Published in final edited form as:

Laryngoscope. 2008 March ; 118(3): 559–563. doi:10.1097/MLG.0b013e31815bac17.

Effect of Aging on Blood Flow in Rat Larynx

John A. Russell, MS, Hiromi Nagai, MD, PhD, and Nadine P. Connor, PhD

Department of Surgery, Division of Otolaryngology Head and Neck Surgery (J.A.R., N.P.C.), University of Wisconsin, Madison, Wisconsin, U.S.A.; the Department of Otolaryngology Head and Neck Surgery (H.N.), Kitasato University School of Medicine, Kanagawa, Japan; and the Department of Communicative Disorders (N.P.C.), University of Wisconsin, Madison, Wisconsin, U.S.A.

Abstract

Objective/Hypothesis—Age-associated muscular changes and fatigue have been shown to affect phonatory function. Reductions in blood flow with aging could translate to reductions in oxidative capacity within laryngeal muscles and increased fatigability. We tested the hypothesis that there would be increased capillary red blood cell (RBC) velocity and a reduction of capillary density in the thyroarytenoid (TA) muscle of senescent rats.

Study Design/Methods—Ten male Fisher 344/Brown Norway rats in two age groups were used: young adult (9 mo) and old (28–30 mo). Sixteen additional young and old rats were used in a fluorescent microsphere experiment that examined blood flow rates before and after a surgical manipulation. With use of a specially equipped intravital microscope, in vivo measurements of capillary geometry and flow were obtained, including RBC velocity, capillary density, tortuosity, and number of branch points.

Results—There was an age-related reduction in capillary surface area as evidenced by reduced lineal density of capillaries. In addition, reduced RBC transit time was suggested by the reduction in branch points found with age. There was no change in RBC velocity with aging. The surgical method used to expose the TA muscle for blood flow recordings did not significantly affect resultant blood flow measurements.

Conclusions—We developed a method to evaluate in vivo laryngeal microvasculature. We found age-related changes in microvascular geometry within the TA muscle of the rat that could affect blood flow to this critical muscle of phonation and airway protection. These micro vascular changes could contribute to age-related laryngeal dysfunction.

Keywords

Aging; larynx; thyroarytenoid; blood flow; in vivo model

INTRODUCTION

The larynx is responsible for protecting the lower airway and permitting ventilation and is used for the production of the voice. These critical laryngeal functions are altered by aging, which may be caused by mechanisms related to sarcopenia and the loss of skeletal muscle mass, organization, and strength.¹ However, the underlying biological mechanisms that may cause laryngeal sarcopenia are unclear.

Several recent studies have contributed to our understanding of factors that may be associated with sarcopenia in muscles of the larynx, including muscle fiber atrophy,² denervation-like changes at the neuromuscular junction (NMJ),³ and a transformation or replacement of rapidly contracting myosin heavy chain isoforms with more slowly contracting forms.⁴ These age-related alterations may have functional consequences because abnormal laryngeal kinematics have also been identified in aged animals and human subjects.^{5,6} Therefore, although investigation of age-related manifestations within laryngeal muscles, NMJs, and movements has been fruitful, there remain unanswered questions regarding possible etiologies.

In addition to muscle fiber type changes, senescent individuals exhibit a pattern of structural and functional adaptations within the vascular system that may contribute to fatigue and weakness. Specifically, senescence results in a reduction in skeletal muscle blood flow⁷ and decreased capillary density.⁸ All of these changes have been reported in other muscular systems within the body and may compromise muscle O₂ delivery and the matching of O₂ delivery to O₂ requirements within muscle. This mismatch of O₂ delivery and O₂ requirement can lead to fatigue and loss of capacity for full muscle function. Very little data concerning vascular supply to laryngeal muscle are available, and even fewer data exist on age-related changes in laryngeal blood flow. As stated by Lyon and Barkmeier-Kraemer,⁹ “No systematic structural studies of the laryngeal microvasculature have been conducted.” Therefore, further investigations are necessary to understand laryngeal blood flow and how blood flow may be altered by aging.

Intravital microscopy has been used previously to study the effect of aging on skeletal muscle. Therefore, using a new in vivo preparation to expose the thyroarytenoid (TA) muscle of the rat larynx, we tested the hypothesis that there would be an increased capillary red blood cell (RBC) velocity (V_{RBC}) and a reduction of capillary density in the TA muscle of senescent rats.

METHODS

Animals: Intravital Microscopy Experiments

Ten male Fischer 344/Brown Norway rats were included in this study for intravital microscopy. Five of the rats were old (32 mo; 606 ± 43 g), and five were young adult rats (9 mo; 426 ± 30 g). All surgical procedures were conducted under general anesthesia with sodium pentobarbital (30–50 mg/kg intraperitoneally) supplemented as necessary before the animal was positioned on the observation platform of the microscope. The femoral artery was cannulated with polyethylene-50 tubing (IntraMedic polyethylene tubing, Clay Adams, Sparks, MD) to monitor arterial blood pressure and to administer fluids and a fluoroprobe (fluorescein isothiocyanate [FITC]). All animal procedures were approved under University of Wisconsin animal care and use policies.

Fluorescent Microsphere Experiments

As a control in a separate experiment, a different group of eight old (32 mo; 573 ± 9 g) and eight young adult (9 mo; 407 ± 16 g) Fischer 344/Brown Norway rats was used for examination of blood flow rates to the larynx and kidney prior to and after surgical manipulation using a fluorescent microsphere technique. We performed this control to allow evaluation of the effect of our surgical manipulation on laryngeal blood flow. Blood flow to the kidney was included in this experiment as an additional control condition because the kidney is located remotely from the larynx and no pre/postlaryngeal surgery differences should be found in the kidney.

Vocal Fold Preparation

Exposure of the TA muscle was achieved using the following method: 1) the skin at the midline of the anterior neck was incised from the lower edge of the mandible to the top of the sternum; 2) the sternohyoid and omohyoid muscles were cut at the attachment to the hyoid bone,

elevated, and sutured in a position lateral to the larynx; 3) after the larynx and trachea were exposed, a tracheotomy was performed caudally to the larynx by sectioning the trachea and suturing the proximal tracheal section to skin near the sternum, without damage to the esophagus or cricopharyngeus muscle; 4) the superior tracheal section and the larynx were then dissected free from surrounding tissue, and then the thyroid and cricoid cartilages were incised at the midline, without contact or damage to the intrinsic laryngeal muscles; 5) sutures were placed at the anterior edges of the cricoid cartilage and on the anterior edge of the thyroid cartilage. Slight tension was applied to the sutures to spread apart the tracheal incision, as shown in Figure 1. The time to complete this procedure was approximately 45 minutes. After the surgical procedure, all animals rested for 45 minutes to allow stabilization prior to intravital imaging. The left vocal fold was kept moist with warm saline during this stabilization period.

Intravital Digital Video Microscopy

After the surgery and stabilization period, the rat was placed on a custom-designed polycarbonate observation platform maintained at 37°C attached to a hybrid Olympus (Tokyo, Japan) microscope. Microcirculatory fields were randomly selected with a noncontact illuminated objective ($\times 20$, numerical aperture 0.8). A 75 W xenon lamp equipped with a 420 nm to 490 nm excitation filter provided the source for fluorescence epi-illumination. All observations were viewed on a LCD monitor (Hitachi CML174SXW, Tokyo, Japan), and digital images were captured using Simple PCI digital software (Compix, Sewickley, PA) and stored for future analysis. To insure visualization of the microcirculation of the TA muscle, the fine focus knob on the microscope was used to focus at a depth of at least 200 μm , which was previously found to be the average thickness of the mucosa covering the TA muscle.¹⁰

A 0.2-mL bolus of FITC-Dextran 150 (Sigma, St. Louis, MO) was administered via the femoral artery for visualization of the microcirculatory field. Six fields that demonstrated good clarity on the monitor, as determined by the microscopist (J.A.R.), were chosen and recorded for subsequent analysis. Observation periods ranged from 20 to 30 seconds. Total experimental duration was no longer than 2.0 to 2.5 hours. Of the six images recorded, the two that demonstrated the greatest clarity were analyzed.

Capillary Structural and Functional Measurements

Capillaries were operationally defined as vessels with the width of an RBC. Illustration of all capillary structural measurements are given in Figure 2. Lineal capillary density (capillaries/mm) was determined by counting the number of capillaries that crossed a horizontal line drawn through the middle of each screen and was expressed as capillaries per millimeter of tissue.

Capillary geometry was determined by measures of capillary tortuosity and number of branch points, which are measures that have been previously described.⁸ These measurements are expressions of capillary length (CL) arising from the nonaniso-tropic components of the capillary bed.¹¹ Capillary tortuosity was calculated as the ratio of CL to shortest distance (SD) between branch points (CL/SD). In addition, the number of branch points per millimeter was counted and recorded for each of the two fields of view per animal.

V_{RBC} was observed in real time and with playback and frame-by-frame analysis techniques. V_{RBC} (μs) was determined in all capillaries that were continuously flowing with RBCs and that could be continuously monitored over several frames. V_{RBC} was calculated as the distance traveled by an individual RBC in between frames. On the average, capillary V_{RBC} was measured twice per capillary for accuracy.

Fluorescent Microspheres

Regional blood flow was studied in the exposed vocal fold with the use of fluorescent microspheres. Our goal was to determine whether the vocal fold preparation surgery affected our measures of laryngeal blood flow characteristics. Both the left and right femoral arteries were exteriorized and cannulated with polyethylene-50 tubing for the withdrawal of a reference blood sample and monitoring of blood pressure. In addition, a cannula was placed in the ascending aorta via the right carotid artery for the injection of the microspheres.

Yellow and red polystyrene microspheres that were 15 μm in diameter were used (NuFlow Ultraspheres, IMT, Irvine, CA). The microspheres were sonicated and vortexed immediately prior to injection. Approximately 1×10^6 microspheres of the yellow microsphere in 0.2 mL of 0.02% Tween were injected into the left atrium followed by 0.3 mL saline injected into the aorta. Starting 30 seconds before and continuing for 120 seconds after injection of the microspheres, a reference blood sample was withdrawn (Harvard Pump, PHD 2000, Harvard Apparatus, Holliston, MA) from the left femoral artery. After the first microsphere injection, the vocal fold preparation surgery was performed as stated above. The animal was then given a 45-minute stabilization period, and a second injection of red microspheres was performed according to the same procedures previously described.

At the end of the experiment, the animals were euthanized, and the left vocal fold and left kidney were extracted and weighed. Processing of tissues and counting of microspheres were performed in a blind fashion by Interactive Medical Technologies, Ltd. (Irvine, CA). In so doing, tissues were digested in an alkaline reagent overnight and centrifuged at 1,500g, after which the supernatant was collected, mixed with a microsphere counting reagent and then sonicated. The effluent was then passed through a 50- μm filter and centrifuged and the supernatant aspirated to leave a volume of 200 μL . The sample was then briefly sonicated and analyzed by flow cytometry to determine the number of microspheres in the tissue of interest.

Calculation Blood Flow Rate

Blood flow values via the microsphere technique were calculated according to the following equation: $Q_T = (C_T * Q_{Ref}) / C_{Ref}$, where Q_T is the tissue blood flow per gram (mL/min/g), C_T is the microsphere count per gram of tissue, Q_{Ref} is the withdrawal rate of the reference sample (mL/min), and C_{Ref} is the microsphere count in the reference blood sample.

Statistical Analysis

Descriptive summary data (mean, standard deviation) were calculated for all variables in the old and young groups. Results were analyzed using nonparametric statistics (Mann-Whitney *U* test) using commercially available statistical software (GB-STAT, Silver Spring, MD). The critical value for obtaining statistical significance was set at $\alpha = .05$.

RESULTS

Intravital Microscopic Findings

There was no significant difference ($P < .05$) in mean arterial blood pressure between young and old rats (young = 105 ± 6 ; old = 109 ± 16). Data from one young animal were excluded because of poor image quality that did not allow visualization and measurement of V_{RBC} or capillary morphology. Group comparisons for measures of V_{RBC} , lineal capillary density, branch points per area, and tortuosity are shown in Table I. Table II contains the rates of blood flow to the vocal fold obtained via the fluorescent microsphere method.

No differences between age groups were found in measurements of V_{RBC} and tortuosity. However, the number of branch points per square millimeter of tissue was significantly reduced

in the old animals ($P = .01$). Similarly, lineal density (capillaries/mm) was also significantly reduced in the old animals ($P < .05$)

Fluorescent Microsphere Measures

Previous research has determined that at least 400 microspheres must be recovered from a tissue sample to determine blood flow within an accuracy of 10% error at the 95% confidence level.¹² With use of these criteria, insufficient microsphere counts were obtained in three young and three old animals. Accordingly, blood flow comparisons were based on data from five young and five old animals. Presurgery blood flow rates (mL/min/g) to the larynx or kidney were not significantly different in young versus old animals (Table II) ($P < .05$). As expected, we did not find significant pre/postlaryngeal surgery blood flow rates (mL/min/g) to the kidney within young and old groups ($P > .05$; Wilcoxon rank sum test for correlated samples). Although blood flow to the larynx was reduced after surgery in both the young and old groups, the pre-versus postsurgery differences were not statistically significant ($P > .05$). However, because of our small sample size, the possibility remains that the vocal fold preparation surgery influenced blood flow measurements in the larynx.

DISCUSSION

The hypothesis of this study was that there would be increased capillary V_{RBC} and reduced lineal density of capillaries in the TA muscle of senescent rats. Our results indicated that there was a reduction of lineal density. However, there was no change in capillary V_{RBC} between young and old rats. In addition, it was discovered that there was a decrease in branch points in old rats, further suggesting an age-related alteration in capillary morphology in the TA muscle. Accordingly, it appears that capillary structure in the TA muscle is altered by aging, whereas V_{RBC} is maintained. Our results demonstrate that aging induces significant vascular changes within the microcirculation of the TA muscle.

Aging lowers the overall number of capillaries surrounding skeletal muscle fibers.¹³ In the current investigation, there was no change in tortuosity with age; however, there was a significant decrease in branch points with age. A reduction in branch points in the capillary bed may reduce the amount of time that an RBC remains in the tissue and thus reduce the opportunity for oxygen offloading. Therefore, in aging tissue, at any given blood flow rate, there may be a decrease in RBC transit time within the capillary bed and thus the potential for a reduction in oxygen extraction. This concept is supported by previous work in which the relationship of fractional oxygen extraction and blood flow has been described.¹⁴ Specifically, fractional O_2 extraction is determined by the relationship between O_2 diffusing capacity (DO_{2m}) and blood flow (Q) such that $VO_{2m} = QO_{2m} (1 - e^{-DO_{2m}/\beta Qm})$ and therefore $VO_2/QO_2 = O_2 \text{ extraction} = 1 - e^{-DO_2/\beta Q}$, where β is the slope of the O_2 dissociation curve in the physiologic range.¹⁴ As such, there may be an alteration in the characteristics of O_2 offloading and extraction with age in the TA muscle that may compromise mitochondrial adenosine triphosphate production and contractile function.

It has been shown recently by McMullen and Andrade¹⁵ that the aged rat TA muscle has significant contractile deficits, including a decrease in force production and an increase in contraction time. These results correspond to previous work from our laboratory illustrating a shift from a faster to slower type 2 fiber myosin heavy chain isoforms in rat TA muscles.¹⁶ In addition, McMullen and Andrade¹⁵ reported abnormal mitochondrial accumulations in the aged rat TA muscle that have been reported previously with aging in other muscle systems¹⁷ and an increase in the number of glycogen-positive fibers. In the current study, there was reduced capillary (lineal) density and the potential of reduction in RBC transit time because of the reduction in branch points. Under these circumstances, either fractional O_2 extraction would be compromised or intracellular PO_2 levels would have to fall to a lower level in older

rats to generate the greater O₂ flux density necessary to maintain a given level of oxygen exchange. A reduction in intramyocyte PO₂ causes the stimulation of glycolysis and thus enhances glycogen degradation and acid-base disturbances associated with contractile impairments.¹⁸ Therefore, the mitochondrial abnormalities and increased glycogen content in the aged TA muscle discovered by McMullen and Andrade¹⁵ may represent a diminished ability for aerobic capacity and may be indicative of the reduced capillary supply for proper oxygen exchange.

The V_{RBC} values in this study were much higher in the TA muscle than previously reported using other microcirculatory models.⁸ Considering the microsphere data, it is difficult to attribute these high velocities to surgical intervention. However, our surgical method required the surgeon to cut and spread the thyroid and cricoid cartilages, therefore possibly applying tension to the TA muscle. This tension on the TA muscle may have caused a change in metabolic rate of the TA muscle in our in vivo preparation and therefore causing an increase in V_{RBC} . Therefore, we cannot rule out the effect of surgery on V_{RBC} within the capillaries of the TA muscle.

In nonlaryngeal skeletal muscle systems, it has been shown that chronic or intermittent bouts of ischemia cause a decrease in the size of both type I and IIb muscle fibers.¹⁹ In addition, ischemia causes a shift away from type IIb toward more type IIa muscle fibers, which are more slowly contracting.¹⁹ Recent data indicate that there are age-related changes to human and rat TA muscle along these same lines.²⁰ Although a reduction in bulk blood flow to the vocal fold was not discovered within the current study, significant age-related reduction in laryngeal blood flow has been discovered in the rat,⁹ with the greatest reduction in blood flow in the TA muscle. A reduction in blood flow to the TA, along with the microvascular structural differences discovered in the current study, could impair normal metabolic activity by decreasing the normal supply of nutrients and oxygen, which may help to explain the muscle fiber type changes previously found in the TA muscle.²⁰

In conclusion, our hypothesis that V_{RBC} would be reduced in old rat TA muscle was not supported. However, our data supported the initial hypothesis that the TA muscles of aged rats would evidence a reduction of capillary density. We also found a reduction in branch points in old rat TA. These findings suggest that microcirculatory changes present in the aged TA muscle may potentially affect the aerobic capacity of laryngeal muscle and therefore the endurance of laryngeal muscle contraction.

Acknowledgments

The assistance of Dr. Diane Bless, Nick Weber, and Jessica Tiede is gratefully acknowledged.

This study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committees (IACUC) of University of Wisconsin and William S. Middleton VA Hospital.

This work was supported by research grants from the National Institute of Deafness and Other Communication Disorders (R01DC005935; R01DC008149).

BIBLIOGRAPHY

1. Doherty T. Aging and sarcopenia. *J Appl Physiol* 2003;95:1717–1727. [PubMed: 12970377]
2. Schindler JS, Kelly JH. Swallowing disorders in the elderly. *Laryngoscope* 2002;112:589–602. [PubMed: 12150508]
3. Connor NP, Suzuki T, Lee K, Sewall GK, Heisey DM. Neuromuscular junction changes in aged rat thyroarytenoid muscle. *Ann Otol Rhinol Laryngol* 2002;111:579–586. [PubMed: 12126012]

4. Nagai H, Ota F, Connor NP. Effect of deficits in laryngeal sensation on laryngeal muscle biochemistry. *Ann Otol Rhinol Laryngol* 2005;114:352–360. [PubMed: 15966521]
5. Suzuki T, Connor NP, Lee K, Levenson G, Ford CN. Laryngeal-respiratory kinematics are impaired in aged rats. *Ann Otol Rhinol Laryngol* 2002;111:684–689. [PubMed: 12184588]
6. Baker KK, Ramig LO, Sapir S, Luschei ES, Smith ME. Control of vocal loudness in young and old adults. *J Speech Lang Hear Res* 2001;44:297–305. [PubMed: 11324652]
7. Irion G, Vasthare US, Tuma RF. Age related change in skeletal muscle blood flow in the rat. *J Gerontol* 1987;42:660–665. [PubMed: 3680885]
8. Russell JA, Kindig CA, Behnke BJ, Poole DC, Musch TI. Effects of aging on capillary geometry and hemodynamics in rat spinotrapezius muscle. *Am J Physiol Heart Circulation Physiol* 2003;285:H251–H258.
9. Lyon, M.; Barkmeier-Kraemer, J. Vascular supply of the Larynx. In: Sapienza, CM.; Casper, JK., editors. *Vocal Rehabilitation for Medical Speech-Language Pathology*. Pro-Ed; Austin, TX: 2004. p. 69-107.
10. Kurita, S.; Nagata, K.; Hirano, M. A comparative study of the layer structure of the vocal fold. In: Bless, DM., editor. *Vocal Fold Physiology: Contemporary Research and Clinical Issues*. College-Hill Press, Inc.; San Diego: 1983.
11. Mathieu-Costello O. Capillary tortuosity and degree of contraction or extension of skeletal muscles. *Microvasc Res* 1987;33:98–117. [PubMed: 3550394]
12. Nose Y, Nakamura T, Nakamura M. The microsphere method facilitates statistical assessment of regional blood flow. *Basic Res Cardiol* 1985;80:417–429. [PubMed: 4051944]
13. Proctor DN, Sinning WE, Walro JM, Sieck GC, Lemon PW. Oxidative capacity of human muscle fiber types: effects of age and training status. *J Appl Physiol* 1995;78:2033–2038. [PubMed: 7665396]
14. Roca J, Agustí AG, Alfonso A, et al. Effects of training on muscle O₂ transport at Vo₂max. *J Appl Physiol* 1992;73:1067–1072. [PubMed: 1400019]
15. McMullen CA, Andrade FH. Contractile dysfunction and altered metabolic profile of the aging rat thyroarytenoid muscle. *J Appl Physiol* 2006;100:602–608. [PubMed: 16239605]
16. Suzuki T, Connor NP, Lee K, Bless DM, Ford CN, Inagi K. Age-related alterations in myosin heavy chain isoforms in rat intrinsic laryngeal muscles. *Ann Otol Rhinol Laryngol* 2002;111:962–967. [PubMed: 12450167]
17. Bua EA, McKiernan SH, Wanagat J, McKenzie D, Aiken JM. Mitochondrial abnormalities are more frequent in muscles undergoing sarcopenia. *J Appl Physiol* 2002;92:2617–2624. [PubMed: 12015381]
18. Wilson DF, Erecinska M, Drown C, Silver IA. Effect of oxygen tension on cellular energetics. *Am J Physiol Cell Physiol* 1977;233:C135–C140.
19. Dodd S, Vrabas IS, Stetson DS. Effects of intermittent ischemia on contractile properties and myosin isoforms of skeletal muscle. *Med Sci Sports Exerc* 1998;30:850–855. [PubMed: 9624642]
20. Malmgren LT, Fisher PJ, Jones CE, Bookman LM, Uno T. Numerical densities of myonuclei and satellite cells in muscle fiber types in the aging human thyroarytenoid muscle: an immunohistochemical and stereological study using confocal laser scanning microscopy. *Otolaryngol Head Neck Surg* 2000;123:377–384. [PubMed: 11020171]

Fig. 1.
Laryngofissure surgery and preparation for intravital microscopy.

Fig. 2. Measurements taken by intravital microscopy. Capillary tortuosity defined as capillary length (CL) divided by the shortest distance (SD) across the screen. Lineal density defined as capillaries/mm of tissue. Branch points (illustrated by circles)/mm² of tissue.

TABLE I

Results From Intravital Microscopic Measures in Four Young and Five Old Rats.

	Young, Mean ($\pm$ SD)	Old, Mean ($\pm$ SD)
RBC velocity (μ m/s)	1243.3 (242.8)	993.5 (362.0)
Tortuosity (% > shortest distance)	31.5 (2.9)	26.3 (8.9)
Branch points (per mm ²)	38.7 (8.4)	18.0 (6.6)*
Lineal density (capillaries/mm)	17.0 (6.0)	9.35 (2.2)*

* $P < .05$ in comparison of young and old rats with Mann-Whitney U test.

RBC = red blood cell.

TABLE II

Results From Fluorescent Microsphere Measures in Four Young and Five Old Rats Prior to Laryngeal Surgery Required for Intravital Microscopy and Also After Surgery.

	Young, Mean (SD)		Old, Mean (SD)	
	Presurgery	Postsurgery	Presurgery	Postsurgery
Blood flow to kidney (mL/min/g)	5.4 (1.7)	4.2 (2.9)	5.0 (2.6)	4.7 (3.0)
Blood flow to larynx (mL/min/g)	2.4 (1.4)	1.5 (0.5)	1.7 (1.5)	1.0 (0.6)

No statistically significant differences ($P < .05$) were found on comparisons of young versus old rats or pre- versus postsurgery on Wilcoxon rank sum tests for correlated samples.