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Ex Vivo Perfused Larynx Model of Phonation: Preliminary Study

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Objectives: Although excised laryngeal models and physical models of the larynx are important in the study of laryngeal dynamics, they cannot be used to study the influence of neuromuscular contraction on vocal fold vibration, especially with regard to the thyroarytenoid muscle. Our aim was to develop an ex vivo larynx model of phonation, and combine the benefits of the in vivo and excised laryngeal models to the ex vivo situation.

Methods: Three canine larynges were surgically removed and perfused ex vivo with modified Krebs-Henseleit reperfusion solution. The laryngeal nerves were stimulated, and an assessment of neuromuscular viability, phonation, and vocal fold vibration was made.

Results: Neuromuscular stimulation, phonation, and experimental manipulation were possible for several hours after the onset of ex vivo perfusion. Repeatable periodic phonation in short bursts was achieved. Perfusion appears critical to maintain ex vivo viability, as adductory force was almost immediately lost upon cessation of ex vivo perfusion.

Conclusions: The ex vivo larynx model has the potential to facilitate the measurement of glottal variables in a neuromuscularly correct model. We propose that the further development of this laryngeal model may be useful in the study of laryngeal dynamics, particularly when invasive measurements, such as that of glottal exit flow, are required.

Key Words: ex vivo model, larynx, perfusion, physiology.

INTRODUCTION

The use of in vivo models has proven to be critical in the study of laryngeal physiology. The canine laryngeal model is the animal model most widely used in voice research because of its similarities to the human larynx with regard to its shape, size, and neuromuscular anatomy.[1] The in vivo model permits physiologic stimulation of the laryngeal nerves, thus allowing the study of a neuromuscularly correct system. However, the in vivo canine model is limited by a narrow superior view of the larynx as visualized from the oral cavity. This narrow viewing angle limits experimental protocols that require multiple viewing angles, such as multidimensional, high-speed imaging of the larynx and glottal exit flow measurements that require a lateral viewing angle.

An excised larynx model is better suited for unencumbered access to the larynx for invasive measurements. However, the excised larynx model has its own limitations. Although excised laryngeal models and physical models of the larynx are important in the study of laryngeal physiology, they cannot be used to study the role of physiologic thyroarytenoid muscle contraction on laryngeal dynamics. In particular, although the actions of extrinsic laryngeal muscles can be simulated in the excised larynx by use of external forces, the simultaneous contraction, stiffening, and medial surface bulging of intrinsic laryngeal muscles, such as the thyroarytenoid muscle, cannot be duplicated in an excised larynx setup. Therefore, we continue to regard in vivo models as essential in the study of laryngeal physiology. To combine the benefits of unencumbered access to the larynx and neuromuscular intactness, we have sought ways to develop a laryngeal model that can be excised from the body with its neuromuscular apparatus intact and functioning, as in the in vivo state.

In other words, our aim was to combine the ben-

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efits of both the in vivo and excised laryngeal models into an ex vivo larynx model of phonation. The larynx is removed completely from the body, and its neuromuscular apparatus is kept fully functional by arterial perfusion of a nutrient-rich, pH-balanced physiologic solution, thus allowing extended neuromuscular stimulation, phonation, and experimental manipulation. We propose that this new laryngeal model could be further developed and would be useful in laryngeal physiology research. Herein, we report our preliminary results.

MATERIALS AND METHODS

The canine larynx was used to develop this model because of its close size match to the human larynx and because the neuromuscular and vascular anatomy is appropriately sized for surgical manipulation and ease of experimentation.

Anatomic Considerations. To prepare the ex vivo larynx, the attachments of the larynx are divided in a stepwise manner until it is completely separated from the body under extracorporeal perfusion. The recurrent and external laryngeal nerves are identified first, but are not dissected off the tissue bed. The arterial and venous supply is dissected next, but is divided only after the mucosal attachments are divided. The arterial blood supply to the canine larynx comes from the superior thyroid artery, which is a branch of the common carotid artery. The venous drainage is via a hyoid venous arch that drains bilaterally into the internal jugular veins. Preservation of the hyoid venous arch is essential and requires that the pharyngeal mucosal cuts be performed at the suprahyoid level. The mucosal attachments of the larynx are divided starting with resection of the suprahyoid muscles from the hyoid bone, followed by division of the pharynx circumferentially at the level of the hyoid bone. The mucosal edges are tied with sutures before cutting; this important step prevents leakage of blood and perfusate through the mucosal cuts during the experiment. Inferiorly, the trachea is transected at the level of the sternum, allowing adequate length to place an endotracheal tube directed rostrally to provide airflow for phonation. The esophagus is divided completely at the same level, and the proximal end is suture-tied to prevent fluid leakage. After these mucosal cuts, the larynx remains attached to the body only by the blood vessels and laryngeal nerves.

The internal jugular veins are then cannulated bilaterally at a level below the larynx, and all venous attachments are divided except the connection to the larynx via the jugular veins and the hyoid arch. At this point, the 2 internal jugular vein cannulas are combined into a single cannula and the combined venous output is measured (milliliters per minute). This information is used to determine the rate of the perfusate. The arterial connections are then sequentially divided, and the procedure is repeated on each side. First, the common carotid artery is divided distal to the superior thyroid artery branch and then cannulated proximal to the superior thyroid artery. The artery is then flushed with heparinized perfusate and then connected to the perfusate bag. The procedure is repeated on the opposite side. The perfusate return is now measured again at the venous cannula, and the height of the perfusate bag is adjusted to obtain the rate of the venous return measured earlier in the in vivo state. The vascular cannulas are sutured to the external trachea to prevent inadvertent kinking of the vessels during transportation and experimental setup. Finally, the nerves are divided as proximally as possible, and the ex vivo perfused larynx is placed in the experimental apparatus (Fig 1).

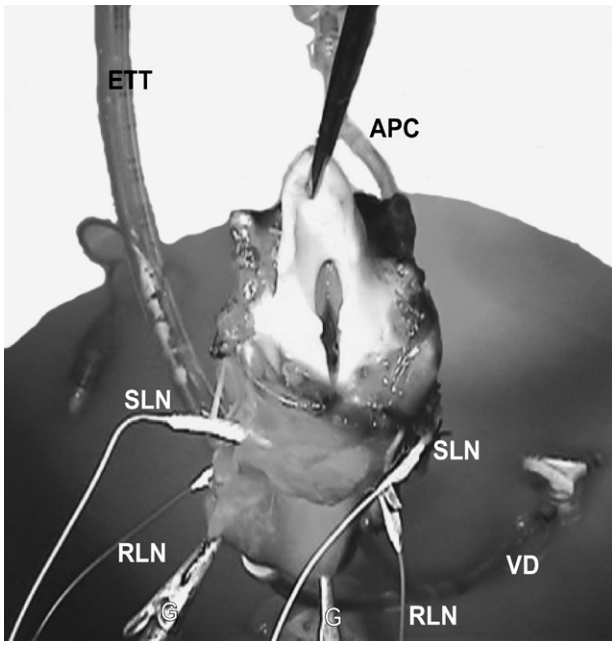

Fig 1. Ex vivo perfused larynx. ETT — endotracheal tube to provide rostral airflow; APC — arterial perfusion cannula; SLN — superior laryngeal nerve electrodes; RLN — recurrent laryngeal nerve electrodes; VD — venous drainage cannula; G — ground electrodes.

Perfusate Selection. We used a modified Krebs-Henseleit buffer solution (mmol/L: sodium chloride, 118; potassium chloride, 4.7; sodium bicarbonate, 21; calcium chloride, 2.5; magnesium sulfate, 1.2; monopotassium phosphate, 1.2; glucose, 11; and creatine, 1.0) as perfusate. It was gassed with 100% oxygen throughout the experiment and maintained at body temperature by passing the tubing through a heating chamber. Mannitol was added to a concentration of 1.25% for the second experiment to

reduce the tissue swelling that was observed in the first experiment. Dexamethasone sodium phosphate 10 mg was given to the animals 30 minutes before the onset of surgery.

Ex Vivo Stimulation, Phonation, and Videostroboscopy. An 8.0-mm cuffed endotracheal tube was placed via the distal trachea and directed rostrally to provide airflow to drive phonation. Custom-designed monopolar electrodes with silicone insulation were applied to the previously isolated superior and recurrent laryngeal nerves. The electrodes were attached to a constant-current nerve stimulator (model 2SLH, WR Medical Electronics Co, St Paul, Minnesota). The nerves were stimulated at 80 Hz with 0 to 3.0 mA for a 1.5-ms pulse duration to achieve complete adduction of the vocal folds. When the vocal folds were adducted bilaterally, airflow from a wall-mounted source drove phonation, and sound was generated. The airflow was regulated between 500 and 700 mL/min, and was heated to 37°C and humidified by passing through a heater-humidifier. The larynx was also kept moist during the experiment by intermittent wetting of the vocal folds with normal saline solution and by keeping moist gauze over the laryngeal inlet between phonation trials.

Laryngeal phonation trials were conducted as follows. Airflow was directed rostrally from the tracheal side toward the vocal folds via the endotracheal tube. A constant current stimulus causing brisk cricothyroid muscle contraction was applied to the superior laryngeal nerves. Both recurrent laryngeal nerves were then stimulated, and the current was gradually increased until stable periodic phonation was achieved. Recording of phonation continued until the phonation lost periodicity, at which point we attempted to regain periodic phonation by decreasing and then increasing the recurrent laryngeal nerve stimulating current. If no periodic phonation was achieved, the larynx was allowed to rest 10 minutes before the next phonation trial. Videostroboscopy was performed when periodic phonation was achieved, with a 0° endoscope and a videostroboscopic light source (model RLS9100, KayPENTAX, Lincoln Park, New Jersey). The phonation protocol, nerve stimulation parameters, and videostroboscopic techniques were identical to those used in in vivo canine experiments.[2]

RESULTS

Three mongrel dogs were used for this study. The first animal was used to develop the concept and to assess the length of time the neuromuscular system remained intact in the ex vivo perfused state. The other animals were used for the phonation experiments.

Excellent control of perfusion, without leakage, was achieved after suture ligation of the mucosal edges. The venous outflow rate of the larynx was 15 mL/min for dog 1 (body weight, 25.5 kg), 11 mL/min for dog 2 (body weight, 27 kg), and 12 mL/min for dog 3 (body weight, 25 kg). It was feasible to change the rate of venous outflow by changing the height of the bags holding the perfusate solution. However, a pump was used for the third experiment for more precise control of the perfusion rate. There were other perfusion-related considerations. First, to prevent clotting of the blood within the cannula and the laryngeal vessels, we used a heparin flush in the arterial cannulas before connecting them to the perfusate. Second, we anticipated that the small-caliber arterial vessels coming off at right angles to the common carotid artery and connected to the plastic perfusion cannulas could twist and kink during experimental manipulation of the larynx, and thus we suture-tied the arterial perfusion cannulas to the trachea. Thus, the entire laryngotracheal complex moved with the perfusion cannulas, and inadvertent kinking of the vessels did not occur.

The Krebs-Henseleit buffer solution alone was used for the first experiment. Brisk muscular contraction in response to both recurrent and superior laryngeal nerve stimulation was present for 4 hours, and the adductory contraction was felt to be strong as judged by subjective palpation of vocal fold adduction with a digit placed into the laryngeal inlet. However, supraglottic and postcricoid tissue edema was evident after 1 hour, and there was pitting edema after 2 hours. Edema was most evident in the pharyngeal muscles, followed by that in the postcricoid larynx. The first experiment was terminated at 4 hours, because we had achieved our goal of keeping the larynx responsive to neural stimulation, and this length of time would be adequate to perform most experiments on laryngeal physiology. After perfusion was stopped, the larynx quickly lost the ability to respond to neural stimulation.

In the second experiment, addition of mannitol to the Krebs-Henseleit perfusate solution appeared to control tissue edema better, as no gross pitting tissue edema was observed for up to 2 hours. Phonation experiments were performed with this larynx. Short bursts of periodic phonation lasting 2 to 3 seconds were achieved during each of the phonation sessions with this larynx. These bursts of periodic phonation were preceded and followed by strained and noisy phonation. A recovery period of 5 to 10 minutes was required between phonation trials. A spectral analysis of a typical phonation, achieved 1 hour after the

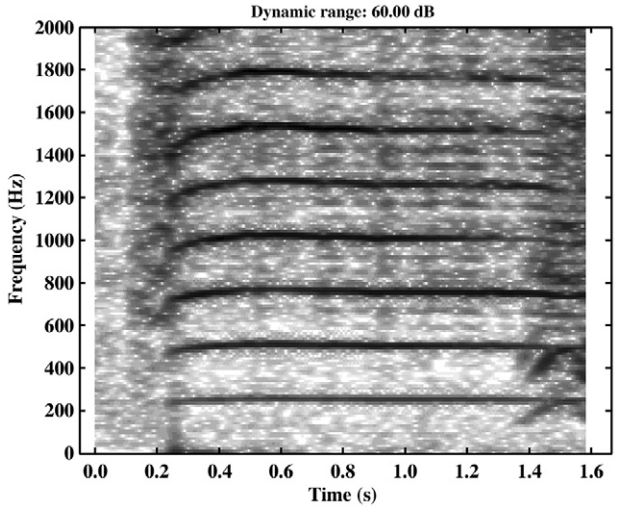

Fig 2. Spectral analysis of periodic phonation achieved with neuromuscular stimulation 1 hour after initiation of ex vivo perfusion shows fundamental frequency of 250 Hz and rich harmonic spectrum.

initiation of ex vivo perfusion, is shown in Fig 2. A fundamental frequency of approximately 250 Hz is shown, as well as a rich harmonic spectrum. The mucosal waves had complete excursion. The perceptual impression of phonation was quite similar to that in in vivo experiments. These short bursts of periodic phonation were possible for up to 2 hours, and the experiment was then terminated 30 minutes later because periodic phonation was not achieved again. It appeared that with increasing passage of time it was harder to obtain periodic phonation with each phonation trial. The muscles continued to contract in response to nerve stimulation; however, there was increasing tissue edema and the adductory force appeared weak on digital palpation, and the phonation achieved was aperiodic and noisy. After perfusion was stopped, the larynx again quickly lost its ability to respond to neural stimulation.

In the third experiment an intravenous perfusion pump was used to regulate the flow of the perfusate. Periodic phonation was possible up to 30 minutes in the ex vivo state; one phonation lasted 20 seconds. At 30 minutes, the perfusion pump malfunctioned and could not be fixed in a timely manner. This larynx lost all adductory force almost immediately. Without the perfusate, the adductory force became quite weak within 5 minutes, although cricothyroid muscle activity remained brisk for another 10 minutes. Without ex vivo perfusion, the intrinsic laryngeal muscles quickly lost their ability to contract in response to neuromuscular stimulation, and phonation was not possible.

DISCUSSION

In this preliminary study, we developed an ex vivo perfused laryngeal model to enhance the study of laryngeal physiology. In this model, the larynx is removed from the body and is kept functional by continuous perfusion with a nutrient-rich, pH-balanced physiologic solution to maintain its neuromuscular viability. We were able to achieve periodic phonation for at least 2 hours after the onset of ex vivo perfusion. Vocal fold movement with neural stimulation was possible for at least 4 hours, but we have not yet achieved phonation beyond 2 hours. However, most laryngeal experiments could be designed to occur within this time frame.

The duration of periodic phonation achieved at each trial was short and typically lasted 3 to 4 seconds, although in 1 experiment it lasted 20 seconds. However, phonation was achieved with laryngeal adduction based solely on neural stimulation and without the use of arytenoid adduction sutures or adduction clamps. Ex vivo perfusion appears to be critical to maintaining the neuromuscular contractility of the larynx. When perfusion was turned off at the end of experiments 1 and 2, there was quick cessation of all neuromuscular activity. The dramatic cessation of activity in the third larynx after the pump malfunctioned further reinforces this view.

The decreasing ability to obtain periodic phonation with time is likely due to a combination effect including tissue edema, inadequate maintenance of nutrients, tissue shock, and muscle fatigue. The choice of perfusate could perhaps be further optimized to lengthen organ viability. The perfusate should provide an appropriate buffer, an energy source, colloidal osmotic pressure, and oxygen-carrying capability. The Krebs-Henseleit buffer solution was chosen because it has a proven track record in perfusion studies in rat hearts and livers.[3] After the first experiment, the addition of 1.25% mannitol to the buffer solution appeared to adequately prevent tissue edema and allowed short periods of periodic phonation. Other oncotic agents, such as albumin and blood, could also be tested. Further studies could also be performed to provide maximal tissue oxygenation to the ex vivo perfused larynx. However, if the ability to phonate the larynx periodically is used as a criterion, then we have achieved the goal for at least 2 hours in the ex vivo state. In addition, the periodic phonation achieved is similar in acoustic quality to that achieved in in vivo canine larynx experiments.

In using the ex vivo perfused larynx to study laryngeal physiology, it seems appropriate to keep the time between the onset of ex vivo perfusion and the phonation experiments as short as possible. In our preliminary experiments, it took about 20 to 30 min-

utes to set up the larynx for phonation. This timing could be further shortened with further experience in vascular cannulation and experimental setup.

CONCLUSIONS

We have developed an ex vivo perfused laryngeal model with the canine larynx. This model is designed to allow unencumbered study of a neuromuscularly intact larynx, thus combining the benefits of both the in vivo and excised laryngeal models. Phonation was achieved for at least 2 hours with use of a modified Krebs-Henseleit reperfusion solution. Additional studies are needed to fully optimize this technique for the study of laryngeal physiology, especially in regard to the length of periodic phonation that can be achieved. If successfully developed, this model would allow unencumbered access for laryngeal imaging studies, such as digital particle imaging velocimetry and high-speed photography, to examine the effects of neuromuscular stimulation on laryngeal physiology.

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