Phonation Analysis Combined with 3D Reconstruction of the Thyroarytenoid Muscle in Aged Ovine *Ex Vivo* Larynx Models

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Summary: Objective. The aim of the study was to establish a basic data set of combined functional and anatomical measures of aged sheep larynges using *ex vivo* models. Combining these two approaches in one and the same larynx is an unmet goal so far yet is important as newer treatment strategies aim to preserve the organ structure and new assessment tools are required. Ovine larynges were used as their dimensions, and muscle fiber type distribution highly resemble the human larynx.

Study Design. *Ex vivo* animal study.

Methods. Larynges of six sheep (~9 years of age) were subjected to *ex vivo* functional phonatory experiments. Phonatory characteristics were analyzed as a function of longitudinal vocal fold (VF) prestress. Anatomical measurements of the same larynges comprised micro-computed tomography scans followed by three-dimensional (3D) reconstructions. Using specially adapted radiological scan protocols with subsequent 3D reconstruction, muscle volumes, surface areas, and anatomical measurements were computed.

Results. Increasing longitudinal prestress yielded higher subglottal pressure (P_S) for the same airflow. Quantitative differences to previous studies—such as the increased P_S and increased phonation threshold pressure—were detected. We achieved excellent visualization of the laryngeal muscles and framework, resulting in accurate 3D reconstructions for quantitative analysis. We found no significant intraindividual volume differences of the thyroarytenoid muscles.

Conclusion. The established protocol allows precise functional and anatomical measures. The data created provide a reference data set for upcoming therapeutic strategies (eg, growth factor therapy, functional electrical stimulation) that target essential structures of the VFs such as the laryngeal muscles and/or the VF mucosa.

Key Words: *Ex vivo* phonation–Laryngeal muscles–3D reconstruction–Micro-computed tomography–Presbylarynx.

INTRODUCTION

In vivo research of the larynx and especially the vocal folds (VFs) is hampered by the inaccessibility of its structures. This is even truer for research of the human larynx, as invasive procedures can often only be carried out in general anesthesia. Biopsies of the laryngeal structures (mucosa, muscles) carry the unbearable risk of long-lasting damage of these structures with a consecutive dysphonia. As a consequence, most exploration in humans is restricted to mere perception (voice analysis) and visualization.[1] The latter can only display the visible parts of the larynx. The complex microarchitecture of the VF (consisting of multilayered epithelium and the trilayered lamina propria) as well as the underlying thyroarytenoid muscle (TAM) cannot be displayed or measured in detail. This is also true for other laryngeal muscles, eg, the cricothyroid muscle, the posterior cricoarytenoid muscle, or the lateral cricoarytenoid muscle. However, these muscles play crucial roles in coordinating the sophisticated VF movements. In addition, the aforementioned muscles are very small, and their courses lie in different geometrical planes, making them difficult to display *in vivo* even with high-resolution imaging methods, such as magnetic resonance imaging (MRI) or computer tomography (CT) scans.

Newer therapeutic treatment options in the realm of laryngology will focus more on causal rather than symptomatic treatment, requiring new methods of evaluation and documentation. The consequences of these target-orientated interventions need to be studied carefully on animal models by an analysis of morphological and functional (biomechanical) changes before going into larger clinical trials.

For example, presbyphonia is such a disease where new treatment strategies appear on the horizon.[2] Presbyphonia is caused by an age-related atrophy of the laryngeal muscles—mainly the TAM—that leads to a glottal gap and consequently to a hoarse and dysphonic voice.[3,4]

Recent studies by our group using aged sheep demonstrated that by functional electrical stimulation (FES) of the recurrent laryngeal nerves, a significant change in an aged laryngeal muscle (as reflected by the mean fiber diameter of the TAM) could be achieved in a short time.[2] Aged sheep (~9 years of age) were employed because the ovine larynx proved to be a suitable model for several reasons: muscle fiber types and distribution as well as tissue histochemistry resemble the human TAM[5]; ovine VF lengths and laryngeal dimensions at the level of the glottis and

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of the subglottic space are within the range of the human larynx and were therefore deemed as one of the models for physiological studies of phonation.[6–8] The intervention left the laryngeal structures intact; still, there is a need to assess and objectify the changes elicited by the electrical stimulation. The aim of this study was to establish a protocol that combines functional (by phonatory experiments) and morphometric (by micro-CT scans and three-dimensional [3D] reconstruction) parameters of one and the same larynx in order to obtain a basic reference data set of untreated ovine larynges. A 3D reconstruction of the intrinsic laryngeal muscles of aged healthy untreated sheep should show symmetry in shape and volume, especially of the TAM, to establish an evaluation tool for future studies in which unilateral FES leads to a muscle hypertrophy of the treated muscle to reverse the aging process. A successful realization would allow studying the effects of an intervention on the whole organ level and would thus add additional outcome parameters.

MATERIALS AND METHODS

Tissue harvest and sample processing

As the animal model, we employed six female sheep of the breed merino mountain.[2,9] For animal welfare reasons, we tried to keep the number of animals as low as possible and in accordance with other studies in the field.[10–13] Perioperative management and anesthesiological procedures were carried out by the veterinarians at the Medical University of Graz. All procedures were approved by the Federal Ministry of Science, Research and Economy and complied with the institution's animal care guidelines.

For euthanasia, an intravenous injection of thiopental (50 mg/kg) followed by T61 (Merck, 10 mL i.v.) was given in deep general anesthesia. For general anesthesia, the sheep were premedicated with midazolam (0.2 mg/kg) and ketamine (5 mg/kg). Anesthesia was induced by using propofol (4 mg/kg). General anesthesia was maintained with sevoflurane in oxygen, delivered from a standard circle rebreathing system, and fentanyl in a continuous rate infusion at 20 µg/kg/h i.v. as well as propofol 2 mg/kg/h.

Directly after euthanasia, larynges were dissected and quick-frozen in liquid nitrogen. Subsequently, the specimens were stored in a freezer at −80°C. This ensured that, after thawing, the viscoelastic tissue characteristics of the larynges were maintained as similar as possible to freshly excised larynges.[14]

Phonatory experiments and analysis

The experiments were performed *ex vivo* with the cadaver sheep larynges. The larynx preparation was as follows: the day before the experiment, the larynx was slowly thawed overnight in a refrigerator.

The larynges were dissected to expose the VFs (Figure 1A). The experimental setup is based on the work of Alipour and Jaiswal.[15] The larynges were mounted on an artificial trachea with a diameter of 16 mm, dimensioned for sheep larynges, including a drilling for the subglottal pressure (P_S) sensor 130 mm below the glottis (Figure 1B). A custom-made PVC tube prevented unintentional displacement of the larynx. Five screws were used to hold the larynx in position.

Three different weights ($w_1 = 20$ g, $w_2 = 40$ g, $w_3 = 60$ g) were attached to the thyroid cartilage to induce prestress forces toward the TAM (by tilting the thyroid cartilage against the cricoid cartilage) and to simulate longitudinal tension of the VF (TAM) as described in previous studies.[16] After the weights were mounted, two iron rods were symmetrically applied to adduct the posterior part of the larynx till the point of nearly complete glottis closure, ie, approximation of the vocal processes to bring the VF in phonatory position.[17] For each larynx, up to 16 runs per prestress level w_i were performed. By slowly increasing the airflow, the phonation onset level was detected. From there, the applied airflow was successively increased in steps of 2.5 L/min or 5.0 L/min. The experiments were stopped when the larynx did not vibrate any longer (eg, L5 did not vibrate at all for prestress level w_3) or sustained vibrations of the larynx could not be achieved, ie, audible frequency jumps. This yielded altogether 253 phonatory experimental runs for further analysis. For each run, 500 ms of sustained oscillation was analyzed. All experiments were performed with left-right symmetric prestress force. The entire experimental setup is displayed in Figure 1B.

The dynamics were recorded with a high-speed camera Phantom V2511 (Vision Research Inc., New Jersey) (4,000 fps, 512 × 512 pixels). The experiment was illuminated with a high-power LED flashlight (TK15; Fenix New Energy Co., Shenzhen, China). The acoustical signal was recorded 30 cm above the larynx

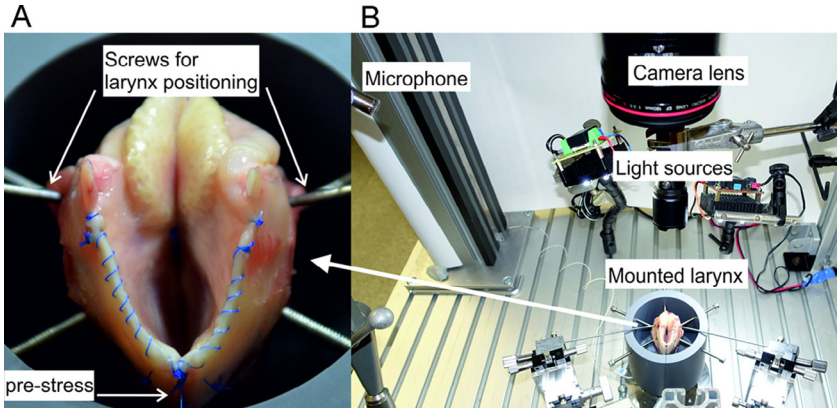

FIGURE 1. Phonatory setup with mounted sheep larynx.