Does Pepsin Play a Role in Etiology of Laryngeal Nodules?

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Summary: Objectives. Vocal fold nodules (VFNs) are benign disorders affecting the superficial lamina propria of the true vocal folds. The etiology of VFNs still remains unclear but laryngeal trauma caused by vocal abuse, tobacco, alcohol, and laryngopharyngeal reflux (LPR) plays a crucial role on the pathogenesis. The aim of this study was to assess the presence of pepsin in formalin-fixed, paraffin-embedded (FEPE) specimens of VFNs to evaluate the role of LPR as a risk factor for VFNs.

Materials and Methods. A total of 28 pathology specimens of patients suffering from VFNs who had undergone laser microsurgery under general anesthesia were evaluated. The specimens were maintained in paraffin blocks in the pathology department. Western blot (WB) and enzyme-linked immunosorbent assay (ELISA) analyses were used to measure pepsin enzyme levels in the VFNs tissue specimens. Signs of LPR were assessed according to the reflux finding score.

Results. The mean reflux finding score of the patients was 13.6 ± 2.89 (8−21). According to WB and ELISA analyses, pepsin was detected with both the WB the ELISA tests in positive controls, but there was no pepsin enzyme in any of the 28 laryngeal FEPE VFNs specimens.

Conclusion. The pepsin enzyme was not detected in any of the FEPE VFNs specimens, and it is concluded that further studies are needed to reveal the role of pepsin in the etiology of VFNs.

Key Words: Vocal fold−Nodules−Laryngopharyngeal reflux−Pepsin−Reflux finding score.

INTRODUCTION

Vocal fold nodules (VFNs) are benign, callous-like, vocal mucosal lesions that affect the superficial layer of the lamina propria.[1] VFNs are usually bilateral, symmetric, sessile, pearl-colored lesions located between the anterior and middle third of the true vocal folds, and they are typically immobile during phonation.[2] The prevalence of VFNs in the general population is still unknown, but it is encountered in 23.4% of pediatric patients with hoarseness[3] and predominantly occurs in female population.[4] The symptoms most commonly seen are hoarseness, throat discomfort, and pain that can easily and seriously affect the patients' quality of life. They are traditionally thought to result from vocal abuse or misuse, and individuals who use their voices professionally, excessively, or incorrectly are at a higher risk for developing VFNs.[5] Other factors still held to be responsible for the pathogenesis are chronic and local irritants, tobacco and alcohol consumption, and laryngopharyngeal reflux (LPR).

LPR is defined as the backflow of the gastric contents beyond the esophagus and into the laryngopharynx. One of the most important gastric contents and the primary proteolytic enzyme of the digestive system is pepsin, which plays a crucial role in the pathogenesis of LPR. Pepsin is also a reliable clinical marker for LPR and can be detected easily from tissue or swab samples of the laryngopharynx with immunologic methods (Western blot [WB] and enzyme-linked immunosorbent assay [ELISA]).[6] Previous studies have suggested that LPR is associated with various laryngeal pathologies, such as reflux laryngitis, subglottic stenosis, and vocal granuloma[7]; however, the relationship between VFNs and LPR remains unclear.

The aim of this study was to assess the presence of pepsin in formalin-fixed, paraffin-embedded (FEPE) specimens of VFNs to evaluate the role of LPR as a risk factor for the development of VFNs.

MATERIALS AND METHODS

A total of 28 pathology specimens of patients suffering from VFNs who had undergone laser microsurgery under general anesthesia were evaluated between September 2013 and July 2016 in the otolaryngology, head, and neck surgery department. The specimens were confirmed histopathologically as VFNs by a pathologist and maintained in paraffin blocks in the pathology department. WB and ELISA analyses were used to measure pepsin enzyme levels in the VFNs tissue specimens. The presence of pepsin in positive controls was investigated with the WB and ELISA to ensure the accuracy of the method used in the study.

The preoperative videolaryngoscopy examination videos of all the patients were evaluated by a senior laryngologist. Signs of LPR were assessed according to the reflux finding score (RFS),[8] a standardized clinical index. The RFS is an eight-item scale that reveals the presence of LPR and documents the clinical severity of disease. The scale ranges from 0 (no abnormal findings) to a maximum of 26 (worst score possible). Statistically, an individual with a score greater than 7 has LPR.

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Each of the authors has contributed to, read, and approved this manuscript. This manuscript is original and it, or any part of it, has not been previously published; nor is it under consideration for publication elsewhere. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

All authors declare that they have no conflict of interest.

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WB analysis

First, the VFNs tissue samples were deparaffinized. Following the deparaffinization, total protein was extracted from the tissue specimens in a RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology, Dallas, Texas). After homogenization and centrifugalization, the supernatants were prepared to discover pepsin by means of immunologic methods (WB and ELISA), and protein content was measured using the Bradford assay. Twenty micrograms of total protein from each specimen (less in the case of low-concentration specimens) were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis protocol and transferred to a polyvinylidene fluoride membrane (GE Healthcare, Piscataway, New Jersey). The proteins were probed with mouse monoclonal antibodies (sc-101405 Pepsin A [2F5], Santa Cruz Biotechnology, Inc., Dallas, Texas). Also, positive and negative controls run with the samples to ensure the accuracy of the method. The blots were probed with appropriate alkaline phosphatase conjugated secondary antibody diluted 1:5000 (Invitrogen Corporation, Carlsbad, California). Finally, the blots were exposed to enhanced chromogenic substrate reagents (sc-2048, Santa Cruz Biotechnology, Dallas, Texas) and then submitted to radiographic exposure and development. If the band is not observed even at any amount, it is concluded that there is no pepsin in the tissue.

ELISA analysis

The pepsin assay was developed based on a sandwich ELISA principle. First, 100 μL of protein content prepared from malignant tissue specimens were added into the microplate wells of an ELISA kit (SN:201-12-0923, Human Pepsin, PP ELISA Kit, SunRedBio, Baoshan District, Shangai) so the target antigens could bind to the capture antibody. A biotin-conjugated detection antibody was incubated for 90 minutes at 37° C, binding the antibody to the captured antigen. After wash steps, a streptavidin-horseradish peroxidase conjugate was added and bound to the biotin. Tetramethylbenzidine substrate was then added. Tetramethylbenzidine reacts with the horseradish peroxidase enzyme, resulting in color development. The color change wavelength was measured at 450 nm and compared with a reference curve.

Statistical analyses

Statistical analyses were performed using the *Statistical Package for Social Sciences* software (SPSS 17.0 for Windows, SPSS Inc., Chicago, Illinois). The results are presented as the mean, standard deviation, or the number (percentage) of patients.

RESULTS

The FEPE pathology specimens of 28 patients affected by VFNs were included in this study. Twenty-two of the 28 patients were male (78.5%), six of the 28 patients were female (21.5%), and the mean patient age was 47.4 ± 14.3 (24−81) years. The mean RFS of the patients was 13.6 ± 2.89 (8−21) (Table 1).

All the pathology specimens of the laryngeal tissues were identified as VFNs. Eighteen of the 28 VFNs (64.2%) were excised from the right true vocal fold, nine of the 28 (32.1%) were excised from the left true vocal fold, and one of the 28 (3.5%) were excised from the anterior commissure.

According to WB and ELISA analyses, pepsin was detected with both the WB the ELISA tests in positive controls (Figures 1 and 2), but there was no pepsin enzyme in any of the 28 laryngeal FEPE VFNs specimens (Figures 2 and 3).

DISCUSSION

The etiology of VFNs remains unclear, but laryngeal trauma caused by vocal abuse, local irritants, and/or tobacco and alcohol intake plays a crucial role in their pathogenesis.[1] There are a limited number of studies emphasizing that other factors may play a role in the pathogenesis of VFNs. The role of personality in the pathogenesis of VFNs has also been discussed. Ratajczak et al examined two groups with VFNs and controls and suggested that the patients with VFNs showed greater social activity, aggression, and impulsivity.[9] Czerwonka et al discovered in their theoretical and physical model that intravascular pressure rises significantly during vocal fold vibration, especially because of vocal abuse and local irritants. This may lead to vessel damage and extravasation of erythrocytes, which is commonly seen in benign vocal fold lesions such as VFNs.[5] Tiba et al assessed the presence of helicobacter pylori (HP) in 14 patients with minimal vocal fold lesions, including nodules, and they detected HP in 10 of the 14 patients. They concluded that HP may be an etiological factor for vocal fold minimal lesions and recommended that eradication be considered as treatment.[10] The relationship between inhalational and/or nutritional allergens and VFNs has also been examined. Allergies are not considered an independent risk factor for the development of VFNs, but they may have a synergistic effect with other factors, such as smoking and vocal abuse.[11]

LPR is another potential factor in the pathogenesis of VFNs because it leads to chronic inflammation of the lamina propria.[1] It has recently been shown that reflux affects 62% of otolaryngologic patients with laryngeal and voice disorders.[8] However, there are only a few studies examining the role of LPR in the development of VFNs, and no causal relationship has been established. Chung et al analyzed the prevalence of LPR in two groups: a control group including 200 patients, and 110 patients with benign mucosal lesions (nodules, polyps, and/or Reinke edema). The prevalence of LPR was 65% in the control group, 66% in the vocal nodule group, 75% in the vocal polyp group, and 90% in the Reinke edema group. Chung et al concluded that LPR was significantly associated with Reinke edema but not with nodules or polyps.[1] Another study by Kantas et al aimed to evaluate the effects of LPR on the healing process of surgical laryngeal trauma and examined 112 patients suffering from benign lesions of the vocal folds (laryngeal polyps/nodules and/or Reinke edema).[12] Half of these patients with LPR were randomly chosen for therapy with proton pump inhibitors (PPI), and another 50 LPR-free subjects with benign lesions were selected as a control group. All these

TABLE 1.
The Demographic Characteristics of Patients, Morphological Features of VFNs, RFS Results, and Investigation of Pepsin Enzyme in VFNs With WB and ELISA

					Pepsin Level	
				Morphological Features of VFNs	Immunologic Method	
Patient Number	Age (y)	Sex	RFS	Anatomic Localization (True Vocal Fold)	WB	ELISA
1	35	M	12	Right	NA	NA
2	32	M	15	Right		
3	45	F	19	Right		
4	31	M	13	Right		
5	46	M	14	Right		
6	43	F	17	Right		
7	53	M	14	Left		
8	40	F	12	Right		
9	37	M	12	Left		
10	65	M	8	Left		
11	53	M	14	Right		
12	70	M	13	Left		
13	24	M	9	Right		
14	73	M	14	Right		
15	36	M	11	Left		
16	34	F	13	Right		
17	45	M	9	Right		
18	43	M	12	Right		
19	68	F	10	Left		
20	47	M	17	Left		
21	81	M	21	Right		
22	40	M	15	Right		
23	53	M	16	Right		
24	61	M	14	Left		
25	57	F	13	Right		
26	29	M	15	Left		
27	44	M	15	Right		
28	43	M	14	Ant. Comm.		
Mean	47,4 ± 14,3 (24–81)		13.6 ± 2.89 (8–21)			

Abbreviations: Ant. Comm., anterior commissure; F, female; M, male; NA, not available.

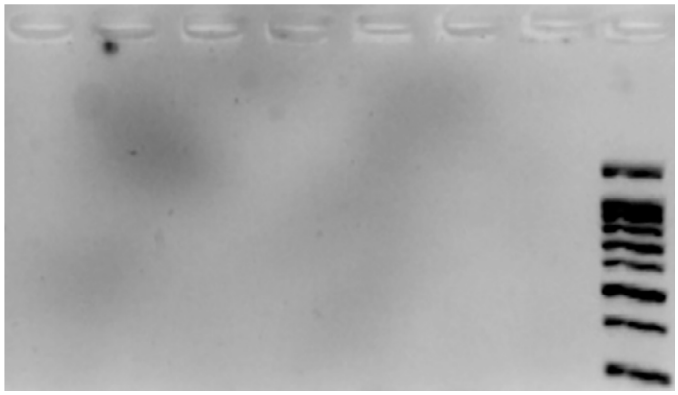

FIGURE 1. According to WB analysis, there was no presence of pepsin enzyme in any of VFNs specimens.

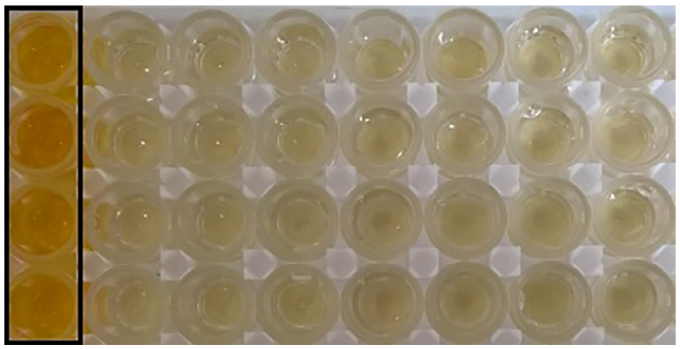

FIGURE 2. According to ELISA analysis, the dark yellow-colored wells observed in the black frame showed the presence of pepsin enzyme in positive controls; the light yellow-colored wells indicate that no pepsin enzyme was detected in VFNs specimens. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

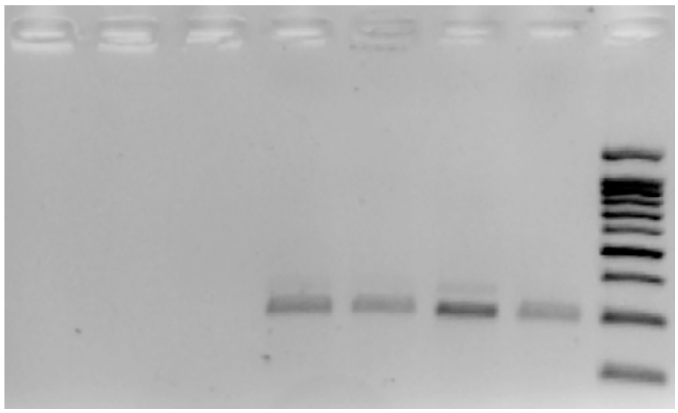

FIGURE 3. According to WB analysis, pepsin enzyme was detected in the positive controls.

groups, except the six patients who recovered after the PPI treatment, had undergone surgery, and the clinical features of the groups were compared with subjective tests to analyze the severity of vocal lesions in each group. The entire control group and all the patients who had undergone surgery after the PPI treatment showed complete and proper healing of the vocal folds. Furthermore, granulation tissue or recurrence occurred in six patients who had not used PPI, and revision surgery was required for two of them. In addition, the subjective test scores showed significant improvement postoperatively across all three groups, but the greatest improvement was in the group of patients who had been administrated PPI. Kantas et al concluded that LPR influences epithelialization and recurrence of benign laryngeal lesions after surgery.[12] In the present study, the laryngeal VFNs specimens were examined for the presence of the pepsin enzyme by WB and ELISA to reveal the role of LPR in the pathogenesis of VFNs, but there was no evidence of pepsin enzyme in these specimens.

Pepsin can be easily detected in tissue specimens of the laryngopharynx with immunologic methods (WB and ELISA), and the presence of this enzyme can be used to diagnose LPR.[6] The sensitivity and specificity of ELISA depends on the affinity and specificity of the antibodies employed, and sensitivity and specificity can reach up to 100% and 89%, respectively. However, a cross-reaction with pepsinogen has been described in many pepsin proteins, and usually an alternative immunologic test (WB) is preferred to improve the specificity of the results.[4]

Kim et al investigated pepsin in laryngeal sputum and saliva samples with WB for the diagnosis of LPR, and the sensitivity and specificity were 89% and 68%, respectively.[13] In the present study, both immunologic methods (ELISA and WB) were used to detect the pepsin enzyme in biopsy specimens of patients suffering from VFNs. Because the authors of the present study had not encountered this technique in the literature, the specimens were confirmed histopathologically as VFNs and maintained in paraffin blocks. According to the results of WB and ELISA, there was no presence of pepsin enzyme in any of the 28 laryngeal FEPE VFNs specimens. It is clearly accepted that the proteins can be extracted from FFPE tissues by antigen retrieval methods and can be suitable for proteomic analysis. The similarity between FFPE specimens and fresh tissues could be up to 90%.[14] The absence of the pepsin enzyme can be explained by the fact that the proteins may not overlap with each other in complete similarity.

Limitations

The patients were not evaluated with ambulatory, 24-hour, double-probe pH monitoring, which is a valuable diagnostic tool of LPR. But the reproducibility of proximal pH testing could be as low as 55% for patients with proximal esophageal acid reflux.[15] Although 24-hour, double-probe pH monitoring could not be used in this study, high RFS results demonstrated symptoms of LPR. Finally, future studies may provide these results, if the controls include other vocal cord samples, esophageal, or pharyngeal specimens of the same patients.

CONCLUSION

In this study, the pepsin enzyme was not detected in any of the FEPE VFNs specimens, and it is concluded that further studies are needed to reveal the role of pepsin in the etiology of VFNs.

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