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ANÁLISE MORFOLÓGICA QUALITATIVA E QUANTITATIVA DO NERVO LARÍNGEO RECORRENTE E DO MÚSCULO TIREOARITENÓIDEO HUMANO

Tese de Doutorado

Deivis de Campos

Porto Alegre
2012

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Neurociências da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Doutor em Neurociências.

Orientadores:
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Prof. Dr. Léder Leal Xavier

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DEDICATÓRIA

À minha adorável filha, Laura.

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“Se vi mais longe, foi por estar colocado nos ombros de gigantes”

Sir Isaac Newton, Matemático Inglês,
1643-1727

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RESUMO

Uma das questões mais intrigantes acerca da mobilidade vocal é o paradigma morfológico/funcional que existe sobre a organização do músculo tireoaritenóideo (TA) em relação à prega vocal. Funcionalmente, o músculo TA é responsável por ajustes seletivos em diferentes partes da prega vocal; contudo, esse padrão funcional somente seria possível se suas fibras não se situassem paralelamente em toda a extensão da prega vocal. Dessa forma, as fibras do músculo TA não teriam uma única orientação. Esse padrão morfológico se assemelharia a organização histológica da musculatura da língua. Portanto, a primeira hipótese desta tese é de que assim como a língua apresenta inúmeras possibilidades de movimento em função de sua organização muscular, o músculo TA também poderia apresentar uma organização muscular similar à musculatura da língua, assim como sua inervação; e dessa forma, propiciaria inúmeras possibilidades de movimento à prega vocal. Portanto, o objetivo do primeiro trabalho desta tese foi investigar em humanos as similaridades na organização histológica entre as fibras do músculo TA e da musculatura da língua, assim como diferentes parâmetros histomorfométricos em seus respectivos nervos, laríngeo recorrente (NLR) e hipoglosso (XII); com relevância clínica para uma técnica de reinervação da prega vocal, baseada na anastomose XII-NLR. Com o auxilio de técnicas estereológicas específicas, nós verificamos em doze cadáveres adultos que a organização histológica do músculo TA é similar à organização histológica da musculatura da língua. No entanto, não existem semelhanças nos parâmetros histomorfométricos quantificados entre o NLR e o XII. Dessa forma, no primeiro trabalho desta tese, conclui-se que a organização histológica das fibras do músculo TA, assemelha-se a musculatura da língua em termos de orientação. Presumivelmente, essa característica morfológica propicia uma maior diversidade/possibilidade de movimentos (ajustes seletivos em diferentes partes) à prega vocal. Baseado na diversidade do padrão vocal de um recém nascido, a segunda hipótese desta tese é de que já no período fetal todas as estruturas associadas à prega vocal (prega vocal falsa, ventrículo da laringe, epitélio, glândulas mucosas, vasos sanguíneos e ligamento vocal) já estão completamente estabelecidas, e assim como no adulto, as fibras do músculo TA não estão situadas paralelamente e lateralmente em toda a extensão da prega vocal. Desse modo, seguindo os mesmos protocolos morfométricos, nós também demonstramos que em um feto humano de 25 semanas de idade, além das estruturas associadas à prega vocal já estarem completamente estabelecidas, as fibras do músculo TA fetal, assim como no adulto, apresentam diferentes orientações: transversal, indefinida e longitudinal, ao longo de toda a extensão da prega vocal. Adicionalmente, o aspecto clínico/fisiológico nas diferenças de timbres sonoros entre homens e mulheres e suas especificidades, é um tema que ainda permanece pouco esclarecido. Embora alguns autores tenham reportado a existência do dimorfismo sexual em estruturas nervosas envolvidas com o controle vocal no sistema nervoso central; na atual literatura não existe nenhum estudo que evidencie a presença ou ausência do dimorfismo sexual no sistema nervoso periférico, especialmente no NLR e no músculo TA. Da mesma forma, existem vários estudos em animais descrevendo o dimorfismo sexual em regiões do sistema nervoso envolvidas com o controle da vocalização; no entanto, pouco se conhece sobre esse tema em humanos. Desse modo, a terceira hipótese desta tese é de que os tecidos (NLR e TA) que controlam as pregas vocais também poderiam apresentar dimorfismo sexual. Portanto, o objetivo do terceiro trabalho foi investigar, em humanos, através de análises morfométricas a presença do dimorfismo sexual no NLR e músculo TA. Análises com 14 cadáveres adultos revelaram que existe dimorfismo sexual em relação aos aspectos histomorfométricos do NLR; contudo, não foi verificado dimorfismo sexual em relação à organização histológica das fibras do músculo TA. Frente a esses resultados, conclui-se que os humanos, assim como outras espécies, também apresentam dimorfismo sexual em estruturas nervosas envolvidas com o controle vocal, tanto no sistema nervoso central, já relatada por outros autores, quanto no sistema nervoso periférico (NLR), mostrada em nosso estudo. Finalmente, nós podemos supor que as diferenças entre os timbres sonoros de homens e mulheres e suas especificidades, talvez não possam ser explicadas somente por diferenças na massa das pregas vocais ou no tamanho do trato vocal; mas também por diferenças que abrangem a organização de todo o sistema nervoso.

ABSTRACT

One of the most intriguing questions concerning vocal mobility is the morphological/functional paradigm regarding the organization of the thyroarytenoid muscle (TA) in relation to the vocal fold. Functionally, the TA is responsible for selective adjustments in different parts of the vocal fold. Yet, such a functional pattern would be only possible if its fibers were situated throughout the entire length of the vocal fold. Thus, TA muscle fibers would need to have more than one orientation. This morphological pattern would resemble the histological organization of the tongue musculature. Thus, the first hypothesis of this thesis is that as the tongue presents numerous opportunities for movement due to its muscular organization, muscle organization as well as innervation in the TA could be similar to that of the tongue, which would thus provide numerous opportunities for vocal fold motion. Therefore, the aim of the first study of this thesis was to investigate in humans the similarities between the histological organization of the TA muscle fibers and the tongue muscle, as well as different histomorphometric parameters in their respective nerves, the recurrent laryngeal nerve (RLN) and the hypoglossal nerve (XII); clinically relevant for a vocal fold reinnervation technique based on XII-RLN anastomosis. With the help of specific stereological techniques, we found the histological organization of the TA muscle to be similar to the histological organization of the tongue musculature in twelve cadavers. However, there are no similarities between the histomorphometric parameters quantified in the RLN and XII. Thus, in the first study of this thesis, it was concluded that the histological organization of the TA muscle fibers is similar to that of the tongue muscles in terms of orientation. Presumably, this morphological characteristic provides the vocal fold with a greater diversity/possibility of movement (selective adjustments in different parts). Based on the diversity of vocal patterns of a newborn, the second hypothesis of this thesis is that during the fetal period all the structures associated with the vocal folds (false vocal fold, ventricle of the larynx epithelium, mucous glands, blood vessels and vocal ligament) are already fully established and, as in adults, the TA muscle fibers are not situated in parallel and laterally throughout the length of the vocal fold. Hence, using the same stereological protocols, we also show that in a human fetus aged 25 weeks, besides the structures already associated with vocal fold being fully established, the TA fetal muscle fibers, as in adults, have different orientations: transverse, undefined and longitudinal, throughout the length of the vocal fold. Additionally, the role of clinical/physiological aspects in relation to differences in the tonal qualities of men and women voices and their specificities remains to be clarified. Although some authors have reported the existence of sexual dimorphism in the neural structures involved in vocal control at the level of the central nervous system, in the current literature there is no study that shows the presence or absence of sexual dimorphism at the level of the peripheral nervous system, especially the RLN and the TA muscle. Likewise, while there are numerous animal studies describing sexual dimorphism in the nervous system of regions involved with vocalization control, little is known about this aspect in humans. Thus, the third hypothesis of this thesis is that the tissues (RLN and TA) that control the vocal folds could also present sexual dimorphism. Therefore, the aim of the third study was to use morphometric analysis to investigate the presence of sexual dimorphism in the NLR and TA muscle in humans. Analyses of fourteen cadavers show that there is sexual dimorphism in relation to histomorphometric aspects of the RLN, although no such sexual dimorphism was observed in relation to the histological organization of the TA muscle fibers. Given these results, we conclude that humans, like other species, also exhibit sexual dimorphism in the neural structures involved in vocal control, both at the central level, as reported by other authors, and at the peripheral level (RLN), as shown in our study. Finally, we can assume that the differences between tonal qualities men and women voices and their specificities, may be not only explained by differences in the vocal fold mass or vocal tract size, but also by differences that include the organization of full the nervous system.

1 INTRODUÇÃO

1.1 Aspectos neurobiológicos do controle vocal

A vida, ao que parece, começou nos mares. Os animais primitivos, à semelhança do que fazem os peixes, retiravam da água o oxigênio de que necessitavam para viver. Para isso, utilizavam guelras ou brânquias. Quando, por especiação ou por defesa, alguns desses animais passaram a viver em águas rasas e, posteriormente, em solos pantanosos, houve a necessidade de que fosse desenvolvido um órgão para extrair oxigênio diretamente da atmosfera. O novo órgão (pulmão) passou a necessitar de válvulas para impedir a entrada da lama. Os evolucionistas acreditam que foi nessa fase que surgiu a laringe (HENEINE, 1996).

Embora a função respiratória da laringe seja básica, suas adaptações à fala e derivados da fala são imprescindíveis no surgimento da sociedade e cultura humanas. A linguagem, em todas as suas permutações, primeiramente na fala para as integrações dos esforços tribais imediatos, depois no registro das idéias e a experiência com toda a acumulação e transmissão consequente de conhecimento através do tempo e espaço, é à base da pré-eminência humana. Nesse contexto, a aquisição da linguagem é talvez a mais complexa realização sensório-motora na vida do indivíduo. Além do sistema auditivo e do intrincado aparelho que produz a fala, esta última envolve não apenas a laringe e outros órgãos da respiração, mas também extensas vias nervosas sob influência de regiões encefálicas específicas (Figura 1) (WILLIAMS et al., 1995).

Portanto, a produção vocal depende da interação dos diversos níveis do sistema nervoso central e periférico, além da ação coordenada e programada dos receptores sensoriais. Embora nas últimas décadas a neurolaringologia, tenha contribuído com importantes informações relativas à fala, ainda pouco se conhece sobre os aspectos neurobiológicos da laringe (BEHLAU et al., 2001).

A visualização intra-operatória dos nervos da laringe tem permitido uma significante redução da paralisia laríngea pós-operatória (THOMUSCH et al., 2003). No entanto, o sucesso para o uso adequado desses métodos ainda depende do conhecimento minucioso da inervação da laringe (KRUSE et al., 2006).

A complexa inervação da laringe, aliada as dificuldades na comprovação funcional dos ramos nervosos para a laringe, tem motivado um vasto número de pesquisas, especialmente àquelas que correlacionam à histoquímica, microscopia e histologia com a clínica. Nesse contexto, essas pesquisas indubitavelmente são imprescindíveis para a confirmação ou reformulação de novas teorias que possam demonstrar com mais detalhes as bases anatômicas e fisiológicas do comportamento vocal humano (NAVARRO et al., 2003; MARANILLO et al., 2005).

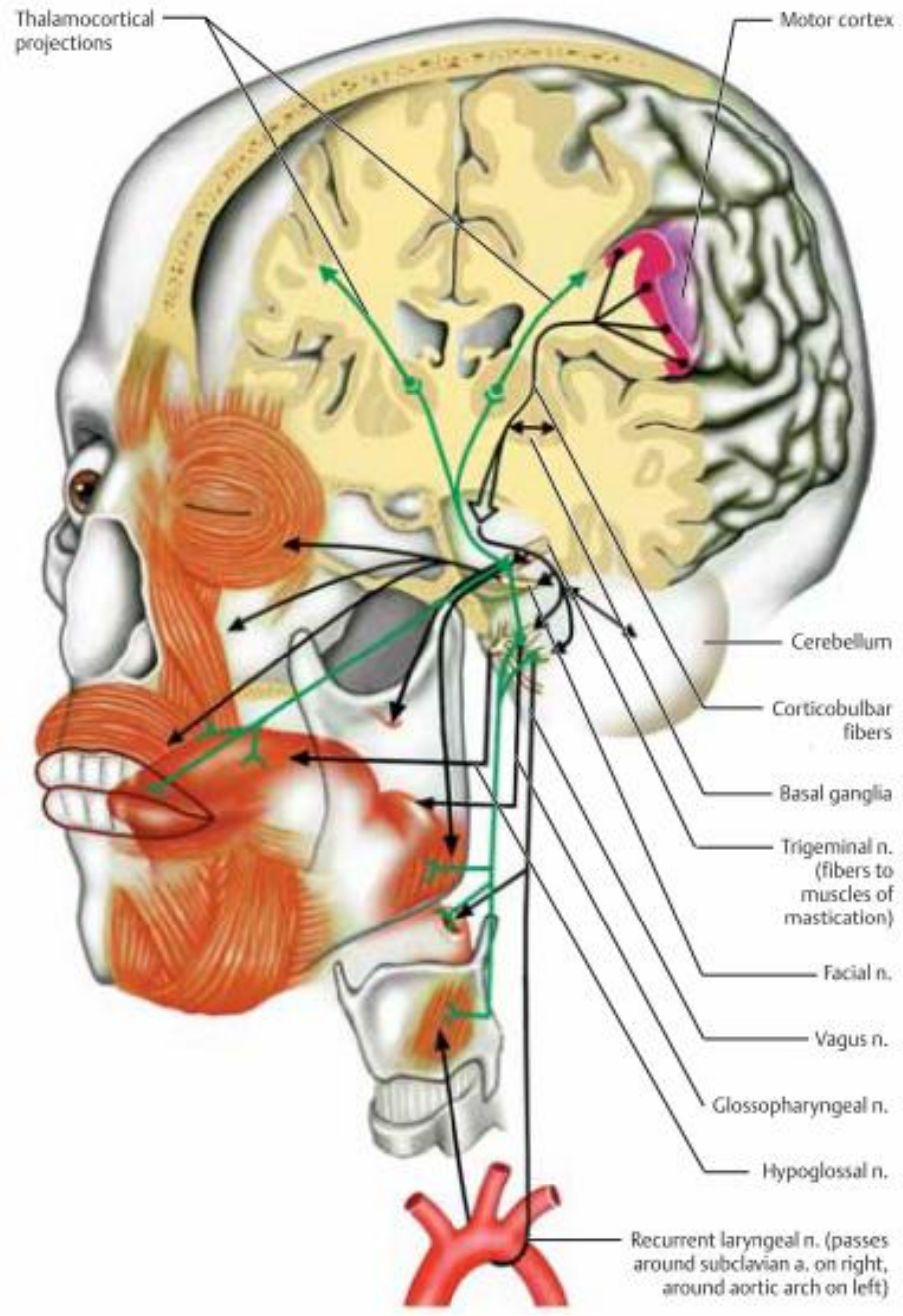

Figura 1 – Representação esquemática do controle neural da voz. Observa-se que o córtex motor por meio das vias corticopontina e corticobulbar faz projeções bilaterais para os núcleos motores dos nervos cranianos no tronco encefálico. O nervo trigêmeo controla os músculos que abrem e fecham a mandíbula. O nervo facial controla a musculatura da expressão facial e a articulação labial. O nervo hipoglosso controla a musculatura que da mobilidade a língua. O nervo glossofaríngeo controla a mobilidade do palato mole e da faringe. O nervo vago, através do nervo laríngeo recorrente controla os músculos intrínsecos da laringe. As conexões com os núcleos basais e o cerebelo são importantes para a adequada coordenação da fala. Através dos nervos trigêmeo, glossofaríngeo e vago os impulsos sensoriais a partir da pele, membranas mucosas e músculos retornam para o encéfalo. Estes impulsos são processados por uma rede neural (formação reticular, tálamo e córtex pré-central) que modulam o controle de *feedback* da fala (ROHKAMM, 2004).

1.2 Inervação da laringe e pregas vocais

Os nervos da laringe são fornecidos por dois ramos do Nervo Vago: Nervo Laríngeo Superior (NLS) e Nervo Laríngeo Recorrente (NLR) (WILLIAMS et al., 1995); este último, o NLR é motor, sensorial e parassimpático (ARDITO et al., 2004). Os nervos laríngeos recorrentes dos dois lados têm essencialmente a mesma distribuição; contudo, recorrem (curvam-se em torno) estruturas diferentes e em níveis diferentes nos dois lados. O NLR direito forma uma alça por baixo da artéria subclávia direita; aproximadamente no nível vertebral de T1/T2 e, o NLR esquerdo forma uma alça por baixo do arco da aorta, aproximadamente no nível vertebral T4/T5. Após formarem alças, ambos os nervos recorrentes ascendem superiormente até a face póstero-medial da glândula tireoide, onde sobem no sulco traqueoesofágico (Figura 2) (MOORE et al., 2011).

Após percorrerem o sulco traqueoesofágico ambos os nervos recorrentes penetram na laringe para inervarem todos os músculos intrínsecos da laringe exceto o músculo cricotireóideo. Os músculos intrínsecos da laringe movem as partes laríngeas, fazendo alterações no comprimento e tensão das pregas vocais e no tamanho e formato da rima da glote (MOORE et al., 2011), motivo pelo qual, ambos os nervos desempenham uma grande importância na patogenia dos distúrbios da mobilidade vocal (FIGÚN & GARINO, 1994).

1.3 Anatomia das pregas vocais

As duas pregas vocais são anteparos musculomembranáceos móveis de cor branco-pérola, localizados abaixo das pregas ventriculares e

medialmente a elas. Estendem-se do ângulo da cartilagem tireóidea, anteriormente, até o processo vocal das cartilagens aritenóideas, posteriormente. Cada uma contém o ligamento vocal, que consiste de tecido elástico derivado de cone elástico (GARDNER et al., 1988).

As pregas vocais são estruturas dotadas de elasticidade e mantêm-se tensas principalmente pela ação dos músculos intrínsecos da laringe, especialmente o músculo tireoaritenóideo (TA), também chamado de músculo vocal. O músculo TA situa-se paralelamente e lateralmente a prega vocal; além disso, diz-se que ele é mais espesso na região posterior do que na anterior, porque muitas fibras mais profundas começam a partir do ligamento vocal e, assim, não alcançam a cartilagem tireóidea. Por outro lado, outros autores consideram que todas as suas fibras curvam-se e se entrelaçam a partir da cartilagem tireóidea para a cartilagem aritenóidea (Figura 3) (WILLIAMS et al., 1995).

O músculo TA produz pequenos ajustes dos ligamentos vocais, tensionando e relaxando seletivamente as partes anteriores e posteriores das pregas vocais, durante a fala (MOORE et al., 2011).

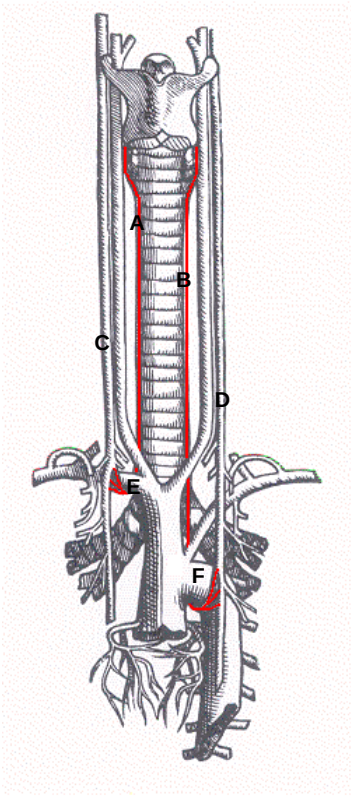

Figura 2 – Organização do nervo laríngeo recorrente direito (A) e esquerdo (B). (C) Nervo vago direito (C) e esquerdo (D). (E) Artéria subclávia e (F) Arco da aorta. Modificada de *De humani corporis fabrica* epítome: tabulae sex. Quarto livro, gravura 52, figura 2 (VESALIUS, 2002).

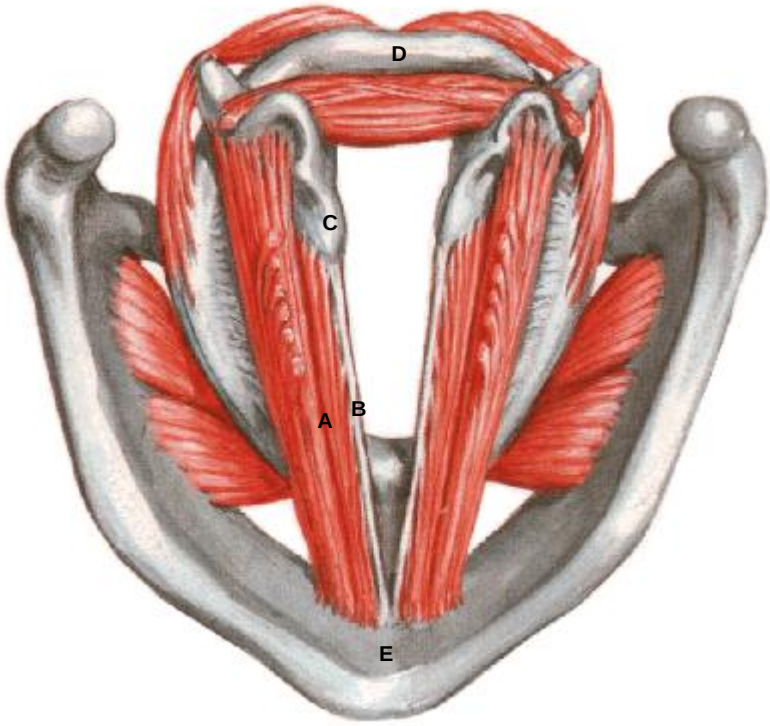

Figura 3 – Vista superior da laringe. (A) Músculo tireoaritenóideo; (B) Ligamento vocal; (C) Cartilagem aritenóidea; (D) Cartilagem cricóidea e (E) Cartilagem tireóidea (NETTER, 2000).

2 HIPÓTESES

2.1 Primeira hipótese

Uma das questões mais intrigantes acerca da mobilidade vocal é o paradigma morfológico/funcional que existe sobre a organização do músculo TA em relação à prega vocal (WILLIAMS et al., 1995). Funcionalmente, o músculo TA é responsável por ajustes seletivos em diferentes partes da prega vocal (MOORE et al., 2011). Contudo, esse padrão funcional somente seria possível se suas fibras não se situassem paralelamente e lateralmente em toda a extensão da prega vocal. Dessa forma, as fibras do músculo TA não teriam uma única orientação. Esse padrão morfológico se assemelharia a organização muscular da língua.

Desse modo, nossa primeira hipótese é de que assim como a língua apresenta inúmeras possibilidades de movimento em função de sua organização muscular (NAPADOW et al., 1999), o músculo TA também poderia apresentar uma organização muscular similar à musculatura da língua, assim como sua inervação; e dessa forma, propiciaria inúmeras possibilidades de movimento à prega vocal. Isso explicaria como o músculo TA fornece ajustes seletivos em diferentes partes da prega vocal. Os resultados da investigação relativa a essa primeira hipótese estão apresentados na forma de artigo científico publicado no periódico internacional indexado *Journal of Voice* - Fator de Impacto (JCR 2011): 1.39.

2.2 Segunda hipótese

Adicionalmente, nossa segunda hipótese é de que essa característica anatômica também seja evidenciada no período gestacional. De acordo com alguns estudos recentes (BRANCO et al., 2005; NICOLLAS et al., 2006; ZESKIND et al., 2011) o choro do recém nascido é rico em sons e propriedades acústicas que apresentam grande importância clínica no momento do nascimento, como por exemplo, características para determinar as condições de saúde do recém nascido como também a possível detecção precoce de doenças congênitas que levam à intervenção médica imediata. Em função disso, nós acreditamos que essa diversidade no padrão vocal pode ser explicada pelo fato de que as estruturas associadas à prega vocal já estão completamente estabelecidas antes do nascimento e as fibras do músculo TA, também poderiam não estar situadas paralelamente e lateralmente em toda a extensão da prega vocal.

Desse modo, este estudo também investiga as estruturas associadas à prega vocal (prega vocal falsa, ventrículo da laringe, epitélio, glândulas mucosas, vasos sanguíneos e ligamento vocal) e a organização histológica do músculo TA em um feto feminino de 25 semanas. Os resultados da investigação relativa a essa segunda hipótese estão apresentados na forma de artigo científico, aceito para publicação (“*in press*”) no periódico internacional indexado *Journal of Voice* - Fator de Impacto (JCR 2011): 1.39.

2.3 Terceira hipótese

A falta de conhecimento científico específico sobre os aspectos neurobiológicos do controle vocal humano deve-se muito a dificuldade da realização de estudos padronizados em seres humanos. O aspecto

clínico/fisiológico nas diferenças de timbres sonoros entre homens e mulheres e suas especificidades, é um tema que ainda permanece pouco esclarecido.

Embora alguns autores (HARASTY et al., 1997) tenham reportado a existência do dimorfismo sexual em estruturas nervosas envolvidas com o controle vocal a nível do sistema nervoso central; na atual literatura não existe nenhum estudo que evidencie a presença ou ausência do dimorfismo sexual no sistema nervoso periférico, especialmente o NLR e o músculo TA. Entretanto, existem vários estudos (DEVICHE & GULLEDGE, 2000; GAHR, 2007; RHODES et al., 2007) em animais descrevendo o dimorfismo sexual em regiões do sistema nervoso envolvidas com o controle vocal/vocalização; no entanto, pouco se conhece sobre este tema em humanos.

Desse modo, nossa terceira hipótese é de que os tecidos (NLR e TA) que controlam as pregas vocais também poderiam apresentar dimorfismo sexual. Os resultados da investigação relativa a essa terceira hipótese estão apresentados na forma de artigo científico aceito para publicação no periódico internacional indexado *Journal of Voice* - Fator de Impacto (JCR 2011): 1.39.

3 OBJETIVOS

3.1 Primeiro artigo

- Investigar em humanos as possíveis semelhanças na organização histológica do músculo tireoaritenóideo com a musculatura da língua e seus respectivos nervos, laríngeo recorrente e hipoglosso.

3.2 Segundo artigo

- Investigar em um feto humano de 25 semanas as estruturas associadas à prega vocal (prega vocal falsa, ventrículo da laringe, epitélio, glândulas mucosas, vasos sanguíneos e ligamento vocal) e a organização histológica do músculo tireoaritenóideo.

3.3 Terceiro artigo

- Investigar em humanos a presença do dimorfismo sexual em nervos (laríngeo recorrente) e músculos (tireoaritenóideo) envolvidos no controle vocal.

4 CONSIDERAÇÕES ÉTICAS

Respeitando todos os preceitos da lei que regem sobre a utilização de tecidos humanos em pesquisas biomédicas; este estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal do Rio Grande do Sul (*PROPESQ/UFRGS – 17547*) e pelo Departamento Médico Legal – Seção de Ensino e Pesquisa (*DML – 2403/09*). Todos os cuidados foram tomados no intuito de respeitar os princípios de autonomia, beneficência, justiça, equidade e sigilo de todos os indivíduos que compuseram este estudo.

5 RESULTADOS

5.1 Primeiro artigo (Publicado no *Journal of Voice* – Fator de Impacto no JCR 2011: 1.39)

Histological Organization is Similar in Human Vocal Muscle and Tongue—A Study of Muscles and Nerves

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Summary: One of the most exciting questions about the human voice is how the vocal fold produces and modulates different sounds. One hypothesis to explain the wide range of movements found in the vocal fold is based on the variety of muscle fiber orientations in the thyroarytenoid (TA) muscle. The tongue (TO) muscle is considered the most complex structure in the body in terms of muscle fiber orientation and movements. Thus, possible similarities between these two muscles and their innervations, the recurrent laryngeal nerve (RLN) and hypoglossal nerve (XII), could explain the complex movements executed by the focal fold. Moreover, such studies help us to understand some microanatomical aspects of vocal fold reinnervation, based on XII-to-RLN anastomosis. Therefore, this study investigates the histological organization of TA and TO muscles and their innervations (n = 12 subjects). The muscle fibers were classified into three categories according to their orientation (transverse, undefined, and longitudinal). To quantify the percentage of fibers in each category in the TA and TO, the shape coefficient (shape Z) was estimated. Qualitative analysis and estimation of fiber area and shape Z show that the histological organization of TA and TO muscle is similar. Both muscles present the same percentage of transversal (~72%), undefined (~15%), and longitudinal fibers (~10%). By contrast, the authors' analysis of the morphometric parameters of the RLN and XII shows that there is no correlation between these nerves. In conclusion, in humans, TA and TO muscles present similar histological organization and this finding could help to explain interesting questions about human phonation.

Key Words: Thyroarytenoid muscle–Tongue muscle–Recurrent laryngeal nerve–Hypoglossal nerve–Histology.

INTRODUCTION

Perfect harmony between the muscles of the tongue (TO) and the larynx is indispensable for the good operation of different functions, such as voice for speech communication, emotional expression when laughing and crying and breathing, swallowing, and coughing.[1,2]

The tongue is known to be composed of extrinsic (genioglossus, styloglossus, hyoglossus, and palatoglossus) and intrinsic (superior and inferior longitudinal, transverse, and vertical) muscles. It is generally described that the TO muscles in mammals are innervated by the hypoglossal nerve (XII).[2]

The larynx, an organ of phonation, contains many small intrinsic muscles, namely the cricothyroid, posterior cricoarytenoid, lateral cricoarytenoid, transverse arytenoid, oblique arytenoids, aryepiglotticus, thyroarytenoid (TA), and thyroepiglotticus. These muscles are innervated by two branches of the vagus nerve (X): the external branch of the superior laryngeal nerve innervates the cricothyroid and the recurrent laryngeal nerve (RLN) innervates the remaining intrinsic muscles.[3]

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Furthermore, these muscles are clinically important, and alterations in their normal function produce voice alterations, dysarthria, dysphagy, and dyspnea.[4,5]

Histological studies have described how the TO muscle is able to produce a large number of different tongue shapes and movements. This capability is widely attributed to diversity in the orientation of the muscle fibers.[4,6–9] However, little is known about exactly how the vocal muscle—TA muscle—also provides the large variety of movements found in the vocal fold.[1,10] One hypothesis to explain these wide range of movements found in the TA and TO muscles could be based on possible histological similarities between these two muscles.

Additionally, the most frequent cause of vocal fold paralysis is injury to the RLN,[11] which can also be reinnervated using XII-to-RLN anastomosis.[12] This technique provides positive effects on vocal fold paralysis in terms of acoustic, perceptual, electromyographic, and visual outcomes.[13]

Gathering data on the morphometric features of the RLN and XII is certainly very important for understanding and improving the techniques used for vocal fold reinnervation (XII-to-RLN anastomosis). There are some data on this topic in the literature.[14,15] However, there is little detailed information on the morphological parameters of RLN and XII.

Thus, the aim of the authors' study was to compare the histological organization of TA and TO muscles, and their respective nerves (RLN and XII), to establish the degree of similarity between these tissues.

MATERIALS AND METHODS

Human tissue

This study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Rio Grande do Sul, Brazil. The histological material of muscles and nerves

were obtained from the collection of the Laboratory of Histology and Pathology of the Universidade de Santa Cruz do Sul, Rio Grande do Sul, Brazil. These tissues were collected from necropsies done in the Department of Legal Medicine in the same university. Twelve subjects were used in their study. Information on the sex, age, weight, height, and body mass index of the subjects is presented in Table 1.

Tissue collection

The TA muscle was bilaterally removed (~10 mm) from the larynx, the middle region of the TA muscle was chosen for their study. This choice was based on a previous study,[10] which showed that this region presents more and better defined muscle fibers when compared with the other regions of the TA muscle (Figure 1).

The tongue samples were collected and processed as described in a previous study.[8] Slices were obtained (~10 mm) from the posterior, median, and the anterior parts of the tongue. These slices presented a complete transverse view of the tongue, from the glossoepiglottic fold (dorsal) to the apex (ventral) of the tongue (Figure 1).

The dissection of the RLN extended deeply toward the point where it entered the larynx (cricothyroid joint). The segments (~10 mm) of RLN used in the authors' histological analysis were obtained 1 cm below the cricothyroid joint, bilaterally. The same procedure was used in a previous study[16] (Figure 1).

The XII nerve was dissected from the point where it crosses the internal carotid artery to its ending at the tongue. Based on previous protocols,[14] the segments (~10 mm) were taken from the XII nerve at the level of its passage over the crest of the greater horn of the hyoid bone, bilaterally (Figure 1).

Histology of muscles and nerves

The samples from each muscle (TA and TO) were fixed in 10% formalin solution, dehydrated in a graded series of ethanol, and embedded in paraffin. Sections of the TO and TA muscles (10 μm) were obtained using a microtome (Leica, Nussloch, Germany); these sections were mounted onto slides; deparaffinized with xylene; rehydrated; and stained with hematoxylin-eosin, washed in water, dehydrated in a graded series of ethanol, cleared with xylene, and covered with entellan and coverslips.[8,10] Five sections with intervals of 100 μm were collected and analyzed.

Based on the previous studies,[15] the specimens (RLN and XII) were fixed by immersion for 24 hours in a modified Karnovsky solution (Sigma Chemical Company, St Louis, MO). Then, they were bathed in 0.05 mol/L of sodium cacodylate solution, postfixed in 1% osmium tetroxide (Sigma Chemical Company) for 2 hours, dehydrated in an increasing graded series of acetone (Electron Microscopy Sciences, Hatfield, PA), and embedded in epoxy resin (araldite, durcupan; Fluka, Buchs, Switzerland), which was then polymerized at 60°C. Three semithin cross-sections (1 μm) were obtained using an ultramicrotome (MT 6000-XL; RMC, Tucson, AZ) with intervals of 100 μm and stained with 1% toluidine blue (Merck, Darmstadt, Germany) in 1% sodium tetraborate (Ecibra, Curitiba, Brazil).

Digitized images of the muscles and nerves were obtained using an Olympus B×50 microscope (4×, 10×, and 100×; Olympus, Tokyo, Japan) coupled to a video camera (Leica DC 300F) interfaced by *Leica Image 50* (*IM50*) software (Leica, Wetzlar, Germany) (Figure 2A and B). The images obtained were measured using *Image Pro Plus* Software (*Image Pro-Plus 6.0* [*IPP 6.0*]; Media Cybernetics, Silver Spring, MD).

Morphometric parameters

In their study, the authors classified and estimated the percentages of transverse, undefined, and longitudinal fibers in the TA and TO muscles and their respective nerves using two histological methods: hematoxylin-eosin and toluidine blue. To estimate the percentage of muscle fiber orientated in each of the three categories the mathematical and stereological tools, the point-counting method[17] and shape Z[18–21] were used to calculate the area and shape coefficient, respectively.

TABLE 1.
Parameters of the Subjects, Namely Sex, Race, Age, Weight and Height, Body Mass Index (BMI), Period Between Death and the Collection of the Fragment (Δt), and Cause of Death

Subjects/Sex	Race	Age (y)	Weight (kg)	Height (m)	BMI (kg/m^2)	Δt (min)	Cause of the Death
1/Male	White	73	55	1.74	18.21	120	Sudden death
2/Woman	White	85	50	1.55	20.83	180	Sudden death
3/Male	White	71	60	1.64	22.38	180	Sudden death
4/Male	White	52	75	1.71	25.68	120	Sudden death
5/Woman	White	85	56	1.60	21.87	135	Coronary thrombosis
6/Male	White	56	72	1.63	27.16	155	Sudden death
7/Male	White	82	55	1.60	21.48	120	Sudden death
8/Woman	White	45	70	1.60	27.34	140	Sudden death
9/Male	Black	50	75	1.70	25.95	160	Sudden death
10/Male	White	60	65	1.75	21.24	175	Sudden death
11/Male	Black	40	80	1.80	24.69	145	Sudden death
12/Male	White	46	65	1.65	23.89	150	Sudden death
Total	—	62.08 ± 16.43	64.83 ± 9.67	1.66 ± 0.07	23.39 ± 2.83	148.33 ± 22.49	—

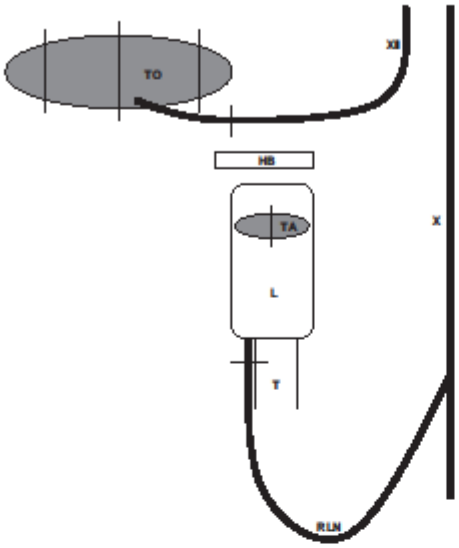

FIGURE 1. Schematic representation showing the regions where the tissues (muscles and nerves) were collected (indicated by a *dotted line*). *Abbreviations:* TA, thyroarytenoid muscle; TO, tongue muscle; L, larynx; T, trachea; HB, hyoid bone; X, vagus nerve; RLN, recurrent laryngeal nerve; XII, hypoglossal nerve.

Morphometric measurements in the RLN and XII nerves included the (1) myelinated fiber density (number of fibers/mm^2), (2) intraperineural area (mm^2), (3) total number of fibers, (4) average myelinated fiber area (μm^2), (5) average myelinated fiber diameter (μm), (6) average axon diameter (μm) of the myelinated fiber, (7) average myelin sheath thickness (μm), (8) *g* ratio (degree of myelination), (9) percentage areas of myelinated fibers (%), and (10) percentage of endoneurial connective tissue (%).

Morphometric measurements of muscles

The classification of the muscle fibers was based on a pilot study performed by two blinded researchers. Pearson's correlation coefficients (*r*) were calculated to determine the relationship between the results obtained by the two researchers. The *r* values obtained for the transverse, undefined, and longitudinal fibers of the TA muscle were 0.9852, 0.7169, and 0.8553, respectively. In relation to the TO muscle, the *r* values found were 0.9909 for transverse, 0.9112 for the undefined, and 0.7877 for longitudinal fibers. These values demonstrate the high level of reliability of the observations made by the blinded researchers.

Equal-sized areas of interest (AOIs) and a grid masks with an area/point value of 400 μm^2 were laid over the images to estimate both the number of fibers per area and the area of muscle fiber to enable the classification of the fibers as transversal, undefined, or longitudinal according to their orientation.[17] The area of muscle fiber was obtained using the following equation: $\hat{A} = \sum p \cdot a/p$, where $\hat{A}$ is the area, $\sum p$ is the sum of points counted, and a/p is the area/point value.

After the pilot study, the criteria for this classification were obtained after detailed observation and measurements taken by three histology specialists who were also blinded to the source of the images. The fibers were classified using the following criteria: the TA muscle fibers were identified, according to their areas, as transverse between 0 and 2000 μm^2, undefined between 2000 and 3200 μm^2, and longitudinal more than 3200 μm^2. The TO muscle fibers were identified, according to their areas, as transverse between 0 and 400 μm^2, undefined between 400 and 1200 μm^2, and longitudinal more than 1200 μm^2 (Figure 2C and D).

The shape coefficient, also known as the shape *Z*,[18–21] was also used to define the fiber types. This parameter is obtained using the following equation: $Shape\ Z = P/\sqrt{\hat{A}}$, where Shape *Z* is the shape coefficient, *P* is the perimeter, and $\hat{A}$ is the area value.

Based on a comparison of the Shape *Z* values obtained with the classification made by the observers, the TA fibers were classified as: transverse, between 0 and 4.61; undefined, between 4.62 and 5.30; and longitudinal, between 5.31 and 6.32. For TO, the fibers were classified as: transverse, between 0 and 4.48; undefined, between 4.49 and 5.28; and longitudinal, between 5.29 and 7.32.

These classifications were used to estimate the percentages of transverse, undefined, and longitudinal fibers in all the analyzed sections.

Morphometric measurements of nerves

The fiber density (number of fibers/mm^2) was estimated using a previously described protocol[16] of planar morphometry. Briefly, five squares named AOIs measuring 532 μm^2 were overlaid onto each obtained image. These five AOIs were arranged according to a pattern in which four AOIs were placed equidistantly from a central AOI (Figure 3A and B). The fibers located inside this square or which intersected with the upper and/or left edge of the AOI were counted; the fibers which intersected with the lower and/or right edge of the AOI were not counted (Figure 3C and D). The fiber density was obtained using the following equation: Fiber density = fibers counted per AOI/AOI area.

To estimate the intraperineural area, the digitized images of the sections were outlined and the parameters were measured (three images were measured for each nerve) (Figure 3A and B). To estimate the axonal area, the same delineation procedure was carried out in the fibers previously counted within the AOIs (Figure 3C and D). The total number of fibers in each nerve was estimated by multiplying the fiber density by the total intraperineural area. Myelin sheath thickness was calculated using a specific software tool (Figure 3E and F).

To estimate the axonal diameter, the axonal area of each individual fiber was measured and the value obtained was converted to the diameter of a circle with an equivalent area. The sum of the axonal diameter and myelin sheath represent the myelinated fiber diameter. A measurement of the degree of myelination (*g* ratio) was estimated by dividing the axon diameter by the myelinated fiber diameter. The percentage of

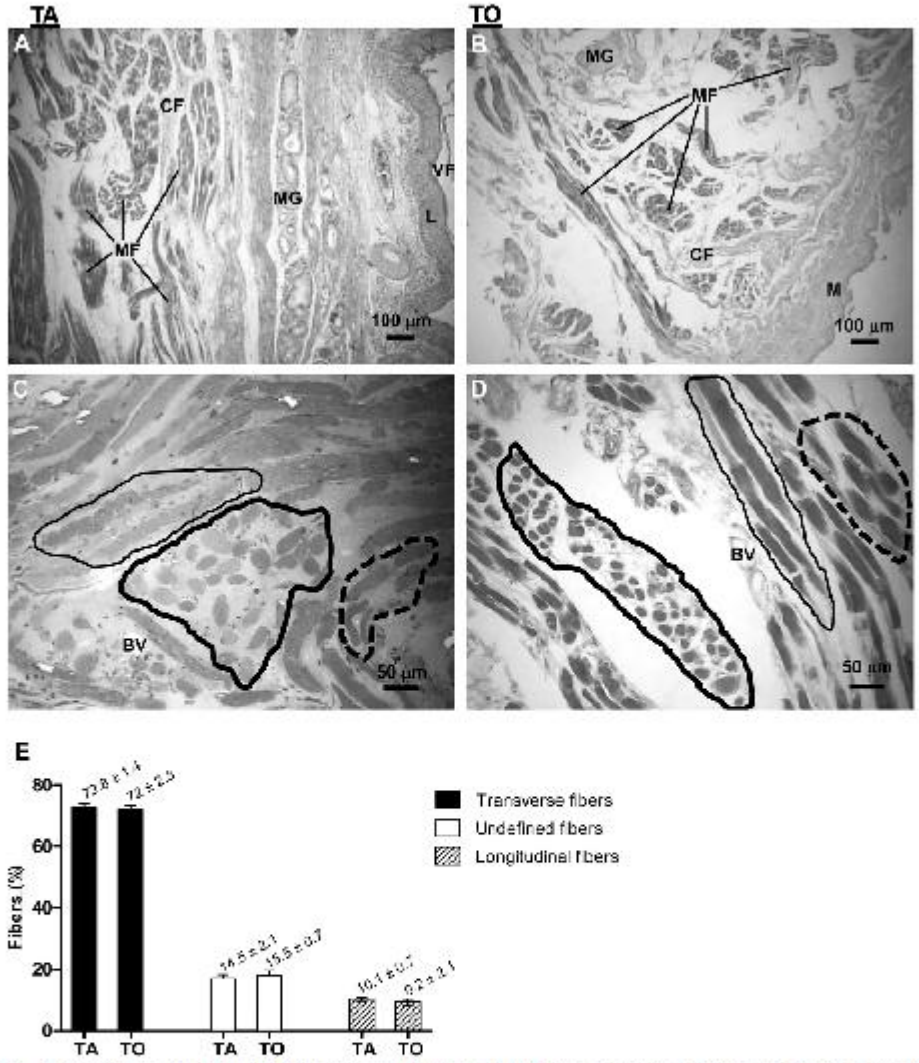

FIGURE 2. Digitized images of sections of the TA muscle and TO muscle. **A** and **B**. Images demonstrating the clear similarity between the TA and TO in terms of muscle fiber organization. **C** and **D**. Digitized images showing transverse (*thick delineation*), undefined (*dotted delineation*), and longitudinal (*thin delineation*) fibers. **E**. Comparison between the TA and TO of the percentage of transverse ($P = 0.678$), undefined ($P = 0.627$), and longitudinal ($P = 0.438$) fibers. *Abbreviations:* TA, thyroarytenoid muscle; TO, tongue muscle; MF, muscles fibers; VF, free margin of vocal fold; L, vocal ligament; M, mucosa; MG, mucous glands; CF, collagen fibrils; BV, blood vessel (hematoxylin-eosin stain).

the nervous tissue (areas of myelinated fibers) and endoneurial connective tissue also were estimated using *IPP 6.0*.

Statistical analysis

Comparisons were made of the TA and TO muscles and their respective nerves (RLN and XII) from each subject using an independent samples *t* test ($P < 0.05$). Pearson's correlation coefficient (r) was used to estimate variations between the results obtained by two blinded researchers in the pilot study. All statistical analyses were performed using the *Statistical Package for the Social Sciences 15.0* software (IBM Co., Armonk, NY).

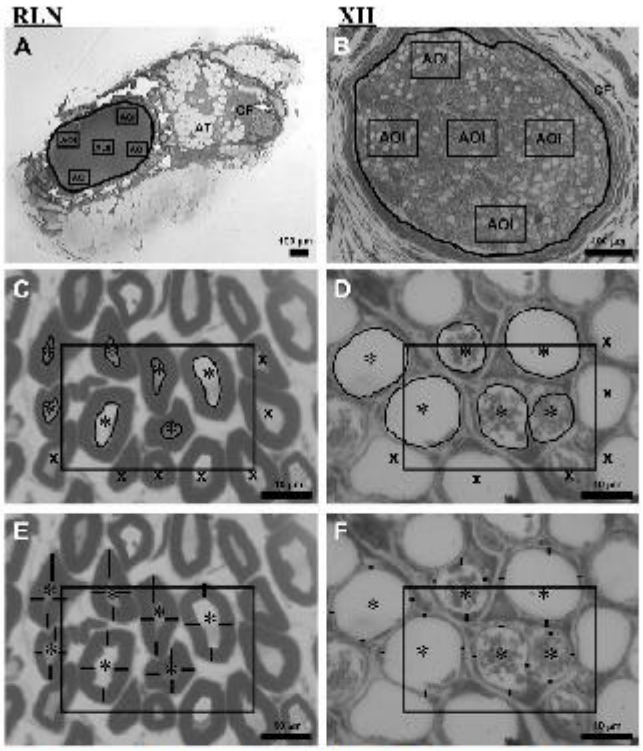

FIGURE 3. Digitized images of sections of the recurrent laryngeal nerve and hypoglossal nerve. **A** and **B**. Images of intraperineural area (*black delineation*) showing the arrangement of AOIs (532 μm^2) overlaying the image of the nerves. **C** and **D**. Representation of one of the fields (AOIs) used in the study to evaluate the fiber density, the total number of fibers, and axonal area (*black delineation*). The fibers located inside this square or intersected by the upper and/or left edge of the AOI were counted (*); the fibers intersected by the lower and/or right edge of the AOI were not counted (×). **E** and **F**. Images demonstrating the myelin sheath thickness of fibers were counted within the AOIs, calculated by means of a specific software tool (*four vertical and horizontal lines in each fiber*). *Abbreviations:* AOIs, areas of interest; AT, adipose tissue; CF, collagen fibrils (toluidine blue stain).

RESULTS

Hematoxylin-eosin staining showed that the measured muscle fibers had well-defined boundaries. In all the histological sections, the qualitative analysis of the organization of the transverse, undefined, and longitudinal fibers of the TA and TO demonstrates that the TA and TO muscles present a significantly similar histological organization (Figure 2A–D).

Quantitative comparison of the transverse, undefined, and longitudinal fibers demonstrated no statistically significant difference between the TA and TO muscles (Figure 2E).

Analysis using toluidine blue staining showed that the myelinated fibers were well preserved in all specimens and their number was not altered by postmortem dimensional changes. In the authors' study, analysis of the all morphometric measurements showed a statistically significant difference between the RLN and XII (Figure 4A–J).

DISCUSSION

Qualitative analysis of histological sections of the TA and TO muscles showed the exact organization of muscle fascicles in relation to the free margin of the vocal ligament, collagen fibrils, and mucous glands. Furthermore, some blood vessels were seen together with the collagen fibrils and muscle fibers, which may represent ramifications of the inferior thyroid artery and lingual artery of the TA and TO muscles, respectively. Thus, in all collected nerves, large portions of the epineurium and adipose tissue were visible. One hypothesis that the authors developed is that this extra padding surrounding the RLN and XII could help to prevent damage resulting from, for example, blows to the neck; compression; and elongation related to movements of the head, such as flexion, extension, and rotational movements.

From the similarities between the TA and TO muscles, the authors conclude that the distribution of the TA fibers along

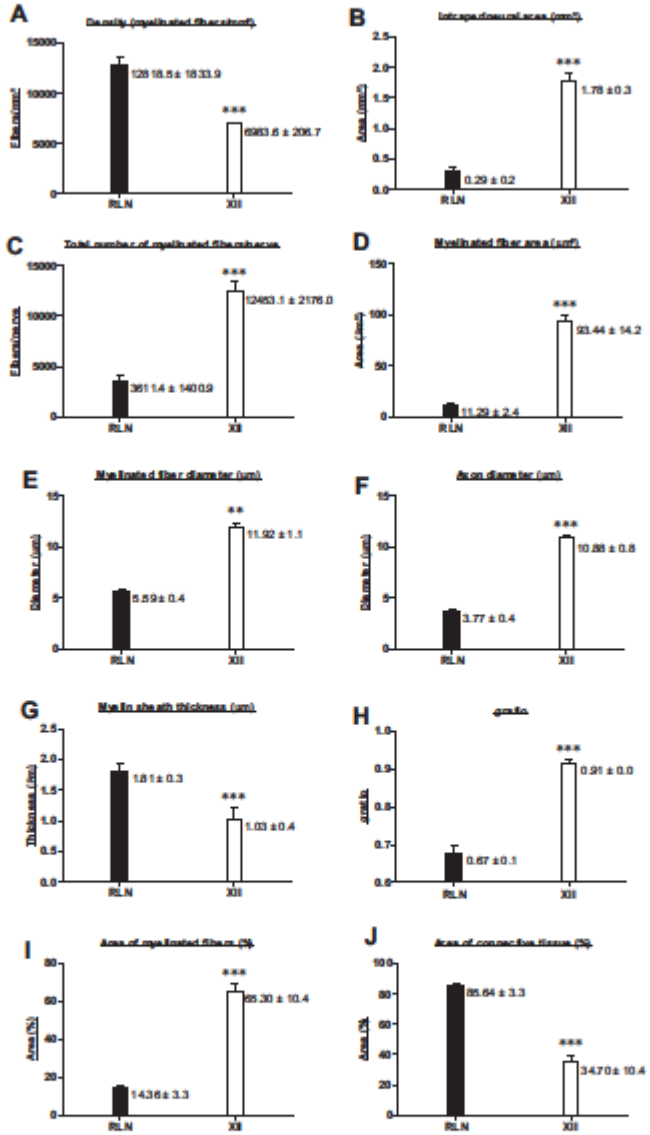

FIGURE 4. Comparison of myelinated fibers density (**A**), intraperineural area (**B**), total number of myelinated fibers (**C**), myelinated fiber area (**D**), myelinated fiber diameter (**E**), axon diameter (**F**), myelin sheath thickness (**G**), *g* ratio (**H**), area of myelinated fibers (**I**), and area of connective tissue (**J**) between the RLN and the XII. *Abbreviations:* RLN, recurrent laryngeal nerve; XII, hypoglossal nerve. $^{**}P < 0.01$ and $^{***}P < 0.001$.

the entire length of the vocal ligament is not parallel. Thus, these findings suggest the hypothesis that the fibers in the TA are orientated differentially to perform different movements, similarly to the TO, as a previous study[4] has shown that despite the great mobility of the tongue and the highly complex arrangement of the TO muscles, its movements can be explained in terms of the activation of a small number of independent muscle groups, each corresponding to an "elementary" or

"primitive" movement. This would help explain how a small number of TO muscles can cause a large number of different tongue shapes and movements.[22]

This fact is very important, because some physiological evidence suggests that during phonation, the TA muscle can potentially affect the biomechanics of the vibrating vocal fold in various ways.[10]

The authors' results are confirmed by other studies in cats, in which the authors reported that the anatomic individualization of the divisions of the TA muscle may suggest that they play distinct physiological roles and may imply that they should not be considered as a single functional unit.[23] Similarly, other authors have reported that for the TA muscle, fiber attachment to the thyroid cartilage is curved, so that fiber length is not uniform (nearly a 12% variation). Thus, when the insertion and origin surfaces of a muscle are not parallel to each other, the fibers on one side of the sample are different in length from those on the other side.[24]

Innervation is another very important aspect of the TA and TO muscles. Classical studies report that in addition to innervating the TA muscle, the X also innervates the palatoglossus, an important muscle of the tongue.[25]

Given their results, it can be surmised that the orientation of muscle fibers in the TA muscle allows the central nervous system to monitor the length changes along the most mobile part of the vibrating vocal fold. Thus, the TA muscle produces small adjustments in the vocal fold, tensioning, and relaxing selective the parts of the vocal fold. Future research could test this hypothesis using a combination of different methods, such as electromyographic and voice analysis.

In their study, the quantitative analysis of the RLN and XII is more than a morphological description of the fibers that control the TA and TO muscle. This study is unique in the literature as it fully describes the morphometric measurements of these nerves using mathematical and stereological tools in humans. This knowledge could be essential to understanding some important aspects of laryngeal reinnervation.

The XII is often used to restore function following facial paralyses in the absence of spontaneous recuperation. A number of different ways of transferring the XII and suturing it to the distal segment of the facial nerve have been described.[14] Similarly, previous investigations[26,27] have proposed several possible advantages of *ansa cervicalis*-to-RLN laryngeal reinnervation. In addition, recent studies[13,28] have shown that the XII-to-RLN anastomosis laryngeal reinnervation technique offers numerous advantages for patients with vocal fold paralysis.

The XII-to-RLN anastomosis techniques should be within the skills of any head and neck surgeon with experience in neurorrhaphy.[28] Given that the success of these reinnervation techniques also depends greatly on understanding the microscopic anatomy of the nerves,[14,15] the data presented in the authors' study may be essential for perfecting such procedures.

The significant difference between these nerves shown in their study is not clearly discussed in the literature, but they believe that this difference can be explained by the location where the analysis of nerves is made. Because of this, the authors have been careful to examine the same locations previously studied by other authors in the RLN[16] and XII[14] to minimize the influence of these anatomical differences in their results.

The main morphological parameter measured in their study that could be useful in respect to XII-to-RLN reinnervation is the nerve area. Some studies provide measurements of nerve diameter but fail to measure nerve area. The authors believe that nerve area is a more reliable parameter and presents less bias when compared with nerve diameter. However, comparisons between these studies could be made, considering that, in general, nerve sections present a circle-like shape. Thus, the average nerve diameters, in the authors' case, RLN and XII, could be easily extracted from their study using the circle formula.[17]

In addition, their study confirms that when compared with the XII, the parameters of the RLN are significantly lower, as shown by the intraperineural area (83.4%), total number of fibers (71%), myelinated fiber area (88%), myelinated fiber diameter (53.8%), axon diameter of the myelinated fiber (65.8%), *g* ratio (26.4%), and percentage areas of myelinated fibers (78.1%).

On the other hand, their results also show that the RLN has a higher myelinated fiber density (45.6%), average myelin sheath thickness (43.1%), and percentage of endoneurial connective tissue (59.5%). One of the interesting findings in the present study was the smaller *g* ratio values in the RLN. This fact is very important as it shows that although the RLN fibers have a smaller area and axon diameter, they exhibit a greater degree of the myelination and this can explain a larger quantity of endoneurial connective tissue in the RLN when compared with XII.

Finally, the simple comparison of the histological organization of the TA and TO muscles carried out in their study may help explain one of the most important questions about human speech: how the TA muscle selectively controls different parts of the vocal fold. Furthermore, the present study also provides morphometric data on the RLN and XII, which may be essential to the success of an important reinnervation technique. Additionally, for future studies, it would be interesting to compare the results from their study with those in which the histology of the same nerves is evaluated after palsy.

CONCLUSION

This study represents the first complete light microscopic comparison of the human TA and TO muscles and their respective nerves (RLN and XII), using a stereological method in a well-defined region of these muscles and nerves, and as such, contributes toward enhancing our understanding of phonation in general. It is well known that the complexity of the tongue movements depends on the organization of muscle fibers.[2,4,6–9,29] The vocal fold presents a more complex structural environment, where multiple agonist/antagonist muscle groups are present, but it is important to note that the TA muscle is the main muscle in this environment and presents a more prominent action when compared with the

other muscles. Moreover, the TA muscle is the only muscle that selectively controls different parts of the vocal fold. Thus, the selective control of different regions of the vocal fold can be explained, at least in part, by fact that the organization of muscle fibers in the TA[10,23,24] is similar to that seen in the TO muscles. Furthermore, their results can be considered as general reference points for future studies aimed at further elucidating the relationship between peripheral nerve regeneration and any possible relation to the pathogenesis of diseases, such as idiopathic laryngeal hemiparalysis and hemiatrophy of the TO muscles.

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REFERENCES

1. Ludlow CL. Central nervous system control of the laryngeal muscles in humans. *Respir Physiol Neurobiol*. 2005;147:205–222.
2. Mu L, Sanders I. Human tongue neuroanatomy: nerve supply and motor endplates. *Clin Anat*. 2010;23:777–791.
3. Nakai T, Goto N, Moriyama H, Shiraishi N, Nonakan N. The human recurrent laryngeal nerve during the aging process. *Okajimas Folia Anat Jpn*. 2000;76:363–367.
4. Hiiemae KM, Palmer JB. Tongue movements in feeding and speech. *Crit Rev Oral Biol Med*. 2003;14:413–429.
5. Ishii K, Adachi H, Tsubaki K, Ohta Y, Yamamoto M, Ino T. Evaluation of recurrent nerve paralysis due to thoracic aortic aneurysm and aneurysm repair. *Laryngoscope*. 2004;114:2176–2181.
6. Langdon HL, Khaeber KM, Barnwell YM. The morphology of M. palatoglossus in the 15-week human fetus. *Anat Anz*. 1979;146:10–17.
7. Zur KB, Mu L, Sanders I. Distribution pattern of the human lingual nerve. *Clin Anat*. 2004;17:88–92.
8. Tourè G, Vacher C. Anatomic study of tongue architecture based on fetal histological sections. *Surg Radiol Anat*. 2006;28:547–552.
9. Stone M, Lundberg A. Three-dimensional tongue surface shapes of English consonants and vowels. *J Acoust Soc Am*. 1996;99:3728–3737.
10. Sanders I, Han Y, Wang J, Biller H. Muscle spindles are concentrated in the superior vocalis subcompartment of the human thyroarytenoid muscle. *J Voice*. 1998;12:7–16.
11. Crumley RL. Unilateral recurrent laryngeal nerve paralysis. *J Voice*. 1994; 8:79–83.
12. Paniello RC. Laryngeal reinnervation. *Otolaryngol Clin North Am*. 2004; 37:161–181.
13. Aynehchi BB, McCoul ED, Sundaram K. Systematic review of laryngeal reinnervation techniques. *Otolaryngol Head Neck Surg*. 2010;143:749–759.
14. Vacher C, Dauge MC. Morphometric study of the cervical course of the hypoglossal nerve and its application to hypoglossal facial anastomosis. *Surg Radiol Anat*. 2004;26:86–90.
15. Asaoka K, Sawamura Y, Nagashima M, Fukushima T. Surgical anatomy for direct hypoglossal-facial nerve side-to-end "anastomosis". *J Neurosurg*. 1999;91:268–275.
16. Jotz GP, de Campos D, Rodrigues MF, Xavier LL. Histological asymmetry of the human recurrent laryngeal nerve. *J Voice*. 2011;25:8–14.
17. Ilha J, Araujo RT, Malysz T, Hermel EE, Rigon P, Xavier LL, Achaval M. Endurance and resistance exercise training programs elicit specific effects on sciatic nerve regeneration after experimental traumatic lesion in rats. *Neurorehabil Neural Repair*. 2008;22:355–366.
18. Matheron G. *The Theory of Regionalized Variables and its Applications. Les cahiers du Centre de Morphologie Mathématique de Fontainebleau*. 5th ed. Fontainebleau, France: Ecole Nationale Supérieure des Mines de Paris; 1971.
19. Mathieu O, Cruz-Orive LM, Hoppeler H, Weibel ER. Measuring error and sampling variation in stereology: comparison of the efficiency of various methods for planar image analysis. *J Microsc*. 1980;121:75–88.
20. Kiêu K, Souchet S, Istas J. Precision of systematic sampling and transitive methods. *J Stat Plan Inference*. 1999;77:263–279.
21. García-Piñana M, Cruz-Orive LM, Mackay CE, Palkenberg B, Roberts N. Comparison of MR imaging against physical sectioning to estimate the volume of human cerebral compartments. *Neuroimage*. 2003;18:505–516.
22. Napadow VJ, Chen Q, Wedeen VJ, Gilbert RJ. Intramural mechanics of the human tongue in association with physiological deformations. *J Biomech*. 1999;32:1–12.
23. Imamura R, Yoshida Y, Fukunaga H, Nakashima T, Hirano M. Thyroarytenoid muscle: functional subunits based on morphology and muscle fiber typing in cats. *Ann Otol Rhinol Laryngol*. 2001;110:158–167.
24. Hunter EJ, Alipour F, Titze IR. Sensitivity of elastic properties to measurement uncertainties in laryngeal muscles with implications for voice fundamental frequency prediction. *J Voice*. 2007;21:641–650.
25. Standring S. *Gray's Anatomy—The Anatomical Basis of Clinical Practice*. 40th ed. New York, NY: Churchill Livingstone; 2009:1576.
26. Crumley RL. Update: ansa cervicalis to recurrent laryngeal nerve anastomosis for unilateral laryngeal paralysis. *Laryngoscope*. 1991;101:384–388.
27. Su WF, Hsu YD, Chen HC, Sheng H. Laryngeal reinnervation by ansa cervicalis nerve implantation for unilateral vocal cord paralysis in humans. *J Am Coll Surg*. 2007;204:64–72.
28. Paniello RC. Laryngeal reinnervation with the hypoglossal nerve: II. Clinical evaluation and early patient experience. *Laryngoscope*. 2000;110: 739–748.
29. Mu L, Sanders I. Neuromuscular organization of the canine tongue. *Anat Rec*. 1999;256:412–424.

5.2 Segundo artigo ("*In Press*" no *Journal of Voice* – Fator de Impacto no JCR 2011: 1.39)

Morphology of Fetal Vocal Fold and Associated Structures

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Summary: This study is the first detailed qualitative morphologic description of the vocal fold and its associated structures (false vocal fold, larynx ventricle, epithelium, mucous glands, blood vessels, and vocal ligament) of a human fetus aged 25 weeks. In addition, a quantitative analysis of thyroarytenoid (TA) muscle fiber orientation is presented to investigate similarities with adult TA. Histologic cross sections from the vocal fold and the anterior, middle, and posterior regions of the TA muscle were examined bilaterally, and both qualitative and quantitative analyses show that the vocal fold and most of the associated structures are completely established in the studied sample.

Key Words: Thyroarytenoid muscle–Fetus–Microanatomy–Vocal fold.

INTRODUCTION

Although a great deal has been written regarding the structure of the vocal fold in adults, few studies have investigated this structure in children and neonates. One classic example of this lack of information about newborn and fetal vocal fold is the presence or absence of the vocal ligament at birth.[1] For example, one study has reported that this structure is absent at birth,[2] whereas another has shown the presence of adult-like vocal ligament at 7–9 months of gestational age.[1]

Although little has been written about the fetal vocal ligament, even less is known regarding the fetal thyroarytenoid (TA) muscle, also referred to as the vocal muscle.[3,4] TA is responsible for selective adjustments in different parts of the vocal fold in adults.[3–5] This ability is probably related to the fact that the TA muscle fibers present the same microanatomic organization seen in the tongue, where transverse (T) and longitudinal (L) fiber orientations are seen. Thus, this arrangement of muscle fibers allows the vocal fold and tongue to present numerous possibilities of movement.[6]

Moreover, the TA muscle is the only muscle that selectively controls different parts of the vocal fold. Thus, the selective control of different regions of the vocal fold can be explained, at least in part, by the fact that the organization of muscle fibers in TA is similar to that seen in the tongue muscles.[6] In this context, considering the complexity of mobility/frequency of the vocal patterns of a newborn,[7–9] it is plausible to deduce that the TA muscle fiber orientation is similar in newborns and adults. Nevertheless, there are no data in the literature about TA muscle fiber orientation in newborns or fetuses.

Thus, using a very rare anatomic piece, a fetus aged 25 weeks, the goals of our study were (1) to perform a detailed morphologic/histologic description of the vocal fold and its associated structures (false vocal fold, larynx ventricle, epithelium, mucous glands, blood vessels, and vocal ligament) and compare the findings with previous morphologic descriptions made in adults and (2) to analyze the TA fiber orientation to identify any similarities with patterns found in adults.

MATERIALS AND METHODS

Human tissue

The histologic sample of TA muscle along with its vocal fold was obtained from the collection of the Laboratory of Histology and Pathology of the *Universidade de Santa Cruz do Sul*, Rio Grande do Sul, Brazil. The present study is based on one female fetus aged 25 weeks after conception, with the cause of death unknown (Figure 1A), and based on only one female fetus because this material is extremely rare.

Tissue collection

The TA muscle along with its vocal fold was completely and bilaterally removed (3 mm) from the larynx, which had been previously fixed in buffered formalin 10%. Thus, the structures associated with the vocal fold and the TA muscle fibers have not been distorted or compartmentalized during processing. To understand the organization of muscle fibers throughout the length of the TA muscle and vocal fold, the anterior, middle, and posterior regions were extracted (1 mm in length each) for morphometric evaluation (Figure 1B). Thus, these samples (anterior, middle, and posterior) presented a complete cross-sectional view of the vocal fold and TA, from the thyroid cartilage, anteriorly, to the vocal process of the arytenoid cartilage, posteriorly.

Histology

The samples from each region were fixed in 10% formalin solution, dehydrated in a graded series of ethanol, and embedded in paraffin. Three sections (7 μm each) with intervals of 300 μm

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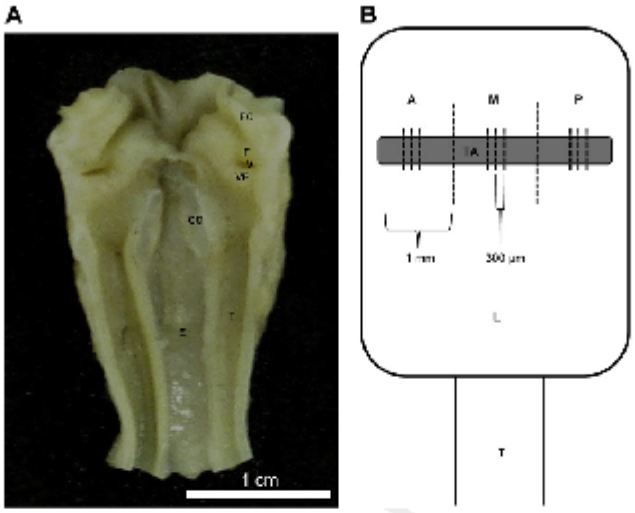

FIGURE 1. A. Midsection of the larynx showing the internal structures such as EC, F, V, VF, CC, T, and E. B. Schematic representation showing the following regions: A, M, and P, where the thyroarytenoid muscle was collected (*indicated by a dotted line*) bilaterally. EC, epiglottis cartilage; F, false vocal fold; V, ventricle of larynx; VF, vocal fold; CC, cricoid cartilage; L, larynx; T, trachea; E, esophagus; A, anterior; M, middle; P, posterior.

were obtained from each of the three regions (anterior, middle, and posterior) using a microtome (Leica, Germany) (Figure 1B). These sections were mounted onto slides, deparaffinized with xylene, rehydrated and stained with hematoxylin-eosin, washed in water, dehydrated in a graded series of ethanol, cleared with xylene, covered with entellan, and coverslipped.

Qualitative analysis

The different sections of the vocal fold were analyzed by three experts in the histology of the adult larynx, and the morphologic findings were discussed by these observers. Thus, all the histologic sections were checked for the presence or absence of structures associated with the vocal folds such as false vocal fold, larynx ventricle, epithelium, mucous glands, blood vessels, and vocal ligament.

Morphometric measurements of the TA muscles

Digitized images of the muscles were obtained using an *Olympus BX 50* microscope (400×) (Olympus, Japan) coupled to a video camera (Leica DC 300F) interfaced by *Leica Image 50* (IM50) software. The images obtained were measured using Image-Pro Plus Software (*Image-Pro Plus 6.0*; Media Cybernetics, Silver Spring, MD).

The TA muscle fibers (vocalis portion) were classified, as described in a previous study,[6] into three categories according to their orientation: T, undefined (U), and L. Briefly, 10 equal-sized areas of interest and grid masks with an area/point value of 400 μm^2 were laid over the images (distributed equidistantly) to estimate both the number of fibers per area and the area of muscle fiber and enable the classification of the fibers as T, U, or L according to their orientation (Figures 2 and 3). The area of muscle fiber was obtained using the following equation: $\hat{A} = \sum p \cdot a/p$, where $\hat{A}$ is the area, $\sum p$ is the sum of points counted, and a/p is the area/point value.[6]

The criteria for the classification were established after detailed observation and measurements taken by three histology specialists who were blinded to the source of the images. The fibers were classified using the following criteria: the TA muscle fibers were identified, according to their areas, as T between 0 and 30 μm^2, U between 31 and 59 μm^2, and L more than 60 μm^2.

The shape coefficient, also known as the Shape Z,[6,10,11] was also used to define the fiber types. This parameter is obtained using the following equation: Shape $Z = P/\sqrt{\hat{A}}$, where Shape Z is the shape coefficient, P is the perimeter, and $\hat{A}$ is the area value.

Based on a comparison of the Shape Z values obtained with the classification made by the observers, the right TA fibers were classified as: T, between 0 and 4.19; U, between 4.20 and 4.28; and L, between 4.29 and 4.64. The left TA fibers were classified as: T, between 0 and 4.09; U, between 4.10 and 4.29; and L, between 4.30 and 4.85. These classifications were also used to estimate the percentages of T, U, and L fibers in all the analyzed sections.

We emphasize that although our morphologic investigation provides innovative and reliable results about the structures associated to vocal fold in the fetus and the orientation of the TA muscle fibers, it is based on the bilateral analysis of only one

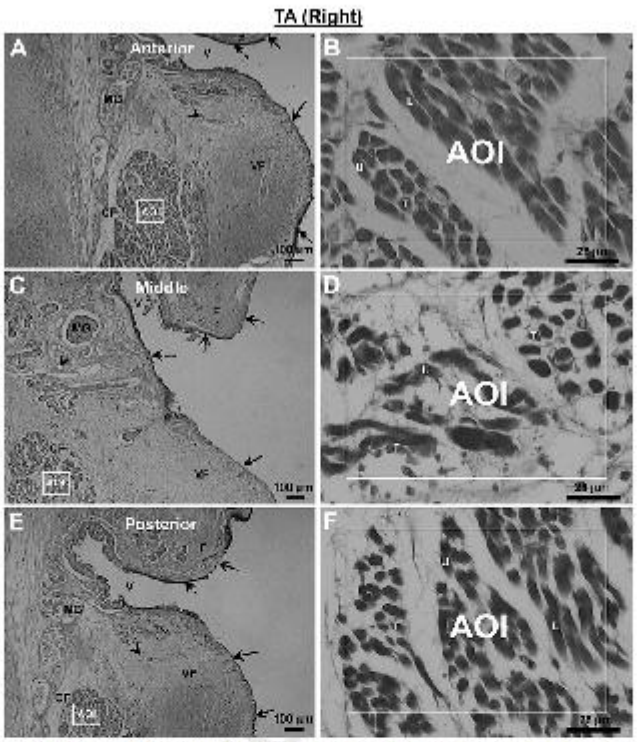

FIGURE 2. Digitized images of histologic cross sections of the right TA muscle. **A**, **C**, and **E**, Images of the anterior, middle, and posterior regions, respectively, showing the arrangement of AOIs (400 μm^2) overlaying the image of the fibers. **B**, **D**, and **F**. Representation of one of the fields (AOIs) used in the study to evaluate the fiber area. AOIs shown in images **A**, **C**, and **E** are merely illustrative and different from AOIs shown in images **B**, **D**, and **F**. The muscle fibers were classified into T, U, and L. Only the fibers with their entire area within AOI were counted. TA, thyroarytenoid; AOIs, areas of interest; T, traverse; U, undefined; L, longitudinal; CF, collagen fibrils; MG, mucous glands; VF, vocal fold; V, ventricle; F, false vocal fold; dotted arrow, pseudostratified ciliated columnar epithelium; thin arrow, non-keratinized stratified squamous epithelium; head arrow, blood vessels (hematoxylin-eosin stain).

subject. Thus, to avoid an overestimation of our results, we suggest that future studies with larger numbers of subjects will be needed to definitively confirm our findings.

Furthermore, the proposals in this article should be considered a point of departure for future studies that may advance the understanding of development and the contributions of variations in the prenatal environment.

RESULTS

In the qualitative analysis of the structures associated with the vocal fold on the right and left sides, the histologic sections showed that at 25 weeks of gestation, the laryngeal mucous membrane already consists of two pairs of folds: false vocal folds, above, and the vocal folds, below. Between these two pairs of folds, a narrow ventricle of the larynx can be observed. The false vocal folds and vocal folds are covered by non-keratinized stratified squamous epithelium, whereas the remaining structures (larynx vestibule, larynx ventricle, and infraglottic cavity) are covered by pseudostratified ciliated columnar epithelium, also non-keratinized (Figures 2 and 3).

The histologic sections from both sides revealed the presence of numerous mucous glands, except in the vocal folds. These glands are mixed type with excretory ducts, which open into the lumen of the larynx and which probably have the function of lubricating the mucosa of the larynx.

Furthermore, in all the analyzed sections, the structure of the vocal fold can be seen to be well formed along its ligament, on the right and left sides. Blood vessels were found in all the histologic sections on both sides organized in different directions (Figures 2 and 3).

In our study, the estimation of fiber area and Shape Z showed that both right and left TA present T, U, and L fibers in each muscle region, the same pattern previously described in adult TA[6] (Figure 4).

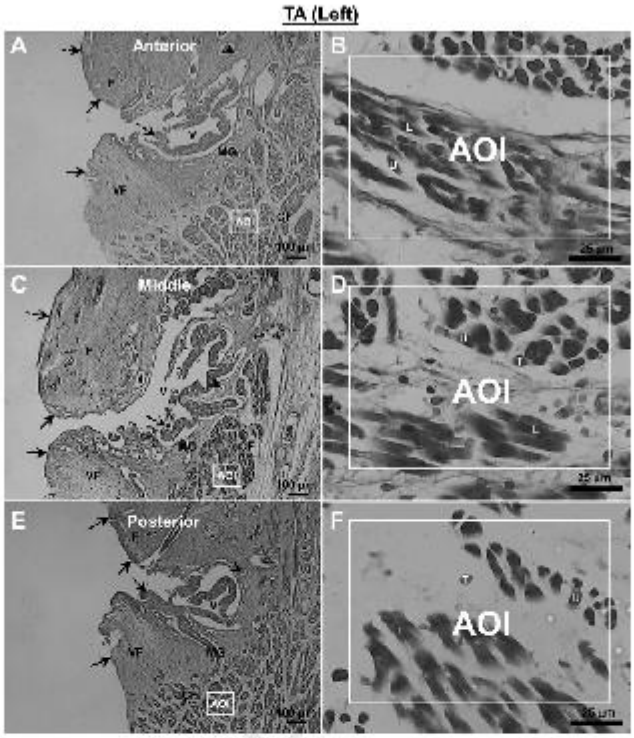

FIGURE 3. Digitized images of histologic cross sections of the left TA muscle. **A**, **C**, and **E**. Images of the anterior, middle, and posterior regions, respectively, showing the AOIs (400 μm^2) overlaying the image of the fibers. **B**, **D**, and **F**. Representation of one of the fields (AOIs) used in the study to evaluate the fiber area. AOIs shown in images **A**, **C**, and **E** are merely illustrative and different from AOIs shown in images **B**, **D**, and **F**. The muscle fibers were classified into T, U, and L. Only the fibers with entire area within AOI were counted. TA, thyroarytenoid; AOIs, areas of interest; T, transverse; U, undefined; L, longitudinal; CF, collagen fibrils; MG, mucous glands; VF, vocal fold; V, ventricle; F, false vocal fold; dotted arrow, pseudostratified ciliated columnar epithelium; thin arrow, non-keratinized stratified squamous epithelium; head arrow, blood vessels (hematoxylin-eosin stain).

In the right TA muscle, the distribution of muscle fiber types was as follows: in the anterior region, 68% of T, 29% of U, and 3% of L fibers; in the middle region, 64% of T, 31% of U, and 5% of L fibers; and in the posterior region, 57% of T, 39% of U, and 4% of L fibers (Figure 4A).

In the left TA muscle, the distribution of muscle fiber types was as follows: in the anterior region, 31% of T, 51% of U, and 18% of L fibers; in the middle region, 48% of T, 38% of U, and 14% of L fibers; and in the posterior region, 57% of T, 33% of U, and 10% of L fibers (Figure 4B). No statistical evaluation of lateral or regional differences was possible because of the reduced number of subjects, n = 1.

DISCUSSION

The qualitative analysis of the structures associated with the vocal fold of a fetus aged 25 weeks conducted in this study demonstrates that all the structures that comprise the adult vocal epithelium exist at this gestational age. An earlier study[12] reported that the structures of the lamina propria in fetuses aged between 13 and 23 weeks differ from those of the adults. Thus, this period between the 23rd and 25th gestational weeks may be crucial for the development of the vocal fold. Moreover, our results have been confirmed by a recent study in which it was reported that certain structures associated with the vocal folds, such as the voice ligament, are present in fetuses aged 7–9 months.[1] Therefore, it is reasonable to suggest that the vocal structures are only established after the 25th gestational week.

Given these results, it can be assumed that the presence of this histologic pattern in the fetus by the 25th gestational week could provide the newborn, at least morphologically, with vocal feasibility. These data suggest a morphologic congruence between the fetal and newborn vocal fold that makes the acoustic proprieties possible.

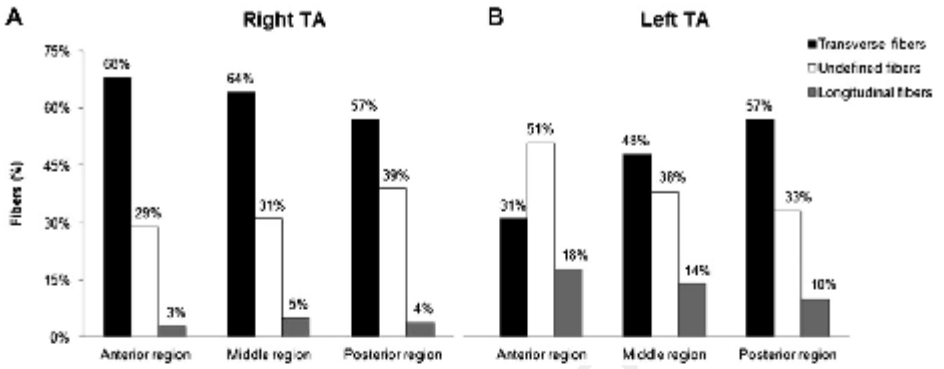

FIGURE 4. Percentage of transverse, undefined, and longitudinal fibers in the different regions: anterior, middle, and posterior of the TA muscle on the (**A**) right side and (**B**) left side. TA, thyroarytenoid.

The hypothesis developed herein is based on a previous study[7] in which the authors showed that in the acoustic characteristics of newborns, not only harmonic, expiratory, acute, and strong emissions are observed but also other emissions involving a wide variety of sounds and types of melody are present.

This fact is very important because the sound of infant crying during the early postnatal period can be likened to a biological siren, an acoustic signal that reflects the current organic condition of the infant and then broadcasts that condition to the social environment in a repetition of high-pitched sounds wavering in both frequency and temporal organization.[8]

Although much of the variability between the voices of adults and infants is because of the differences in the length of the vocal tract,[13] cineradiographic data and magnetic resonance imaging[14–16] have shown that the adult vocal apparatus is a complex remodeling of that of infants. For example, at birth, the overall vocal tract length, determined from the larynx to the lips, is about 8 cm, whereas the adult male vocal tract is about 17 cm long.[17] Moreover, the modifications that occur in the transition from the infant vocal tract to that of the adult are the result of the normal neurodevelopment. Similarly, other authors used an articulated model of the vocal tract based on such measurements and demonstrated that anatomy does not prevent even the youngest speaker from producing all the possible vocalic sounds if motor control were mature.[18] Therefore, the fundamental frequencies of these vocalizations also have different neural and physiological bases and, thus, may reflect different aspects of neurobehavioral function.[9]

In addition, numerous studies[19–21] have reported the importance of using mathematical models to obtain more reliable data for the assessment of the structures associated with the vocal fold, especially the TA muscle.[22] Thus, in our study, we were careful to use the Shape Z as a mathematical method to classify the TA muscle fibers. This procedure was chosen to estimate the shape coefficient of the different fibers and use the value obtained to classify muscular fibers.[6]

Our estimations demonstrate that by fetal age (25 weeks), the TA muscle fibers, both on the right and left sides, are not located parallely or laterally across the length of the vocal fold. These results should be interpreted carefully because the muscles analyzed are still in a developmental stage and may not match those found in newborns. However, the results seem to be consistent with those obtained in previous studies involving adult TA muscles, in which the authors showed that the percentage of T (~72%), U (~15%), and L fibers (~10%) is able to produce a large number of different vocal fold shapes and movements.[6]

In addition, our results regarding the orientation of the TA muscle fibers of a newborn can still be confirmed by other studies using animals, in which the researchers have reported that the anatomic individualization of the divisions of the TA muscle may suggest that they play distinct physiological roles and may imply that they should not be considered a single functional unit.[23] Similarly, previous investigations showed that many laryngeal muscles contain different compartments with a variety of fiber orientations and insertions.[24,25] These compartments have anatomic differences, suggesting they are functionally distinct entities, which may be independently controlled by the nervous system.[5]

Moreover, there is evidence to show that the attachment of the TA muscle to the thyroid cartilage is curved so that the fiber length is not uniform. Thus, when the insertion and origin surfaces of a muscle are not parallel to each other, the fibers on one side of the sample are different in length from the ones on the other side.[26]

Finally, we emphasize that our morphologic investigation provides innovative and reliable results about the structures associated with the vocal fold and orientation of the fetal TA muscle fibers. These data could be used as a point of departure for other studies into the prenatal development of the vocal fold and TA.

CONCLUSIONS

Our study demonstrates that the vocal fold and most of the associated structures are completely established in the sample

studied. These results are consistent with those of the previous studies[1,6,7,23,26] and provide important insights into several morphologic aspects of fetal vocal structures. Future studies with larger numbers of subjects, in different gestational ages, are needed to show all the stages of human vocal fold formation. Thus, our results undoubtedly will be useful to increase the detailed knowledge about the features of the voice in adults and newborns and can be the starting point to understand the complete embryologic development of the vocal fold and TA.

Acknowledgments

L.L.X. is a CNPq investigator.

REFERENCES

1. Nita LM, Battlehner CN, Ferreira MA, Imamura R, Sennes LU, Caldini EG, Tsuji DH. The presence of a vocal ligament in fetuses: a histochemical and ultrastructural study. *J Anat*. 2009;215:692–697.
2. Hartnick CJ, Rehbar R, Prasad V. Development and maturation of the pediatric human vocal fold lamina propria. *Laryngoscope*. 2005;115:4–15.
3. Moore KL, Dalley AF, Agur AMR. *Clinically Oriented Anatomy*. 6th ed. MD: Lippincott Williams & Wilkins; 2009.
4. Standring S. *Gray's Anatomy—The Anatomical Basis of Clinical Practice*. 40th ed. New York, NY: Churchill Livingstone; 2008.
5. Sanders I, Han Y, Wang J, Biller H. Muscle spindles are concentrated in the superior vocalis subcompartment of the human thyroarytenoid muscle. *J Voice*. 1998;12:7–16.
6. De Campos D, Do Nascimento PS, Ellwanger JH, Gehlen G, Rodrigues MF, Jotz GP, Xavier LL. Histological organization is similar in human vocal muscle and tongue—a study of muscles and nerves. *J Voice*. 2012: In press.
7. Branco A, Behlau M, Rehder MI. The neonate cry after cesarean section and vaginal delivery during the first minutes of life. *Int J Pediatr Otorhinolaryngol*. 2005;69:681–689.
8. Nicollas R, Giordano J, Perrier P, et al. Modelling sound production from an aerodynamical model of the human newborn larynx. *Biomed Signal Process Control*. 2006;1:102–106.
9. Zeskind PS, McMurray MS, Garber KA, et al. Development of translational methods in spectral analysis of human infant crying and rat pup ultrasonic vocalizations for early neurobehavioral assessment. *Front Psychiatry*. 2011;2:1–16. article 56.
10. Matheron G. The theory of regionalized variables and its applications. In: *Les Cahiers du Centre de Morphologie Mathématique de Fontainebleau*. 5th ed Fontainebleau, France: Ecole Nationale Supérieure des Mines de Paris; 1971.
11. García-Fiñana M, Cruz-Orive LM, Mackay CE, Pakkenberg B, Roberts N. Comparison of MR imaging against physical sectioning to estimate the volume of human cerebral compartments. *Neuroimage*. 2003;18:505–516.
12. Subotić R, Vecerina S, Knjina Z, Hirano M, Kurita S. Histological structure of vocal fold lamina propria in foetal larynx. *Acta Otolaryngol*. 1984;97:403–406.
13. Smith DRR, Patterson RD. The interaction of glottal-pulse rate and vocal-tract length in judgements of speaker size, sex, and age. *J Acoust Soc Am*. 2005;118:3177–3186.
14. Fitch WT, Giedd J. Morphology and development of the human vocal tract: a study using magnetic resonance imaging. *J Acoust Soc Am*. 1999;106:1511–1522.
15. Callan DE, Kent RD, Guenther FH, Vorperian HK. An auditory-feedback-based neural network model of speech production that is robust to developmental changes in the size and shape of the articulatory system. *J Speech Lang Hear Res*. 2000;43:721–736.
16. Vorperian HK, Kent RD, Lindstrom MJ, Kalina CM, Gentry LR, Yandell BS. Development of vocal tract length during childhood: a magnetic resonance imaging study. *J Acoust Soc Am*. 2005;117:338–350.
17. Serkhane JE, Schwartz JL, Boë LJ, Davis BL, Matyear CL. Infants' vocalizations analyzed with an articulatory model: a preliminary report. *J Phon*. 2007;35:321–340.
18. Ménard L, Schwartz JL, Boë LJ. Role of vocal tract morphology in speech development: perceptual targets and sensorimotor maps for synthesized French vowels from birth to adulthood. *J Speech Lang Hear Res*. 2004;47:1059–1080.
19. Alipour F, Titze IR, Hunter EJ, Tayama N. Active and passive properties of canine abduction/adduction laryngeal muscles. *J Voice*. 2005;19:350–359.
20. Hunter EJ, Titze IR, Alipour F. A three-dimensional model of vocal fold abduction/adduction. *J Acoust Soc Am*. 2004;115:1747–1759.
21. Gunter HE. A mechanical model of vocal-fold collision with high spatial and temporal resolution. *J Acoust Soc Am*. 2003;113:994–1000.
22. Deguchi S, Kawahara Y, Takahashi S. Cooperative regulation of vocal fold morphology and stress by the cricothyroid and thyroarytenoid muscles. *J Voice*. 2011;25:255–263.
23. Imamura R, Yoshida Y, Fukunaga H, Nakashima T, Hirano M. Thyroarytenoid muscle: functional subunits based on morphology and muscle fiber typing in cats. *Ann Otol Rhinol Laryngol*. 2001;110:158–167.
24. Ludlow CL. Central nervous system control of the laryngeal muscles in humans. *Respir Physiol Neurobiol*. 2005;147:205–222.
25. Bryant NJ, Woodson GE, Kaufman K, Rosen C, Hengesteg A, Chen N, Yeung D. Human posterior cricoarytenoid muscle compartments. Anatomy and mechanics. *Arch Otolaryngol Head Neck Surg*. 1996;122:1331–1336.
26. Hunter EJ, Alipour F, Titze IR. Sensitivity of elastic properties to measurement uncertainties in laryngeal muscles with implications for voice fundamental frequency prediction. *J Voice*. 2007;21:641–650.

5.3 Terceiro artigo (Aceito para publicação no *Journal of Voice* – Fator de Impacto no JCR 2011: 1.39)

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Sexual dimorphism in the human vocal fold innervation

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Footnotes to the title: Sexual dimorphism in the vocal fold innervation

Abstract

This study investigated the sexual dimorphism in the recurrent laryngeal nerve (RLN) and thyroarytenoid muscle (TA) which control the vocal fold. The RLN and TA were bilaterally studied in human specimens obtained from necropsies (7 men and 7 women). Analysis of the morphometric parameters showed that the RLN of the men were significantly larger, as shown by the intraperineural area (42.5%) (p= 0.006), total number of fibers (38.0%) (p= 0.0002), axonal area (34.3%) (p= 0.0001), axonal diameter (19.0%) (p= 0.0001) and the area of the nerve occupied by myelinated fibers (34.9%) (p= 0.001). By contrast, in the women, our results showed that the area of the RLN occupied by endoneurial connective tissue was larger (5.7%) (p= 0.001). Estimation of the fiber area and shape coefficient showed that the histological organization of TA is similar in men and women. These results may contribute toward enhancing our understanding about the voice neurobiology.

Key Words: sexual dimorphism - recurrent laryngeal nerve - thyroarytenoid muscle - voice.

INTRODUCTION

Several studies have demonstrated the presence of sexual dimorphism in the organization of the nervous system in different groups of vertebrates, such as amphibians,[1] reptiles,[2,3] birds,[4] and mammals.[5-9] Similarly, numerous studies with animals have shown sexual dimorphism in different regions of the nervous system involved in vocalization/vocal control.[4,10,11] However, little is known about this aspect in humans.

Although some authors have reported the existence of sexual dimorphism in the neural structures involved in vocal control at the level of the central nervous system in humans,[12] to our knowledge there is no study in the current literature that shows the presence or absence of sexual dimorphism in structures related to the peripheral nervous system and muscles related to vocalization, especially in the recurrent laryngeal nerve (RLN) and thyroarytenoid muscle (TA).

In addition, classically, the variability between the voices of men and women has been explained by the differences in the mass of the vocal folds.[13] This sexual dimorphism is attributable to increased testosterone at puberty in males which stimulates growth in the laryngeal cartilages.[14] In the twentieth century, the dominant model of sexual differentiation stated that genetic sex (*XX versus XY*) causes differentiation of the gonads, which then secrete gonadal hormones that act directly on tissues to induce sex differences in function. This serial model of sexual differentiation was simple, unifying and seductive. Recent evidence, however, indicates that the linear model is incorrect and that sex differences arise in response to diverse sex-specific signals originating from inherent differences in the genome and involves cellular mechanisms that are specific to individual tissues or brain regions.[15]

Likewise, studies of songbirds and rodents suggest that male and female brain cells are also intrinsically different because of the sex differences in the expression of sex chromosome genes within the cells.[16] As those differences in gene expression and alterations in brain structures are often responsible for important changes in different body characteristics, such as innervation and morphology, our hypothesis is that sexual dimorphism may be present in the RLN and TA of subjects of different genders. This study aims to investigate this matter.

MATERIALS AND METHODS

Specimens

All the nerves and muscles analyzed were obtained from necropsies of fourteen caucasian subjects who had died suddenly (7 men [age=71.14±8.07] and 7 women [age=75.71±7.83]) (Mean±SD), from the Department of Forensic Medicine. It should be pointed out that that although our analysis was performed in older individuals, due to the difficulty of obtaining younger specimens, this limitation was also found in a previous study, in which subjects, average ages 70 years for men and 75 years for women, were analyzed.[17] Furthermore, in the present study, the men and women were in the same age group. This study was approved by the Ethics Committee of the *Universidade Federal do Rio Grande do Sul*, RS, Brazil.

Dissection

The dissection of the RLN was performed according to Jotz et al.[18] The TA muscle was bilaterally removed (~10 mm) from the larynx, the middle region of the TA was chosen for our study. This choice was based on a previous study[19] which showed that this region presents more and better defined muscle fibers when compared to the other regions of the TA (Figure 1).

Data acquisition

The digitized images of the RLN and TA were captured using an Olympus BX 50 microscope (4X, 10X and 100X) (Olympus, Japan) coupled to a video camera (Leica DC 300F) interfaced by Leica Image 50 (IM50) software. Pearson's correlation coefficients (*r*) were calculated to determine the relationship between the results obtained by the two researchers for all analyses. The values obtained for this correlation demonstrate the high level of reliability of the observations made by the blinded researchers.

Histological and morphometric measurements of the RLN

Based on previous studies,[20] the specimens of the RLN were fixed by immersion for 24 hours in a modified Karnovsky solution (Sigma Chemical Company, St. Louis, MO, USA). Next, they were bathed in 0.05 mol/L of sodium cacodylate solution, postfixed in 1% osmium tetroxide (Sigma Chemical Company) for 2 hours, dehydrated in an increasing graded series of acetone (Electron Microscopy Sciences, Hatfield, PA, USA), and embedded in epoxy resin (araldite, durcupan; Fluka, Buchs, Switzerland) which was then polymerized at 60°C. Three semithin cross-sections (1 μm) were

obtained using an ultramicrotome (MT 6000- XL; RMC, Tucson, AZ, USA) with intervals of 100 μm and stained with 1% toluidine blue (Merck, Darmstadt, Germany) in 1% sodium tetraborate (Ecibra, Curitiba, Brazil) (Figure 1).

According to previous protocols[18,21,22] of planar morphometry, the myelinated fiber density (number of fibers/mm^2), intraperineural area (mm^2), total number of fibers, axonal area (μm^2), axonal diameter (μm), myelin sheath thickness (μm), area of the nerve occupied by myelinated fibers (%) and area of the nerve occupied by endoneurial connective tissue (%) was estimated using the measurement tools in the Image Pro-Plus Software [Image Pro-Plus 6.0; Media Cybernetics, Silver Spring, MD, USA] (IPP 6.0) (Figure 2A-D).

Histological and morphometric measurements of the TA

Based on previous studies[19] the samples from each muscle were fixed in 10% formalin solution, dehydrated in a graded series of ethanol and embedded in paraffin. Five sections (10 μm) with intervals of 100 μm of the TA were obtained using a microtome (Leica, Germany) (Figure 1). These sections were mounted onto slides, deparaffinized with xylene, rehydrated, stained with hematoxylin-eosin, dehydrated in a graded series of ethanol, diaphanized with xylene, and covered with Entellan (Merck, Darmstadt, Germany) and coverslips.

The TA muscle fibers were classified, as described in a previous study,[21] into three categories according to their orientation: transverse (T), undefined (U) and longitudinal (L).

Using the measurement tools in the IPP 6.0, grid masks with an area/point value of 400 μm^2 were laid over the images in order to estimate both the number of fibers per area and the area of muscle fiber to enable the classification of the fibers as T, U or L according to their orientation.[21] The area of muscle fiber was obtained using the following equation: $\hat{A} = \sum p.a/p$. Where $\hat{A}$ is the area, $\sum p$ is the sum of points counted and a/p is the area/point value.

The criteria for this classification were obtained after detailed observation and measurements taken by two histology specialists who were also blinded to the source of the images. The fibers were classified using the following criteria: the TA muscle fibers of male and female were identified, according to their areas, as T between 0 and 2000 μm^2, U between 2000 – 3200 μm^2 and L more than 3200 μm^2 (Figure 2E and F).

In addition, previous studies[23,24] have reported the importance of using mathematical models to obtain more reliable data for the assessment of the structures associated with the vocal fold, specially the TA muscle.[25] Thus, in our study we were careful to use the Shape Z as a mathematical method to classify the TA muscle fibers. This procedure was chosen to estimate the shape coefficient of the different fibers and use the value obtained to classify muscular fibers. This analysis may be important for the fact that recent studies have indicated that the orientations of the fibers (T, U and L) are able to produce a large number of different vocal fold shapes and movements.[21]

Thus, the shape coefficient, also known as the Shape Z,[21,26-29] was also used to define the fiber types. This parameter is obtained using the following equation: $Shape\ Z = P/\sqrt{\hat{A}}$. Where Shape Z is the shape coefficient, P is the perimeter and Â is the area value.

Based on a comparison of the Shape Z values obtained with the classification made by the observers, the TA fibers in males were classified as: T, between 0 and 4.53; U, between 4.53 and 4.78; and L, between 4.78 and 5.22. In females, the TA fibers were classified as: T, between 0 and 4.83; U, between 4.83 and 5.94; and L, between 5.94 and 6.25.

These classifications were used to estimate the percentages of T, U and L fibers in all the analyzed sections in right and left TAs from men and women.

Statistical analysis

The statistical analyses were carried out using an independent samples *t* test ($P<0.05$), to compare the different parameters in men and women. All statistical analyses were performed using *Graph Pad Prism 5.0* software (Graphpad Software Inc., USA).

RESULTS

RLN findings

The main result of our study is the evident sexual dimorphism in the RLN. This study shows that, when compared to the RLN of the women, the parameters of the RLN of the men are significantly larger, as shown by the intraperineural area (42.5%), total number of fibers (38.0%), axonal area (34.3%), axonal diameter (19.0%) and the area of the nerve occupied by myelinated fibers (34.9%) (Table 1 and Figure 2A-D).

On the other hand, our results show that, in the women, endoneurial connective tissue occupied a larger area of the RLN (5.7%). No statistical differences between the two nerves were observed in terms of the myelinated fiber density and average myelin sheath thickness (Table 1 and Figure 2A-D).

TA findings

The percentages of transverse, undefined and longitudinal fibers in muscles from the men were similar to those found in muscles from the women (Table 1 and Figure 2E and F).

DISCUSSION

The importance of the fiber area for nerve impulse conduction has been demonstrated in several studies because the conduction velocity is related to the fiber area/axonal diameter, and increases in these parameters are responsible for increases in the velocity of impulse conduction.[30-32]

This aspect is of great importance due to the fact that any sexual dimorphism would presumably alter nerve conduction velocity and the electrical signal would cause differences in the firing frequency of the TA muscle fibers and consequently in the rate at which the vocal folds open and close, known as the glottal-pulse rate (GPR). Statistical clustering studies have consistently highlighted GPR and vocal tract related variables as explaining most of the variance between the speech sounds of adult males and females.[33,34]

Some authors state that adult males have pitches about an octave lower than adult females primarily because the vibrating segments of the male vocal folds are about 60% longer than those of the female.[35] However, studies indicate that the GPR of the men tends to increase with advancing age, with an evident increase among men between the fifth and eighth decades of life.[36]

These data, at least partially, appear to be consistent with our findings, since our study has provided morphological evidence to show that the male RLN probably confers a higher electrical conduction rate when compared with that of females, and this can increase the GPR in men.

It would seem logical to presume that the greater the number for TA muscle fibers, the greater the number of nerve fibers that would be required.[37] In relation to nerve conduction velocity, axonal diameter is considerably more important than the

nerve fiber density.[30-32] Our results also demonstrate that although the density was not statistically different, the axonal diameter was larger in men, indicating that men can present a higher conduction velocity in relation to women.

Given these results, it can be assumed that the variability between the voices of men and women is probably not solely due to the differences in vocal fold mass,[13] biomechanical properties of the vocal fold tissue,[38] length of the vocal organ/vocal tract[14,39] and in the brain regions involved in vocal control,[12] but also due to structural/cellular differences at the level of the peripheral nervous system. Thus, it would seem to be the case that the peripheral nervous structures of men and women may follow slightly different paths to achieve similar levels of function.

The last decade has seen an exponential increase in evidence for structural, cellular, and molecular sex differences in the nervous system that can be described as true dimorphisms, defined as the occurrence of two forms in the same species. These include regions of human and animal brains that are important for cognition, memory and language.[12,40,41]

Classically, the sexual dimorphism in the voices of men and women has been main attributed to the increased testosterone levels at puberty in males which stimulate growth in the laryngeal cartilages.[14] One highly-salient cue is voice pitch; while in adult men the pitch tends to be low in women it tends to be higher. Pitch is determined by the rate of opening and closing of the vocal folds, also known as the glottal-pulse rate. Another important cue is vocal-tract length, which tends to be longer in adult men than in women.[39]

Currently, there are no human studies in the literature showing sexual dimorphism in the human vocal fold innervation. Therefore, to explain our results, one hypothesis that we developed is that this difference in terms of innervation between men and women could also be explained by the fact that the laryngeal musculature is larger in men than in women,[17] which would thus require the RLN in men to have a larger intraperineural area, more fibers, a larger axonal area and diameter and an increased area of the nerve occupied by myelinated fibers when compared with women.

Therefore, we assume that our results may be supported by previous studie[17] involving vocal musculature. With regard to the nervous control, although some studies[12] demonstrate that language-associated cortical regions are proportionally larger in the female brain, our results show that some peripheral nervous structures are larger in men than in women. The data found in our study are acceptable because there is not

necessarily a correlation between the size of the brain volume dedicated to speech and the morphology of the nerves responsible for controlling vocal muscles.

One important point is that all of the specimens were obtained from an aged population. Thus, the female larynges and nerves were from patients well past menopause. Unfortunately, we have no information regarding hormonal levels and the use of any type of hormone replacement by females who composed our study. Therefore, the females in our study probably presented significant anabolic steroid deprivation while, the males probably presented significant levels of testosterone. We believe that such a difference in hormone levels, between aged men and women,[42-45] is likely to increase the sexual dimorphism of laryngeal muscles and nerves. However we also believe that this sexual dimorphism is present immediately after sexual maturity and is mainly generated by the high levels of testosterone found in men.[42,45] Thus, future studies using young subjects would shed some light on this theory.

Finally, our morphological investigation provides innovative and reliable results about the peripheral vocal mechanisms in humans. Similarly, critical aspects of the other motor systems involved in sound generation and their interactions remain to be investigated in greater detail.

CONCLUSIONS

In summary, this study represents the first complete light-microscopic comparison to investigate the presence of sexual dimorphism in tissues (nerves and muscles) that control the vocal fold, and as such, contributes toward enhancing our understanding of voice neurobiology.

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REFERENCES

1. Rhodes HJ, Yu HJ, Yamaguchi A. Xenopus vocalizations are controlled by a sexually differentiated hindbrain central pattern generator. *J Neurosci*. 2007;27: 1485-1497.

2. O'Bryant EL, Wade J. Sexual dimorphism in neuromuscular junction size on a muscle used in courtship by green anole lizards. *J Neurobiol*. 2002;50: 24-30.

3. Wade J. Sexual dimorphisms in the brainstem of the green anole lizard. *Brain Behav Evol*. 1998;52: 46-54.

4. Wade J, Buhlman L, Swender D. Post-hatching hormonal modulation of a sexually dimorphic neuromuscular system controlling song in zebra finches. *Brain Res*. 2002;929: 191-201.

5. Carrer HF, Cambiasso MJ. Sexual differentiation of the brain: genes, estrogen, and neurotrophic factors. *Cell Mol Neurobiol*. 2002;22: 479-500.

6. De Courten-Myers GM. The human cerebral cortex: gender differences in structure and function. *J Neuropathol Exp Neurol*. 1999;58: 217-226.

7. Huerta-Ocampo I, Mena F, Barrios F, Martínez G, González L, Larriva-Sahd J. Perinatal exposure to androgen suppresses sexual dimorphism in nerve trunk diameter, axon number, and fiber size spectrum: a quantitative ultrastructural study of the adult rat mammary nerve. *Brain Res*. 2005;1060: 179-183.

8. Larriva-Sahd J, Rondán-Zárate A, Ramírez-Degollado M. Sexually dimorphic contribution from the fornix to the ventromedial hypothalamic nucleus: a quantitative electron microscopic study. *Neurosci Lett*. 1995;200: 147-150.

9. Segovia S, Garcia-Falgueras A, Carrillo B, Collado P, Pinos H, Perez-Laso C, Vinader-Caerols C, Beyer C, Guillamon A. Sexual dimorphism in the vomeronasal system of the rabbit. *Brain Res*. 2006;1102: 52-62.

10. Deviche P, Gulledge CC. Vocal control region sizes of an adult female songbird change seasonally in the absence of detectable circulating testosterone concentrations. *J Neurobiol*. 2000;42: 202-211.

11. Gahr M. Sexual differentiation of the vocal control system of birds. *Adv Genet*. 2007;59: 67-105.

12. Harasty J, Double KL, Halliday GM, Kril JJ, McRitchie DA. Language-associated cortical regions are proportionally larger in the female brain. *Arch Neurol*. 1997;54: 171-176.

13. Smith DRR, Patterson RD. The interaction of glottal-pulse rate and vocal-tract length in judgements of speaker size, sex, and age. *J Acoust Soc Am*. 2005;118: 3177-3186.

14. Beckford NS, Rood SR, Schaid D, Schanbacher B. Androgen stimulation and laryngeal development. *Ann Otol Rhinol Laryngol*. 1985;94: 634-640.

15. McCarthy MM, Arnold AP. Reframing sexual differentiation of the brain. *Nat Neurosci*. 2011;14: 677-683.

16. Arnold AP. The gender of the voice within: the neural origin of sex differences in the brain. *Curr Opin in Neurobiol*. 2003;13: 759-764.

17. Happak W, Zrunek M, Pechmann U, Streinzer W. Comparative histochemistry of human and sheep laryngeal muscles. *Acta Otolaryngol*. 1989;107: 283-288.

18. Jotz GP, De Campos, D, Rodrigues MF, Xavier LL. Histological asymmetry of the human recurrent laryngeal nerve. *J Voice*. 2011;25: 8-14.

19. Sanders I, Han Y, Wang J, Biller H. Muscle spindles are concentrated in the superior vocalis subcompartment of the human thyroarytenoid muscle. *J Voice*. 1998;12: 7-16.

20. Asaoka K, Sawamura Y, Nagashima M, Fukushima T. Surgical anatomy for direct hypoglossal-facial nerve side-to-end "anastomosis". *J Neurosurg*. 1999;91: 268-275.

21. De Campos D, Do Nascimento PS, Ellwanger JH, Gehlen G, Rodrigues MF, Jotz GP, Xavier LL. Histological organization is similar in human vocal muscle and tongue - A study of muscles and nerves. *J Voice*. 2012;26: 811.e19-811.e26.

22. Ilha J, Araujo RT, Malysz T, Hermel EE, Rigon P, Xavier LL, Achaval M. Endurance and resistance exercise training programs elicit specific effects on sciatic nerve regeneration after experimental traumatic lesion in rats. *Neurorehabil Neural Repair*. 2008;22: 355-366.

23. Alipour F, Titze IR, Hunter EJ, Tayama N. Active and passive properties of canine abduction/adduction laryngeal muscles. *J Voice*. 2005;19: 350-359.

24. Gunter HE. A mechanical model of vocal-fold collision with high spatial and temporal resolution. *J Acoust Soc Am*. 2003;113: 994-1000.

25. Deguchi S, Kawahara Y, Takahashi S. Cooperative regulation of vocal fold morphology and stress by the cricothyroid and thyroarytenoid muscles. *J Voice*. 2011;25: 255-263.

26. Matheron G. ***The theory of regionalized variables and its applications***. 5th ed. Les cahiers du Centre de Morphologie Mathématique de Fontainebleau. Ecole Nationale Supérieure des Mines de Paris: Fontainebleau;1971.

27. Mathieu O, Cruz-Orive LM, Hoppeler H, Weibel ER. Measuring error and sampling variation in stereology: comparison of the efficiency of various methods for planar image analysis. *J Microsc*. 1981;121: 75-88.

28. Kiêu K, Souchet S, Istas J. Precision of systematic sampling and transitive methods. *J Stat Plan Inference*. 1999;77: 263-279.

29. García-Fiñana M, Cruz-Orive LM, Mackay CE, Pakkenberg B, Roberts N. Comparison of MR imaging against physical sectioning to estimate the volume of human cerebral compartments. *Neuroimage*. 2003;18: 505-516.

30. Gasser HS, Grundfest H. Axon diameters in relation to the spike dimensions and the conduction velocity in mammalian a fibers. *Am J Physiol*. 1939;127: 393-414.

31. Sanders FK, Whitteridge D. Conduction velocity and myelin thickness in regenerating nerve fibres. *J Physiol*. 1946;105: 152-174.

32. Schlaepfer WW, Myers FK. Relationship of myelin internode elongation and growth in the rat sural nerve. *J Comp Neurol*. 1973;147: 255-266.

33. Bachorowski J, Owren MJ. Acoustic correlates of talker sex and individual talker sex identity are present in a short vowel segment produced in running speech. *J Acoust Soc Am*. 1999;106: 1054-1063.

34. Childers DG, Wu K. Gender recognition from speech. Part II: fine analysis. *J Acoust Soc Am*. 1991;90: 1841-1856.

35. Titze IR. Physiologic and acoustic differences between male and female voices. *J Acoust Soc Am*. 1989;85: 1699-1707.

36. Harnsberger JD, Shirivastav R, Brown Junior WS, Rothman H, Hollien H. Speaking rate and fundamental frequency as speech cues to perceived age. *J Voice*. 2008;22: 58-69.

37. Santos Neto H, Marques MJ. Estimation of the number and size of motor units in intrinsic laryngeal muscles using morphometric methods. *Clin Anat*. 2008;21: 301-306.

38. Chan RW, Fu M, Young L, Tirunagari N. Relative contributions of collagen and elastin to elasticity of the vocal fold under tension. *Ann Biomed Eng*. 2007;35: 1471-1483.

39. Fitch WT, Giedd J. Morphology and development of the human vocal tract: a study using magnetic resonance imaging. *J Acoust Soc Am*. 1999;106: 1511-1522.

40. Gillies GE, McArthur S. Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. *Pharmacol Rev*. 2010;62: 155-198.

41. Schlaepfer TE, Harris GJ, Tien AY, Peng L, Lee S, Pearlson GD. Structural differences in the cerebral cortex of healthy female and male subjects: a magnetic resonance imaging study. *Psychiatry Res*. 1995;61: 129-135.

42. Tobias ML, Marin ML, Kelley DB. The roles of sex, innervation, and androgen in laryngeal muscle of Xenopus laevis. *J Neurosci*. 1993;13: 324-333.

43. Jung-Testas I, Baulieu EE. Steroid hormone receptors and steroid action in rat glial cells of the central and peripheral nervous system. *J Steroid Biochem Mol Biol*. 1998;65: 243-251.

44. Nunez JL, Nelson J, Pych JC, Kim JHY, Juraska JM. Myelination in the splenium of the corpus callosum in adult male and female rats. *Dev Brain Res*. 2000;120: 87-90.

45. Gillies GE, McArthur S. Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. *Pharmacol Rev*. 2010;62: 155-198.

TABLE AND FIGURES LEGENDS

TABLE 1. Comparison of all morphometric parameters estimated in the nerves and muscles of the men (♂) and women (♀). SD, standard deviation; *P*, level of significance.

FIGURE 1. Schematic drawing showing the regions where the tissues (nerves and muscles) were collected (indicated by a dotted line in different sections), bilaterally.

TA, thyroarytenoid muscle; L, larynx; T, trachea; X, vagus nerve; RLN, recurrent laryngeal nerve.

FIGURE 2. Digitized images of the sections showing the comparison of the intraperineural area (*black delineation*) of the recurrent laryngeal nerve (RLN) between men (**A**) and women (**B**) presenting evident differences. Images demonstrating the differences between the nerves of the men (**C**) and women (**D**) in terms of myelinated fiber diameter. By contrast, there is similarity in the orientation/organization of the fibers of the thyroarytenoid muscle (TA) in men (**E**) and women (**F**). AT, adipose tissue; CF, collagen fibrils; Mf, myelinated nerve fiber; * (asterisk), endoneurial connective tissue; T, transverse fibers; U, undefined fibers; L, longitudinal fibers; BV, blood vessel (RLN/toluidine blue stain; TA/hematoxylin-eosin stain).

TABLE 1

	♂	♀	
MORPHOMETRIC PARAMETERS	MEAN ± SD	MEAN ± SD	*P*
Myelinated fiber density (number of fibers/mm^2)	13530±951.4	13760±394.0	*0.829*
Intraperineural area (mm^2)	0.3031±0.03	0.1743±0.01	***0.006***
Total number of fibers	3736±263.2	2318±203.0	***0.0002***
Axonal area (µm^2)	10.8±0.44	7.1±0.36	***0.0001***
Axonal diameter (µm)	3.7±0.20	3.0±0.07	***0.0001***
Myelin sheath thickness (µm)	2.0±0.14	2.3±0.09	*0.135*
Area of the nerve occupied by myelinated fibers (%)	14.9±1.35	9.7±0.46	***0.001***
Area of the nerve occupied by endoneurial connective tissue (%)	85.1±1.35	90.2±0.46	***0.001***
Transverse fibers (%)	62.7±2.1	64.0±2.8	*0.721*
Undefined fibers (%)	17.5±1.2	17.7±1.8	*0.949*
Longitudinal fibers (%)	20.3±1.7	18.2±1.6	*0.399*

FIGURE 1

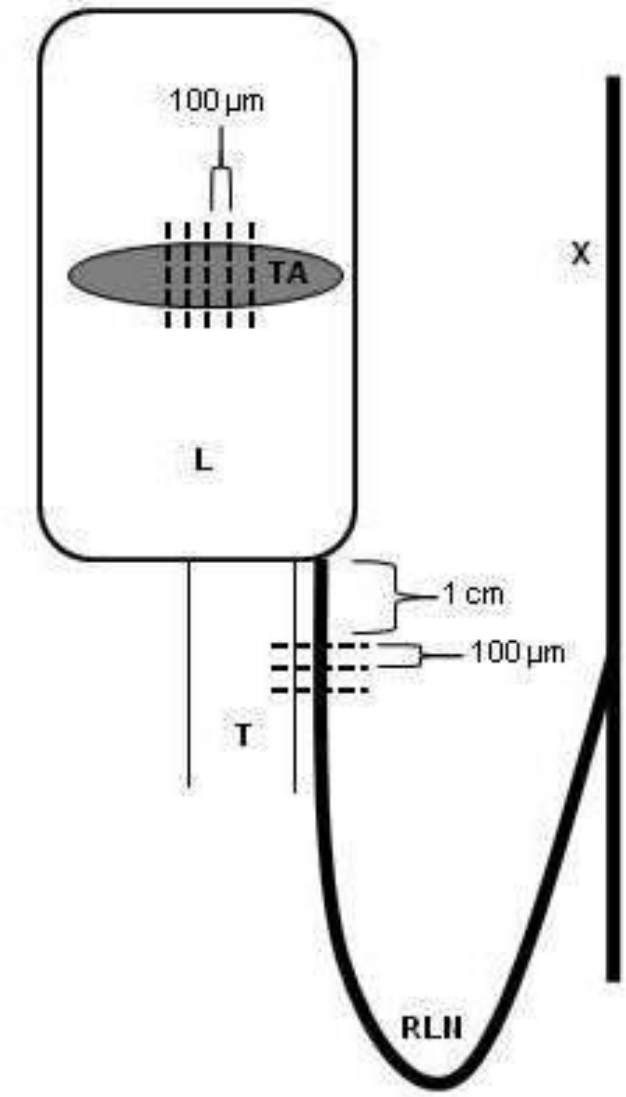
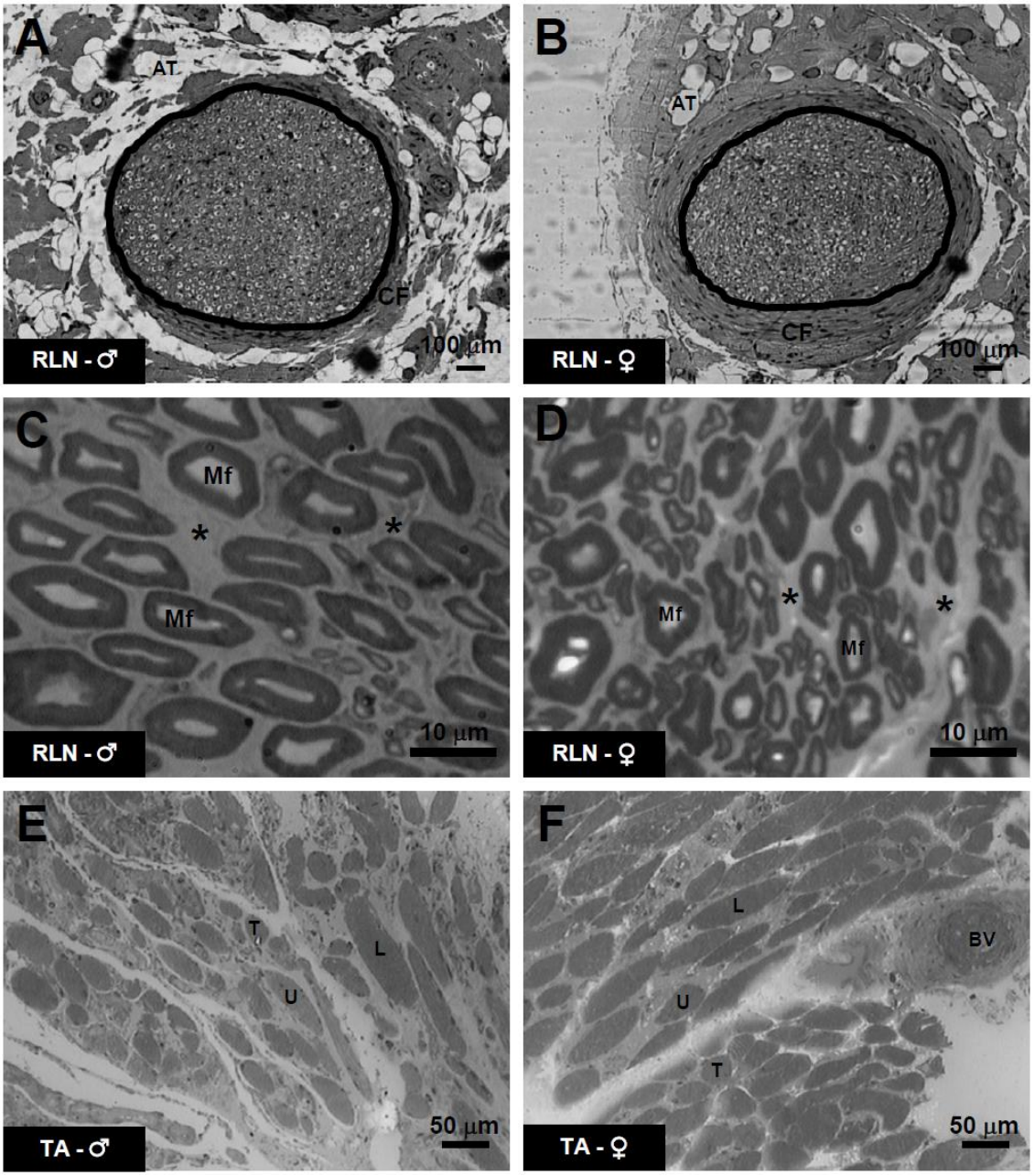


FIGURE 2


A
AT
CF
100 µm
RLN - ♂
B
AT
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RLN - ♀
C
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L
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TA - ♂
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TA - ♀

6 CONCLUSÕES

Os dados referentes à nossa primeira hipótese revelam que em humanos existe uma evidente similaridade na organização muscular entre as fibras do músculo TA e a musculatura da língua. Neste sentido, pode-se supor que o controle seletivo de diferentes partes da prega vocal se justifica, ao menos em parte, pelo fato de que as fibras do músculo TA não se situam paralelamente em toda a extensão da prega vocal e, dessa forma, não têm uma única orientação. Desse modo, essa organização muscular, assemelha-se a organização da musculatura da língua e, presumivelmente pode fornecer uma maior diversidade/possibilidade de movimentos (ajustes seletivos em diferentes partes) à prega vocal.

Adicionalmente, não se evidenciou semelhanças em nenhum dos parâmetros morfométricos quantificados entre o NLR e o XII; no entanto, esses dados morfométricos, serão imprescindíveis para o sucesso de uma importante técnica de reinervação da prega vocal.

Os resultados da nossa segunda hipótese, embora obtidos a partir de um único indivíduo, demonstram que todas as estruturas associadas à prega vogal, direita e esquerda, já estão completamente estabelecidas no período de 25 semanas de gestação. Adicionalmente, as estimativas referentes à organização histológica do músculo TA direito e esquerdo demonstram que assim como nos adultos, as fibras musculares não se situam paralelamente e lateralmente em toda a extensão da prega vocal. Esses resultados podem explicar, pelo menos em parte, a grande variedade de sons emitidos pelo recém-nascido. Além disso, nós enfatizamos que essa investigação

morfológica fornece resultados inovadores sobre as estruturas associadas à prega vocal e a orientação das fibras do músculo TA fetal que indubitavelmente serão úteis para aumentar o conhecimento acerca da funcionalidade vocal em diferentes estágios do desenvolvimento embriológico.

Adicionalmente, nós sugerimos que futuros estudos envolvendo um número maior de indivíduos serão necessários para comprovar definitivamente esses achados.

Os dados referentes à nossa terceira hipótese evidenciam que em humanos existe um notável dimorfismo sexual nos nervos (NLR) que controlam a mobilidade vocal; contudo, isso não se evidencia na organização histológica das fibras do músculo TA. Esses dados fornecem evidências de que os humanos, assim como outras espécies (DEVICHE & GULLEDGE, 2000; GAHR, 2007; RHODES et al., 2007), também apresentam dimorfismo sexual em estruturas nervosas envolvidas com o controle vocal, tanto no sistema nervoso central, como já relatado previamente (HARASTY et al., 1997), quanto no sistema nervoso periférico (NLR), como mostrado em nosso estudo.

Frente a esses resultados, podemos supor que as diferenças entre os timbres sonoros de homens e mulheres e suas especificidades, talvez não possam ser explicadas somente por diferenças na massa das pregas vocais (SMITH & PATTERSON, 2005) ou no tamanho do trato vocal (BECKFORD et al., 1985; FITCH & GIEDD, 1999); mas também por diferenças que abrangem a organização de todo o sistema nervoso. Desse modo, estruturas nervosas de homens e mulheres, tanto centrais quanto periféricas, possivelmente seguem caminhos sutilmente diferentes para atingir níveis semelhantes de função.

Em conjunto, os achados do presente trabalho revelam importantes descobertas sobre alguns aspectos neurobiológicos de nervos e músculos

envolvidos no controle vocal humano, complementando e enriquecendo a literatura atual, com resultados inovadores que ampliam e reforçam o conhecimento mais detalhado acerca da inervação e funcionalidade vocal.

7 BIBLIOGRAFIA ADICIONAL

BECKFORD, N. S.; ROOD, S. R.; SCHAID, D. Androgen stimulation and laryngeal development. *The Annals of Otology, Rhinology, and Laryngology*. 94:634–640, 1985.

BEHLAU, M.; AZEVEDO, R.; MADAZIO, G. Anatomia da Laringe e Fisiologia da Produção vocal. In: BEHLAU, M. (Org.). *Voz: o livro do especialista.* Vol. I. Rio de Janeiro: Revinter, 2001.

BRANCO, A.; BEHLAU, M.; REHDER, M. I. The neonate cry after cesarean section and vaginal delivery during the first minutes of life. *International Journal of Pediatric Otorhinolaryngology.* 69:681-689, 2005.

DEVICHE, P.; GULLEDGE, C. C. Vocal control region of an adult female songbird change seasonally in the absence of detectable circulating testosterone concentrations. *Journal of Neurobiology*. 42:202-211, 2000.

FIGÚN, M. E.; GARINO, R. R. *Anatomia Odontológica Funcional e Aplicada*. 3ª ed. São Paulo: Panamericana, 1994. 668p.

FITCH, W. T.; GIEDD, J. Morphology and development of the human vocal tract: a study using magnetic resonance imaging. *The Journal of the Acoustical Society of America*. 106:1511–1522, 1999.

GAHR, M. Sexual differentiation of the vocal control system of birds. *Advances in Genetics*. 59:67-105, 2007.

GARDNER, E.; GRAY D. J.; RAHILLY R. *Anatomia: estudo regional do corpo humano*. 4ª ed. Rio de Janeiro: Guanabara, 1988. 815p.

HARASTY, J.; DOUBLE, K. L.; HALLIDAY, G. M.; KRIL, J. J.; MCRITCHIE, D. A. Language-associated cortical regions are proportionally larger in the female brain. *Archives of Neurology*. 54:171–176, 1997.

HENEINE, I. F. *Biofísica básica*. 2ª ed. São Paulo: Atheneu, 1996. 391p.

KRUSE, E.; OLTHOFF, A.; SCHIEL, R. Functional anatomy of the recurrent and superior laryngeal nerve. *Langenbecks Arch Surg*. 391:4-8, 2006.

MARANILLO, E.; LEON, X; ORUS, C.; QUER, M.; SANUDO, J. R. Variability in nerve patterns of the adductor muscle group supplied by the recurrent laryngeal nerve. *Laryngoscope*. 115:358-362, 2005.

MOORE, K. L.; DALLEY, A. F.; AGUR, A. M. R. *Anatomia orientada para a clínica*. 6ª ed. Rio de Janeiro: GEN-Guanabara Koogan, 2011. 1104p.

NAPADOW, V. J.; CHEN, Q.; WEDEEN, V. J. GILBERT, R. J. Intramural mechanics of the human tongue in association with physiological deformations. *Journal of Biomechanics*. 32:1-12, 1999.

NAVARRO, J. A. C.; FILHO, J. T.; ANDREA, J. C.; SILVA, H. S.; BANHARA, M. R. Contribuição ao estudo anatômico da inervação dos músculos cricoaritenóideos posterior e lateral. *Revista Brasileira de Otorrinolaringologia*. 69 (6):740-743, 2003.

NETTER, F. H. *Atlas de anatomia humana*. 2ª ed. Porto Alegre: Artes Médicas, 2000. 525p.

NICOLLAS, R.; GIORDANO, J.; PERRIER, P.; BURTSCHELL, Y.; MEDALE, M.; GIOVANNI, A.; TRIGLIA, J. M.; OUAKNINE, M. Modelling sound production from an aerodynamical model of the human newborn larynx. *Biomed Signal Process Control*. 1:102-106, 2006.

RHODES, H. J.; YU, H. J.; YAMAGUCHI, A. Xenopus vocalizations are controlled by a sexually differentiated hindbrain central pattern generator. *The Journal of Neuroscience*. 27:1485-1497, 2007.

ROHKAMM, R. *Color Atlas of Neurology*. New York: Thieme, 2004. 448p.

SMITH, D. R. R.; PATTERSON, R. D. The interaction of glottal-pulse rate and

vocal-tract length in judgements of speaker size, sex, and age. *The Journal of the Acoustical Society of America*. 118: 3177-3186, 2005.

THOMUSCH, O.; SEKULLA, C.; TIMMERMANN, W.; NEUMANN, H. J.; KRUSE, E.; MUHLIG, H. P.; HAMELMANN W. H.; RICHTER, C.; VO, J.; DRALLE, H. Intraoperative neuromonitoring in thyroid surgery – results of the German prospective multicentre study. *Eur Surg*. 35:240-245, 2003.

VESALIUS, A. *De humani corporis fabrica: epítome: tabulae sex*. São Paulo: Ateliê Editorial, 2002. 262 p.

ZESKIND, P. S.; MCMURRAY, M. S.; GARBER, K. A.; NEUSPIEL, J.M.; COX, E. T.; GREWEN, K. M.; MAYES, L. C.; JOHNS, J. M. Development of translational methods in spectral analysis of human infant crying and rat pup ultrasonic vocalizations for early neurobehavioral assessment. *Frontiers in Psychiatry.* 2, article 56:1-16, 2011.

WILLIAMS, P. L.; WARWICK, R.; DYSON, M.; BANNISTER H. L. *Gray Anatomia*. 37ª ed. Vol. 2. Rio de Janeiro: Guanabara Koogan, 1995. 1489p.