Improving the Utility of Laryngeal Adductor Reflex Testing: A Translational Tale of Mice and Men

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Abstract

Objectives. Evaluation of the laryngeal adductor reflex (LAR) entails delivering air through an endoscope positioned 1 to 2 mm from the arytenoid mucosa to elicit bilateral vocal fold (VF) closure. This short working distance limits visualization to only the ipsilateral arytenoid and results in quantification of a single LAR metric: threshold pressure that evokes the LAR. Our goal was to evolve the LAR procedure to optimize its utility in clinical practice and translational research.

Study Design. Prospective translational experiment.

Setting. Academic institution.

Subjects. Young healthy human adults (n = 13) and 3 groups of mice: healthy, primary aging mice (n = 5), a transgenic mouse model of amyotrophic lateral sclerosis (ALS; n = 4), and young healthy controls (n = 10).

Methods. The VFs were visualized bilaterally during supra-maximal air stimulation through an endoscope. Responses were analyzed to quantify 4 novel metrics: VF adduction phase duration, complete glottic closure duration, VF abduction phase duration, and total LAR duration.

Results. The 4 LAR metrics are remarkably similar between healthy young humans and mice. Compared to control mice, aging mice have shorter glottic closure durations, whereas ALS-affected mice have shorter VF abduction phase durations.

Conclusions. We have established a new LAR protocol that permits quantification of novel LAR metrics that are translatable between mice and humans. Using this protocol, we showed that VF adduction is impaired in primary aging mice, whereas VF abduction is impaired in ALS-affected mice. These preliminary findings highlight the enhanced diagnostic potential of LAR testing.

Keywords

laryngeal adductor reflex, sensory testing, laryngeal reflex, aspiration, dysphagia, laryngoscopy, mouse models, amyotrophic lateral sclerosis

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The laryngeal adductor reflex (LAR) entails brief bilateral closure of the true vocal folds (VFs) in response to mechanical or chemical stimulation of the laryngeal mucosa.1,2 This defensive airway reflex provides protection from material inappropriately entering the lungs. It is therefore no surprise that LAR impairment correlates with pharyngeal dysphagia and aspiration,3,4 which are highly correlated with morbidity and mortality.5–11

The clinical procedure for evaluating the LAR is air-pulse laryngopharyngeal sensory testing, performed as part of a flexible endoscopic evaluation of swallowing. The current procedure entails transnasal passage of a flexible laryngoscope, typically without topical anesthesia, to deliver air pulses to the arytenoid mucosa.12,13 An air-pulse device (AP-4000; Vision Sciences, Pentax, and Medtronic) is used to generate pulses of air between 50-millisecond to 1-second durations at incrementally increasing pressures ranging from approximately 2 to 10 mm Hg. Air pulses are delivered through the endoscope working channel or side channel sheath, with the endoscope tip positioned 1 to 2 mm away from the arytenoid mucosa. This extremely short working
distance limits visualization to only the ipsilateral arytenoid, which restricts quantification to a single metric: threshold air pressure that evokes ipsilateral arytenoid medialization, determined separately for each side. Requiring pressures >4 mm Hg to evoke a response or having an asymmetric or absent response is suggestive of sensory pathology, although it is not pathognomonic for any specific disease process.3

LAR impairment has been identified in numerous conditions, such as Parkinson’s disease,14 cerebrovascular accident,15 chronic cough,16,17 adductor spasmodic dysphonia,18 and acid reflux disease.16 In amyotrophic lateral sclerosis (ALS), 54% of cases have elevated sensory thresholds (>4 mm Hg), which suggests that LAR impairment may be a major contributing factor of aspiration pneumonia,19 a leading cause of death in this disease.20 LAR impairment has also been identified in healthy aging individuals, with progressive increases in pressure thresholds occurring each decade of life.21 This finding corresponds with increased incidence of silent aspiration in healthy older individuals, placing them at risk for aspiration pneumonia.22-24 Despite the negative outcomes associated with LAR impairment, effective treatments are lacking, most likely due to limitations of the clinical procedure to evaluate the LAR and the limited scientific knowledge of underlying pathological mechanisms.

We propose that LAR testing has been markedly underutilized by focusing solely on air pressure thresholds, which have known limitations of false-positive results and poor interobserver reliability between endoscopists.25 We hypothesized that increasing the endoscopic field of view during LAR testing would provide additional metrics to improve the usefulness of LAR testing in clinical practice and research. Most important, we recognize that investigations of the underlying neural mechanisms involved in the LAR are extremely difficult, if not impossible, to conduct in humans because of the invasive nature of experimental procedures. We have therefore begun investigating the LAR in laboratory mice, which are widely recognized as the premier animal model for studying human biology and disease.26,27 Numerous mouse models of human diseases associated with LAR impairment currently exist; however, LAR studies have not yet been conducted in this species. Examples include the SOD1-G93A transgenic mouse (ie, the most widely studied and best characterized mouse model of ALS28) and healthy aging C57BL/6 mice, commonly called B6 mice (ie, the most popular laboratory rodent for biomedical research26,27). We have been studying the swallow function of these mouse models for several years and have shown that they do indeed develop dysphagia similar to humans with these conditions.29-31 Given the strong association between dysphagia and LAR impairment in humans,3,4 as well as published reports of LAR impairment in human ALS19 and primary aging,21 we hypothesized that SOD1-G93A transgenic mice and healthy aging B6 mice would also develop LAR impairment.

The goal of this study was to identify novel metrics for quantifying the LAR in humans and mice to facilitate translational research efforts between these species. A secondary goal was to test the utility of our novel metrics in detecting LAR impairment in ALS-affected mice and primary aging B6 mice, thereby laying the foundation for subsequent preclinical experiments aimed at improving LAR dysfunction for these conditions. We initially planned to use an AP-4000 device for air-pulse delivery. However, this device is no longer commercially available, and we were unable to obtain a working model. Therefore, we designed and constructed a prototype air-pulse system. We developed protocols to evoke, visualize, and quantify bilateral LAR responses in mice and humans. This accomplishment in mice required innovative techniques, given the magnitude of the size obstacle to overcome between the mouse and human larynx (Figure 1). This article focuses on the test protocol and resultant novel LAR metrics rather than the development of the air-pulse device, which will be described in detail elsewhere.

**Methods**

**Human LAR Test Procedure**

The human LAR protocol was approved by the University of Missouri Institutional Review Board. Twenty healthy nonsmoking human subjects (7 men and 13 women) aged 20 to 40 years were recruited and tested between May and October 2014. Exclusionary criteria included the following self-reported medical conditions: laryngeal pathology, neurologic conditions, heart disease, gastroesophageal reflux disease, recent or current upper respiratory symptoms, and current use of anticoagulant medications. Informed consent was obtained by the principal investigator.
The LAR test procedure entailed nasal administration of aerosolized oxymetazoline, followed by transnasal passage of a flexible 3.7-mm outer-diameter endoscope with a 1.5-mm inner-diameter working channel (11302BD2, Karl Storz). The endoscope interfaced with a Storz Tele Pack X system to provide real-time visualization and digital recordings. The endoscope tip was positioned at a typical level for viewing laryngeal pathology to permit visualization of the bilateral VFs throughout the procedure (Figure 2). Polyethylene (PE) tubing (1.22-mm outer diameter, 0.76-mm inner diameter, cut into uniform lengths of 100 cm) was inserted through the working channel of the endoscope and advanced until the distal tip was visible in the endoscopic field of view. The proximal end of the PE tubing was connected to the air-pulse delivery system via a 22-gauge blunt-tip Luer-lock needle. The purpose of the PE tubing was to amplify the pressure of each air pulse to enable supramaximal air-pulse stimulation of the arytenoid mucosa from a greater working distance. The tubing could also be advanced closer to the arytenoid mucosa as needed, without altering the field of view. Air pulses were delivered to the left arytenoid mucosa at a rate of approximately 1 pulse every 10 seconds until 5 to 10 bilateral LAR responses were elicited. The entire procedure was digitally recorded at 30 frames per second and stored as MPEG-4 video files.

Bilateral LAR responses were evoked and recorded from 13 subjects (6 men and 7 women). As this was a research study on healthy volunteers and did not directly benefit the subjects, any discomfort resulted in termination of the procedure. This was the case for the remaining 7 subjects who experienced discomfort with passage of the endoscope.

Mouse LAR Test Procedure

The LAR protocol for mice was approved by the University of Missouri Animal Care and Use Committee. Three strains of mice from established colonies at the University of Missouri were included in this study: transgenic SOD1-G93A mice on a C57/SJL background, nontransgenic C57/SJL mice, and wild-type C57BL/6 (or B6) mice. All mice were tested only once. Transgenic mice were tested after reaching disease onset, between 4 and 8 months of age. Nontransgenic littermates were age matched with transgenic mice (ie, between 4 and 8 months old). B6 mice were divided into 2 age groups: young (4-8 months) and old (12-18 months). Young B6 mice and nontransgenic SOD1-G93A mice served as a combined healthy control group for comparison with transgenic SOD1-G93A and aging B6 mice. Approximately 50 mice were utilized in this study, the majority for design and development of our air-pulse system and LAR protocol rather than data collection.

Unlike humans, mice are unable to tolerate laryngoscopy without sedation. Therefore, we administered subcutaneous injections of ketamine/xylazine/acepromazine dosed at 80/10/2 mg/kg. This anesthesia regimen resulted in a rapid induction time (<10 minutes) that persisted for ~1 hour on room air with extinguished deep pain reflexes (eg, pedal withdrawal reflex). Anesthetized mice were tested in dorsal recumbency on a custom test platform after an overnight food restriction (6-12 hours) to prevent gastric reflux. Eyes were lubricated to prevent drying. The head was immobilized in ear bars secured to the test platform. Core body temperature was maintained at 37.0 ± 0.2°C using a rectal thermocoupler heating pad system. A schematic of the LAR test environment is shown in Figure 3.

Laryngoscopy was performed using a 0° sialendoscope (R11573A; Karl Storz) with a 1.1-mm outer-diameter fiberoptic shaft and 0.5-mm inner-diameter working channel. The working channel was connected to the air-pulse system via PE tubing (1.58-mm inner diameter). A customized miniature laryngoscope was attached to the endoscope base and secured to a manual control micromanipulator. The tongue was pulled outward with a gentle finger grip, and the endoscope was inserted transorally (Figure 4) and positioned so that the VFs filled the entire field of view. Supramaximal air pulses were delivered to the interarytenoid area approximately once every 10 breaths for up to 20 minutes. The
procedure was digitally recorded at 30 frames per second using a Storz Tele Pack X system. Upon procedure completion, mice were given 1 subcutaneous injection of Banamine (2.2 mg/kg) for inflammation and pain prophylaxis and transferred to a 37°C heating pad until complete emergence from anesthesia. Only mice with bilateral LAR responses were included in data analysis: healthy control mice (n = 10), aging B6 mice (n = 5), and ALS-affected mice (ie, transgenic SOD1-G93A; n = 4).

**Video Analysis of Bilateral LAR Responses**

Videos were analyzed on a computer using video editing software (Pinnacle Studio 14; Pinnacle Systems Inc). Using frame-by-frame analysis methods, we identified 4 novel LAR metrics: VF adduction phase duration, complete glottic closure duration, VF abduction phase duration, and total LAR duration. Our operational definitions are described in Table 1. Quantification of these metrics required identification of 4 distinct events within each bilateral LAR response: (1) rest frame preceding the first frame of VF adduction, (2) first frame of complete glottic closure (ie, absent or smallest glottal gap), (3) final frame of complete glottic closure (ie, frame preceding glottal gap reemergence at the onset of VF abduction), and (4) final frame of VF abduction (ie, maximum glottal gap). Various combinations of paired comparisons of the 4 LAR events corresponded to the 4 novel LAR metrics of interest to this study. For example, the duration of time (milliseconds) between events 1 and 2 corresponded to VF adduction phase duration; events 2 and 3, to complete glottal closure duration; events 3 and 4, to VF abduction phase duration; and events 1 and 4, to total LAR duration, as shown in Figure 5.

All videos were initially viewed by the first author (L.A.S.) to identify and analyze up to 10 episodes of bilateral LAR responses for each subject. A second reviewer (T.E.L., project principal investigator) then independently reanalyzed each response in a blinded fashion, using only the LAR starting frames identified by the first reviewer. All value discrepancies were subjected to group consensus to resolve reviewer error.

**Statistical Analysis**

Statistical analysis entailed independent t tests for group comparisons of the means for each of the 4 LAR metrics.

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**Table 1. Operational Definitions of Novel LAR Metrics.**

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<tr>
<th>LAR Metrics</th>
<th>Operational Definitions</th>
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<tr>
<td>VF adduction phase duration</td>
<td>The duration of time that it takes the VFs to adduct during a bilateral LAR response. The start frame is the “rest frame” that immediately precedes VF adduction after delivery of air pulse stimulation. The end frame is when the VFs approximate along the entire medial edge. In cases of incomplete VF medialization, the frame of maximal medialization (ie, smallest glottal gap) serves as the end frame.</td>
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<tr>
<td>Complete glottic closure duration</td>
<td>The duration of time that the VFs remain approximated along the entire medial edge during a bilateral LAR response. The start frame is identical to the end frame described above for VF adduction phase duration (ie, when the VFs approximate along the entire medial edge; smallest glottal gap). The end frame is 1 frame preceding the emergence of a glottal gap between the medial edges of the VFs.</td>
</tr>
<tr>
<td>VF abduction phase duration</td>
<td>The duration of time that it takes the VFs to abduct during a bilateral LAR response. The start frame is identical to the end frame described above for glottic closure duration (ie, one frame preceding VF abduction). The end frame is when the VFs reach maximum abduction prior to resuming the next rest breathing cycle. In cases of VF abduction phase hesitation for more than 3 consecutive frames, followed by additional abduction, the end frame is the first frame of VF abduction hesitation.</td>
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<tr>
<td>Total LAR duration</td>
<td>The duration of time between the VF adduction phase start frame and the VF abduction phase end frame.</td>
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Abbreviations: LAR, laryngeal adductor reflex; VF, vocal fold.

*aThe number of frames between the start and end frames for each LAR metric is divided by 30 frames per second and converted to milliseconds.*
Two-sided tests were performed with IBM SPSS Statistics 21. A P value <.05 defined statistical significance.

Results

Young Healthy Humans and Mice

Multiple bilateral LAR responses (n = 3-10) were elicited from each young human subject who tolerated nasolaryngoscopy (n = 13; 6 men and 7 women) and from young control mice (n = 10; 5 males and 5 females). The timing of the 4 metrics resulted in a stair-step pattern that is remarkably similar between both species (Figure 6). There were no significant differences identified between mice and humans for each LAR metric.

Mouse Models

Figure 7 shows comparisons among the 3 groups of mice: healthy controls (n = 10), aging B6 (n = 5), and ALS affected (n = 4). Three to 10 bilateral responses were elicited from each mouse. Of the 4 LAR metrics, only glottic closure duration was significantly different between young and old B6 mice (T = 2.37, P = .037), which was shorter for old B6 mice compared to young controls. Mean VF adduction phase duration was noticeably longer for aging B6 mice compared to controls; however, statistical significance was not reached (T = −1.90, P = .079). Comparisons between controls and ALS-affected mice revealed a significant difference for VF abduction phase duration (T = 3.86, P = .002), which was shorter for ALS-affected mice. Mean LAR duration was shorter for ALS-affected mice compared to controls; however, statistical significance was not achieved (T = 1.97, P = .072).

Discussion

The primary goal of this study was to improve the clinical utility of LAR testing, which is currently limited to quantification of a single LAR metric: threshold air pressure that evokes the LAR.12,13 We accomplished this goal by making 2 major modifications to the LAR test protocol: (1) the working distance was increased by positioning the endoscope tip more rostrally to visualize the bilateral VFs during the entire procedure; (2) air pulses were delivered to the arytenoid mucosa through small-diameter PE tubing inserted through the endoscope working channel, which produced higher air pressures to compensate for the larger working distance. These simple modifications enabled quantification of 4 novel LAR metrics: VF adduction phase duration, glottic closure duration, VF abduction duration, and total LAR duration. Using this modified protocol, we successfully evoked bilateral LAR responses and quantified the 4 new LAR metrics in 13 healthy young adults, thus demonstrating that quantification of LAR metrics beyond threshold air pressure levels is indeed possible.

A second goal was to adapt LAR testing for use with mice to facilitate translational research efforts in laryngology. Using a modified sialendoscope and customized test
platform, we successfully evoked bilateral LAR responses in 10 healthy control mice between 4 and 8 months of age, thus providing the first reported evidence that this airway protective reflex is conserved in this small rodent. LAR metrics were readily quantified for each mouse for comparison with our human data. Results showed that LAR profiles were remarkably similar between young healthy mice and humans. This preliminary finding suggests that mice are well suited for translational LAR research.

We tested the diagnostic utility of our LAR protocol by comparing the normative LAR metric values obtained from control mice to 2 mouse models: the B6 model of primary aging and the SOD1-G93A transgenic mouse model of ALS. Compared to controls, glottic closure duration was significantly shorter for aging B6 mice, and VF abduction duration was significantly shorter for aging B6 mice, and VF abduction duration was significantly shorter for aging B6 mice, and VF abduction duration was significantly shorter for aging B6 mice, and VF abduction duration was significantly shorter for ALS-affected mice. The shorter glottic closure duration in aging mice corresponds with glottal incompetence associated with advanced aging in humans, which results in increased episodes of laryngeal penetration and aspiration.22-24 The novel finding of VF incompetence associated with advanced aging in mice corresponds with glottic closure duration in aging mice.

Compared to controls, aging B6 mice and amyotrophic lateral sclerosis–affected mice demonstrate significantly short durations of glottic closure and vocal fold (VF) abduction, respectively. *P < .05. Error bars: ±1 standard error of the mean.

**Figure 7.** Laryngeal adductor reflex (LAR) impairment. Compared to controls, aging B6 mice and amyotrophic lateral sclerosis–affected mice demonstrate significantly short durations of glottic closure and vocal fold (VF) abduction, respectively. *P < .05. Error bars: ±1 standard error of the mean.

rationale for further investigations of LAR impairment in aging and ALS-affected mice, as well as other mouse models of human diseases that are specifically associated with compromised airway protection and dysphagia.

As with any new methodology, areas for improvement have been identified. For example, we limited our analysis of murine LAR to only mice with bilateral responses. Unilateral (ie, asymmetric) responses were frequently noted in ALS-affected mice, which corresponds with a study showing asymmetric LAR threshold values in 75% of patients with ALS.19 We also noted several cases of absent LAR responses in ALS-affected mice. However, we were uncertain if the unilateral and absent responses were due to ALS pathology or a limitation of our LAR technique. To elucidate this distinction, we modified our protocol to also include tactile stimulation of the laryngeal mucosa, which we found produces strong bilateral responses in control mice. Our future studies will quantify asymmetric and absent responses in mouse models for correlation with findings in humans.

Another limitation entailed delivering air pulses only to the left arytenoid mucosa of human subjects to establish proof of concept. However, future studies should include testing of left and right sides in conditions of health and disease to detect asymmetry of LAR responses. Additionally, only supramaximal air-pulse stimulation was used to evoke LAR responses in both species. Our future studies with larger sample sizes will investigate the effects of systematically varying air pressure levels and air-pulse durations while synchronizing air-pulse delivery to different phases of the respiratory cycle. We also expect to find additional LAR metrics that may be of diagnostic value. For instance, the time from air-pulse delivery to the start of the LAR response (ie, LAR onset latency) may facilitate detection of subtle VF adductor pathology. Ultimately, we foresee these novel LAR metrics serving as functional biomarkers to quantify treatment outcomes in preclinical trials with mice and clinical trials with patient populations. Using a higher frame rate camera (eg, ≥60 frames per second) would likely improve the precision of LAR analysis, which would open the possibility of automated LAR quantification. Finally, investigating the underlying pathologic mechanisms of LAR impairment could be achieved by combining our LAR test protocol with brainstem-evoked potential testing in mouse models of human diseases. We have already begun working toward this long-term goal. We expect that this work will lay the foundation for subsequent experiments and possible treatments aimed at improving LAR dysfunction in numerous disease conditions.

While only in its preliminary stages, our research showcases the rapid scientific advances that can be achieved by innovative researchers perfectly poised between the bench and clinical medicine. Moreover, we have experienced firsthand that overcoming barriers to technology opens new and exciting avenues of scientific and therapeutic exploration and discovery. A prime example is the recent miniaturization of endoscopes—namely, the sialendoscope, which
allowed us to identify and quantify novel LAR metrics previously impossible to visualize in small rodents. Another example is the unavailability of commercial air-pulse devices, which have recently been rendered obsolete in clinical practice. Our attempts to fill this technology gap resulted in the design and construction of an air-pulse system specifically tailored to our translational research goals with mice and humans. Our new system has overcome numerous limitations of preexisting air-pulse devices. We are currently optimizing this system and LAR test protocol for maximum diagnostic value in clinical practice.

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Teresa E. Lever, conception and design, data acquisition and analysis, article drafting, revision and final approval of submitted version; Leslie A. Farmer, data acquisition and analysis, article drafting, revision and final approval of submitted version; Brandon C. Gallemore, data acquisition and analysis, article drafting, revision and final approval of submitted version; Cameron J. Hinkel, design, data acquisition and analysis, article drafting, revision and final approval of submitted version; Marlena M. Szewczyk, data acquisition and analysis, article drafting, revision and final approval of submitted version; Mitchell J. Allen, data acquisition, article drafting, revision and final approval of submitted version; Lori A. Thombs, statistical analysis, article drafting, revision and final approval of submitted version.

Disclosures
Competing interests: Teresa E. Lever, patent application 15UMC008prov; Lever Scientific LLC, CEO of start-up company (not related to the technology described in this manuscript); Cameron J. Hinkel patent application 15UMC008prov.
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