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Salivary Levels of Human Neutrophil Peptide (HNP) 1-3 in Patients with Recurrent Aphthous Stomatitis and Behçet's Disease: A Cross-sectional Study in Iran

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Abstract

Background: Recurrent oral ulcers are the most common complaint of patients with Behçet's disease (BD) and recurrent aphthous stomatitis (RAS). Enhanced innate immune response and neutrophilic activity might be a possible etiopathogenesis of BD. This study aimed to determine the significance of salivary human neutrophil peptide (HNP 1-3) in BD and RAS patients and detect their correlation with different clinical presentations, disease activity, and characteristics of oral ulcers.

Methods: This cross-sectional study included 25 BD patients and 25 RAS patients as well as 25 healthy participants. Five cubic centimeters of unstimulated saliva was collected and levels of HNP 1-3 were measured by ELISA. Other data were obtained through interviews, examinations, and reviews of medical records. Finally, data analysis was performed using SPSS 25.0 software.

Results: Salivary HNP 1-3 levels were not significantly different between the study groups ($P=0.282$). Duration of oral ulcers did not correlate with HNP 1-3 levels in RAS and BD patients ($P>0.05$). Also, BD patients with involvements other than oral ulcers were not found to have different levels of HNP 1-3 compared to those who did not manifest these conditions.

Conclusion: The validity of HNP 1-3 to be used as a probable biological marker for evaluation, diagnosis, and estimation of disease activity in patients with BD and RAS is still questionable according to our results.

Keywords: Saliva, Human neutrophil peptide, HNP 1-3, Behçet's disease, Recurrent aphthous stomatitis

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Introduction

Behçet's disease (BD) is a chronic multisystemic vasculitis of unknown origin with unpredictable exacerbations and remissions, which is more prevalent in Turkey, Iran, Japan, and Korea (1). It is accompanied by recurrent mucocutaneous lesions, uveitis, arthritis, and presentations involving the skin, central nervous system, and gastrointestinal system. Oral ulceration is the most common and initial symptom of BD, which is clinically quite similar to recurrent aphthous stomatitis (RAS) (2,3). Although the precise mechanism explaining the etiopathogenesis of BD is still unclear, autoimmune reactions triggered by infectious and environmental exposure in a genetically predisposed host have been proposed (4). Colonization of microorganisms such as streptococci in the oral environment can stimulate immune responses and consequently ulcer formation in patients with BD and RAS (5,6). Changes in oral flora and microbial plaque accumulation due to poor oral health are implicated in the severity and pathogenesis of BD (7,8).

Salivary antimicrobial peptides (AMPs), as gene-encoded natural antibiotics, play a central role in regulating immune functions, preventing biofilm formation, and protecting the oral cavity from microbial invasions. Among AMPs, α-defensin human neutrophil peptide (HNP) 1-3 take part in non-oxidative killing mechanisms. HNP 1-3 is secreted by submandibular duct cells and neutrophils migrating through the gingival sulcus into the oral environment (9-11). Increased neutrophil functions, as well as tissue accumulation of neutrophils, have been reported to cause tissue damage in BD (12-14).

Several lines of evidence measured and compared the

salivary levels of HNP 1-3 in BD, oral mucosal diseases, and oral infections. It has been suggested that an increase in HNP 1-3 might result in enhanced innate immune responses in BD both locally and systemically (15). Also, BD patients with decreased HNP 1-3 levels were found to be more predisposed to oral ulcers (16).

Although immune dysregulation and neutrophil hyperactivity have been reported as probable etiopathogenesis of oral ulceration, only a limited number of studies evaluated salivary HNP 1-3 in BD and RAS and reported controversial results. Therefore, this study, for the first time in Iran, aimed to investigate the salivary levels of HNP 1-3 in BD and RAS patients and compare them with healthy individuals. This study also provides HNP 1-3 levels in BD based on different oral and extra-oral presentations and disease activity, which were not reported in previous studies. We hope our results will allow a better insight into HNP 1-3 as a possible diagnostic and prognostic biomarker for BD and RAS.

Materials and Methods

Subjects

This analytic cross-sectional study included 25 BD patients and 25 RAS patients who were referred to Imam Reza and Ghaem hospitals' rheumatology clinics and researchers' offices as well as 25 healthy controls (HC) during 2018-2020. The BD patients were diagnosed according to the International Criteria for Behçet's Disease (ICBD) (17). RAS was defined as oral ulcers that occur at least three times a year. Patients with connective tissue diseases and oral diseases other than RAS were excluded. All participants were asked to complete the questionnaire and sign the informed consent. The primary data were collected through interviews, examinations, and reviews of medical records. Behcet's disease activity was measured based on Behçet's Disease Current Activity Form (BDCAF). The number and duration of oral ulcers during the last month were also recorded.

Collection of saliva samples

Five cc of unstimulated saliva was collected from each participant within 15 minutes between 9 AM to 12 AM, due to the circadian rhythm of saliva secretion. Saliva was frozen at -70 °C. Drinking, eating, brushing teeth, and chewing gum was avoided 60 minutes before sampling.

Determination of HNP 1-3 concentration in saliva samples and assay of HNP 1-3

Saliva samples were centrifuged for 10 minutes and supernatants were collected. HNP 1-3 levels were measured using sandwich enzyme-linked immuno-sorbent assay (ELISA) technology. According to the manufacturer's instructions, test samples, standards, and biotin-conjugated reagents were added to the wells and incubated. The HRP-conjugated reagent was then added, and the whole plate was incubated. Unbound conjugates were removed using a wash buffer at each stage. Tetramethylbenzidine (TMB) substrate was used to quantify the HRP enzymatic reaction. After the TMB substrate was added, only wells containing sufficient HNP 1-3 produced a blue-colored product, which then changed to yellow after adding the acidic stop solution. The optical density was measured spectrophotometrically with a microplate reader at 450 nm, by which the concentration of HNP 1-3 (μg/dL) was calculated.

Statistical analysis

The obtained data were analyzed using SPSS 25.0 software. Normal distribution was measured using the Kolmogorov-Smirnov test. The differences among groups were evaluated for statistical significance using one-way analysis of variance (ANOVA) (normal distribution data) or Mann-Whitney and Kruskal-Wallis (non-normal distribution data). Fisher's exact test or chi-square test was used to study qualitative data in groups. Spearman's correlation coefficient test was used to examine the correlation between quantitative non-normal variables. P value < 0.05 was considered statistically significant.

Results

Seventy-five participants including 25 BD patients, 25 RAS patients, and 25 healthy individuals were recruited in the present research. The male-female ratio was 10/15 in BD and RAS groups, and 8/17 in the HC group, which did not indicate a significant difference ($P = 0.796$). The mean age was 35.5 ± 8.3, 34.7 ± 11.7, and 45.8 ± 17.1 years in the BD, RAS, and HC groups, respectively. The Bonferroni correction was performed to compare the groups two by two and suggested that healthy individuals were significantly older than the two other groups ($P < 0.05$). Also, the study groups were not matched for occupation ($P = 0.024$). As shown in Table 1, our study groups were not statistically different in terms of their laboratory values ($P > 0.05$). Salivary HNP 1-3 levels were 4.8 ± 2.4 μg/dL in the BD group, 4.9 ± 3.4 μg/dL in the RAS group, and 9.1 ± 9.2 μg/dL in the HC group. Therefore, HNP 1-3 was revealed to be unaffected by the presence of aphthae ($P = 0.282$). Table 2 represents the association between oral manifestations of BD and salivary HNP 1-3. The levels of HNP 1-3 were not correlated with the duration of oral ulcers neither in BD patients nor in the RAS group ($P = 0.692$ and 0.120, $r = 0.080$ and 0.319, respectively). Additionally, RAS patients with different numbers of oral ulcers did not show significantly different HNP 1-3 levels ($P = 0.684$, $r = 0.086$). BD activity and salivary levels of HNP 1-3 were not found to be correlated ($P = 0.365$, $r = 0.189$).

Extra-oral presentations of BD patients were as follows: skin disease, genital ulcers, and articular, ocular, neurologic, vascular, and gastrointestinal involvements.

Table 1. Comparison of demographic and laboratory parameters of patients with Behçet's disease, recurrent aphthous stomatitis, and healthy controls

Variable		BD (n=25)	RAS (n=25)	HC (n=25)	*P* value
Gender (Female/Male)		15/10	15/10	17/8	0.796 [a]
Age, Mean±SD		35.5±8.3	34.7±11.7	45.8±17.1	0.005 [b]
Occupation, No. (%)	Housekeeper	14 (56)	11 (44)	8 (32)	0.024 [c]
	Employee	1 (4)	5 (20)	4 (16)	
	Self-employed	8 (32)	8 (32)	4 (16)	
	Unemployed	2 (8)	1 (4)	9 (36)	
WBC, Mean±SD (x1000/mm^3)		8.7±3.1	7.0±2.3	6.9±2.8	0.072 [d]
ESR, Mean±SD (mm/h)		18.5±17.6	9.8±8.4	13.8±9.6	0.103 [d]
CRP, No. (%)	Positive	9 (36)	7 (28)	5 (20)	0.452 [a]
	Negative	16 (64)	18 (72)	20 (80)	
HNP 1-3, Mean±SD (mg/dL)		4.8±2.4	4.9±3.4	9.1±9.2	0.282 [d]

Abbreviations: WBC, white blood cells; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; HNP, human neutrophil peptide; BD, Behçet's disease; RAS, recurrent aphthous stomatitis, HC, healthy control; N, number.
[a] Chi-square test; [b] One-way ANOVA; [c] Fisher's exact test; [d] Kruskal-Wallis test.

Table 2. Correlation analysis of HNP 1-3 levels and number and duration of oral ulcers and disease activity in patients with Behçet's disease and recurrent aphthous stomatitis

		BD		RAS	
		r	*P* value [a]	*r*	*P* value [a]
Oral ulcers	Duration	0.080	0.692	0.319	0.120
	Number	NA	NA	0.086	0.684
Disease activity [b]		0.189	0.365	NA	NA

Abbreviations: NA, not applicable; BD, Behçet's disease; RAS, recurrent aphthous stomatitis.
[a] Spearman correlation coefficient; [b] measured according to Behçet's Disease Current Activity Form.

The number and percent of BD patients who had these involvements are shown in Figure 1. Also, 17 out of 25 BD patients had positive pathergy test results. The data in Figure 1 presents no difference in HNP 1-3 levels between BD patients with and without the aforementioned presentations ($P>0.05$). Also, the levels of HNP 1-3 between RAS patients with and without articular involvement were compared and no significant association was found (4.2±2.0 vs 5.5±4.3, respectively, $P=0.397$).

Discussion

Immunologic abnormalities are recognized as a crucial player in the pathogenesis of BD and RAS (4,18). The presence of circulatory immune complexes, complement activation, and endothelial dysfunction have been found in BD patients (19,20). Prior researchers have noted the importance of altered neutrophil functions such as enhanced chemotaxis and phagocytosis, excessive production of oxygen radicals, and overexpression of adhesion molecules in tissue injury observed in BD (12,13). Additionally, a higher neutrophil-to-lymphocyte ratio (NLR) in the sera of active BD patients compared to those in the remission phase and controls supports the hypothesis that neutrophils may be involved in the pathophysiology and inflammatory cascade of BD (14,21). Azurophilic granules of neutrophils migrating into the oral environment contain several types of AMPs delivered to saliva and might be influenced by various oral mucosal disorders (22). Studies by Kucukkolbasi et al have reported significantly higher concentrations of human β-defensins (hβD-1 and hβD-2) and HNP-1 in the saliva of patients with BD, RAS, and oral lichen planus (OLP) than in healthy volunteers (23,24). Similarly, hβD-1 production was considerably higher in OLP patients compared to non-age-matched healthy subjects (25). According to a study by Mizukawa et al (26), the saliva of patients with OLP, oral leukoplakia, and glossitis associated with iron deficiency contain more HNP-1 than controls while patients with glossodynia, oral discomfort, and healthy controls have similar HNP-1 salivary levels.

However, in contrast to the above-mentioned investigations, we found no significantly different HNP 1-3 between our study groups. This result also differs from the previous studies conducted in Turkey and Egypt which revealed higher salivary HNP 1-3 in BD patients compared to controls (15,16,27). It had been proposed that over-reactive neutrophils migrating through the junctional epithelium into the oral cavity might be responsible for higher levels of AMPs such as HNP 1-3 in the saliva (15,16). However, these increased levels are not necessarily related to BD pathogenesis and might be due to the natural defensive response of neutrophils to a possible higher microbial colonization in patients with oral mucosal diseases. The discrepancy found may be also attributable to the differences in racial characteristics, oral hygiene habits, socioeconomic status, and dietary and smoking habits of the target populations. The accuracy

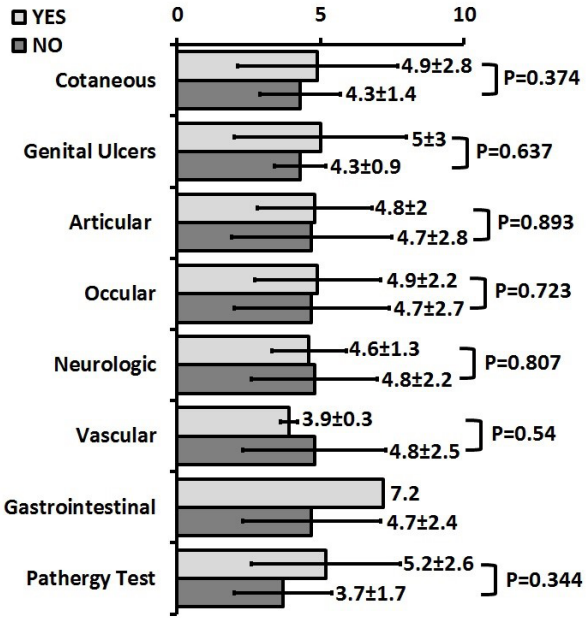

Figure 1. Salivary levels of HNP 1-3 in patients with Behçet's syndrome based on extra-oral manifestations and pathergy test results

Table 3. Salivary levels of HNP 1-3 in patients with Behçet's syndrome based on extra-oral manifestations and pathergy test results

Clinical feature	No. (%)	HNP 1-3 if the feature (or test) was:		*P* value [a]
		Present (or positive)	Absent (or negative)	
Cutaneous	17 (68)	4.9±2.8	4.3±1.4	0.374
Genital ulcers	16 (64)	5.0±3.0	4.3±0.9	0.637
Articular	11 (44)	4.8±2.0	4.7±2.8	0.893
Ocular	10 (40)	4.9±2.2	4.7±2.7	0.723
Neurologic	2 (8)	4.6±1.3	4.8±2.5	0.807
Vascular	2 (8)	3.9±0.3	4.8±2.5	0.540
Gastrointestinal	1 (4)	7.2	4.7±2.4	NA

Abbreviation: NA, not applicable; HNP, human neutrophil peptide
[a] Mann-Whitney test.

of this hypothesis should be evaluated by subsequent research.

Another notable finding of our study was that BD and RAS patients with different durations of oral ulcers and disease activity had statistically similar HNP 1-3 salivary levels. In accordance with the present result, Mumcu et al (16) found no significant difference in HNP 1-3 levels between BD patients with active and inactive oral ulcers. On the contrary, when patients with HNP-1 levels lower than 1000 µg/mL and 1500 µg/mL were studied, patients with active oral ulcers had higher levels of HNP-1 than patients with inactive oral ulcers. Therefore, it has been concluded that a decrease in HNP 1-3 might be a possible predisposition factor for oral ulcers in BD patients (16).

Salivary HNP 1-3 levels had not been observed to contribute to the presence of different extra-oral manifestations as described in Table 3. This outcome is consistent with the study by Ahn et al which indicated no significant association between the clinical presentations of BD and α-defensin-1 protein level. However, BD patients with musculoskeletal symptoms were found to express significantly higher α-defensin-1mRNA than those without these symptoms (28). The association between disease severity due to extra-oral presentations and HNP 1-3 levels was also previously reported (15).

We also observed statistically similar white blood cells (WBC), erythrocyte sedimentation (ESR), and C-reactive protein (CRP) levels in patients with BD, RAS, and healthy subjects. This differs from the recent investigation by Zhang et al which demonstrated significantly higher levels of these laboratory parameters in BD patients (21). Serum levels of CRP, high-sensitivity CRP (hsCRP), ESR, and homocysteine were found to be increased in BD patients compared to controls and also in active BD patients compared to those in remission (28-30).

Limitations

The current study has several limitations. Due to the cross-sectional nature of our study, we could not conduct multiple measurements on a single participant in different disease activity states. Besides, there is a potential for bias from the relatively small sample size, non-age- and occupation-matched groups, and possible differences between individuals in terms of other confounding variables (e.g. oral and dental health status, nutrition, smoking habits, and unrecognized diseases). Although most of the BD patients were newly diagnosed, some of them were treated with low-dose anti-inflammatory or immunosuppressive medications that seem unlikely to affect the results.

Conclusion

In this comparative study, the initial objective was to explore the potential of salivary α-defensin HNP 1-3 to be used as a future biomarker for BD and RAS screening, detection, and estimation of disease progression. The study results lead us to question the validity of this hypothesis as we found no significant difference in salivary HNP 1-3 between the study groups. Furthermore, the duration of oral ulcers, disease activity, and various clinical presentations of BD do not affect the HNP 1-3 levels in the saliva. Further research should be carried out in Iran and other BD-endemic areas with extended sample size and frequency of measurements. Patients can be monitored and compared before and after receiving treatment. To provide a better view of the relationship

between HNP 1-3 and its main secretion source which is neutrophils, the determination of neutrophil count in the saliva or NLR is recommended.

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Visualization: Simin Saffari, Hanieh Gholamalizadeh, Hasan Mehrad-Majd, Mahmoud Mahmoudi.
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Competing Interests
The authors declare that there are no conflicts of interest.

Ethical Approval
The present research was conducted according to the principles of the Declaration of Helsinki and was approved by the Medical Ethics Committee of Mashhad University of Medical Sciences (99/561591). Accordingly, the confidentiality of information was maintained.

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