Neuromuscular induced phonation in a human *ex vivo* perfused larynx preparation

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Abstract: Considering differences in laryngeal anatomy, degree of control, and range of voice qualities between animals and humans, investigations of the neuromuscular process of voice control are better conducted using a living human larynx in which parametric stimulation of individual laryngeal muscles is possible. Due to difficulties in access and monitoring of laryngeal muscle activities, such investigations are impossible in living human subject experiments. This study reports the recent success in developing an *ex vivo* perfused human larynx model, which allows parametric muscle stimulation and observation of its influence on phonation of a virtually living human larynx in a well-controlled laboratory environment.

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1. Introduction

The position of the larynx deep within the pharynx and its status as a reflexive guardian of the airway severely limit the direct study of the subtle vocal fold posturing or other performance variables in living human beings (Solomon *et al.*, 2007). Therefore, most information about phonation and laryngeal biomechanics has been derived indirectly, via imaging of vocal fold motion or computational and physical modeling; inferentially, via vocal acoustics; or through the use of *ex vivo* animal and human larynges or *in vivo* animal models. *Ex vivo* models imitate some muscular actions required for phonation, such as closure of the cartilaginous glottis, by mechanically adducting the arytenoid cartilages to simulate interarytenoid and lateral cricoarytenoid muscular contraction. Similarly, increased vocal pitch can be modeled by mechanically rotating the thyroid onto the cricoid cartilage, thereby stretching the vocal folds to simulate cricothyroid muscle contraction. However, *ex vivo* models cannot simulate the essential functions of the thyroarytenoid muscle, which include increasing vocal fold body stiffness, relaxation of the cover, and medial bulging of the medial vocal fold surface for successful membranous glottal adduction during phonation. Moreover, *ex vivo* models utilize non-living tissue and therefore include effects of post mortem deterioration.

In contrast, *in* vivo animal models are attractive because of their ability to reliably and systematically model the intrinsic laryngeal muscular contraction that provides the great flexibility of loudness, pitch, and vocal quality that characterize human phonation during speech and singing. Although *in vivo* animal models do allow realistic physiologic stimulation of the laryngeal muscles (as demonstrated in a series of parametric studies of individual muscular function on phonation; e.g., Choi *et al.*, 1993a,b; Nasri *et al.*, 1994; Chhetri *et al.*, 2010, 2012) and utilize living tissue, concerns about external validity arise because it remains unclear whether animal larynges are sufficiently similar to humans for meaningful generalization to human phonation (Hirano and Kakita, 1985). For example, although the canine larynx is most often used for the study of

laryngeal physiology, the canine larynx has a thicker and taller superficial and intermediate lamina propria of the vocal fold cover, a less-developed vocal ligament, a proportionally longer cartilaginous glottis, and a much more pronounced posterior commissure than the human larynx (Hirano and Kakita, 1985; Titze and Hunter, 2004). The relative sizes of the different laryngeal muscles also differ between canines and humans. Such differences in anatomy may lead to different patterns in the regulating of vocal fold stiffness and geometry (the two most important variables governing human phonation) by laryngeal muscle activation. Thus, studies of voice quality would unquestionably be better conducted in a living human laryngeal model to reach the goal of modeling the manner in which humans control voice quality.

Because of the desirability of direct control over the laryngeal muscles in the *in vivo* canine model as compared to mechanical manipulation in *ex vivo* models and the concern about the external validity of animal models, we have developed a human, *ex vivo* perfused living laryngeal model that has the best features of both approaches, by providing experimental control over muscular activation of the human larynx in an animated donor organ. This letter describes the development of this model and provides preliminary evidence of its functionality.

2. Method

Permission to obtain human research larynges from transplant donors was obtained through One Legacy, the Southern California transplant distribution center. Permission was granted after approval of a research protocol to study *ex vivo* perfusion of composite tissue organs, specifically the larynx. No institutional review board approval was required because the medical history of the patients was not required for this study. Families of donors must sign a separate consent permitting laryngeal harvest for research purposes. To date seven laryngeal research organs have been harvested following removal of the transplantable organs. The larynx, esophagus, trachea, strap muscles, thyroid, carotid arteries, vagus nerves and branches, and the internal jugular veins were harvested as one composite tissue unit. The organ was then placed in a sterile plastic bag on ice and transferred to the laboratory.

In the laboratory, the recurrent and superior laryngeal nerves were identified and the carotid branches not related to laryngeal perfusion were tied off. The carotid arteries were cannulated by 12-French cannulae (DLP Pediatric One-Piece Arterial Cannulae, Medtronic, Minneapolis, MN). Venous outflow was via gravity drainage into the RM3 collection system (RM3 Renal Preservation System, Walters Medical Systems, Rochester, MN). Type-specific whole blood diluted with Ringers Lactate to a hematocrit of 40 was used to perfuse the organ. Approximately 150 cc of blood was required to prime the RM3. The RM3 perfusion pump provided pulsating intra-arterial blood pressure and flow, based on experimental pumps developed for extended renal preservation. Our initial experiments using canine larynges showed that sustained active neuromuscular function in the *ex vivo* perfused larynx requires a pulse pressure. Larynges that were arterially infused with intra-arterial Krebs reperfusion solution plus or minus colloid in a constant flow system did not produce sustained phonation beyond 3 min and the organ quickly became non-viable. When the RM3 was utilized to provide pulsating intra-arterial blood pressure and systolic/diastolic flow, larynges with whole blood perfusion demonstrated strong phonation for over 12 h without fatigue. This pump was modified to permit additional oxygenation to the venous drained blood. The unit was also modified to remove blood for exchange transfusion. Finally, a warming device was added along with additional heat lamps to warm the organ.

Between the pump and pressurized blood collecting chamber, prior to the arterial cannulation, a hemoconcentrator with a dialysis filter was installed (Minntech-Hemocor HPH 400 HemoConcentrator, Metronic, Minneapolis, MN). Dialysate solution (PrismasateBgk 4-2.5, Gambro Renal Products, Stockholm, Sweden) was infused to maintain electrolyte homeostasis and to remove additives (paralytic agents required during the harvesting of organs) and the hyperkalemia from the Wisconsin solution

which had been infused during the organ collection procedure. Sugammadex at a dose range of 1 to 16 mg/kg IV was used to chelate off any remaining paralyzing agent.

During the experiment, the pH, blood gases, and electrolytes were monitored and adjusted if needed at 15 min intervals and the RM3 was adjusted to provide a systolic pressure of approximately 90 mm Hg with a pulse pressure of about 25 mm Hg, at a pump rate of 60 pulses/min.

Muscular contraction was elicited by nerve or direct muscle stimulation. Nerve stimulators (custom designed carbon coated) or mono or bipolar needle electrodes (Natus Medical Incorporated, San Carlos, CA) were inserted directly into the laryngeal muscles. For the results shown below, the method of direct muscle stimulation was used. The cricothyroid and intrinsic laryngeal muscles were electrically stimulated by two constant current stimulators (Model WPI 301-T, World Precision Instruments, Sarasota, FL) set at 60 Hz. Current ranged from 0.5 to 10 milliamps depending on the desired degree of muscle contraction. The needle electrodes were placed directly through the thyroid cartilage into the thyroarytenoid muscles and into the lateral cricoarytenoid muscles. The cricothyroid, posterior cricoarytenoid, and interarytenoid muscles were also stimulated using needles placed under direct vision of the anterior/superior laryngeal surface. Warmed, 37°, 100% humidified air was flowed rostrally through the larynx at approximately 300 to 500 ml/s, utilizing an endotracheal tube with an inflated cuff.

3. Results

With the pulsating blood pressure supply, phonation in the *ex vivo* human larynx was achieved approximately 3 h following harvest of the organ. Audio and video examples of phonation elicited with this model are given below. Mm. 1 demonstrates unilateral and bilateral stimulation of the intrinsic laryngeal muscles, without airflow. Complete glottal closure was achieved with bilateral intrinsic laryngeal muscle stimulation. Stimulation of the cricothyroid muscles is demonstrated in the beginning part of Mm. 2, which elongated the vocal folds but slightly adducted the vocal fold.

Mm. 1. Unilateral and bilateral stimulation of the intrinsic laryngeal muscles. This is a file of type "mov" (4.29 Mb).

Mm. 2. Modulation of voice quality during breathy phonation. This is a file of type "mov" (1.07 Mb).

The larynx was successfully phonated with airflow at 400 ml/s over a period of 90 min intermittently every 10 to 15 min for approximately 1 min per activation. Perceptually, the sound was quite different from canine phonation, with a distinct human voice quality. An example of the sound production is included in Mm. 3.

Mm. 3. Outside sound produced by the *ex vivo* perfused human larynx. This is a file of type "wav" (95 Kb).

Mm. 2 demonstrates the ability of the model to modulate muscle stimulation and the influence of such modulations on the resulting voice. In this case, breathy phonation was simulated. Modulation of voice quality was achieved by varying the stimulation level to the left intrinsic adductor muscles. Despite the poor sound recording condition (due to the use of a home video recorder located directly above the glottis), changes in voice quality with stimulation variation can be distinguished.

4. Discussion

This paper presents our recent success of neuromuscularly-induced phonation in an *ex vivo* perfused human larynx. For voice research, this model opens the possibility of the parametric study of human laryngeal neuromuscular physiology outside the human body. For example, the effects of actual human laryngeal muscle activation on vocal fold stiffness and geometry can be experimentally observed. More importantly, activating individual laryngeal muscles would make it possible to investigate muscular interactions, such as those between the thyroarytenoid and cricothyroid muscles, to provide

new insights into synergies and interactions among muscles (if any) in the control of pitch, loudness, and voice quality. Combined with acoustic analysis, imaging, aerodynamic analysis, and perceptual testing, this model will allow the development of a comprehensive model of the precursors and correlates of changes in voice quality. Finally, this research has implications extending beyond laryngeal modeling to eventual composite tissue transplantation and re-implantation following removal and repair for treatment of trauma and malignancies.

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