Quantitative Distinction of Unique Vocal Fold Subepithelial Architectures Using Optical Coherence Tomography

Stephen Maturo, MD; Fouzi Benboujja, PhD; Caroline Boudoux, PhD; Christopher Hartnick, MD

Objectives: The primary objective of this study was to quantitatively analyze ex vivo porcine, fetal human, and adult human vocal folds by use of optical coherence tomography (OCT). A secondary objective was to quantitatively discriminate among 1-, 2-, and 3-layer lamina propria structures.

Methods: We performed an analysis of the vocal folds of 10 adult pig, 3 adult human, and 2 fetal human vocal fold specimens using OCT and histologic techniques. We present a quantitative comparison of the OCT results and histologic findings.

Results: We found that OCT allowed for the visualization of the subepithelial vocal fold architecture of all imaged tissue, and that it revealed distinct characteristic signal intensities for each type of specimen.

Conclusions: Optical coherence tomography was developed for in vivo imaging of biological microstructures. This study demonstrates the ability of OCT to differentiate between the vocal fold architectures of 3 histologically distinct types of vocal folds. Future studies aim to develop a quantitative optical imaging algorithm that can be used to facilitate an in vivo longitudinal clinical investigation of the changes that occur in this layered structure over time and maturation.

Key Words: development, maturation, pediatrics, vocal fold.

INTRODUCTION

Optical coherence tomography (OCT) is a noninvasive imaging modality that uses interferometry to produce in vivo resolution of biologic tissue to the order of 10 μm. Similar in concept to ultrasonography, OCT provides cross-sectional images without the need for tissue penetration. Optical coherence tomography has been increasingly investigated for the evaluation of the vocal fold and its subepithelial structure, as it provides for high resolution up to a depth of approximately 2 mm and allows for qualitative differentiation between normal and abnormal tissues. Optical coherence tomography provides the potential, as in vivo technology, to differentiate, without biopsy, benign from malignant lesions. In the growing field of pediatric laryngology, OCT has the potential to provide insight into the development of the vocal fold, for which the lack of adequate numbers of histologic specimens has prevented definitive answers. Pediatric voice and airway disorders have garnered increased attention over the past 20 years. There still remain significant gaps in knowledge, especially in terms of invasive vocal fold procedures, due mainly to the lack of insight into the development of the pediatric vocal fold from infancy to adulthood.

The currently established view of the structure of the human vocal fold is based on the work of Sato and Hirano describing a superficial epithelial layer with a deep trilayer lamina propria that includes superficial, middle, and deep structures. Further work by Sato and Hirano described the “cover and body” theory in which the epithelium and superficial layer of the lamina propria make up the “cover” and the middle and deep lamina propria layers and muscle make up the “body.” Their findings are based on histologic specimens on which qualitative identification of tissue layers was made by examining histologic specimens. Although the trilaminar structure of the vocal fold is widely accepted, there are still some unknowns regarding the precise delineation of each layer and how these layers develop from infancy to adulthood. Sato and Hirano demonstrated a lack of a layered structure in the newborn vocal fold. Hartnick et al demonstrated a gradual progression from a monolayer to a...
bilayer to a trilayer lamina propria structure that was present by 7 years of age. Yet, although a trilaminar organization was seen in the pre-adolescent years, the mature adult trilaminar structure, in which the elastin in the middle layer and the collagen in the deep layer align with the vocalis muscle in a parallel fashion, was not seen until the teenage years. Although these studies provided insight into the makeup of the pediatric vocal fold, they only described a qualitative analysis of the developing vocal folds, as all of these studies were based on visual inspection of histologic slide preparations. Additionally, there are currently no data describing any gender differences in the microstructure of the pediatric vocal fold. There are objective voice analysis data demonstrating certain ages at which the fundamental frequency changes in boys and girls, but the cause is still unknown. It is assumed that changes in the vocal fold length are the cause, but no one has investigated the possible differences in the layers of the vocal fold in boys and girls.

Our group has previously used optical microscopy techniques to evaluate both in vivo and ex vivo porcine and human vocal folds. These studies provided qualitative descriptions of the layers of the vocal fold and demonstrated that the transition from the laboratory to the operating room for in vivo evaluation was possible. We believed that OCT provided the optimal imaging depth, but were unsure whether it provided enough anatomic detail to discriminate between the layers of the vocal fold. It is difficult to obtain pediatric vocal fold specimens, because of the paucity of cadaveric specimens and the frequent artifacts encountered in postmortem analysis of pediatric larynges. Optical coherence tomography provides the potential for in vivo analysis of pediatric vocal fold development with limited patient risk. Previous studies have qualitatively demonstrated a proof of principle, but the ability to accurately discriminate between the layers of the lamina propria has not been demonstrated. Furthermore, most studies focus on the adult epithelial-superficial lamina junction, as this area has been shown to be of significant importance to functional voice outcome. Because of the unanswered questions of pediatric vocal fold development, along with the possible long-term effects of surgery on a child’s vocal folds, there is a need to determine the developmental characteristics of the pediatric vocal fold. The primary objective of this study was to develop a technique that could quantitatively analyze ex vivo porcine, fetal human, and adult human vocal folds by using OCT. A secondary objective was to quantitatively discriminate between 1-, 2-, and 3-layer lamina propria structures.

**METHODS**

**Specimen Preparation.** This study received Institutional Review Board approval from Massachusetts Eye and Ear Infirmary. The true vocal folds, ventricle, and false vocal folds of adult pig, adult human, and fetal human specimens were analyzed. Adult pig specimens were chosen, as they have been shown in previous studies to have a 2-layer lamina propria, whereas fetal human specimens have been shown to have a monolayer. The porcine specimens were thawed from frozen specimens, whereas the human specimens were analyzed on the day of procurement. The adult specimens were taken from total organ donors free of any laryngeal disease. The analyzed vocal fold was free of gross disease, and was confirmed free of cancer on histologic analysis; thus, only normal tissue structure was analyzed. In all specimens, the thyroid cartilage and cricoid cartilages were split vertically and the glottis was dissected free from the thyroid cartilage. Each specimen was imaged immediately after harvesting.

The true vocal fold scanning area of interest was marked with a tuberculin syringe and India ink to accurately define the area of interest on histologic processing (Fig 1). A 0.15-mm-thick coverslip was placed over the specimen, and water-soluble gel was placed on top of the coverslip. The coverslip acts as a fiducial marker for imaging depth and also provides a slight compression to the specimen that provides for a constant focal range. On OCT scanning, India ink results in a black void that is easily identifiable. Two India ink dots were placed, one anterior and one posterior, approximately 3 to 5 mm from each other. Immediately after imaging, the specimens were placed in formalin. The area between the India ink dots was processed and stained with hematoxylin and eosin along with Masson trichrome.
stain. The images were analyzed by a head and neck pathologist, and digital photos were taken.

**OCT Imaging.** The OCT imaging took place on a Thorlabs OCS 1300SS (Thorlabs, Newton, New Jersey). This OCT system uses a 55-kHz swept source laser with a center wavelength of 1,325 nm providing deep imaging up to 3 mm. Two-dimensional (2-D) and 3-dimensional (3-D) imaging capabilities are available and were bundled into a data control and acquisition software package that was installed on a personal computer. In the 2-D imaging mode, the probe beam is scanned in one direction and reflectivity images are displayed in real time. The software provides flexible control of scanning parameters, which were set at $1,024 \times 512 \times 512$ pixels with 3-mm intervals in width, length, and depth. In the 3-D imaging mode, the probe beam sequentially scans across the sample as a series of 2-D cross-sectional images are acquired and built into a
3-D image. Display of the cross-sectional images is provided as they are being acquired to build the 3-D volume data set. In this mode, the 3-D volume data set can be displayed in 1 of 3 orthogonal planes (XY, YZ, or XZ) or in a combination of the 3 planes. The user can zoom, rotate, and define the orthogonal planes in which to display. The 3-D data sets are saved as a series of 2-D cross-sectional scans.

RESULTS

Twenty porcine, 4 fetal human, and 6 adult human vocal fold specimens were analyzed. Histologic sections and OCT imaging were carried out on each specimen. Figures 2-4 demonstrate selected images of the porcine, fetal human, and human adult specimens along with specific quantitative analysis of light intensity changes of the corresponding layers.

Figure 2 shows a representative porcine evaluation. Figure 2A is the hematoxylin and eosin stain, and Fig 2B is the trichrome stain. The black areas on the edges of the histologic figures correspond to the India ink markings. The signal voids on the edges of the OCT image are also the India ink marks, which have no effect on the imaging acquisition. The epithelium and bilayer lamina propria are delineated more clearly in the trichrome stain, in which there is a darker area under the epithelium that transitions to a lighter area. The square box labeled “mark 1” on Fig 2A demonstrates a ductlike structure in the center that corresponds to the same structure as in Fig 2C on the OCT image. Corresponding layer measurements are made in which the epithelium approximates a thickness of 50 μm and the subsequent layers each have a depth of approximately 200 μm. Figure 2D demonstrates the change in light intensity at increasing depths of the specimen.

Figure 3 shows a representative fetal evaluation. Figure 3A, the trichrome stain, demonstrates a monolayer subepithelial structure, and Fig 3B is an OCT image. As with the histologic images, there is no striking change in the light intensity as one progresses deep from epithelium. This finding is reflected in C, as there is a constant slope of light intensity.

Figure 4 shows a representative adult human specimen. Figure 4A is the hematoxylin and eosin
stain. The trilayer lamina propria is delineated. Figure 4C demonstrates the change in light intensity at increasing depths of the specimen. The individual slopes correspond to the light intensity changes at each subepithelial layer.

When we compared all porcine specimens' change in light intensity graphs (OCT graphs), there was high variability between specimens, but overall, there was a characteristic signal that was different from that of the human fetus and human adult specimens. There was also high variability among the human adult specimens.

DISCUSSION

Similar to other published results of OCT, we demonstrate in this report the ability to visualize the subepithelial vocal fold architecture of different laryngeal tissues. The unique aspect of our study is the attempt to develop a method to quantitatively differentiate the layers of the lamina propria in 3 distinct tissue specimen types on the basis of changes in light intensity. Previous reports evaluating adult vocal folds have attempted to delineate the interface between the epithelium and the superficial layer of the lamina propria. Kaiser et al reported the mean epithelial thickness of adult glottal structures, but there was no report on the depths of the lamina layers. Identifying the epithelium and the superficial layer of the lamina is important, as most adult vocal fold damage occurs at this level, but it does not provide a quantitative analysis of the levels of the lamina propria. A more detailed evaluation of the deeper levels, along with a quantitative analysis, is necessary to determine the structural development of the pediatric vocal fold and the changes that occur as a child matures. Ridgway et al reported on in vivo OCT analysis of pediatric airway struc-
tures, but this, again, was a qualitative analysis focusing on the delineation of the epithelium, lamina propria, muscular layers, and underlying cartilage, rather than a sub-analysis of the layers of the lamina propria.

Our OCT evaluation and postimaging process technique suggests the possibility of quantitatively analyzing the layers of the lamina propria in vocal folds that have been previously histologically confirmed to have a set amount of layers. We found that although each corresponding specimen class (i.e., pig, fetal, and adult) had variable signal characteristics, there was a distinct difference in light intensity characteristics between classes. This intraspecimen variability is not surprising, as each specific vocal fold is not uniform in terms of its tissue content throughout its entire length, as evidenced by visual inspection of histologic specimens. It should be stressed, however, that we are not commenting on the type of tissue present in the distinct layers. Our use of optical intensity to help discriminate among 1-, 2-, and 3-layer structures is just one variable of OCT technology. Histologic analysis suggests that the difference in the lamina propria layers in the adult human vocal fold is due to increasing amounts of collagen. Our purpose in this study was not to correlate the light intensity characteristics of the various layers, although Burns et al. have suggested that polarization-sensitive OCT may be a technology that can help identify more definitively the subepithelial layers on the basis of collagen fiber orientation.

Recently, we demonstrated that boys and girls undergo significant changes in voice fundamental frequency at certain times in their development. In boys, this occurs around 12 and 16 years of age; in girls, this occurs at around 11 and 14 years of age. This baseline pediatric acoustic database provides objective data demonstrating functional changes over time. We believe that the next step in determining why these changes occur is to attempt to correlate anatomic and subepithelial developments. To determine this, our group is in the process of measuring in vivo vocal fold length in boys and girls over a wide age range. It is our hope that analyzing changes in length along with changes in the subepithelial layered structure seen on OCT imaging may provide for a multivariate analysis that can correlate with the objective functional acoustic data.

There are some weaknesses of this study that are not uncommon in comparisons of histologic and noninvasive imaging techniques. Tissue preparation is subject to sectioning and staining artifacts. Furthermore, ex vivo imaging may result in OCT distortion that may not be visualized on in vivo imaging; we did try to control for this by implementing a technique in which we matched the India ink dots on the OCT images and the histologic specimens. Our ex vivo imaging avoids direct contact, whereas intraoperative probes may require direct contact, thus changing the tissue characteristics. Finally, it is difficult to ascertain the changes in vocal fold structure that occur after patients have died. Also, along these lines, there may be alterations in tissue structure after thawing of the porcine specimens. Finally, no sample size analysis was performed before this study was organized. Because of the inherent difficulty in obtaining an appropriate number of specimens, especially fetal tissue, and our goal of technique development, we decided that our number of representative samples was adequate on the basis of previous OCT reports in the otolaryngology literature. Although the sample size is small, our results suggest that OCT can be used to help differentiate the subepithelial layers of the lamina propria. An ideal next step would be to evaluate healthy pediatric and adult vocal folds in vivo and compare their optical qualities.

Optical coherence tomography provides a noninvasive means of analyzing the subepithelial architecture of the vocal fold. In the future, OCT may be the optimal technology for determining the development of the pediatric vocal fold. Many reports in the otolaryngology literature focus on OCT’s theoretical ability to evaluate benign versus malignant lesions in adults. One drawback that has been identified is OCT’s inability to evaluate bulky lesions, as its depth of penetration is not adequate. Some have suggested that OCT’s greatest utility is in evaluating thin lesions at the periphery of tumors. Given that OCT’s application in pediatrics is unlikely to involve malignant tumors, this technology may be optimally used in defining normal subepithelial characteristics in vivo. Currently, it is unclear why children have changes in their voice as they develop. There are specific time points at which both boys and girls experience decreases in their vocal fundamental frequency. Changes in vocal fold length that occur as a child matures have been accepted by many as the most plausible explanation, but significantly less thought has been given to changes in subepithelial vocal fold composition. It is our hope that future OCT advances may be able to correlate tissue changes with objective computerized vocal analysis.

Besides having the advantage of helping to elucidate the development of the pediatric vocal fold, OCT technology may also be able to help in determining whether operative intervention is needed in a child with dysphonia. Optical coherence tomogra-
phy may provide the ability to determine in real time the depth of a vocal fold lesion and provide for precise excision of a lesion, thus avoiding the potential for postoperative scarring. We believe that this study will help in developing additional techniques and in vivo technology to identify subepithelial changes of the human vocal fold in pathologic processes and aid in determining the changes of the maturing pediatric vocal fold as it develops from a monolayer to a trilayer structure. Moving forward, the goal is to develop an accurate in vivo probe that can evaluate the vocal fold subepithelial architecture in children of all ages. With this knowledge, we would hope to elucidate the timing of surgical intervention for children with vocal fold disorders resulting in poor voice.

REFERENCES


