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Histological evaluation of soft palate tissues in patients with sleep-disordered breathing

Authors' Contribution:
A – Study Design
B – Data Collection
C – Statistical Analysis
D – Data Interpretation
E – Manuscript Preparation
F – Literature Search
G – Funds Collection

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ABSTRACT:

Introduction: Sleep is a physiological state essential for the proper functioning of the body. One of the reasons for its disturbance is obstructive sleep apnea syndrome (OSAS).

Aim: The aim of this research is the histological evaluation of the soft palate in patients who suffered from various types of OSAS.

Material and method: The study group consisted of patients with sleep-disordered breathing (SDB) in the form of primary snoring or OSAS. Patients with chronic tonsillitis, without a history of SDB, were included in the comparative group. Fragments of the mucous of the uvula (study group) and the palatoglossal pillar (comparative group) were obtained during surgery for histological evaluation. Using histological, histochemical and immunohistochemical methods, we assessed the inflammation and its severity (CD3, CD20, CD68), the structure of nerve fibers (S-100) and the size of blood vessels (CD34) in the examined tissue.

Results: Patients with OSAS developed a local inflammatory process in the palatal tissues (stronger expression of CD3, CD20, CD68 in patients with OSAS). The severity of the immunohistochemical reaction with CD3 correlated with the stage of OSAS. A higher degree of fibrosis and a higher expression of CD34 and S-100 receptors were observed in patients with OSAS compared to snoring patients and patients from the comparative group.

Conclusion: Most likely, snoring due to chronic tissue vibration leads to damage to the nerve fibers in the soft palate, which can intensify episodes of hypopneas during and increase the chance for sleep apneas.

KEYWORDS: CD20, CD3, CD34, S-100 protein, snoring, soft palate, obstructive sleep apnea syndrome

ABBREVIATIONS

AHI – apnea-hypopnea index
HIF-1 – hypoxia-inducible factor 1
OSAS – obstructive sleep apnea syndrome
VEGF – vascular endothelial growth factor

INTRODUCTION

Obstructive sleep apnea syndrome (OSAS) is a form of sleep-disordered breathing most commonly affecting middle-aged people [1]. Due to recurring episodes of collapse of the upper respiratory tract, OSAS leads to hypoventilation, apnea, desaturation, and hypercapnia [2]. The vibration of the oropharyngeal and laryngopharyngeal tissues is observed in patients who suffered from sleep-disordered breathing in the form of primary snoring. Recurring episodes of hypoxia are responsible for the development of systemic inflammation [3]. Mediators of inflammation are released, such as histamine, serotonin, kinins, prostaglandins and thromboxane [4]. The daily rhythm of cytokine secretion and their concentration in the blood changes [5]. Cells of the leukocytes system migrate from the bloodstream to tissues affected by the inflammatory process [6]. Hypoxia also produces a hypoxia-inducible factor-1 (HIF-1) that induces an inflammatory response. HIF-1 is a potent stimulator of gene transcription for a vascular endothelial growth factor (VEGF) and erythropoietin. The action of HIF-1 causes the intensification of angiogenesis and erythropoiesis as a defense response to a decrease in arterial oxygenation. The CD 34 protein is the antigen of hematopoietic progenitor cells. It occurs on endothelial cells of blood vessels.

Inflammatory infiltration of tissues can be assessed based on the number of inflammatory cells (T lymphocytes, B lymphocytes, macrophages) in the examined tissue. The major marker of T cells is considered to be the extracellular domain of the CD3 receptor. In contrast, the CD20 receptor is a molecule that marks B cells.

Chronic tissue vibration which occurs due to persistent snoring most likely leads to damage to the nerve fibers in the soft palate, which can intensify episodes of hypopneas during sleep and increase the occurrences of sleep apnea. The structure of nerve endings is assessed based on the expression of the S-100 protein.

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PURPOSE

The study aimed to assess the inflammation, nerve fibers and blood vessels in the soft palate tissues obtained from snoring and OSAS patients.

HYPOTHESIS

The vibration of the soft palate tissues caused by snoring develops local inflammation within their tissues.

MATERIAL AND METHOD

The study included adult patients who were divided into two groups: study and comparative. All patients underwent surgery. 22 people from the study group underwent injection palatoplasty, 16 – laser-assisted palatoplasty, and 8 underwent palatopharyngoplasty performed. Bilateral tonsillectomy was performed in patients from the comparative group. The average age of patients enrolled in the study was 43: the oldest patient was 69, while the youngest was 27. The average AHI index was 14.9/h in the study group.

A fragment of the mucous membrane of the uvula and the palatoglossal pillar obtained during surgery was evaluated histologically, histochemically and immunohistochemically.

The study group was divided into the following three subgroups:

1. G1 – 22 patients with habitual snoring with no history of sleep apnea. None of them reported the drowsiness, tiredness or need for daytime naps. Polygraphy confirmed primary snoring. Sleep parameters were within the range of standards (AHI index ranged from 0.0 to 4). The average age of patients in this group was 44 years; the oldest was 69 years old, and the youngest – 29 years old;

2. G2 – 16 patients who complained of persistent snoring and sleep apnea observed by a bed partner. A mild form of OSAS was diagnosed based on sleep examination. The AHI index ranged from 5.5 to 15. The average age in this group was 43; the oldest was 75 years old and the youngest was 27 years old;

Patients from the G1 and G2 subgroups underwent injection or laser-assisted palatoplasty.

3. G3 – 8 patients with moderate to severe OSAS syndrome. Polygraphy showed AHI ≥ 15. The patients underwent palatopharyngoplasty or expansion sphincter pharyngoplasty under general anesthesia. The AHI index ranged from 16 to 35. The average age in this group was 46; the oldest was 57 years old and the youngest was 28 years old.

The comparative group – G0 – consisted of patients who had no history of snoring or daytime sleepiness. The patient's bed partner did not observe sleep apnea. Patients included in the comparative group were operated due to chronic tonsillitis. They did not report any comorbidities. A fragment of the palatoglossal pillar collected during tonsillectomy was examined.

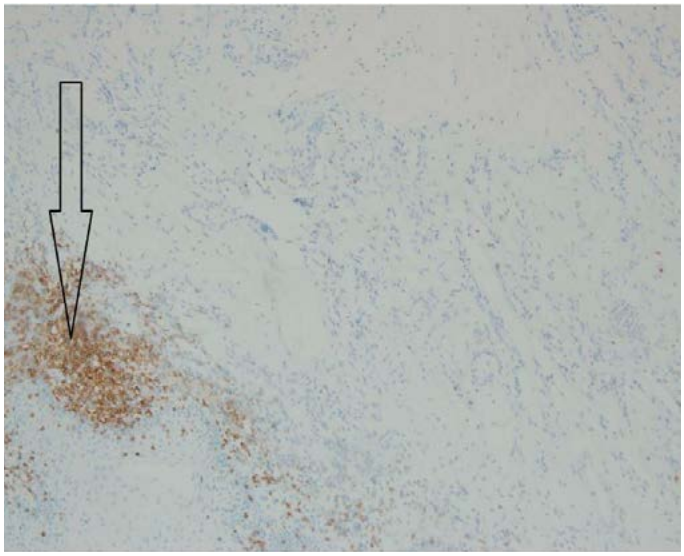


Fig. 1. Specimen of the mucosa obtained from a patient from the group G2. Numerous cells with signs of CD3 receptor expression (arrow). Immunohistochemical method. Magnification x200.

Morphological assessment methods

The histopathological examination included the assessment of fibrosis and inflammatory infiltration, according to the following criteria.

In the case of fibrosis (assessed using Masson trichrome staining):

- (0) no tissue fibrosis,
- (1) focal fibrosis (visible fibrosis in more than 10% but less than 50% of fields in 10 representative fields),
- (2) diffused fibrosis (fibrosis visible in more than 50% of fields in 10 representative fields of view).

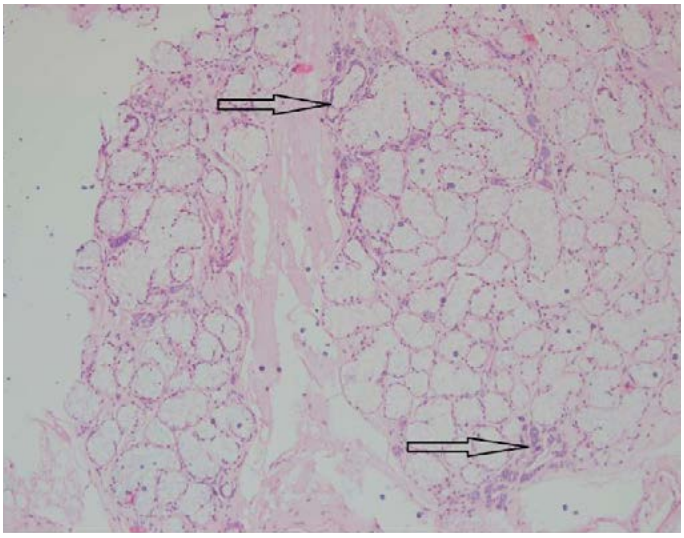
Immunohistochemical assessment methods

To assess the presence of inflammatory infiltrate, we used an immunohistochemical method with antibodies against T and B lymphocyte receptors and macrophages: anti-CD3, anti-CD20, anti-CD68. The expression of the CD34 antigen served as a marker for assessing the presence of vascular endothelium in the examined tissue. In contrast, expression of the S-100 protein indicated the presence of nerve fiber endings. Immunohistochemical tests were performed using Agilent (DAKO) antibodies:

1. mouse monoclonal antibody to CD3 protein at a dilution of 1:50 (Monoclonal Mouse Anti-Human CD3, Clone F7.2.38);

2. monoclonal mouse antibody to CD20 protein at a dilution of 1:50 (Monoclonal Mouse Anti-Human CD20cy, Clone L26);

3. mouse monoclonal antibody to CD34 protein at a dilution of 1:50 (Monoclonal Mouse Anti-Human, CD34 Class II, Clone QBEnd 10);



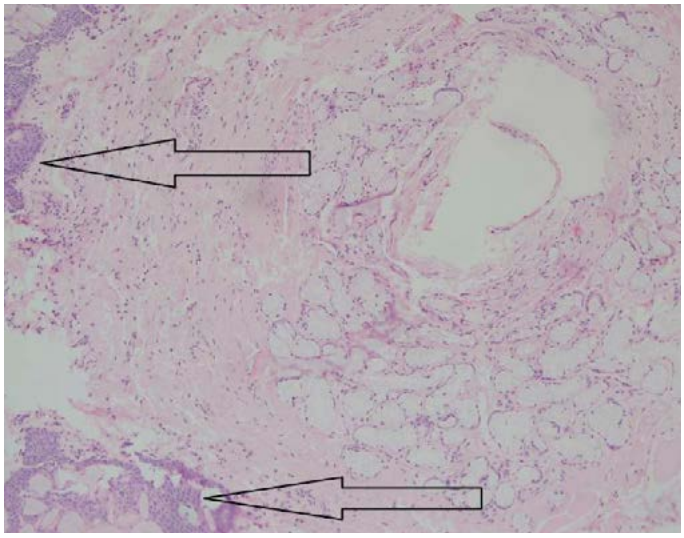


Fig. 2. and 3. A specimen of the mucosa obtained from a patient from the group G3. Mucosa with signs of intense infiltration of inflammatory cells in the examined tissue (arrows). H&E staining. Magnification x200.

4. polyclonal rabbit antibody to S-100 protein at a dilution of 1:50 (Code N1573, Ready-to-use N-Series Primary Antibody);

5. monoclonal mouse antibody to CD68 protein (Monoclonal Mouse Anti-Human CD68, Clone PG-M1) at a concentration of 1:100.

The obtained specimens were dewaxed for 2 hours in an incubator at 56 degrees C, followed by dewaxing in xylene and hydrated with a gradient of ethyl alcohol (99.9%, 96%, 70%). To expose the antigen to antibodies directed against the tested proteins, the tissues underwent an antigen retrieval technique in PT Link at high pH (pH 9.0). Next, the specimens were stained in the Autostainer Link system (DAKO). Visualization of antigen-antibody complexes was done using the visualization system and stained with DAB (diaminobenzidine).

The immunohistochemical reaction in the examined specimens was evaluated using a light microscope at 200x magnification.

The following assessment scale was used:

- (0) no immunohistochemical reaction – no expression or expression in less than 10% of cells,
- (1) moderate expression – expression visible in 10–30% of cells,
- (2) strong expression – expression in more than 30% of cells.

The evaluation of CD34 and S-100 protein expression consisted of finding the places most "rich" in blood vessels and nerve endings (hot spots) in a light microscope at 40 times magnification and then at x200 magnification. We assessed the percentage of the expression of vascular endothelial markers and expression of the S-100 protein as a marker of nerve endings in one field, the presence of expression even in the smallest blood vessels (microvessels) and nerve endings, followed by the expression on a 0–2 scale, according to the following criteria:

- (0) expression of CD34 and S-100 in 1–10% of cells in the examined field,
- (1) expression of CD34 and S-100 in 10–30% of cells in the examined field,
- (2) expression of CD34 and S-100 in more than 30% of cells in the examined field.

The obtained results were then averaged to 10 tested fields.

RESULTS

The results were developed using SPSS Version 24 software. Statistical analysis of the study was started by comparing the age distribution and the AHI index in the study groups. Frequency distributions and differentiation of the G1–G3 groups in terms of age and the AHI index can be considered suitable for further research (despite the small size of groups). No statistical differences were found between the G1, G2, G3 subgroups due to the age of the subjects (U-Mann-Whitney test G1 vs G2 – $p = 0.609$, G1 vs G3 – $p = 0.534$, G2 vs G3 – $p = 0.490$).

The obtained results were supplemented with a chi-square test of independence between the examined groups and factor evaluation. The contingency coefficient is presented in Tab. I.

The results of our research indicate significant statistical relationships between the study group and any factor ($p \leq 0.01$). Values of contingency coefficients based on the chi-square test, describing the strength of the relationship between the examined characteristics, range from 0.511 for factor CD20 to 0.753 for CD3. The strongest relationship occurs between the expression of the CD3 receptor and belonging to a given group (the greater the level of expression, the greater the severity of the disease). The least T-lymphocytes are found in the tissues of the comparative group and G1 group, the most in patients from the G3 group. The weakest relationship was observed between CD20 receptor expression and belonging to a given group. On the basis of the Mann-Whitney U test, we

Tab. I. Descriptive statistics of obtained test results.

FACTOR	EVALUATION	GROUP 0 (G0) N = 4	GROUP I (G1) N = 22	GROUP II (G2) N = 16	GROUP III (G3) N = 8	CHI-SQUARE TEST STATISTICS (SIGNIFICANCE LEVEL)	CONTINGENCY COEFFICIENT (SIGNIFICANCE LEVEL)
CD3	0	4	1	0	0	$x^2 = 65.411$ (p = 0.000)	0.753 (p = 0.000)
	1	0	21	5	2		
	2	0	0	11	6		
CD20	0	4	18	10	1	$x^2 = 17.650$ (p = 0.007)	0.511 (p = 0.007)
	1	0	4	6	6		
	2	0	0	0	1		
CD68	0	2	0	1	0	$x^2 = 21.288$ (p = 0.002)	0.546 (p = 0.002)
	1	2	14	9	3		
	2	0	0	6	5		
S-100	0	4	9	0	0	$x^2 = 28.909$ (p = 0.000)	0.605 (p = 0.000)
	1	0	13	13	3		
	2	0	0	3	5		
trichotomy	0	4	17	0	0	$x^2 = 36.790$ (p = 0.000)	0.651 (p = 0.000)
	1	0	5	8	4		
	2	0	0	8	4		
CD34	0	2	4	0	0	$x^2 = 27.154$ (p = 0.000)	0.593 (p = 0.000)
	1	2	18	6	4		
	2	0	0	10	4		

found significant differences ($p < 0.05$) between groups G1 and G3 due to the assessment of each of the examined factors; groups G1 and G2 due to the assessment of five factors (except for CD20, where p = 0.326); groups G2 and G3 only due to the assessment of CD20 (p = 0.034).

The weakest relationship was found between CD20 receptor expression and belonging to a given group.

In the examined soft palate tissues obtained from patients with SDB (G2 and G3) an inflammatory reaction was observed (Fig. 2. and 3.).

Correspondence analysis was used to graphically present the relationships between the studied groups and the assessment of the two selected CD3 receptors (the highest contingency value) and CD20 (the lowest contingency value) [7]. The correspondence analysis graph shows how group membership differentiates the scores for each of these factors. In the G0 group, to a greater extent than in the other groups, there are patients for whom CD3 receptor expression was assessed as 0, in G1 as 1, while in G2 and G3 as 2. In contrast, in the G1 group, to a greater extent than in the case of the other groups, there are patients for whom CD20 receptor expression was assessed at 0, while in the G1 and G2 group at 1 (Fig. 6. and 7.).

Based on the statistical analysis, the following results were obtained:

- the higher the CD3 receptor expression, the greater the severity of the disease;
- higher CD20 receptor expression in the G3 group (moderate and severe OSAS) compared to patients in the G0 group (comparative), G1 (patients with primary snoring), G2 (patients with mild OSAS). Expression of the CD20 receptor was least associated with the severity of the disease (snoring, daytime sleepiness);
- higher CD68 receptor expression in patients with OSAS (groups G2 and G3) compared to patients with primary snoring (group G1) (Fig. 4.);
- higher expression of S-100 protein in patients with moderate to severe OSAS (G3 group) compared to patients with mild OSAS (G2 group) and primary snoring (G1 group);
- a higher degree of fibrosis in the group of patients with OSAS (group G2 and G3) compared to patients from the comparative group and with primary snoring (groups G0 and G1) (Fig. 5.);
- higher expression of CD34 protein in patients with OSAS (groups G2 and G3) compared to patients with primary snoring (group G1).

DISCUSSION

The vibration of the soft palate tissues in people with sleep-disordered breathing causes the formation of an inflammatory infiltrates. The dominant inflammatory cells within the soft palate tissues are T lymphocytes and macrophages, and B lymphocytes are also present in patients with AHI index over 15. In the study of Sekosan [8], the leukocyte infiltration of OSAS patients was compared with the control group (fragments of the mucous membrane of the soft palate taken from persons without a history of sleep-related breathing disorders). The tissues of the study group were shown to have a greater number of inflammatory cells. Pulsen [9] performed an analysis of inflammatory infiltrate in the palate area and proved that the majority of inflammatory cells in the soft palate mucosa were CD3 lymphocytes, which is consistent with our study results. Most likely the presence of inflammatory cells is re-

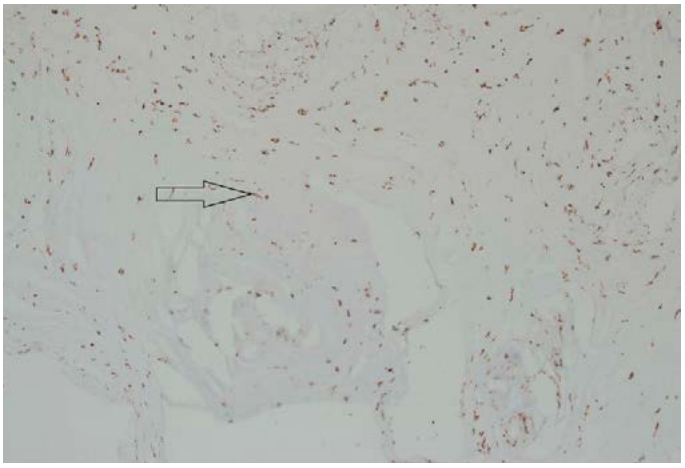


Fig. 4. A specimen of the mucosa obtained from a patient from the group G2. Mucosa with signs of infiltration of numerous macrophages (membrane expression of CD68 protein, arrows). Immunohistochemical method. Magnification x200.

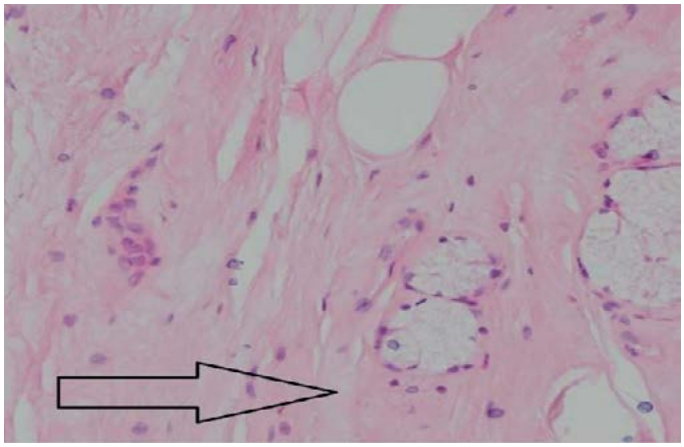
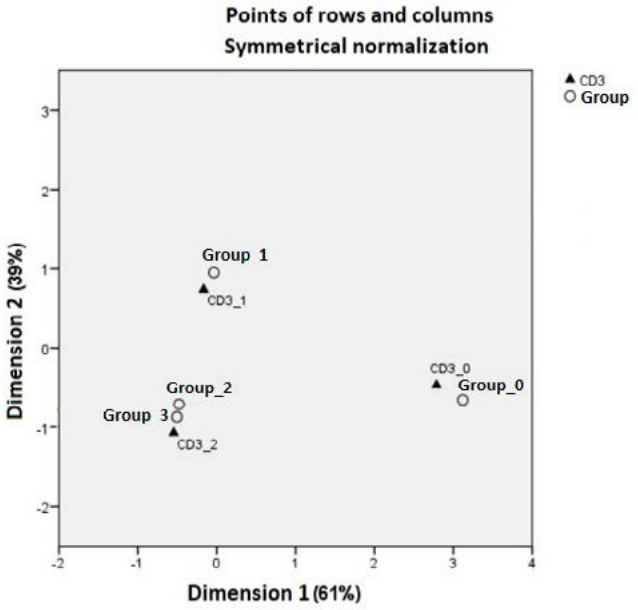


Fig. 5. Mucosa with signs of high degree of fibrosis, with the presence of numerous collagen fibers (arrow). A specimen of the mucosa obtained from a patient from the group G3. H&E staining. Magnification x200.



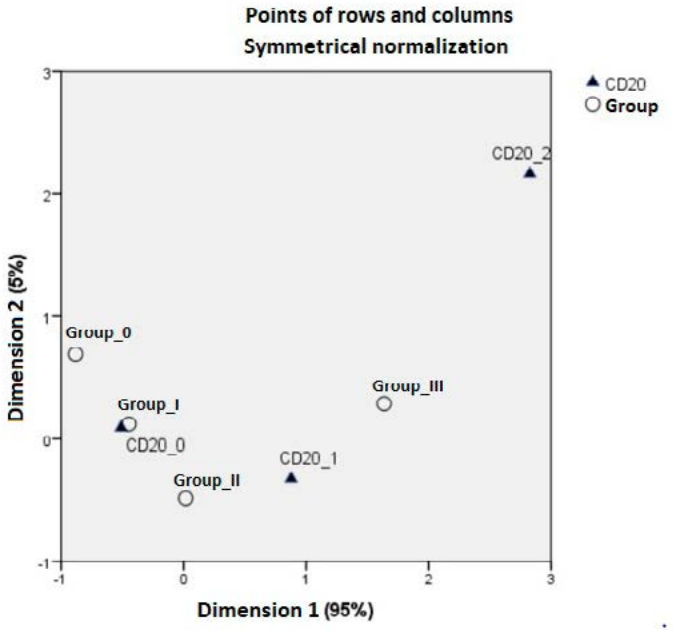


Fig. 6. and 7. The result of using correspondence analysis showing the relationship between the study group and the assessment of CD3 and CD20 (Group 0 defines G0, Group I – G1, Group II – G2 and Group III – G3).

lated to an increase in the level of pro-inflammatory factors released as a result of hypoxia, which was caused by sleep apnea [10]. The exacerbation of inflammation correlated with the phase of OSAS. Greater local inflammatory infiltration is observed in patients with severe and moderate obstructive sleep apnea syndrome than in patients with a mild form of this disease. A small inflammatory reaction occurs in snoring patients with AHI < 5.

Besides, systemic inflammation was also found in people with OSAS. Studies on pro-inflammatory cytokines IL-6 and TNF alpha have confirmed that OSAS affects the occurrence of systemic inflammation in both adults and children [11].

Significant differences were also shown in the area of fibrosis in soft palate tissues. Increased fibrosis in the palate tissues has been observed in patients with OSAS compared to those without the condition. Other results are presented by Berger et al. [12] who, when examining the soft palate tissues of patients with OSAS and taken from cadavers (no history of SDB during lifetime) did not show differences in fibrosis, inflammation, edema, and vasodilation within the examined tissues.

Using an electron microscope, Woodson [13] demonstrated the demyelination of nerve fibers and axons of nerve cells in the soft palate area. Our research has demonstrated a positive correlation between the density of nerve endings in 10 fields of view, expressed by the expression of the S-100 protein, and the severity of the disease. Interesting is also the research of Shah et al. [14] who found damage to nerve fibers in patients with obstructive sleep apnea based on the increased concentration of BDNF (a neurotrophic

protein belonging to the family of nerve growth factors). Uvula tissue was collected for evaluation using a biopsy.

Snoring and OSAS are affected by the length of the soft palate and the size of the uvula. Based on an analysis of many works, Chang et al. [15] established the parameters of an enlarged uvula – length above 15 mm and width above 10 mm. The size of the uvula may be associated with tissue swelling resulting from chronic vibration exposure during snoring [16]. In the examined tissues, we observed edema and the widening of blood vessels in patients with sleep-disordered breathing.

Damage to the nerve endings results in impairing the upper respiratory tract's ability to respond to inspiratory hypotension, and the risk of obstructive apnea increases. Damage to the motor nerves in the soft palate weakens the response and tension of muscle tissue. Damage to muscle fibers can also contribute to an increased risk of airway collapse during sleep. Based on immunohistochemical studies, Shah et al. [17] showed disorders in the structure of the cytoskeleton of muscle cells in the area of the soft palate.

In the systematic review, which analyzed about 900 publications, Patel et al. [18] emphasize the role of neuropathy causing a loss in the tone of the palate and soft palate in OSAS, which contributes to the progression of the disease.

More and more research is being conducted to analyze the relationship between inflammation, hypoxia and sleep-related breathing disorders. Pena-Ortega [19] emphasized the need to clarify cellular mechanisms between these processes. Further studies can contribute to a better understanding of the molecular processes involved in the development of OSAS, and in the future lead to the detection of OSAS therapeutic points.

CONCLUSIONS

Patients with sleep-disordered breathing (SDB) develop local inflammation in tissues of the soft palate. Of the factors studied, the inflammatory infiltrate, mainly composed of T lymphocytes, is the most closely related to the severity of OSAS. This means that the more episodes of hypoxia occur, the more T lymphocytes accumulate in the soft palate tissues. Local inflammation in patients with OSAS may lead to increased blood vessel permeability and tissue edema in this area. The volume of the mucosa increases, which enhances the obstructive mechanism of SDB. A higher expression of the S-100 protein in patients with moderate to severe OSAS compared to patients with mild disease and primary snoring may indicate damage to nerve fibers, which probably contributes to the impaired upper respiratory response to "negative" inspiratory pressure.

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