned for the animal with fully and symmetrically mobile vocal folds. Grade two was given when the right vocal fold had minimum

adduction as compared to the control side. Lastly, grade one was for animals with totally immobile right vocal fold.

3.3 Sample size calculation

For the primary end point, we determined that a median difference of two grades on the LEMG scale between the two groups is desirable. This required a sample size of six animals in each group. Fifteen percent was added as the sample size was based on a difference of means (no sample size calculation available for non-parametric tests). Thus the sample size desired was fourteen.

Another sample size was calculated based on estimated proportion of spontaneous recovery of laryngeal function after surgical trauma of 10% based on two reports and our unpublished data on neonates. We decided that the desired resolution rate in the test group would be 80%. Accepting a *p*-value of 0.05 and a power of 80%, the sample size is twenty. Allowing for unforeseen morbidity, we added four animals, thus twenty-four animals divided into two groups were required.

We decided to adopt the larger sample size for the animal ethics application, to cater for attrition of animals while acquiring experience with the procedures.

3.4 Randomization and blinding

A computer generated random list numbers (Microsoft Excel® for Mac 2011) was used for randomization. The key was kept by one of the investigator who was not involved in the evaluation process, and who was responsible for marking the animals and concealing the intervention drugs (i.e. preparing the intervention drugs in identical unmarked 1 ml syringes with transparent solution). The surgeon and

electrophysiologist performing the final evaluation were blinded to animal allocation. Investigators performing the laryngoscopy and LEMG data analysis were blinded for animal allocation as well, and the key was held by an investigator not involved in the process, and was only broken at the end of the analysis.

3.5 Statistical analysis

SPSS 21 software for Microsoft OS was used to perform the statistical analysis. The Fisher exact test was used to compare the proportions of the recovered and the paralyzed right sides of the larynges of both groups. Mann-Whitney U test was used to compare the medians of LEMG grade, amplitude and burst duration of the right side muscles of the intervention and the control groups. The results are expressed as median (25^{th}-75^{th} percentile), and Monte Carlo *p*-value was used for statistical significance. Additionally, in a post hoc analysis, the Fisher exact test was used for comparing the proportions of animals that attained an unfavorable (grade 0-1), intermediate (grade 2), and favorable LEMG grade (grade 3-4) of right sided PCA, TA and CT muscles at the final evaluation of the intervention and the control groups. Student t-test was used for comparing the means of the basic group parameters (weight and age) and the operative data (duration between procedures, volume of injection, surgery duration, and intraoperative heart rate and respiratory rate). The results are expressed as mean (±SD).

4 Result

Thirty Sprague-Dawley (SD) rats were used. Six animals were used initially to establish a reproducible model and to optimize the experimental conditions, (i.e.

optimizing the anesthetic and endoscopic techniques, identifying the anatomical landmarks, and determining the most advantageous EMG electrode needle insertion method). Twenty-four rats were used in the study (twelve animals were randomized to each group). The basic parameters, and the operative data for the two groups are shown in Table 2-1. There was no statistically significant difference between the two groups regarding body weight, time intervals between procedures, duration of the surgery, and intra-operative vital signs. Only the median age of the control group was significantly lower than the intervention group (9.8 ± 0.4 months versus 10.6 ± 0.3 months, respectively, p= 0.0001) (Table 2-1).

Only nineteen animals were available for the final evaluation, nine from the control group and ten from the intervention group. Four animals died intra-operatively due to anesthesia related complication, three animals developed cardio-respiratory arrest after ten, fifteen, and thirty-five minutes from the beginning of the procedure, and one after forty minutes during LEMG needle insertion following a prolonged episode of laryngeal spasm. The fifth animal died on the seventh post-operative day, but post-mortem study did not reveal any specific cause of death. The remaining animals did well post-operatively, and did not develop any sign of distress.

4.1 Laryngeal electromyography

4.1.1 PCA muscle

Data from five left and four right-sided muscles were available from the final evaluation for the control group, and from ten pairs of muscle for the intervention group. Recordings were obtained endoscopically in all but three animals due to the high background electrical activity. Comparison between the LEMG variables of the

endoscopic and the laryngofissure recordings revealed a decrease in the latter's amplitude and burst duration, but without a change in the burst timing or the LEMG grade. The difference was not statistically significant (see appendix Table 2-18).

Upon comparing the final LEMG grades of the right PCA muscles (Table 2-2, Figure 2-3), the intervention group's median grade was significantly higher than the control group (4 [2.75-4] versus 1 [0.25-3.25], respectively, *p* 0.02 [95% CI 0.017-0.023]). The proportion of the animals that acquired LEMG grade of 3-4 was 80% (eight out of ten) in the intervention group, and 25% (one out of four) in the control group. The remaining 20% (two out of ten) of the intervention group attained grade 2, and 75% (three out of four) of the control group attained grade 0-1. The difference between the two groups was statistically significant *p* 0.015 (95% CI 0.012-0.017) (Table 2-3)

With respect to the other variables, the amplitude and burst duration of the right PCA of the intervention group was lower than that of the control group, however the difference was not statistically significant. (Table 2-4, 2-5, Figure 2-4 2-5)

4.1.2 TA muscle

Baseline readings were available for nine pairs of muscles from the control group, and for eleven right and ten left muscles from the intervention group. Data from eighteen pairs of muscle were available for the final evaluation (eight from the control group, and ten from the intervention group).

After right RLN transection, the grade, amplitude, and burst duration of the right TA muscle were lower than the contralateral side and its baseline reading in both

groups. The difference was significant for the grade and the amplitude (*p* 0.02, *p* 0.03, respectively), but not for the burst duration (Table 2-6, 2-8, 2-9).

Upon comparing the final LEMG grades of the right TA of the two groups, the median grades of both groups were similar (control 3 [1.5-3.75], intervention 3 [2.75-3.25]) (Table 2-6, Figure 2-6). The proportion of animals that acquired grade 3-4 was 80% (eight out of ten) and 75% (six out of eight) in the intervention and control groups, respectively. Ten percent (one out of ten) of the intervention group had grade 2. Grade 0-1 was found in 10% (one out of ten) of the intervention and 25% (two out of eight) of the control. The difference in the proportion of grades was not statistically significant (Table 2-7).

For the other variables, the amplitude and burst duration of right sided muscles of the control group in the final evaluation were higher and longer, respectively. However, there was no statistically significant difference (Tables 2-8, and 2-9, Figure 2-7, and 2-8).

4.1.3 CT muscle

Baseline readings were available for twenty pairs of muscle; nine in the control group, and eleven in the intervention group. Data from eighteen pairs of muscle were available from the final evaluation; eight in the control group, and ten in the intervention group.

When we compared the final LEMG grades of the right CT of the two groups, the median grades were similar (Table 2-10). Ninety percent (nine out of ten) of the intervention group attained grade 3-4 and 10% (one out of ten) attained grade 2,

while in the control group 100% (eight) of the animals attained grade 3-4. The difference between the two groups was not statistically significant (Table 2-11). Upon comparing the final evaluation variables of the right CT muscle at the final evaluation between the two groups, lower amplitude and burst duration were noted in the intervention group. However, no statistically significant difference was found (Tables 2-12, 2-13).

4.2 Laryngoscopy

Baseline laryngoscopic recoding of twelve control and eleven intervention animals were available. All animals had normal bilateral VFs movement (grade 3). After right RLN transection, all animals had immobile right VF (grade 1). Laryngoscopic recordings of nineteen animals, nine in the control group and ten in the intervention group, were available for final evaluation. The proportion of the animals that recovered VF mobility was forty percent (four out of ten animals) and forty-four percent (four out of nine animals) in the intervention and the control group, respectively. There was no statistically significant difference between the two groups (p 1.00) (Table 2-14, Figure 2-12).

5 Discussion

Our results suggest that BTX-A injection into the external laryngeal and strap muscles altered the recovery of some laryngeal muscles as demonstrated by the quantitative LEMG assessment. According to the LEMG grading system, a significant improvement of the PCA muscle electrophysiological phasic activity took place, demonstrated by the significantly higher median grade in the intervention group,

without a comparable change in the TA muscle. However, this was not paralleled with a difference in VF movement. There were also no significant differences between the amplitudes and burst durations of these muscles. This could be attributed to the short duration of our study that might not have allowed for translation of the improvement into VF mobility.

This study is unique in several respects. To our knowledge, the potential use of BTX-A for recovering laryngeal function in LP has never been previously studied in an animal model. Further, we employed a stringent experimental design, and combined direct laryngoscopy and quantitative LEMG as end points. We shall discuss these point by point.

Most of the previous studies analyzed the LEMG data qualitatively, focusing mainly on reporting the presence or the absence of the denervation or the re-innervation potentials, and the morphological changes in the motor unit potential waveform[200,256,258,379,380]. Although qualitative analysis provides valuable information about the muscle, it tends to be more subjective, being dependent on the experience of the neurophysiologist analyzing the data.

Our method of interpreting the LEMG allowed us to quantitatively determine the changes in electrical activity of the laryngeal muscles. The use of the respiratory belt provided an objective way to correlate the activity of these muscles to the respiratory phase, instead of the subjective visual inspection, thereby identifying synkinetic activity more accurately. The LEMG grades mirrored the change in TA muscle activity after RLN transection. The median grade was 2 (2-2) for both groups after the transection and was different from the contralateral side in both groups.

Therefore, this system has shown diagnostic accuracy, reproducing results previously demonstrated as its inception [287].

Since laryngoscopy is considered the reference standard of diagnosing LP, we used it as our secondary end point to assess the dynamic status of the VFs. In the rat model, several methods were used to assess the VF mobility, from familiar types of laryngoscopy to laryngofissure [203,229,257,258,260,319]. Most of the studies used ketamine-xylazine anesthesia [257,258,260,260,319], and few used isoflurane [203] to provide a state of spontaneous respiration. We used propofol continuous intravenous infusion to simulate the actual clinical situation and avoid the potential suppression of the spontaneous phasic activity of the VFs produced by other anesthetic agents[255].

Previous studies that utilized BTX-A in a rat model investigated its safety and effect on muscle structure and function in the intact larynx [279,280,297-299,371]. In the LP model, BTX-A was used only once, where it was injected into the CT muscle of two mongrel dogs after severing the RLN on one side [242]. The side of injection was ipsilateral to the injury side in one animal, and on the contralateral side in the other. In the same experiment, the investigators compared the effect of unilateral, and bilateral injections of BTX-A, with dose escalation, to bilateral saline injections, in the normal larynx. The end point was measuring the anterior commissure angle on endoscopic photographs of the VFs at rest, maximum adduction and abduction. The authors reported successful lateralization of the VF after BTX-A injection in the LP model, which was more pronounced upon using BTX-A in the same side of the transected RLN. In the remaining groups, widening of the angle was observed in all

animals, and the effect was dose dependent and more pronounced with bilateral injections. But the authors did not report on recovery of function.

Some of the earlier studies, based on Wagner-Grossman theory, trialed eliminating the action of the CT muscle on the airway (i.e. selective denervation or surgical removal of CT) as a static way to enlarge the airway [45,80,82,242,243,365,366]. The CT muscle was reported to be predominantly active during inspiration after the elimination of the PCA activity [45] or under the compromised respiratory conditions (as in the case of LP) [45,46,50,53,54,367]. Although this procedure demonstrated improvement of airway patency in some studies [45,366], the results were not consistently reproducible [80,82,365]. The difference in the reported results could be attributed to the change of the innervation status of the larynx over time (i.e. synkinetic re-innervation), in addition to the difference in the experimental conditions and the end point used in these studies.

Several interventions aiming to restore function have been described in LP animal model. However, none of them was translated successfully into the clinical mainstream practice. One option has been the re-innervation procedure, which can be classified as selective and non-selective. The non-selective type aims mainly at maintenance of the muscle tone and bulk, and rarely reported to result in VF mobility due to the occurrence of synkinesis [354,355,357]. On the other hand, selective re-innervation of the abductor muscles was used to overcome the obstacle of synkinesis associated with the non-selective type, and to restore the appropriate movement. Although it has achieved some success [225-229], the results were not universally reproduced [234,352,353]. Moreover, the technique itself was proved

technically difficult (i.e. required isolation of the abductor branch of RLN), and most of the studies were uncontrolled and done on small number of animals without demonstration of the long-term results [225,226,229]. Another option is laryngeal pacing, or functional electrical stimulation, where an electrical pacer is implanted to deliver electrical impulses to the laryngeal muscles either synchronous with the respiratory phase or at a pre-set rate. Despite the success reported in producing phasic contraction in some studies, so far full functional recovery has not been achieved [237-240]. More importantly, many technical limitations and complications were reported in several studies [240,358-360]. Non-surgical options for functional recovery include pharmacological treatment, stem cell and gene therapy to promote nerve regeneration [203,220,317,319,364]. Although, some studies demonstrated improvement of neural regeneration and preservation of muscle bulk, they failed to prevent the synkinetic innervation, and their safety profiles have not been established yet. BTX-A has many clinical applications and was shown to be safe and effective therapeutic agent for several neuromuscular disorders [291,292,309]. In addition to its temporary paralytic action, the toxin was reported to have a beneficial effect that lasted beyond its expected duration in some conditions, which was likely mediated via a central effect [305,306,308]. Several experimental and clinical studies observed changes at different levels in the neuromuscular system that might play a role in producing this effect. These are namely alteration of the muscle fibers ratio [295-297], proliferative effect [298] and proteomic changes [299] on the muscle, synaptic remodeling[300], alteration of gene expression in the lower motor neuron [301], retrograde transport of the toxin [302,303], alteration of spinal [305,306] and brainstem

reflex mechanism [278,307], and alteration of the motor neuron pool organization [308]. In the larynx, Inagi et al reported histological evidence of denervation of the injected TA muscle and the contralateral muscle in the rat model after injecting BTX-A into the TA muscle on one side [280]. Although this could be related to the spread of the toxin to the nearby muscles, Bielamowicz et al in a clinical study of spasmodic dysphonia reported an improvement of the activity of the injected TA muscle as well as the ipsilateral CT and contralateral TA and CT muscles, which they attributed to modulation of the brain stem central reflex mechanism [278].

Only very few clinical studies utilized BTX-A in humans with LP [244-247,313]. Three of these studies injected BTX-A into the TA muscle, and reported improvement of the airway patency in most of the patients. However, repeated injections were required[244-246]. Only one clinical study used BTX-A injection into the CT muscle and two other extralaryngeal muscles (ST and SH muscles) in a group of children with idiopathic LP [247]. The toxin produced a lasting effect with documented clinical recovery of VFs movement in most of the patients.

It seems that the site of injecting BTX-A has an influence on the produced effect. Temporarily, injecting the TA muscle might reduce its paradoxical activity, thus improving the airway patency. On the long term, when injected into TA, the toxin was not reported to result in long-lasting effect, although functional improvement was reported in spasmodic dysphonia [278]. On the other hand, injecting the CT muscle eliminates its overriding effect and results in relaxation of the glottic aperture temporarily [247], this effect is augmented when combined with elimination of the SH an ST muscles activities, which were reported to have an effect similar to that of CT

on the airway [377]. At the long term, the uninterrupted SLN might provide a route for BTX-A to exert its effect centrally. Although we used these muscles for injection, our model might not be representative of the picture observed in El-Hakim study. First, we induced LP by severing the RLN. Second, for the potential of anatomical and the physiological differences between the rat and the human larynges [195,259,381-384].

The limitations of our study are mainly related to the attrition of the sample size, although the 95% confidence interval of significance remains reassuring that the statistical significance holds. We also performed a post hoc analysis using comparison of proportions, which still demonstrated a significant difference. The attrition was mainly due to the mortality and the poor quality of some LEMG recordings, both could be attributed to our learning curve. We had a mortality rate in our study of 20%, which was mainly due to anesthetic complications (e.g. laryngospasm, respiratory depression). Endoscopic needle insertion in the rat model was used previously with specific maneuvers that helped to provide full visualization of the VFs, such as using the suspension laryngoscope and the epiglottic elevator [256-259,379]. However, it appears that morbidity and mortality associated with airway manipulation is underreported in the experimental literature employing these methods. One study reported a mortality rate of 17% during LEMG recording (trans-cartilagenous approach) in a rat model [204]. Another study that utilized laryngoscopy and LEMG reported a mortality rate of 20%, which was evenly attributed to anesthesia complications and postoperative wound infection and aspiration, despite the use of a larger animal (i.e. dog model) [326]. We also experienced a high background electrical activity (e.g. heart beat activity) in

many LEMG recordings (with all needle insertion approaches; trans-cartilagenous, endoscopic, and laryngofissure). This might be related to the use of the monopolar needle and the small size of the laryngeal muscles. Therefore, we analyzed all of the LEMG recordings manually, and excluded those where muscle bursts are highly contaminated by external activity. Interestingly, we found that laryngofissure approach only decreased the amplitude and burst duration, without an impact on the timing of the muscle activity.

Future studies might benefit from the optimization of the anesthetic technique, and the development of an easily reproducible method for endoscopic needle insertion into the TA and PCA muscles. Longer follow-up might produce more noticeable results, and provides more information about the final effect of the toxin on laryngeal functions. Delaying the BTX-A injection for a period of several weeks after RLN transection could be more reflective of the picture in the clinical setting, where the paralysis is usually discovered postoperatively.

6 Tables

Group	Weight (gm.)	Age (mnth)	Duration between the primary the final procedures (days)	Volume of injection (µl)	Primary surgery			Final procedure		
					Duration (min)	HR (/min)	RR (/min)	Duration (min)	HR (/min)	RR (/min)
Control	761.25 (±81.2)	9.8 (±0.4)	39.3 (±5.6)	194 (±3)	64 (±14.68)	306 (±30)	56 (±5)	30.56 (±3.9)	322 (±20)	59 (±3)
Intervention	781.33 (±79.4)	10.6 (±0.3)	39.9 (±5.6)	172* (±2.6)	52.7 (±11.48)	309 (±20)	58 (±10)	28 (±7.2)	320 (±10)	58 (±3)
***p*-value**	0.55	0.0001	0.81	0.6	0.07	0.83	0.61	0.34	0.84	0.47

Table 2-1: Basic parameters and operative data for the intervention and control groups
Student t-test was used for comparison. Results are expressed as mean (±standard deviation). *Dose 1.7 U (±0.26)
gm.: grams, mnth, months HR: heart ate, RR: respiratory rate, Min: minute, /min: per minute, µl: microliter.

Group	PCA LEMG grade at final evaluation	
	Left side	Right side
Control	4 (4-4)	1 (0.25-3.25)
Intervention	4 (4-4)	4 (2.75-4)
		***p* 0.02**

Table 2-2 Median LEMG grades of the posterior cricoarytenoid muscle (PCA) muscle at final evaluation for the intervention and control groups
Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported.

Group	LEMG grade of right PCA at final evaluation			
	0-1	2	3-4	
Control	3 (75%)	0 (0%)	1 (25%)	*p* 0.015
Intervention	0 (0%)	2 (20%)	8 (80%)	

Table 2-3 Proportion of the right posterior cricoarytenoid (PCA) muscle LEMG grade at final evaluation for the intervention and control group
Fisher exact test was used for comparison. Results are expressed as number of muscles (percentage).

Group	Maximum rectified smoothed amplitude of PCA at final evaluation (mv)	
	Left side	**Right side**
Control	55.22 (24.45-155.09)	27.87 (14.83-114.8)
Intervention	19.97 (11.43-91.95)	19.77 (15.38-37.16)
		***p* 0.57**

Table 2-4 Median of maximum rectified smoothed amplitude measurements of the posterior cricoarytenoid (PCA) muscle at final evaluation for the intervention and control groups

Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported. mv.: millivolt

Group	Burst duration of PCA at final evaluation (ms)	
	Left side	**Right side**
Control	426.56 (281.45-599.77)	467.58 (366.93-550.21)
Intervention	321.15 (235.63-420.9)	295 (189.02-325.29)
		***p* 0.08**

Table 2-5 Median of maximum rectified smoothed amplitude measurements of the posterior cricoarytenoid (PCA) muscle at final evaluation for the intervention and control groups

Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported. ms: millisecond

Group	LEMG grade of TA					
	Baseline		Post-RLN-transection		Final evaluation	
	Left side	Right side	Left side	Right side	Left side	Right side
Control	4 (4-4)	4 (4-4)	4 (2-4)	2 (1.5-2.5)	4 (4-4)	3 (1.5-3.75)
Intervention	4 (4-4)	4 (4-4)	4 (3-4)	2 (1.5-3)	4 (3-4)	3 (2.75-3.25)
						***p* 0.89**

Table 2-6 Median LEMG grade of thyroarytenoid (TA) muscle at baseline, post –RLN transection and final evaluation for the intervention and control groups

Difference between the median grades of the right-sided muscle at baseline and post-RLN-transection was statistically significant for both groups *p* 0.02. Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported.

Group	LEMG grade of right TA at final evaluation			
	0-1	2	3-4	
Control	2 (25%)	0 (0%)	6 (75%)	*p* 0.76
Intervention	1 (10%)	1 (10%)	8 (80%)	

Table 2-7 Proportion of right thyroarytenoid (TA) muscle grades at final evaluation for the intervention and control groups
Fisher exact test was used for comparison. Results are expressed as number of muscles (percentage).

Group	Maximum root mean square amplitude of TA (mv)					
	Baseline		Post-RLN-transection		Final evaluation	
	Left side	Right side	Left side	Right side	Left side	Right side
Control	18.66 (13.83-46.76)	18.55 (12.71-33.99)	14.21 (5.53-51.21)	7.15 (0-11.89)	37.64 (20.3-83.12)	31.14 (21.22-39.86)
Intervention	24.01 (16.04-34.46)	41.35 (15.61-55.6)	33.16 (13.32-52.83)	19.9 (9.37-60.73)	19.08 (15.05-34.06)	25.71 (18.33-34.89)
						***p* 0.46**

Table 2-8 Median of maximum root mean square amplitude measurements of the thyroarytenoid (TA) muscle at baseline, post-RLN-transection, and final evaluation for the intervention and control groups

Difference between the median amplitudes of the right-sided muscle at baseline and post-RLN-transection was statistically significant (control group *p* 0.01, intervention group *p* 0.03). Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported. mv: millivolt.

Group	Burst duration of TA (ms)					
	Baseline		Post-RLN-transection		Final evaluation	
	Left side	Right side	Left side	Right side	Left side	Right side
Control	307.89 (248.94-445.76	236.52 (156.27-329.51)	228.55 (215.88-439.67)	316.61 (275.32-411.2)	395.65 (313.1-511.99)	408.79 (259.63-516.43)
Intervention	444.46 (211.84-600.88)	404.44 (236.93-550.8)	325.62 (225.68-543.4)	452.16 (224.49-548.43)	305.35 (230.06-457.89)	329.02 (306.68-421.35)
						***p* 0.46**

Table 2-9 Median of burst duration measurements of the thyroarytenoid (TA) muscle at baseline, post-RLN-transection, and final evaluation for the intervention and control groups

Difference between the median burst durations of the right-sided muscles at baseline and post-RLN-transection was not statistically significant for both groups (control group *p* 0.81, intervention group *p* 0.63). Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported. ms: millisecond.

Group	LEMG grade of CT			
	Baseline		Final evaluation	
	Left side	Right side	Left side	Right side
Control	4 (4-4)	4 (4-4)	4 (4-4)	4 (3-4)
Intervention	4 (4-4)	4 (4-4)	4 (3-4)	4 (3-4)
				***p* 0.84**

Table 2-10 Median LEMG grade of cricothyroid (CT) muscle at baseline, and final evaluation for the intervention and control groups
Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported

Group	LEMG grade of right CT at final evaluation			
	0-1	2	3-4	
Control	0 (0%)	0 (0%)	8 (100%)	*p* 1.00
Intervention	0 (0%)	1 (10%)	9 (90%)	

Table 2-11 Proportion of right cricothyroid (CT) muscle grades at final evaluation for the intervention and control groups
Fisher exact test was used for comparison. Results are expressed as number of muscles (percentage).

Group	Maximum root mean square amplitude of CT (mv)			
	Baseline		Final evaluation	
	Left side	Right side	Left side	Right side
Control	76.8 (74.99-121.35)	92.08 (77.55-150)	100.49 (76.03-128.15)	98.01 (52.18-139.75)
Intervention	113.4 (71.06-132.9)	87.79 (48.1-98.53)	107.8 (63.84-157.65)	89.51 (37.24-128.61)
				***p* 0.7**

Table 2-12 Median of maximum root mean square amplitude measurements of the cricothyroid (CT) muscle at baseline, and final evaluation for the intervention and control groups

Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported. mv: millivolt.

Group	Burst duration of CT (ms)			
	Baseline		Final evaluation	
	Left side	Right side	Left side	Right side
Control	441.29 (348.9-620.76)	487.28 (401.07-583.17)	492.94 (378.89-531.2)	475.82 (419.97-521.77)
Intervention	346.72 (299.25-488.41)	383.04 (346.71-553.9)	457.81 (408.63-526.11)	406.52 (330.64-519.57)
				***p* 0.7**

Table 2-13 Median of burst duration measurements of the cricothyroid (CT) muscle at baseline, and final evaluation for the intervention and control groups

Mann Whitney U test was used for comparison. Results are expressed as median (25^{th}-75^{th} percentile). Monte Carlo *p*-value is reported. ms: millisecond.

Group	Laryngoscopy grade of right vocal fold at final evaluation		
	1	**2-3**	
Control	5 (55.6%)	4 (44.4%)	***p* 1.00**
Intervention	6 (60%)	4 (40%)	

Table 2-14 Proportion of the laryngoscopic grade of the right vocal fold at final evaluation for the intervention and control groups

Fisher exact test was used for comparison. Results are expressed as number of animals (percentage).

7 Figures

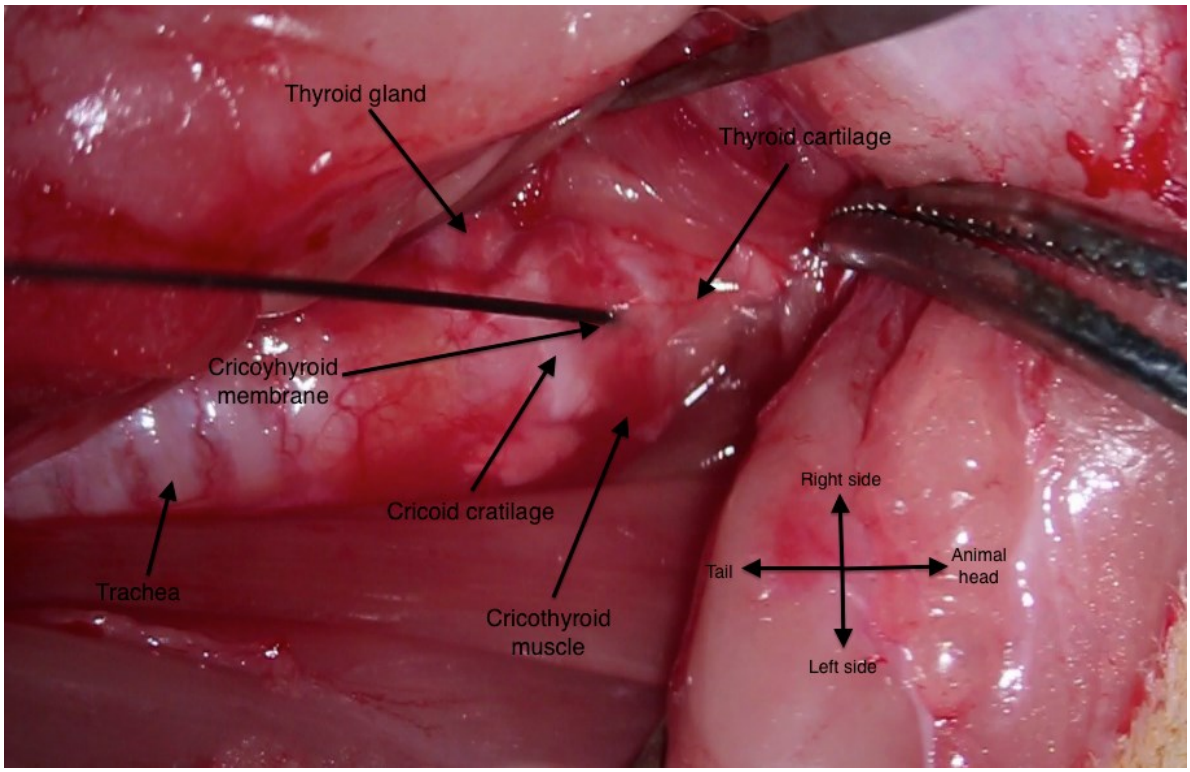

Figure 2-1 Monopolar needle inserted through the cricothyroid membrane into the left TA muscle

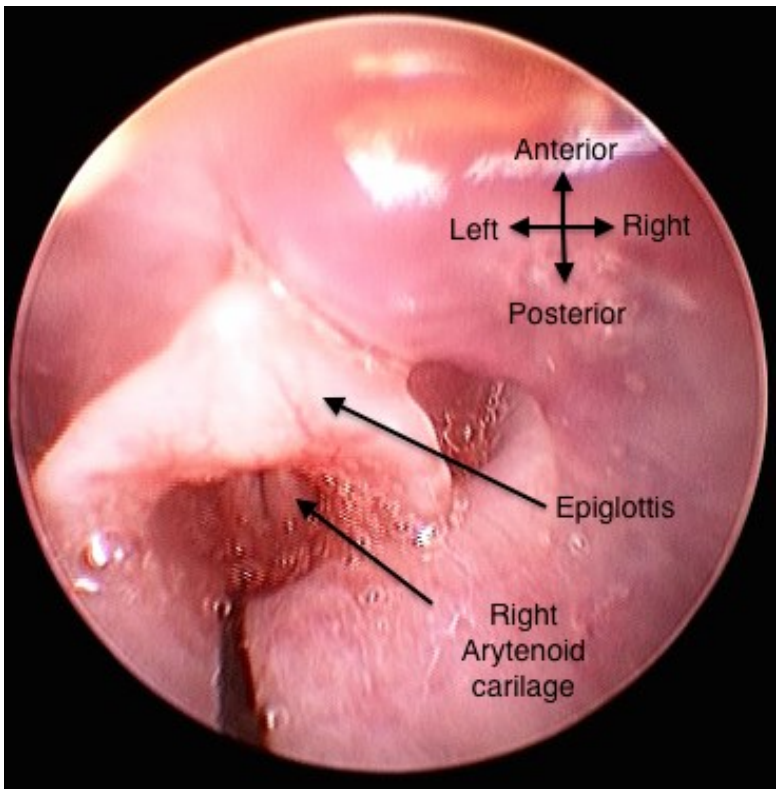

Figure 2-2 Laryngoscopic view of the VFs, showing monopolar needle insertion into the left PCA muscle

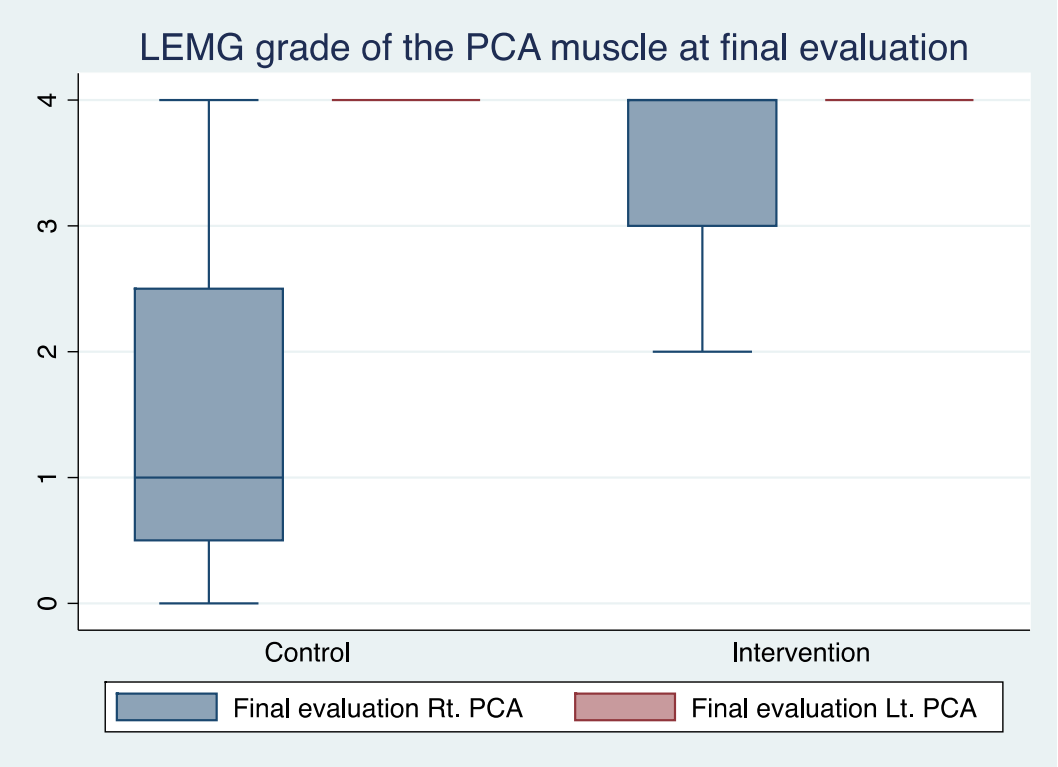

Figure 2-3 LEMG grade of the PCA muscle at final evaluation for the intervention and control groups

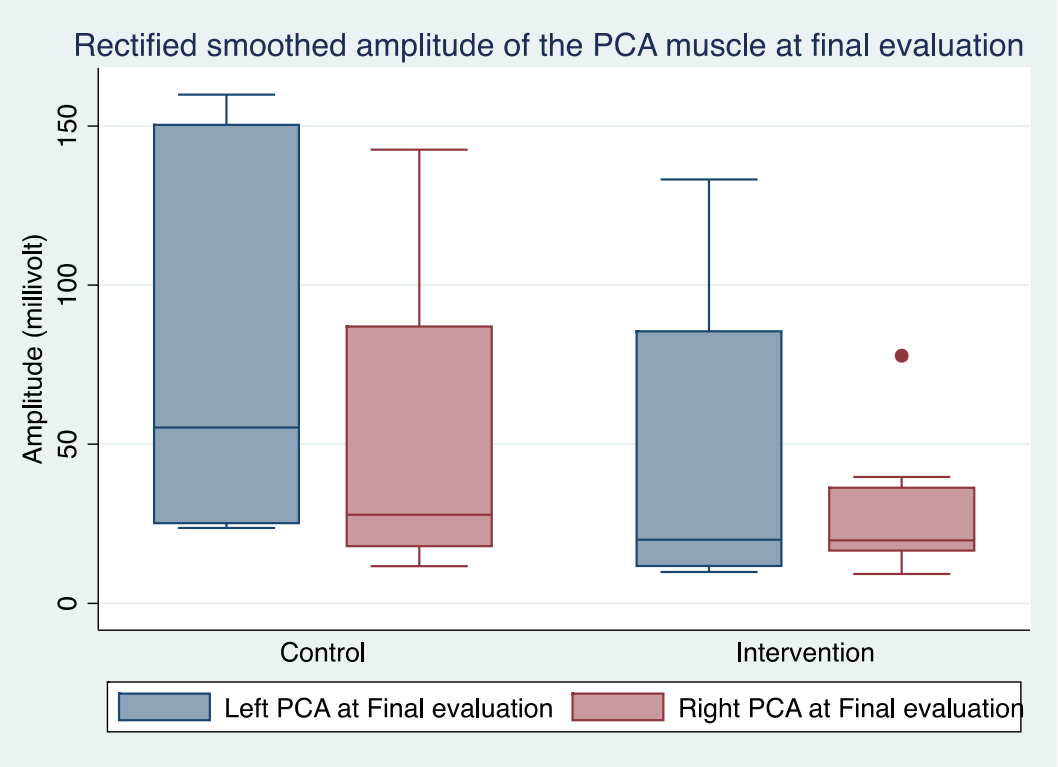

Figure 2-4 Rectified smoothed amplitude of the PCA muscle at final evaluation for the intervention and control groups

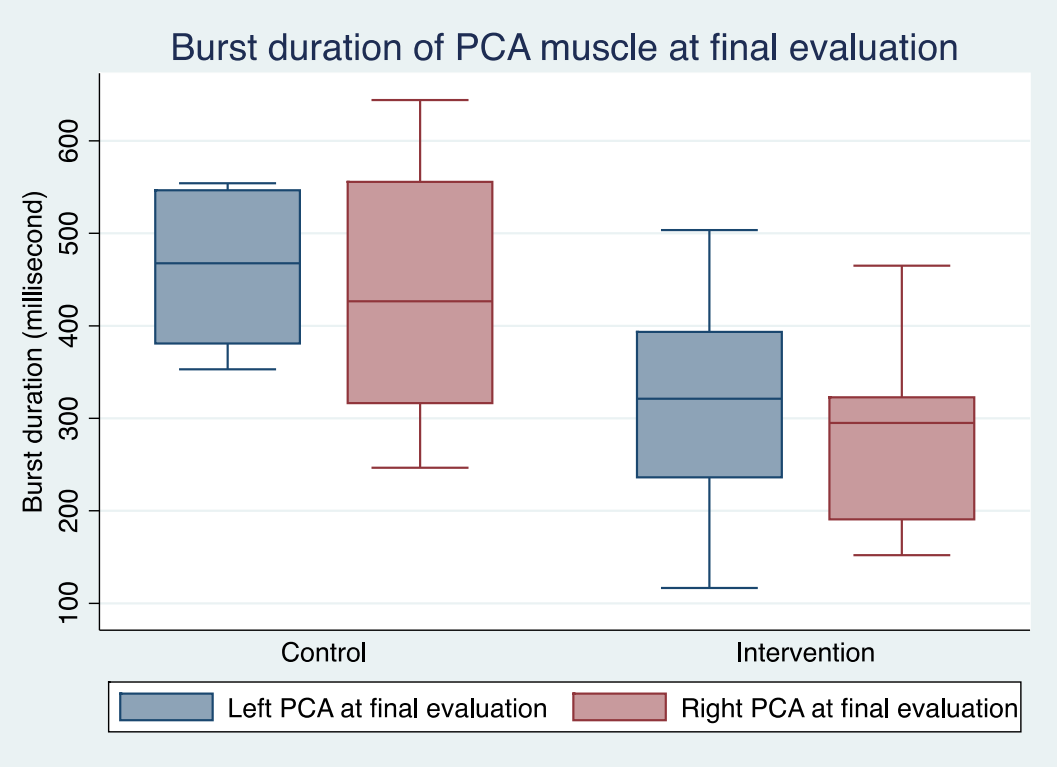

Figure 2-5 Burst duration of the PCA muscle at final evaluation for the intervention and control groups

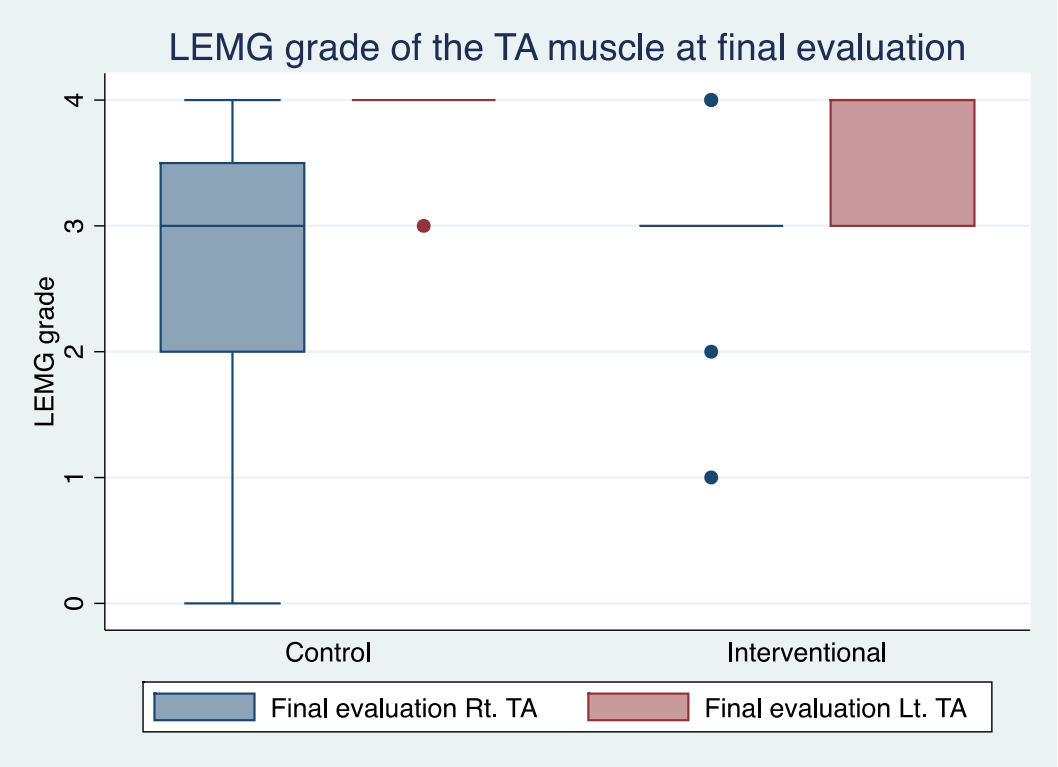

Figure 2-6 LEMG grade of the TA muscle at final evaluation for the intervention and control groups

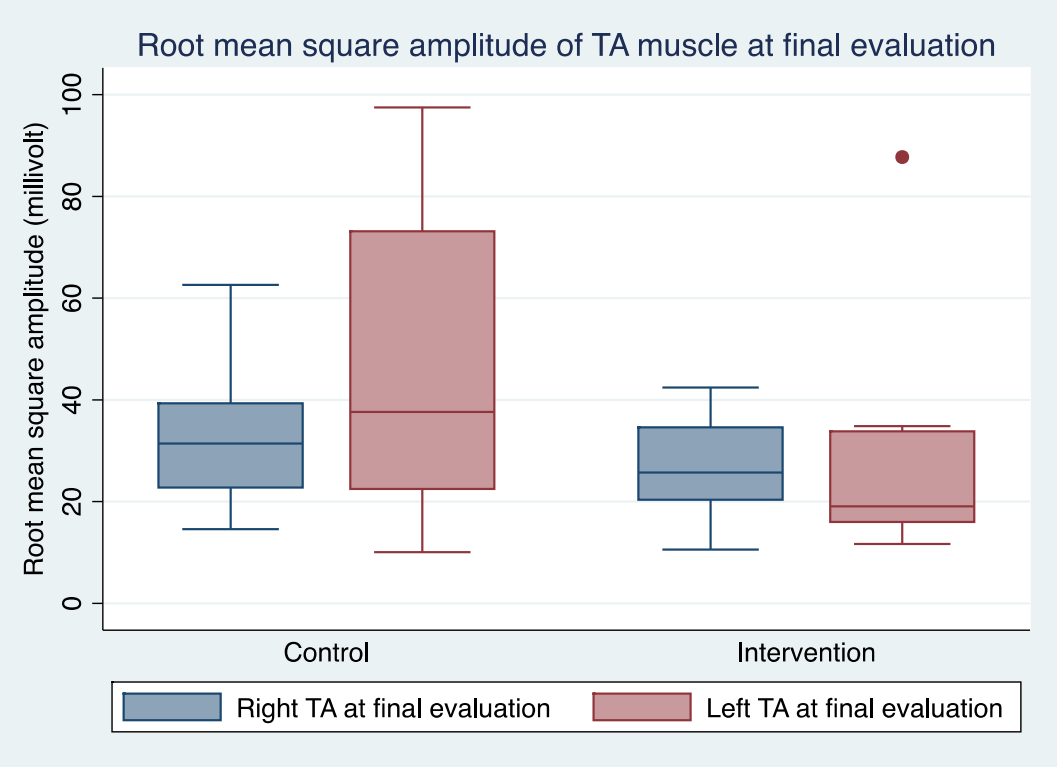

Figure 2-7 Root mean square amplitude of the TA muscle at final evaluation for the intervention and control groups

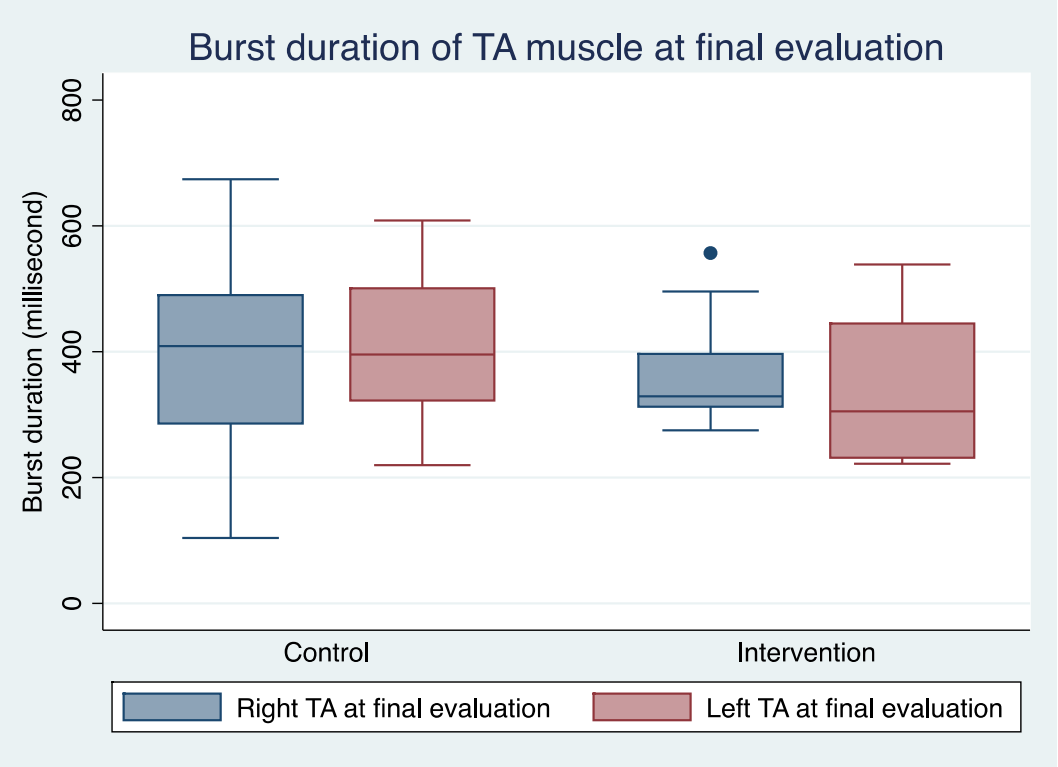

Figure 2-8 Burst duration of the TA muscle at final evaluation for the intervention and control groups

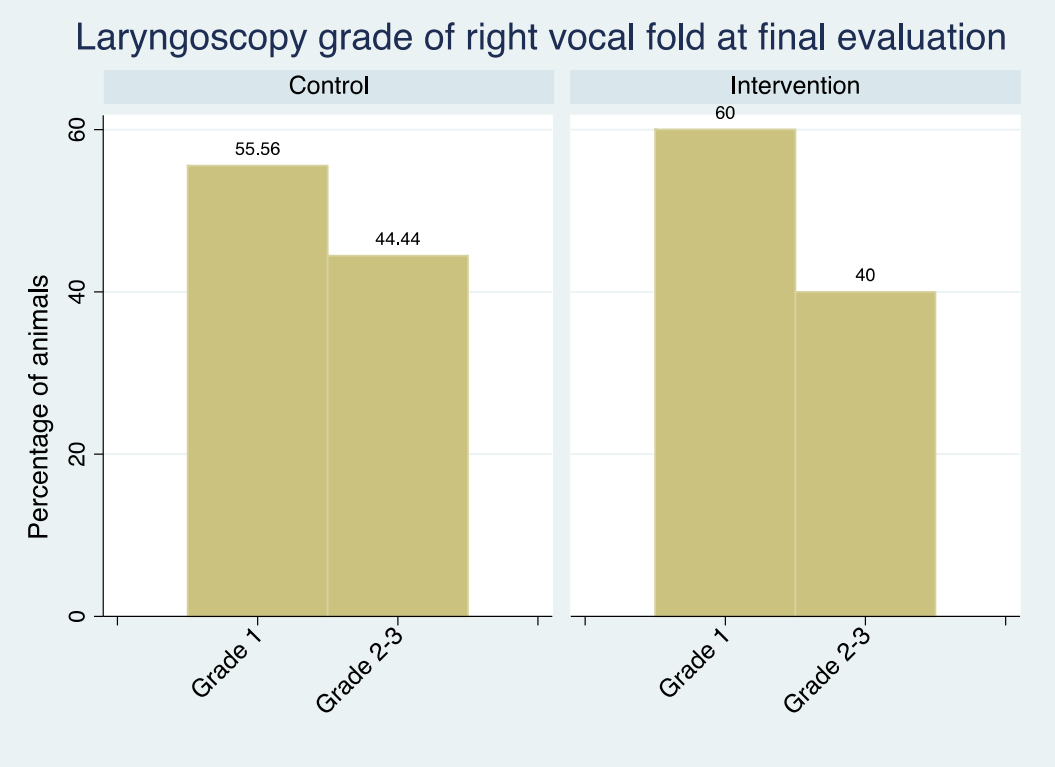

Figure 2-9 Laryngoscopy grade of right vocal fold at final evaluation for the intervention and control groups

8 Appendix

Group	Animal number	LEMG grade at final evaluation	
		Left	Right
Control	1	4	1
	2	N/A	N/A
	3	N/A	N/A
	4	N/A	N/A
	5	4	0
	6	N/A	N/A
	7	N/A	N/A
	8	N/A	N/A
	9	4	1
	10	4	4
	11	4	N/A
	12	N/A	N/A
Intervention	13	4	4
	14	4	4
	15	N/A	N/A
	16	4	2
	17	4	4
	18	4	4
	19	4	4
	20	4	4
	21	4	2
	22	4	3
	23	4	4
	24	N/A	N/A

Table 2-15 Details of the posterior cricoarytenoid (PCA) muscle LEMG grade at final evaluation

Group	Animal number	Maximum rectified smoothed amplitude at final evaluation (millivolt)	
		Left	Right
Control	1	150.4	31.5
	2	N/A	N/A
	3	N/A	N/A
	4	N/A	N/A
	5	159.8	142.6
	6	N/A	N/A
	7	N/A	N/A
	8	N/A	N/A
	9	55.2	11.7
	10	25.2	24.3
	11	23.7	N/A
	12	N/A	N/A
Intervention	13	11.7	36.3
	14	85.5	18.2
	15	N/A	N/A
	16	133.2	16.6
	17	11.9	9.2
	18	16	16.6
	19	9.8	11.8
	20	10.6	7.8
	21	111.3	21.3
	22	24	26.9
	23	35.5	39.7
	24	N/A	N/A

Table 2-16 Details of maximum rectified smoothed amplitude of the posterior cricoarytenoid (PCA) muscle at final evaluation

Group	Animal number	Burst duration at final evaluation (millisecond)	
		Left	Right
Control	1	554	467
	2	N/A	N/A
	3	N/A	N/A
	4	N/A	N/A
	5	467.6	246.6
	6	N/A	N/A
	7	N/A	N/A
	8	N/A	N/A
	9	352.9	644
	10	380.9	386.1
	11	546.4	N/A
	12	N/A	N/A
Intervention	13	236.2	184.1
	14	299.7	333.4
	15	N/A	N/A
	16	503.4	201.2
	17	342.6	322.6
	18	393.4	320.2
	19	239.7	277.2
	20	116.5	190.7
	21	503.6	465.1
	22	390.2	152.1
	23	234	312.7
	24	N/A	N/A

Table 2-17 Details of burst duration measurement of the posterior cricoarytenoid (PCA) muscle at final evaluation

Endoscopic data						Laryngofissure data					
Right PCA (n=7)			Left PCA (n=7)			Right PCA (n=6)			Left PCA (n=6)		
Grade	Amplitude (mv)	Burst duration (ms)	Grade	Amplitude (mv)	Burst duration (ms)	Grade	Amplitude (mv)	Burst duration (ms)	Grade	Amplitude (mv)	Burst duration (ms)
4 (3-4)	18.2 (16.6-39.7)	312.8 (190.7-322.6)	4 (4-4)	23.9 (11.9-85.5)	342.6 (234-393.4)	4 (3-4)	21.6 (21.3-33.3)	240 (184-277.2)	4 (4-4)	11.3 (9.8-14.4)	237.9 (215.1-315.2)

Table 2-18 Comparison between the endoscopic and laryngofiissure variables of the posterior cricoarytenoid (PCA) muscle

Results are expressed as median (25^{th}-75^{th} percentile). ms: millisecond, mv: millivolt

Group	Animal number	LEMG grade					
		Baseline		Post-transection		Final evaluation	
		Left	Right	Left	Right	Left	Right
Control	1	N/A	N/A	N/A	N/A	4	3
	2	N/A	N/A	N/A	N/A	4	0
	3	4	4	4	2	N/A	3
	4	4	4	4	4	4	N/A
	5	4	4	3	3	4	4
	6	4	4	4	1	N/A	N/A
	7	4	4	0	0	4	1
	8	4	4	4	2	N/A	N/A
	9	4	4	4	2	3	3
	10	4	4	2	2	4	4
	11	2	2	2	2	4	3
	12	N/A	N/A	N/A	N/A	N/A	N/A
Intervention	13	N/A	4	3	2	4	3
	14	4	4	3	2	3	3
	15	4	4	N/A	N/A	N/A	N/A
	16	4	4	4	N/A	4	4
	17	4	4	N/A	N/A	3	3
	18	4	4	4	2	4	3
	19	4	4	4	4	4	4
	20	4	4	4	2	4	3
	21	4	4	2	2	4	3
	22	4	4	4	2	4	2
	23	4	4	4	2	3	1
	24	N/A	N/A	N/A	N/A	N/A	N/A

Table 2-19 Details of the thyroarytenoid (TA) muscle LEMG grade at baseline, post-RLN transection and final evaluation

Group	Animal number	Maximum root mean square amplitude (millivolt)					
		Baseline		Post-transection		Final evaluation	
		Left	Right	Left	Right	Left	Right
Control	1	N/A	N/A	N/A	N/A	53.2	25.9
	2	N/A	N/A	N/A	N/A	97.5	40.4
	3	88.2	96.6	279.3	13.1	N/A	62.6
	4	12.8	9.3	14.2	10.3	31.1	N/A
	5	18.7	18.6	11.1	10.6	18.3	19.7
	6	61.1	34.6	59.2	22.9	N/A	N/A
	7	17	16.1	10.6	6.4	44.2	38.2
	8	32.4	33.4	21.9	7.2	N/A	N/A
	9	14.9	24.3	43.2	0	10.1	14.6
	10	22.5	16.2	0.5	0	26.8	28.8
	11	0.1	0.1	0.1	0	93.1	34
	12	N/A	N/A	N/A	N/A	N/A	N/A
Intervention	13	N/A	25.8	12.6	N/A	87.8	28.6
	14	32.4	54	14	0	16	34.6
	15	8.6	7.5	N/A	N/A	N/A	N/A
	16	17.1	41.3	20.4	N/A	19	35.8
	17	118.9	55.6	N/A	N/A	34.9	34.5
	18	40.5	53.4	62.8	13.9	33.8	22.4
	19	12.9	15.6	33.2	25.9	11.7	42.4
	20	21	27.9	45	11.9	19.2	20.4
	21	20.8	13.3	7.5	8.5	17.7	10.6
	22	27	69.6	60.7	0	27.2	12.2
	23	29.3	60.5	43.8	0	12.3	22.9
	24	N/A	N/A	N/A	N/A	N/A	N/A

Table 2-20 Detail of the thyroarytenoid (TA) muscle maximum root mean square amplitude at baseline, post-RLN-transection, and final evaluation

Group	Animal number	Burst duration (millisecond)					
		Baseline		Post-transection		Final evaluation	
		Left	Right	Left	Right	Left	Right
Control	1	N/A	N/A	N/A	N/A	375	410.6
	2	N/A	N/A	N/A	N/A	340.5	104.1
	3	252.7	304.7	178.5	452.2	N/A	337.8
	4	467.3	337.8	496.9	411.2	219.7	N/A
	5	477.6	533.2	329.2	332.8	303.9	233.6
	6	296.5	157.9	215.9	275.3	N/A	N/A
	7	224.2	193.2	228.6	284.9	608.5	674.1
	8	319.3	122.6	439.7	313.6	N/A	N/A
	9	247.7	155.7	223	0	416.3	406.9
	10	381.1	279.8	N/A	N/A	523.2	437.4
	11	N/A	N/A	N/A	N/A	478.5	542.8
	12	N/A	N/A	N/A	N/A	N/A	N/A
Intervention	13	N/A	391	236.4	N/A	226.5	326.1
	14	222.9	256.6	325.6	0	444.7	556.8
	15	292.1	236.1	N/A	N/A	N/A	N/A
	16	148.7	571.7	214.9	N/A	231.2	312.6
	17	178.5	236.9	N/A	N/A	273.8	324.9
	18	627.9	569.9	268.8	552.3	336.9	354.8
	19	482.6	483.8	441.1	536.9	538.7	396.6
	20	577.9	404.4	479.3	453.9	431.2	331.9
	21	406.3	199.7	206.2	198.8	267.4	288.9
	22	591.9	550.8	806.6	0	497.4	495.7
	23	632.3	486.1	607.5	0	221.9	274.9
	24	N/A	N/A	N/A	N/A	N/A	N/A

Table 2-21 Detail of the thyroarytenoid (TA) muscle burst duration at baseline, post-RLN-transection, and final evaluation

Group	Animal number	LEMG grade			
		Baseline		Final evaluation	
		Left	Right	Left	Right
Control	1	N/A	N/A	4	3
	2	N/A	N/A	4	N/A
	3	4	4	4	4
	4	4	4	N/A	3
	5	4	4	4	4
	6	4	4	N/A	N/A
	7	4	4	3	4
	8	4	4	N/A	N/A
	9	4	4	4	3
	10	4	4	4	4
	11	4	2	4	4
	12	N/A	N/A	N/A	N/A
Intervention	13	4	4	3	3
	14	4	4	4	4
	15	4	4	N/A	N/A
	16	4	4	4	4
	17	4	4	3	4
	18	4	4	4	3
	19	4	4	3	2
	20	4	4	4	4
	21	4	4	4	3
	22	4	4	4	4
	23	4	4	4	4
	24	N/A	N/A	N/A	N/A

Table 2-22 Details of cricothyroid (CT) muscle LEMG grade at baseline and final evaluation

Group	Animal number	Maximum root mean square amplitude (millivolt)			
		Baseline		Final evaluation	
		Left	Right	Left	Right
Control	1	N/A	N/A	124.7	51
	2	N/A	N/A	130.3	N/A
	3	75.9	274.9	73.8	177.9
	4	127.1	75.4	N/A	32.3
	5	76.8	99.8	82.8	94.7
	6	115.6	158.6	N/A	N/A
	7	74.1	84.1	43	101.4
	8	156.8	67.7	N/A	N/A
	9	76.7	84.4	103.1	55.5
	10	99.8	124.2	129.3	122.8
	11	30.5	0	97.9	145.4
	12	N/A	N/A	N/A	N/A
Intervention	13	86.6	87.8	45.4	37.8
	14	71.1	48.1	105.6	81
	15	32.9	29.7	N/A	N/A
	16	123.2	88.4	100.1	154.1
	17	53.3	84.3	21.7	120.1
	18	84.6	91.2	158.1	35.6
	19	132.9	25.8	70	11.7
	20	173.4	98.5	141.1	194.7
	21	193.1	173.1	185.5	98
	22	130.4	66.3	157.5	41
	23	113.4	105.9	109.9	106
	24	N/A	N/A	N/A	N/A

Table 2-23 Details of the maximum root mean square amplitude of the cricothyroid (CT) muscle at baseline and final evaluation

Group	Animal number	Burst duration (millisecond)			
		Baseline		Final evaluation	
		Left	Right	Left	Right
Control	1	N/A	N/A	353.3	260
	2	N/A	N/A	327	N/A
	3	636.1	1040	476.9	462.3
	4	688.5	590.3	N/A	461.9
	5	348.6	486.8	534.2	505.2
	6	349.2	434.9	N/A	N/A
	7	430.9	389.8	508.9	406
	8	578.1	561.7	N/A	N/A
	9	441.3	487.8	455.6	489.4
	10	330.3	316	522.3	527.3
	11	605.5	0	682.4	546.3
	12	N/A	N/A	N/A	N/A
Intervention	13	325.3	376.5	422.7	321.4
	14	462.7	346.7	402.9	527.4
	15	299.3	636.1	N/A	N/A
	16	246.8	252.6	481.8	433.7
	17	342.6	383	488.1	406.4
	18	346.7	367.2	372.6	287.2
	19	488.4	538.6	557.6	619.4
	20	574.4	553.9	515.6	517
	21	272.2	316.6	433.8	406.7
	22	618.3	669.9	644.7	333.7
	23	425.6	541.9	410.6	368.2
	24	N/A	N/A	N/A	N/A

Table 2-24 Detail of the cricothyroid (CT) muscle burst duration at baseline and final evaluation

Group	Animal number	Laryngoscopy grade of the right vocal fold movement		
		Baseline	Post-transection	Final evaluation
Control	1	3	1	1
	2	3	1	1
	3	3	1	3
	4	3	1	1
	5	3	1	2
	6	3	1	N/A
	7	3	1	1
	8	3	N/A	N/A
	9	3	1	2
	10	3	1	2
	11	3	1	1
	12	3	1	N/A
Intervention	13	3	1	2
	14	3	1	1
	15	3	N/A	N/A
	16	3	1	2
	17	3	1	2
	18	3	1	2
	19	3	1	1
	20	3	1	2
	21	3	1	1
	22	3	1	1
	23	3	1	1
	24	N/A	N/A	N/A

Table 2-25 Detail of the laryngoscopy grade of the right vocal fold movement at baseline, post-RLN-transection and final evaluation

Chapter 3: General discussion, conclusion and future directions

1 Discussion

The larynx is a complex organ that possesses a delicate and variable neuroanatomical configuration. It performs fundamental functions with high precision, which are controlled by complicated neurophysiological mechanisms. Thus, affection of its neural supply at any level can compromise its functions, and result in a wide range of manifestations that significantly impacts the general health and the quality of life of the affected individuals [184-186]. Many etiological factors have been implicated in aspect of dysfunction. Although they all produce analogous clinical picture, they exhibit different natural histories and require therefore different management approaches. Implicating an etiology requires a thorough investigative work up to rule out other causes of vocal fold immobility.

Laryngeal paralysis in general is a poorly understood condition. Whether it is of unilateral or bilateral variety, the natural history of the disease has not been clearly documented for most of the etiological categories, which explains the absence of consensus on the optimal management options. Most of the currently employed interventions (tracheostomy, glottic expansion and medialization procedures, alternate route of feeding, and minimally invasive ventilation) aim at ameliorating the symptoms of the disease, but do not reestablish the lost laryngeal functions. This is largely due to the lack of understanding of the underlying pathophysiological mechanism of the disease. While many theories have been proposed, the most accepted one is the synkinesis theory, where random, or chaotic, re-innervation of

the laryngeal muscles takes place after interruption of the neural pathway [77]. The net result is a non-functional, paradoxical movement of the vocal folds. It has also been reported that re-organization of the motor neuron pool takes place after synkinetic re-innervation, which further complicate the picture [191,195]. Hence, optimal treatment should focus on decreasing the synkinetic re-innervation, in order to restore purposeful functional mobility to the vocal folds.

The concept of synkinesis is based on the evidence provided by laryngeal electromyography (LEMG), which demonstrated persistence of the laryngeal muscles activity following LP [77,82]. LEMG has a proven clinical application in differentiating between LP and vocal fold fixation [198,252], and some potential in predicting the prognosis, although not based on substantial evidence [201]. Particularly, the lack of consistent methodology of recording and interpretation, and the absence of diagnostic trials on its accuracy, leave us with some skepticism on its clinical utility in LP and other mobility disorders [198,200,252]. In the field of animal experiments, LEMG has been proposed as an objective end point for laryngeal function. Despite the lack of standardized methods, and barely any controlled quantitative LEMG studies, it remains a fundamental outcome measure for assessing functional recovery of the larynx.

Many animal studies focused on alterative modalities to tracheostomy and other symptomatic treatments for LP. Most of these studies used models of surgically induced paralysis, created by interruption of the recurrent laryngeal (RLN) or vagus nerve. Although this model simulates the clinical picture, it represents only one etiological category of the disease (i.e. physical neural interruption). However, since

a representative model for the other categories is not available, this model remains the most feasible and employed for experimental research. Several animal species were used, however none of them is particularly equivalent to the human case. The rat model is being increasingly used lately [203,262,263,317-322], and was reported to be suitable for studying the laryngeal functions by laryngoscopy and LEMG [258,259]. Efforts in general were directed at either ameliorating the symptoms or restoring of the laryngeal functions. However, most of the studies that attempted to restore functional mobility failed to offer a reproducible and practical therapeutic modality that can be safely translated into the clinical practice. Aside from methodological flaws, including power problems and lack of controls, ethical issues, technical problems and common to all inability to resolve synkinesis prevented translation to clinical practice.

In our study we sought to investigate the effect of botulinum toxin type A (BTX-A) in enhancing the laryngeal functional recovery in a rat model of surgically induced LP. BTX-A is a safe drug that has been used widely for treating various neuromuscular disorders. Many studies reported a more durable improvement than expected of the neuromuscular functions after using it, and this sparked interests in its plausible central mechanisms [278,305-307]. There is indeed evidence of different mechanisms of action for the toxin, beyond the local temporary paralytic effect, seen in several functional and structural changes on different regions of the motor system after its use [278,302,303,385,386]. Some of these effects include alteration of the muscle fibers composition ratio [295-297], proliferative effect [298] and proteomic changes [299] on the injected muscle, synaptic remodeling and sprouting [300], alteration of gene

expression in the lower motor neuron [301], retrograde transport of the toxin along the lower motor neuron [302,303], alteration of spinal [305-307] and brainstem reflex mechanism [278], and alteration of the motor neuron pool organization [308]. Of specific interest are the studies on the facial nerve paralysis model, where BTX-A injection into the rat whisker pad (after transection and re-anastomosis of the facial nerve unilaterally) decreased synkinesis. This effect was more significant when the injection was applied to the contralateral whisker pad, with intact facial nerve [386]. Based on these reports, we proposed that BTX-A may decrease the synkinetic re-innervation in LP, and/ or normalizing the organization of the motor neuron pool in the central nervous system.

The present experiment is the first to assess the effect of BTX-A on enhancing functional recovery after LP using a single blind, randomized controlled design. We utilized quantitative methods for LEMG interpretation as a surrogate for functional recovery. The grading system, created by our group and previously used in humans, enabled us to correlate the muscle activity to the respiratory cycle. Therefore, it identified muscles that recovered function, and were re-innervated in a favorable way (indicated by grade three and four), and those where synkinesis might have taken place and lead to paradoxical activity (i.e. grades zero and one). Laryngoscopy was used as a secondary end point to assess whether LEMG findings were translated into functional movement in the short term.

The results of the current experiment demonstrated an improvement in the activity of the main abductor laryngeal muscle (i.e. posterior cricoarytenoid (PCA)) after BTX-A injection into an extra laryngeal muscle (cricothyroid) and two strap muscles

(sternothyroid and sternohyoid). There was no improvement in the corresponding variables of the adductor muscle (tyroarytenoid (TA)), which could be attributed to the short duration of the study. Also, as known from anatomical studies, the central representation and the number of neural axons supplying the adductor muscles are larger than those of the abductors [26,387]. Thus, it is possible that the toxin requires a longer duration to produce its full central effect. Another potential confounder is the uncertainty of accurate and consistent needle insertion into the extremely small TA muscle, as this was not done under direct vision (i.e. trans-cricothyroid-membrane). Hence, different regions or even different muscles might have been mistakenly sampled.

The current experiment, its results, and conditions merit some reflection. Although some relative attrition of the sample size occurred, with respect to the final recordings of the PCA, yet the 95% confidence intervals and the post hoc analysis are reassuring that we have not lost the power altogether. Second, the critic might challenge the use of a grading system that has not been externally validated yet. However, the accuracy of the system in the described human study was 86.3% (sensitivity 100% and specificity 89.7%) [287], and it mirrored the change in muscle activity in the present experiment after nerve section. As to the lack of difference in endoscopic findings, this is probably related to the short interval between the initial surgery (i.e. RLN transection with BTX-A administration) and the final evaluation. After RLN transection, nerve regeneration could take a period of several weeks, with LEMG changes preceding the recovery of vocal folds mobility [86-88,326,378]. There is no doubt that the conditions of this experiment, like many others, do not mimic real

life. The immediate administration of BTX-A after RLN transection, means that the toxin was administered before synkinetic re-innervation has taken place, and as a result the role of the toxin here can only be pre-emptive, differing from the usual clinical scenarios where any intervention is instated after a period of time from the injury.

In several ways the experimental conditions can be improved. We encountered a significant mortality rate in our study (not dissimilar to other experiences), which was mostly related to anesthesia complications. We used propofol continuous intravenous infusion anesthesia in order to simulate the actual clinical picture, and avoid the effect of the other anesthetic agents on muscle activity. However, despite our regular use of an opioid analgesic, NSAID, and topical lidocaine, several animals developed frequent episodes of laryngeal spasms during airway manipulation. Previous studies rarely reported the morbidities and mortality associated with using laryngoscopy or LEMG. One study reported a mortality rate of 20%, where half of the animals expired due to anesthesia complications, despite using a dog model [326]. Another study reported a mortality rate of 17% during LEMG recording in the rat model [204]. This suggests that the mortality and morbidity are under reported in the literature. Further, there are no evidence based reports on an optimal, reasoned anesthetic protocol that provides the most advantageous conditions for this particular experiment, in particular for obtaining ideal LEMG readings and recordings. Our group intends to research this area in future work.

2 Conclusion

BTX-A might enhance the phasic activity of the laryngeal abductor muscle in the rat model at short term; however without being translated into an observable vocal fold movement. Taken with prior observations in humans, BTX-A merits further consideration and research as a promising treatment modality for LP.

3 Future directions

Most of the published clinical studies on LP are of low quality and contained major methodological flaws. Particularly, the diagnosis in most studies is questionable due to the inconsistency or inadequacy of the diagnostic modalities, potentially mislabeling a significant number of cases as LP. Future studies should utilize the reference standard for diagnosis (i.e. direct laryngoscopy with arytenoid palpation[169]), and have a comprehensive systematic protocol of investigations to determine the likely underlying etiology. In that respect, LEMG is a valuable tool that can help reach the diagnosis and potentially predict the prognosis. However, the currently used methods for recording, interpretation and reporting need to be standardized, and validated [198,201,252]. As a consequence to diagnostic inconsistencies, the natural history of the disease and its epidemiology are largely unknown [91]. Therefore prospective multicenter studies are needed to clarify how subgroups behave especially when the insult is removed. These efforts should also help in forming a consensus about the optimal management approaches.

With regards to the experimental literature of LP management, the quality of many studies is poor due to flaws in design and methodology. For example, most studies

lack objectivity, as they were not controlled or blinded. In addition, many studies neither reported the mortality and morbidly rates, nor accounted for the statistical power, which make the reported results questionable.

Future studies on the LP rat model in particular might benefit from optimization of the experimental conditions, namely the anesthetic techniques in order to achieve a reproducible and stable model, and avoid the attrition of the sample size. The effects of the inhalational and injectable anesthetic agents on VF mobility and electrical activity of laryngeal muscles were not studied in depth previously. Therefore, a reproducible and safe anesthetic protocol for laryngoscopy and LEMG with minimal effect on laryngeal muscles activity that closely resembles the clinical setting is needed. A combination of propofol and remifentanil appears to be a reasonable choice, as it can provide a superior analgesic effect and allow for spontaneous breathing [388,389]. In addition, it may reduce the respiratory morbidity associated with airway manipulation [388,389].

BTX-A was shown to enhance, to certain extent, the functional recovery in our experiment. However, this needs to be reproduced, and studied further. Since the impact on laryngeal function was determined based on the LEMG grading system, further validation of this system is required, and correlation to endoscopic findings as well.

Also, in order to closely simulate the clinical situation, an evaluation of the effect of the delayed administration of BTX-A is required. This will allow it a window of time to act on the synkinetically re-innervated muscles.

Our hypothesis rests on mechanisms of action for BTX-A other than that described at the neuromuscular junction. For further understanding of these mechanisms, injection of radiolabelled BTX-A followed by histological examination of the brainstem and laryngeal muscles would be a potentially useful technique to determine whether a central effect of the toxin exists or not. It is also interesting to study whether a histological change reflecting an alteration to the motor neuron pool distribution following BTX-A injection takes place, and if so, whether this change is permanent or temporary, and whether it depends on the BTX-A dose or timing of injection.

To elaborate also on the venue of transport, total denervation of the larynx by interrupting the superior and RLNs followed by BTX-A injection could provide further evidence of the central action of the toxin, and rule out spread of the toxin by other routes.

Finally, a long-term follow up with frequent assessments may clarify the final effect of BTX-A administration on LP. A minimum of six months follow-up with bi-monthly assessment by laryngoscopy and LEMG might be adequate to study the temporal course of the functional changes in laryngeal muscles activity. In order to lower the morbidity associated with repeated surgical exposure, it might be better to use an implanted wire electrodes into laryngeal muscles for LEMG recording.

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