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Axon and Schwann Cell Degeneration in Nerves of Upper Airway Relates to Pharyngeal Dysfunction in Snorers and Patients With Sleep Apnea

Farhan Shah, MDS; Thorbjörn Holmlund, PhD; Eva Levring Jäghagen, PhD; Diana Berggren, PhD; Karl Franklin, PhD; Sture Forsgren, PhD; and Per Stål, PhD

BACKGROUND: The pathophysiologic mechanism of nocturnal obstruction and swallowing dysfunction commonly occurring in patients with sleep apnea is unclear. The goal of this study was to investigate whether nerve injuries in the upper airways of snorers and patients with sleep apnea are associated with pharyngeal dysfunction and severity of sleep apnea.

METHODS: Twenty-two patients undergoing palatal surgery due to snoring and sleep apnea were investigated for a swallowing dysfunction by using videoradiography. Twelve healthy nonsnoring subjects were included as control subjects. Tissue samples from the soft palate at the base of the uvula were obtained in all patients and control subjects. Nerves and muscle were analyzed with immunohistochemical and morphologic methods, and the findings were correlated with swallowing function and degree of sleep apnea.

RESULTS: In the soft palate of patients, nerve fascicles exhibited a significantly lower density of axons (5.4 vs 17.9 × 10^3 axons/mm^2; P = .02), a smaller percentage area occupied by Schwann cells (17.5% vs 45.2%; P = .001) and a larger number of circular shaped Schwann cells lacking central axons (43.0% vs 12.7%; P < 0.001) compared with control subjects. The low density of axons was significantly related to degree of swallowing dysfunction (r = 0.5; P = .03) and apnea-hypopnea index > 5 (P = .03). Regenerating axons were frequently observed in patients compared with control subjects (11.3 ± 4.2% vs 4.8 ± 2.4%; P = .02).

CONCLUSIONS: Axon degeneration in preterminal nerves of the soft palate is associated with pharyngeal dysfunction in snorers and patients with sleep apnea. The most likely cause for the nerve injuries is traumatic snoring vibrations and tissue stretch, leading to swallowing dysfunction and increased risk for upper airway obstruction during sleep.

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KEY WORDS: muscle degeneration; nerve injury; OSA; swallowing dysfunction; upper airways

ABBREVIATIONS: AHI = apnea-hypopnea index; GAP-43 = growth associated protein 43

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Part of the preliminary results was presented at the World Sleep Congress, October 7-11, 2017, Prague, Czech Republic, and in abstract form (Shah F, Berggren D, Levring Jäghagen E, Holmlund T, Franklin K, Stål P. Neuromuscular injuries in the soft palate correlates with pharyngeal dysfunction in sleep apnea subjects. Sleep Med. 2017;40:e302-e303.).

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OSA is a common disorder associated with adverse health consequences. The pathophysiologic mechanism of the nocturnal upper airway obstruction is not entirely understood. Several clinical studies and a few histologic studies have suggested nerve injuries as a cause of the inability of the muscles to maintain airway patency during sleep. Furthermore, previous studies reported an increase in peripheral nerve fibers stained for protein gene product (PGP 9.5) in the soft palate of patients with sleep apnea compared with control subjects. This increase was considered to be nerve sprouting and therefore suggested as a result of traumatic nerve damage due to snoring. Nerve injuries have also been proposed as a cause for the commonly occurring swallowing dysfunction in snorers and patients with sleep apnea. However, to the best of our knowledge, no study has shown any evidence of axon loss in preterminal nerves and its association with pharyngeal dysfunction in these patients.

In the present study, we hypothesized that nerve injuries in the upper airway are linked to the degree of pharyngeal dysfunction in snorers and patients with sleep apnea. Our goal therefore was to investigate for loss of axons and Schwann cells within preterminal nerve fascicles and muscle changes typical for denervation in the soft palate of patients who snore and those with OSA. Immunohistochemistry using specific markers for axons and their associated Schwann cells were used for detecting changes in nerve fascicles; histochemistry was used to reveal morphologic muscle alterations typical for denervation. The findings were compared with those of healthy control subjects and correlated with videoradiographic findings of pharyngeal function and severity of sleep apnea.

Patients and Methods

Patients and Control Subjects

Twenty-two patients (21 male subjects, 1 female subject) referred for upper airway surgery because of snoring and sleep apnea were included in the study. The exclusion criteria were smoking, previous palatal surgery, systemic disease, medications, drug abuse, and obesity. Their mean age was 44 years (range: 29-60 years), and the mean BMI was 28 kg/m² (range: 21-34 kg/m²).

Twelve age-matched subjects were included as control subjects. Their mean age was 46 years (range: 30-75 years), and the mean BMI was 25 kg/m² (range: 21-31 kg/m²). Six male subjects participated as voluntary control subjects recruited through advertisement. They were clinically evaluated in a similar way as patients. Six previously healthy subjects (three male subjects and three female subjects) who had suffered sudden accidental death were also included as control subjects. All tissue autopsies were taken within 8 to 24 h postmortem. Their medical history did not reveal any habitual snoring, sleep apnea, or swallowing dysfunction.

Sleep Apnea Recordings

Ambulatory sleep apnea recordings (Embletta Systems, ResMed) were made for all patients and living control subjects. The procedure comprised continuous records of airflow by using nasal cannula pressure, finger pulse oximetry (Nonin Oximeter Xpod, Nonin Medical Inc.), thoracic and abdominal respiratory effort, and a body position sensor. All recordings were scored manually according to the recommendations of the American Academy of Sleep Medicine.

Swallowing Examination

The pharyngeal swallowing function was examined by using videoradiography (BV 29 C-Arm; Philips; field width: 23 cm). The patients and voluntary control subjects chewed and swallowed a solid bolus of crisp bread with barium sulfate (Mixobar Oesophagus; Astra Laboratories) and a liquid barium sulfate contrast bolus (Mixobar High Density; Astra Laboratories). The swallowing was investigated twice in both lateral and frontal projection (ie, eight times in each subject). The examinations were evaluated at full speed and at slow motion by two investigators blinded for the clinical findings of the subjects. Swallowing function was graded as follows: 1, normal function; 2, mild dysfunction in the presence of repeated premature leakage, velar dysfunction, residual, or laryngeal penetration; 3, moderate dysfunction if repeated deviant features in grade 2 or dysfunction of the upper esophageal sphincter, the epiglottis, or the propagation wave was present (Video 1); and 4, severe dysfunction if the participant aspirated a bolus with aspiration below the vocal cords.

Tissue Samples

In patients, the entire base of the uvula was resected following soft palate surgery. The samples from the voluntary control subjects were acquired from the corresponding site by using the punch biopsy technique, except in one case in which complete surgical resection of the uvula was performed. The autopsy specimens were obtained from a similar area of the soft palate.

Some of the samples were immediately mounted in optimum cutting temperature compound (Tissue-Tek; Miles) and frozen in liquid propane chilled with liquid nitrogen; the other samples were fixed before mounting and freezing. Fixation was performed by using 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0, for 24 h at 4°C and overnight washing at 4°C in Tyrode’s solution containing 10% sucrose. All samples were stored at ~80°C until further processing.

Staining for Basic Histology

Tissue cross-sections, 7 to 8 µm thick, were cut in a cryostat (Leica CM3050; Leica Biosystems) at −20°C. The sections were mounted on glass slides and stained with routine hematoxylin and eosin for demonstration of basic morphology.

Immunohistochemistry

Serial tissue sections (5 µm thick), serial to those used for the hematoxylin and eosin stain, were multistained with well-characterized monoclonal and polyclonal antibodies. The antibodies were directed against neuronal microtubule beta III-tubulin in axons, glial-specific protein S100B in Schwann cells, a growth cone protein (growth-associated protein 43 [GAP-43]) in regenerating axons, and basement membrane protein laminin (Table 1). When required, postfixation of sections from unfixed tissue was performed by using

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Table 1

<table>
<thead>
<tr>
<th>Antibody (Product)</th>
<th>Description</th>
</tr>
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<tr>
<td>PGP 9.5</td>
<td>Protein gene product 9.5</td>
</tr>
<tr>
<td>GAP-43</td>
<td>Growth-associated protein 43</td>
</tr>
<tr>
<td>S100B</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>laminin</td>
<td>Basement membrane protein</td>
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1-3 The pathophysiologic mechanism of the nocturnal upper airway obstruction is not entirely understood. Several clinical studies and a few histologic studies have suggested nerve injuries as a cause of the inability of the muscles to maintain airway patency during sleep. Furthermore, previous studies reported an increase in peripheral nerve fibers stained for protein gene product (PGP 9.5) in the soft palate of patients with sleep apnea compared with control subjects. This increase was considered to be nerve sprouting and therefore suggested as a result of traumatic nerve damage due to snoring. Nerve injuries have also been proposed as a cause for the commonly occurring swallowing dysfunction in snorers and patients with sleep apnea. However, to the best of our knowledge, no study has shown any evidence of axon loss in preterminal nerves and its association with pharyngeal dysfunction in these patients.

In the present study, we hypothesized that nerve injuries in the upper airway are linked to the degree of pharyngeal dysfunction in snorers and patients with sleep apnea. Our goal therefore was to investigate for loss of axons and Schwann cells within preterminal nerve fascicles and muscle changes typical for denervation in the soft palate of patients who snore and those with OSA. Immunohistochemistry using specific markers for axons and their associated Schwann cells were used for detecting changes in nerve fascicles; histochemistry was used to reveal morphologic muscle alterations typical for denervation. The findings were compared with those of healthy control subjects and correlated with videoradiographic findings of pharyngeal function and severity of sleep apnea.
For measurements of quantified shaped Schwann cells without axons within each nerve fascicle was discriminating individual S-100B with the fascicle area occupied by Schwann cells in the nerve fascicle was calculated by cluster was considered as a unit and analyzed separately. The area of the fascicle (axons/c2) was calculated by dividing the number of axons by the cross-sectional size of the fascicle. Due to difficulty in discriminating individual fine-sized axons gathered in clusters, each cluster was considered as a unit and analyzed separately. The area occupied by Schwann cells in the nerve fascicle was calculated by dividing the total Schwann cell area stained for monoclonal antibody S-100B with the fascicle area × 100 (%). The percentage of circular-shaped Schwann cells without axons within each nerve fascicle was quantified.

For measurements of fiber size and amount of connective tissue, four photographs were randomly selected from each muscle sample of patients and autopsy control subjects; two to four photographs from the smaller punch biopsies were included in the quantification. The size of the scanned area in each photo was 276,856 μm² (20× magnification). The mean cross-sectional area of muscle fibers was calculated by tracing the membrane of each muscle fiber stained for laminin on each photo. Variability in muscle fiber size was expressed as the coefficient of variation. The percentage of area occupied by muscle fibers was calculated according to the following formula: total muscle cross-sectional area/total scanned tissue area × 100 (%). The amount of extracellular tissue (ie, connective tissue including glands, vessels, and nerves) was calculated by subtracting the total muscle cross-sectional area from the total tissue area of each scanned photograph. A total of 11,713 muscle fibers were included in the quantification. A mean of 276 muscle fibers were evaluated from each control subject and 382 muscle fibers from each patient. The investigator was blinded regarding the origin of the samples.

**Antibodies Used for Immunohistochemistry**

<table>
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<tr>
<th>Antibody</th>
<th>Product Code</th>
<th>Specificity</th>
<th>Gene</th>
<th>Host/Clone</th>
<th>Dilution</th>
<th>Source</th>
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<td>LAM</td>
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</tr>
</tbody>
</table>

GAP-43 = growth-associated protein 43; mAb = monoclonal antibody; pAb = polyclonal antibody.

**Morphologic and Quantitative Analysis of Nerves and Muscles**

All uvula samples were scanned with a fluorescence microscope (Leica DM6000B; Leica Microsystems CMS GmbH) equipped with a digital high-speed fluorescence charged-coupled device camera (Leica DFC360 FX). Nerves and muscles were analyzed for morphology by using customized morphometric software (Leica QWin Standard version 3.5.1 software; Leica Microsystems Ltd).

Transversely sectioned nerves were identified and scanned at 40× magnification in both the mucosal and muscle regions. Nerve fascicles in patients (n = 132) and control subjects (n = 46) were analyzed for the density of axons and Schwann cells and the proportion of circular-shaped Schwann cells lacking central axons (ie, loss of myelinated axons). The cross-sectional area of each nerve fascicle was measured by tracing the circumference of the nerve membrane stained for the polyclonal antibody Z0097 against laminin. The number of axons stained for beta III-tubulin and GAP-43 was counted in each nerve fascicle, and the density per unit was calculated by dividing the number of axons by the cross-sectional area of the fascicle (axons × 10⁻³ per μm²). Due to difficulty in discriminating individual fine-sized axons gathered in clusters, each cluster was considered as a unit and analyzed separately. The area occupied by Schwann cells in the nerve fascicle was calculated by dividing the total Schwann cell area stained for monoclonal antibody S-100B with the fascicle area × 100 (%). The percentage of circular-shaped Schwann cells without axons within each nerve fascicle was quantified.

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**Statistical Analysis**

All statistical tests were performed by using SPSS version 23 (IBM SPSS Statistics, IBM Corporation). Normality in distribution of the nerve findings from the tissue samples was tested by using the Shapiro-Wilk test. Comparisons between the groups were made by using the independent sample t test for variables with normal distribution; for variables with non-normal distribution, the Mann-Whitney U test was performed. Spearman’s rank order correlation test was performed to detect correlations. Values are presented as mean ± SD. The results were considered significant at P values ≤ .05.

**Approval of the Study**

The regional medical ethical committee in Umeå approved the study (Dnr-05-130M). The patients and voluntary control subjects were informed and provided written consent to participate. All muscle samples were collected in agreement with the Declaration of Helsinki. Autopsy specimens were collected in agreement with Swedish laws and regulations on autopsy and transplantation.

**Results**

All patients snored (mean: 20 years of snoring; range: 6–40 years), and 14 of the 22 patients had OSA with a mean apnea-hypopnea index (AHI) of 24 (range: 5–84). Ten patients had moderate swallowing dysfunction (grade 3), six patients had mild dysfunction (grade 2), and six patients had normal function (grade 1). All the voluntary control subjects had an AHI < 5 and normal swallowing function (grade 1). No significant correlation between severity of AHI and degree of swallowing dysfunction was observed.

**Nerve Morphology**

The mean nerve fascicle area in control subjects were 2,881 ± 1,360 μm²; in patients, it was 2,898 ± 1,486 μm². The density of axons within nerve fascicles was significantly lower in patients (5.4 ± 2.0 × 10⁻³ axons/μm²) than in control subjects (17.9 ± 10.6 × 10⁻³ axons/μm²).
axon/μm²; P = .02) (Figs 1A, 1D, 2A). Schwann cells occupied a smaller percentage area within the nerve fascicles of patients (17.5 ± 6.5%) compared with control subjects (45.2 ± 7.1%; P = .001) (Figs 1B, 1E, 2B). The number of circular-shaped Schwann cells lacking central axons within nerve fascicles was significantly higher in patients than in control subjects (43.0 ± 16.6% vs 12.7 ± 7.6%, respectively; P < .001), highlighting loss of myelinated axons (Figs 2C, 3). Moreover, clusters of fine-sized axons partially embedded with Schwann cells were more frequently seen in nerve fascicles of patients than in control subjects. The density of regenerating axons stained for GAP-43 was significantly higher in patients compared with control subjects (11.3 ± 4.2% vs 4.8 ± 2.4%; P = .02). In general, GAP-43-positive axons were randomly spread in the nerve fascicle, but they were also often localized in the areas showing clusters of fine-sized axons (Fig 4). In the nerve fascicles, the area occupied by connective tissue was 36% larger in patients than in control subjects (P < .001).

Nerve Morphology in Patients With Vs Without Pharyngeal Swallowing Dysfunction

There was a significant correlation between the low density of axons within the nerve fascicles and the degree of pharyngeal swallowing dysfunction in snorers and patients with sleep apnea (r = 0.5; P = .03). The percentage of circular-shaped Schwann cells lacking central axons was significantly higher in patients with pharyngeal swallowing dysfunction compared with patients with a normal function (47.7 ± 14.1% vs 28.0 ± 15.3%; P = .001) (Fig 2D).

Comparison Between Nerve Changes, Swallowing Dysfunction, and AHI

In patients with AHI ≥ 5, the axon density (4.8 ± 1.8 × 10⁻¹ axons/μm²) and arterial oxygen saturation (81 ± 7%) were significantly lower than in those with AHI < 5 (6.6 ± 2.3 axons/μm² [P = .03] and 89 ± 3% arterial oxygen saturation [P = .004]). No significant correlation between AHI and swallowing dysfunction was observed.

Figure 1 – A-F. Nerve fascicles from a control subject and a patient. Nerve fascicles from (A-C) the control subject and (D-F) the patient immunostained for beta III-tubulin in axons (red color; A, D) and S-100B in SCs (green color; B, E). Merged pictures of stained axons (red) and SCs (green) are shown in C and F. Areas devoid of axons (*, D) and SCs (+, E) and circular-shaped SCs lacking central axons (arrowheads, F) in patients. Note the lower density and variability of axons in patients and the clusters of small-sized axons (F, white arrow). Scale bar = 25 μm. SCs = Schwann cells.
Comparison Between Control Biopsy and Autopsy Specimens

No significant differences were observed between the control biopsy specimens and the control autopsy specimens in axon density and Schwann cell area within nerve fascicles.

Muscle Morphology

The muscle samples from the patients differed from those of control subjects ($P = .02$) by having 9% greater fiber size variability (coefficient of variation); that is, a larger number of hypotrophied and hypertrophied fibers, 10% smaller area occupied by muscle fibers and, conversely, 10% larger amount of connective tissue in the scanned muscle cross-sections ($P = .04$, respectively) (Fig 5).

Discussion

This study is the first to show a relation between pharyngeal dysfunction and axon loss in snorers and patients with sleep apnea. We report polymorphic evidence of nerve injury and muscle denervation in the soft palate that was significantly related to pharyngeal swallowing dysfunction and OSA in these patients.

The finding of significantly lower axon density within the nerve fascicles and its relation to pharyngeal swallowing dysfunction and severity of sleep apnea reinforce nerve injuries as a cause for impaired reflexes and inadequate neuromuscular response. In addition, the significantly higher muscle fiber size variability in patients reflects muscle fiber denervation (ie, fiber atrophy) and indicates that muscle weakness also contributes to pharyngeal dysfunction in snorers and patients with sleep apnea. Fiber atrophy is further supported by the significantly increased amount of connective tissue in patients (Fig 5). The increased number of large-sized fibers is probably related to a compensatory process in which the remaining
innervated muscle fibers are more frequently used to uphold the function.

Interestingly, the area occupied by Schwann cells was lower and the number of circular-shaped Schwann cells lacking axons in nerve fascicles was significantly higher in patients. Schwann cells in the peripheral nervous system are responsible for maintaining homeostasis and providing support, nutrition, and protection for axons. Myelinating Schwann cells wrap around axons to form a fatty layered sheath, making the conduction velocity of the nerve faster, whereas nonmyelinating Schwann cells embed small-sized sensory and, to some extent, autonomic sympathetic axons. The significantly smaller Schwann cell area and the large number of thick Schwann cells lacking central large-sized axons reflect both demyelination and loss of motor axons in patients with sleep apnea. Schwann cells also play a major role in axon regeneration by producing neurite-promoting factors that help axon growth cones adhere to endoneurial tubes.\textsuperscript{18} Hence, the reduced Schwann cell area in nerves might adversely affect the regenerative capacity of axons.

Even though the number of axons within the nerve fascicles was lower in patients, the nerves contained a relatively large number of fine-sized axons. Most of these axons were gathered in clusters embedded by Schwann cells, whereas others were randomly scattered throughout the nerve fascicle. Interestingly, a significantly higher number of the axons were immunostained for GAP-43 in patients compared with control subjects. Neuromodulin, or GAP-43, is an intracellular GAP that plays an important role in neurite outgrowth during development. In adulthood, the expression of GAP-43 is low in nerves,\textsuperscript{19} but following damage, an increase in the expression of GAP-43 can be observed in the growth cones of axons. The extensive expression of GAP-43 in axons in snorers and patients with sleep apnea highlights a regenerative attempt following injury. However, the recurrent nature of snoring vibratory trauma and tissue stretch might have the potential to disturb the regenerative and healing process. Treatment strategies to reduce snoring trauma and tissue stretch may allow re-innervation and

Figure 3 – Nerve fascicle from a patient. Nerve fascicle immunostained for beta III-tubulin in axons (red color), S-100B in SCs (green color) and 4’, 6-diamidino-2-phenylindole dihydrochloride (stains nuclei blue). Sections 1 through 3 display magnified regions of the nerve fascicle (white arrows). Section 1 shows a missing central axon within a circular-shaped SC, and section 2 shows a large axon encircled by a SC. Section C shows cluster of fine-sized axons embedded with SCs. Scale bar = 10 \( \mu \)m. See Figure 1 legend for expansion of abbreviation.

Figure 4 – A-C, Regenerating axons in a nerve fascicle from a control subject and a patient. Nerve fascicles immunostained for S-100B (SCs; red color) and GAP-43 (regenerating axons; green color). Note the large number of regenerating axon growth cones in (B) the patient compared with (A) the control subject (white arrows). C, Bar graph shows a significantly higher density of regenerating axons stained for GAP-43 compared with control subjects. \(* P = .02. \) Scale bar = 10 \( \mu \)m. GAP-43 = growth-associated protein 43. See Figure 1 legend for expansion of other abbreviation.
regeneration of muscle tissue. In control subjects, the GAP-43-positive axons might relate to a normal continuous process of plasticity and synaptic remodeling that could occur at nerve endings.20

Although the background mechanism for the neuromuscular changes is unclear, snoring vibratory trauma, tissue stretch, inflammation, and hypoxia have all been reported as potential causes of nerve injuries in the upper airway.4,6,21-24 However, because sleep apnea is frequently preceded by years of snoring, traumatic vibrations and tissue stretch seem to be major initiating factors for the progressive development of sleep apnea.25

Long-standing exposure to mechanical vibrations in limbs is known to cause nerve lesions, vasospasm, reduced blood flow, loss of capillaries, and reduced muscle strength.26-28 Interestingly, a low capillarization in the soft palate muscles of patients who snore and patients with sleep apnea was reported by us,29 suggesting a potential for development of local hypoxia followed by neuropathy. Moreover, animal models have shown that prolonged vibration causes structural damage to the nerve myelin sheath.30 As a result, nerve injuries in the upper airways caused by traumatic snoring might disturb the reflex circuits responsible for normal swallowing function. During sleep, when the tonic activity of muscles is decreased, disturbed reflexes and weakened muscle might render the upper airway more susceptible to collapse.

**Conclusions**

We found novel evidence that neuromuscular injuries in the soft palate of snorers and patients with sleep apnea are significantly related to pharyngeal swallowing dysfunction and sleep apnea. It is suggested that the nerve injuries are due to snoring vibrations and tissue stretch leading to pharyngeal dysfunction and increased risk for upper airway collapse in patients with sleep apnea.
Acknowledgments

Author contributions: P. S. is the guarantor of the study. D. B., E. L. I., and P. S. conceived the study; F. S., E. I. J., T. H., D. B., and P. S. designed the study; F. S., S. F., T. H., E. L. J., and P. S. conducted the acquisition and analysis of the data; and F. S. and P. S. performed the statistical analysis. All authors contributed to the interpretation of the findings. F. S., S. F., and P. S. prepared the first draft of the manuscript, and all authors critically revised the manuscript for intellectually important content. All authors gave final approval for the publication of the work, and all accept responsibility for the integrity of the work.

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Additional information: The Video can be found in the Supplemental Materials section of the online article.

References