The Laryngoscope

Functional Electrical Stimulation Leads to Increased Volume of the Aged Thyroarytenoid Muscle

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Objectives/Hypothesis: To reverse sarcopenia and increase the volumes of atrophied laryngeal muscles by functional electrical stimulation (FES) using a minimal invasive surgical procedure in an aged ovine model.

Study Design: Prospective animal study.

Methods: A stimulation electrode was placed unilaterally near the terminal adduction branch of the recurrent laryngeal nerve (RLN) adjacent to the right cricothyroid joint. The electrode was connected to an implant located subcutaneously at the neck region. Predesigned training patterns were automatically delivered by a bidirectional radio frequency link using a programming device and were repeated automatically by the implant every other day over 11 weeks in the awake animal. Outcome parameters comprised volumetric measurements based on three-dimensional reconstructions of the entire thyroarytenoid muscle (TAM), as well as gene expression analyses.

Results: We found significant increases of the volumes of the stimulated TAM of 11% and the TAM diameter at the midmembranous parts of the vocal folds of nearly 40%. Based on gene expression, we did not detect a shift of muscle fiber composition.

Conclusions: FES of the terminal branches of the RLN is a secure and effective way to reverse the effects of age-related TAM atrophy and to increase volumes of atrophied muscles.

Key Words: Aged larynx, functional electrical stimulation, glottal gap, vocal fold atrophy.

Level of Evidence: NA

***Laryngoscope*, 128:2852–2857, 2018**

INTRODUCTION

The percentage of elderly people has been steadily increasing during the last decades in most Western societies and large parts of Asia. This is accompanied by a steady increase of age-related diseases. These changes do not spare the larynx.[1] Although the term *presbylarynx* denotes the typical age-related morphological changes observed, presbyphonia delineates age-related voice changes that comprise a hoarse and breathy voice and significantly reduced vocal capacity. Implications of these vocal changes may lead to a reduced quality of life with social withdrawal.[2] Up to 30% of elderly people complain about some disorder of the voice, and most of these individuals have a chronic problem.[3] Weakening of the voice has long been neglected in healthcare, but gained consideration more recently, as vocal endurance is required in many professions as the importance of preserved voice quality is recognized. Vocal changes result from a number of alterations including changes of the larynx itself, such as calcification of the cartilaginous structures and joints, increased collagen content of laryngeal mucosa and muscular atrophy, but also from decreased pulmonary function, reduced mucosal secretions, and diminished neuromotor control.[4] A large retrospective study identified vocal fold (VF) atrophy as the most prevalent finding.[5] The noticeable glottal gap and VF bowing are the most prominent videolaryngoscopic findings in these patients and are related to the atrophy of the thyroarytenoid muscle (TAM).[6] This glottal gap is the reason for air loss during phonation leading to a breathy voice. Current treatment builds on conservative speech therapy and surgery of the larynx (phonosurgery). Although the first is time and money consuming, the latter is often refused by the patients themselves, as surgery under general anesthesia is accompanied with an increased risk of complications.

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This work was supported by the Austrian Research Promotion Agency, fund no. 848458.

The authors have no other funding, financial relationships, or conflicts of interest to disclose.

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DOI: 10.1002/lary.27342

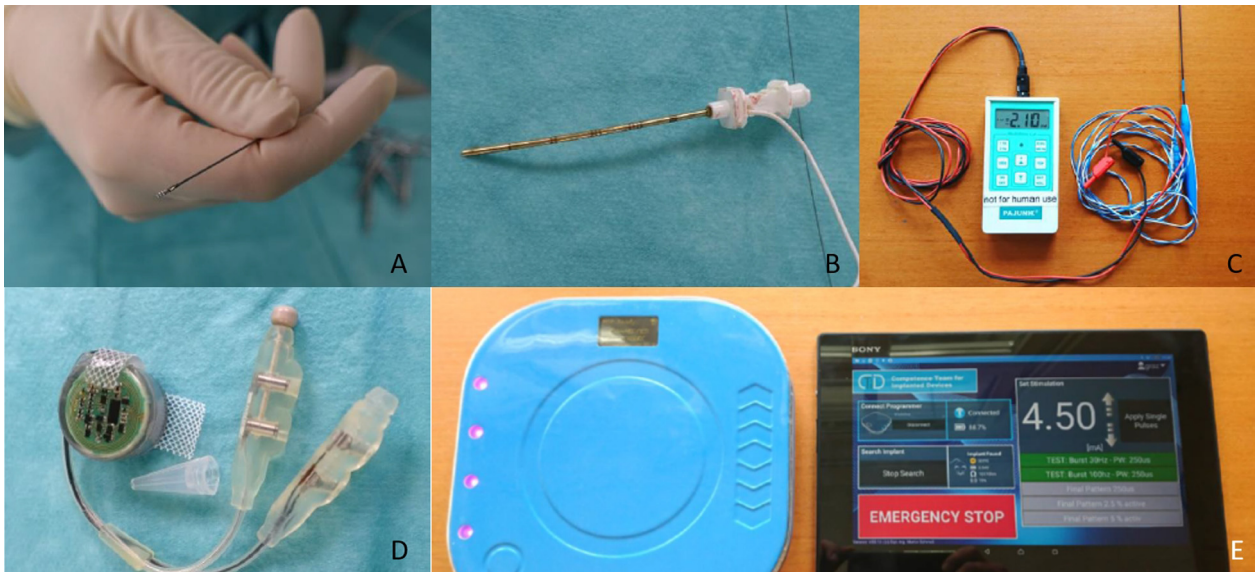

Fig. 1. Technical equipment used. (A) Stimulation electrode. (B) Hollow needle. (C) Stimulator. (D) Implant. (E) Programming device.

Phonosurgical procedures comprise injection laryngoplasties and thyroplasties, and aim to medialize one or both VFs to close the gap.[7] However, both procedures modify either the laryngeal skeleton (thyroplasty) or need to be repeated in case of injection laryngoplasties. It is fair to mention, however, that neither of them provides symptomatic treatment of the underlying pathology.

In a previous trial using an ovine model, we showed that functional electrical stimulation (FES) of the recurrent laryngeal nerve (RLN) led to an increase of the muscle fiber diameters using a very conservative pattern of electrical stimulation.[8] Based on these preliminary results and a recently published method of three-dimensional (3D) reconstructions of the laryngeal muscles,[9] we sought to apply FES in a similar cohort of animals with a minimally invasive surgical approach that is far more clinically orientated. In this trial we implanted the tip of the stimulation electrode near the terminal branch of the RLN innervating the TAM. We hypothesized that FES, by targeting the terminal adduction fibers of the RLN only, can lead to favorable results in terms of increased muscle volume. This surgically easy accessible approach is significant in that it may have future therapeutic potential for human applications.

MATERIALS AND METHODS

Surgical Procedure and Training

Our cohort consisted of 12 female sheep aged 9 years. Given an average life expectancy of 11 years and in accordance with similar studies, these sheep can be considered as old.[10] In general anesthesia, the skin was incised on the right-hand side and the inferior margin of the thyroid cartilage was exposed. The tip of the stimulation electrode (Fig. 1A) was inserted using a hollow-needle (Fig. 1B) that served as a searching probe while being connected to a stimulator (Fig. 1C). After identification of the "hot spot" (i.e., the spot where a unilateral palpable muscular contraction of TAM was elicited), the electrode was screwed in that position. We sought to place the electrode near the right cricothyroid joint, as the terminal branch of the RLN constantly passes this structure (Fig. 2). The position of the tip was visualized in the 3D reconstruction as shown in Figure 3. All technical equipment required for the implantation surgery was purchased at Osypka Inc. (Berlin, Germany). The electrode was connected to the implanted pulse generator (IPG), also referred to as the implant (Fig. 1D), which was placed subcutaneously at the neck region. A long-term stimulation protocol spanning 11 weeks was started 1 week after implantation. Predesigned training patterns were automatically delivered by a bidirectional radio frequency link using a programming device (Fig. 1E) and were repeated every other day. During the training phase, the terminal adductor branch of the RLN was stimulated at exactly the same time of the day with 10 sets of contractions separated by 1 minute.

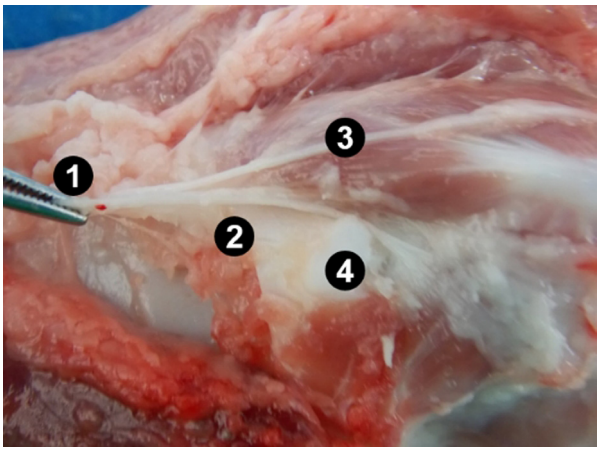

Fig. 2. Sheep larynx from right side with thyroid cartilage removed. 1 = common trunk of the right recurrent laryngeal nerve (RLN). 2 = divided branches of the RLN serving abduction and adduction muscles. 3 = Galen's anastomosis. 4 = articular surface of the cricothyroid joint.

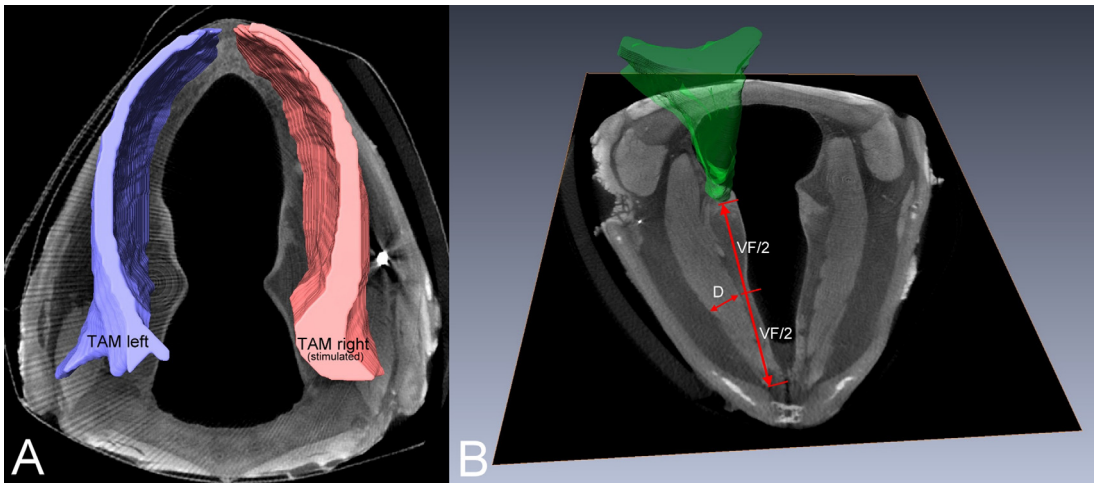

Fig. 3. (A) Three-dimensional reconstruction of bilateral TAM. (B) Axial plane of the larynx. Green: reconstructed arytenoid cartilage. Bright spot near the right TAM indicates the position of the tip.

Each set consisted of 16 contractions (repetitions, 3 seconds on/0.5 seconds off). Stimulation frequency was 100 Hz, pulse width was 250 μs, and the initial amplitude was 0.2 to 3 mA. The total stimulation time was therefore only 480 seconds per day, or 0.54% of total time, calculated from the on time in each set. The initial amplitude was adjusted for each sheep to three times the value that elicited the first detected response obtained endoscopically during the weekly test stimulations. Once the IPG has been programmed, no further anesthesia or sedation was necessary. As in our previous study,[8] the very first training session of awake animals was carefully monitored, and no signs of stress could be observed. During every other week, we performed a transnasal endoscopy in the sedated but awake animal to ensure the implant was functioning correctly by observing VF movement. After 11 weeks of training, the animals were euthanized and their larynges harvested. Six larynges were examined for gene expression and six for morphometric analyses. Animal procedures were carried out using the same methods as described previously.[8] We obtained an approval from the Austrian Federal Ministry of Science, Research and Economy (approval number: BMWFW-66.010/0039-WF/V/3b/2015). All procedures complied with the institution's animal care guidelines and were carried out by experienced veterinarians.

Analysis of Gene Expression

Larynges were excised immediately after euthanasia, opened in the midline, and VF mucosae were removed to expose the TAM. A sample from the central region (i.e., the part of the TAM underlying the VF, between the anterior commissure and the vocal process) was prepared, snap frozen in liquid nitrogen, and stored at −80 °C. RNA isolation, determination of RNA concentration and quality, reverse-transcription and quantitative real-time polymerase chain reaction (RT-qPCR) were performed as previously described.[8] Primer sequences are provided in Table I. Each combination of cDNA sample and mRNA of interest was assayed in triplicate. Quantitation cycle (Cp) values (derived from the LightCycler 480 software [Roche, Vienna, Austria]; AbsQuant/2nd Derivative Max method) from triplicates were averaged. Transformation into relative quantities (RQ) was performed according to the formula $RQ_{i,k} = E_i \,\hat{}\, (Cp_{ref} - Cp_{i,k})$,where E_i is the gene-specific efficiency (previously calculated from RT-qPCR results of a cDNA dilution series), $Cp_{i,k}$ is the Cp value of the respective combination of primer pair i and sample k, and Cp_{ref} is an arbitrarily chosen reference Cp value (measured for primer pair B2M in the sample S34-TA-L).[11] The normalized relative quantities (NRQ) (for each mRNA of interest i and sample k) were obtained according to the formula $NRQ_{i,k} = RQ_{i,k} / NF_k$, where $RQ_{i,k}$ is the relative quantity of the

TABLE I.
Primers Used for Reverse-Transcription Quantitative Polymerase Chain Reaction

Gene Symbol	Also Known As	NCBI Reference Sequence	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
UXT		XM_012183356.1	GACTCCAGGAAGCTAATCACTC	TGAGAGCTTCTGCCAGTGT
B2M		NM_001009284.2	GCCATCCAGCGTATTCCAGA	CCCCGTTCTTCAGCAAATCG
IGF1		NM_001009774.3	GAGACAGGGGCTTTTATTTCAACA	TCCAGCCTCCTCAGATCACA
LOC101111980	MYH4	XM_004012702.2	CTGCAAGACTTGGTGGACAA	TGGAGTTTGCGGAATTTGGA
LOC442994	OMYHC2X, MYH1	XM_004012706.3	GTTCTCTGGCGCAGCATCT	GAGTGCTCCTCAGGTTGGTC
LOC101103165	MYH13	XM_012185735.2	TGCAGAAGCAGGCGACTC	TTTCCCTGAACACAGCGGAC
LOC443471	OMYHC2A, MYH2	XM_015098654.1	AATGGCAGTCTTTGGGGAGG	AAAGATTCCTTGGGCTCGGC
MYH7	OMYHCS	XM_004010325.2	TGCTGACAGACAGAGAAAACCAG	TTTTGCTGCGGTCGCCAAT
PPARGC1A	PGC1; PPARGC1	XM_012179733.2	AAGGCAATTGAAGAGCGCCG	AGCTGTCTCCATCATCCCGC
TFAM		XM_015104510.1	AGCTCAAAACCCAGATGCAAAA	TATACCTGCCAGTCTGCCCT

NCBI = National Center for Biotechnology Information.

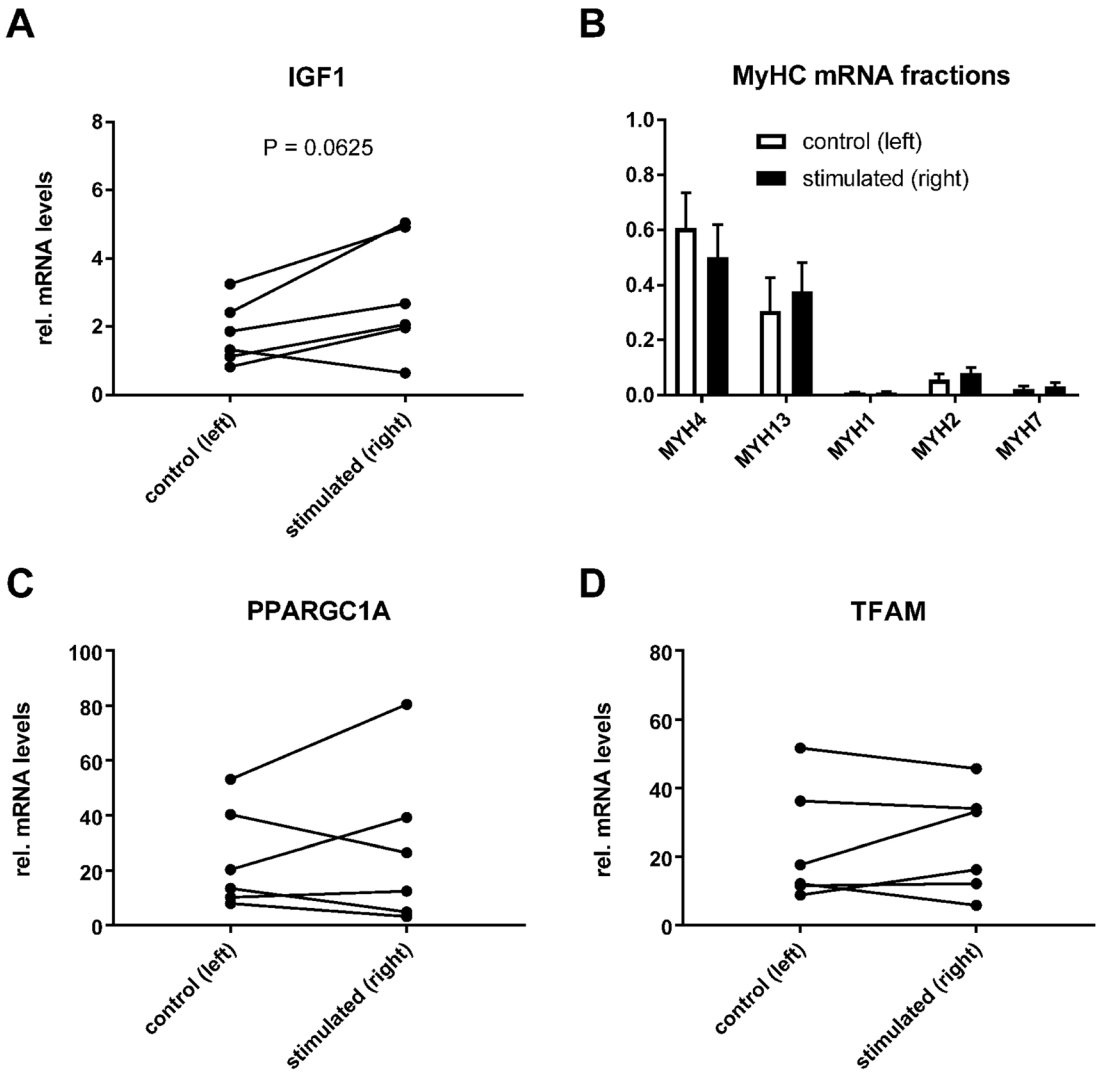

Fig. 4. Results of reverse-transcription quantitative polymerase chain reaction. IGF1 = insulin-like growth factor 1; MyHC = myosin heavy chain; PPARGC1A = peroxisome proliferator-activated receptor gamma coactivator 1-α; TFAM = mitochondrial transcription factor A.

respective mRNA of interest i in the sample k, and NF_k is the geometric mean of the relative quantities of the internal reference RNAs UXT and B2M in the sample k.

Micro Computed Tomography Scans and Reconstruction

All procedures were carried out as described in a previous study.[9] Briefly, the harvested larynges were placed in 3.75% iodine potassium iodide solution as a contrast agent before being scanned. The images were segmented, 3D reconstructed, and the volume determination of the TAM was performed with the 3D visualization and analysis software Avizo 9.3 (Field Electron and Ion Co., Hillsboro, OR). The diameter of the TAM perpendicular to the long axis of the muscle was determined using a standardized procedure. In the horizontal plane, the foremost tip of the vocal process was identified, and a line was drawn to the anterior commissure. The line was divided in half, and the diameter of the TAM was measured (Fig. 3A,B). For estimating the results of FES, we compared the stimulated VF versus the unstimulated VF in one and the same animal. Previous studies in an age-matched cohort showed that in the unstimulated animal right and left VF did not differ in its dimensions.[9]

Data Analysis

For sample size estimation, the effect size was chosen based on the rationale that a 30% increase in TAM diameter D (measured in the horizontal plane perpendicular to the course of the TAM) would be desirable from a clinical perspective. α and β values were chosen as 0.05 and 0.1, respectively, whereas the standard deviation of D was calculated from a previous cohort of aged sheep (mean = 2.54 mm ± 0.43 mm).[9] These parameters resulted in a minimum sample size of n = 6 (power = 92%, effect size: 1.74).

For the statistical analyses, SPSS Statistics version 23 (IBM Corp., Armonk, NY) was used. Nonparametric tests (Wilcoxon signed rank test) were employed, due to the small sample size of animals. A significance level of .05 was set.

TABLE II.
Volumes of the TAM

	L1	L2	L3	L4	L5	L6	Median	Q1–Q3	Test Value	*df*	*P* Value
vol_left	1,516	1,525	1,513	1,418	1,251	1,572	1,514.50	1,536.75–1,376.25	20	6	.046
vol_right (s)	1,649	1,822	1,772	1,620	1,328	1,563	1,634.50	1,784.50–1,504.25			
VF_left	2,8.04	26.23	21.69	22.45	21.62	18.95	22.07	26.68–20.95	4	6	.173
VF_right (s)	28.21	25.01	21.12	21.73	21.71	18.85	21.72	25.81–20.55			
D_left	2.86	2.47	2.69	2.44	2.65	3.56	2.67	3.04–2.46	20	6	.046
D_right (s)	4.49	3.29	3.87	4.96	3.18	3.34	3.61	4.61–3.26			

Volumes are given in cubic millimeters. Morphometric data of the VF: D_left/right: diameter of left/right TAM in a defined cutting plane in millimeters. VF_left/right: length of left/right VF.

TAM = thyroarytenoid muscle; VF = vocal fold; Q = quartile.

RESULTS

All animals tolerated the procedures well and finished the protocol. At the molecular level, we found that the mRNA coding for insulin-like growth factor 1 was increased in the TAM in five out of six animals on the stimulated versus control side, but it failed statistical significance ($P = .06$) (Fig. 4A). Excessive muscle contraction can evoke a switch from fast (glycolytic) to slow (oxidative) fiber types, which would be detrimental for TAM function. Therefore, we investigated the expression of relevant sarcomeric myosin heavy chain (MyHC) isoforms. These analyses confirmed the well-known predominance of fast (MyHC-IIb/*MYH4* gene; MyHC-eo/*MYH13* gene) fiber types in TAM, and also revealed that FES did not evoke significant changes in the relative mRNA levels of any MyHC isoform (Fig. 4B) MYH4: $P = .563$, MYH13: $P = .563$, MYH1: $P = .844$, MYH2: $P = .313$, MYH7: $P = .688$). Furthermore, the expression of two markers of mitochondriogenesis, peroxisome proliferator-activated receptor gamma coactivator 1-α, and mitochondrial transcription factor A remained unchanged (Fig. 4C,D). The results of 3D reconstructions showed significantly different volumes of the TAM on the stimulated versus the unstimulated side (median values = 1514.50 vs. 1634.50, $P = .046$) (Table II). The superficial part of the ovine TAM, corresponding to the vocalis muscle, is far more compartmentalized, which is why we decided to reconstruct and analyze the entire TAM. When comparing the TAM diameters in a horizontal plane perpendicular to the course of the TAM, we again obtained significantly increased mean values, proving the effectiveness of FES (median values = 2.67 vs. 3.61, $P = .046$). It is noteworthy that VF lengths did not differ.

DISCUSSION

Our study proved for the first time that by stimulating terminal adduction fibers of the RLN using an elaborated stimulation protocol, a significant increase in muscle volume could be gained. Vocal fold bowing and the glottal gap are the main laryngoscopic findings in patients suffering from presbyphonia.[4,5] This results from an age-related atrophy of the laryngeal muscles, mainly the TAM. The changes do not only lead to a glottal gap, but are combined with reduced electrical signals and diminished sound pressure levels.[12]

Recently, we demonstrated a new approach to reverse the effects of laryngeal muscular ageing by FES.[8] Electrical stimulation was delivered by a cuff electrode that was coiled around the RLN. After 29 days of daily unilateral stimulation using a very conservative training pattern, we identified a significant increase of the muscle fiber diameters on the stimulated versus unstimulated side. In the current project we chose to implement a more clinical approach in both implantation technique and outcome parameters. The electrode tip was placed near the cricothyroid joint to stimulate the terminal (adduction) branches of the RLN. This approach differs significantly from the previous, where both nerve qualities—adduction and abduction fibers—were stimulated. After 11 weeks of training, we observed significant increases of the entire TAM volume and the TAM diameter in the axial plane. The latter finding is important, as this location of the TAM correlates with the membranous part of the VF, where TAM atrophy leads to the glottal gap. We demonstrated in detail that the increase in total TAM volume was 11% in the stimulated versus the unstimulated muscle. The increase of the TAM diameter (midmembranous part) was even higher and close to 40%. We know from an unstimulated control group of similar aged sheep, that the TAM volumes (right vs. left) do not differ.[9]

It should be noted that, most likely, this desired effect was not accompanied by an undesired switch of skeletal muscle fiber type, as neither myosin heavy chains, nor markers of mitochondrial density exhibited FES-induced changes in expression. This is presumably due to the design of FES, which consisted of high-intensity training sets that were delivered only over a short period of time daily.

FES has proven to be a possible upcoming treatment option for bilateral vocal fold paralysis,[13] and it remains to elucidate if the anatomical site used for stimulation in this trial is a reasonable approach to restore muscle mass in, for example, unilateral vocal fold paralysis in humans.[14] Additionally, this surgical approach did not change the anatomical structures of the larynx in contrast to existing methods (e.g., thyroplasty). Preexisting nerve structures were used to transmit electrical stimulation.

There are, however, shortcomings of our study. The volumetric findings need to be tested functionally to study the effects on vibration characteristics and vocal outcome by ex vivo phonation models. It is also unclear which is

the optimal pattern to achieve the best long-term outcome, with the smallest amount of training and the current required.

CONCLUSION

Our results in an aged ovine model demonstrate that FES of the terminal adduction branch of the RLN lead to a significant increase of both TAM volumes as well as muscle diameters in the midmembranous part. Combining state-of-the-art microelectronics with minimal invasive surgery could provide a new treatment modality not only for age-related muscular atrophy, but also in other states of atrophied laryngeal muscles such as in VF paralysis.

Acknowledgments

The authors thank Anita Leitner for technical assistance and Laura Roche, PhD, for proofreading.

BIBLIOGRAPHY

1. Ruben RJ. Redefining the survival of the fittest: Communication disorders in the 21st century. *Laryngoscope* 2000;110(2 pt 1):241–245.
2. Johnson AM, Ciucci MR, Connor NP. Vocal training mitigates age-related changes within the vocal mechanism in old rats. *J Gerontol A Biol Sci Med Sci* 2013;68:1458–1468.
3. Mau T, Jacobson BH, Garrett CG. Factors associated with voice therapy outcomes in the treatment of presbyphonia. *Laryngoscope* 2010;120: 1181–1187.
4. Bloch I, Behrman A. Quantitative analysis of videostroboscopic images in presbylarynges. *Laryngoscope* 2001;111(11 pt 1):2022–2027.
5. Davids T, Klein AM, Johns MM III. Current dysphonia trends in patients over the age of 65: is vocal atrophy becoming more prevalent? *Laryngoscope* 2012;122:332–335.
6. Martins RH, Benito Pessin AB, Nassib DJ, Branco A, Rodrigues SA, Matheus SM. Aging voice and the laryngeal muscle atrophy. *Laryngoscope* 2015;125:2518–2521.
7. Bradley JP, Hapner E, Johns MM III. What is the optimal treatment for presbyphonia? *Laryngoscope* 2014;124:2439–2440.
8. Karbiener M, Jarvis JC, Perkins JD, et al. Reversing age-related changes of the laryngeal muscles by chronic electrostimulation of the recurrent laryngeal nerve. *PLoS One* 2016;11:e0167367.
9. Gerstenberger C, Dollinger M, Kniesburges S, et al. Phonation analysis combined with 3D reconstruction of the thyroarytenoid muscle in aged ovine ex vivo larynx models [published online September 27, 2017]. *J Voice* doi: 10.1016/j.jvoice.2017.08.016.
10. Chen RL, Kassem NA, Sadeghi M, Preston JE. Insulin-like growth factor-II uptake into choroid plexus and brain of young and old sheep. *J Gerontol A Biol Sci Med Sci* 2008;63:141–148.
11. Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* 2007; 8:R19.
12. Baker KK, Ramig LO, Sapir S, Luschei ES, Smith ME. Control of vocal loudness in young and old adults. *J Speech Lang Hear Res* 2001;44: 297–305.
13. Mueller AH, Hagen R, Foerster G, Grossmann W, Baumbusch K, Pototschnig C. Laryngeal pacing via an implantable stimulator for the rehabilitation of subjects suffering from bilateral vocal fold paralysis: a prospective first-in-human study. *Laryngoscope* 2016;126:1810–1816.
14. Cheetham J, Perkins JD, Jarvis JC, et al. Effects of functional electrical stimulation on denervated laryngeal muscle in a large animal model. *Artif Organs* 2015;39:876–885.