Evaluation of hβD-1 and hβD-2 Levels in Saliva of Patients with Oral Mucosal Diseases

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ABSTRACT

Objective: This study aimed to determine a possible correlation between oral mucosal disease and salivary concentrations of the antimicrobial peptides human beta-defensin-1 (hβD-1) and human beta-defensin-2 (hβD-2).

Method: The present work focussed on the establishment of a reversed phase-high performance liquid chromatography (RP-HPLC) procedure to quantify human beta-defensins (hβD-1 and hβD-2) in saliva samples of patients with oral diseases such as lichen planus (n = 10), Behçet (n = 10) and recurrent apthous stomatitis (n = 10).

Results: Linear calibration range for hβD-1 and hβD-2 defensins was $1.67-200$ μg mL^{-1} and $3.13-100$ μg mL^{-1} with R^2 values of 0.9998 and 0.996, correspondingly. The concentration of beta-defensins in saliva was determined by comparing the peak areas of eluted hβD-1 and hβD-2 with that of their standards. The variation of the amount of beta-defensins was evaluated by comparisons of the results obtained from the patients with oral mucosal diseases before and after treatments and the control subjects. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.62 μg mL^{-1} and 5.39 μg mL^{-1} for hβD-1 and 0.94 μg mL^{-1} and 3.13 μg mL^{-1} for hβD-2, respectively.

Conclusion: The salivary beta-defensin concentration was significantly higher in patients with oral mucosal diseases than in healthy volunteers; furthermore, in patients with oral mucosal diseases, the concentration was significantly higher before treatment than after treatment.

Keywords: Defensin, hβD-1, hβD-2, high-performance liquid chromatography (HPLC), oral disease, saliva

Evaluación de los Niveles de hβD-1 y hβD-2 en la Saliva de Pacientes con Enfermedades de la Mucosa Oral

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RESUMEN

Objetivo: Este estudio tuvo por objeto determinar una posible correlación entre la enfermedad de la mucosa oral y las concentraciones salivales de la beta-defensina humana 1 (hβD-1) y la beta-defensina humana 2 (hβD-2) de los péptidos antimicrobianos.

Método: El presente trabajo estuvo encaminado al establecimiento de un procedimiento de cromatografía líquida de alta eficacia de fase reversa (RP-HPLC) para cuantificar las beta-defensinas humanas (hβD-1 y hβD-2) en muestras de saliva de pacientes con enfermedades orales como el liquen plano (n = 10), Behçet (n = 10), y la estomatitis aftosa recurrente (n = 10).

Resultados: El rango de calibración lineal de las defensinas hβD-1 y hβD-2 fue $1.67-200$ μg mL^{-1} y $3.13-100$ μg mL^{-1} con valores R^2 de 0.9998 y 996, respectivamente. La concentración de beta-

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defensinas en la saliva se determinó utilizando el área de sus estándares. La variación de la cantidad de beta defensinas fue evaluada por comparaciones de los resultados obtenidos de los pacientes con enfermedades de la mucosa oral, antes y después de los tratamientos y los sujetos de control. Se halló que el límite de detección (LDD) y el límite de cuantificación (LDC) fueron 1.62 μg mL^{-1} y 5.39 μg mL^{-1} para hβD-1 y 0.94 μg mL^{-1} y 3.13 μg mL^{-1} hβD-2, respectivamente.

Conclusión: *La concentración de beta-defensina salival fue significativamente mayor en los pacientes con enfermedades de la mucosa oral que en los voluntarios sanos. Además, en pacientes con enfermedades de la mucosa oral, la concentración fue significativamente mayor antes del tratamiento que después del tratamiento.*

Palabras claves: Saliva, HPLC, defensina, hβD-1, hβD-2, enfermedad oral

INTRODUCTION

Common superficial oral lesions include candidiasis, recurrent herpes labialis, erythema migrans, hairy tongue, recurrent aphthous stomatitis and lichen planus. Behçet`s disease is a multisystem inflammatory disorder dominated clinically by recurrent oral and genital ulceration, uveitis and erythema nodosum. They are all chronic autoimmune in-flammatory conditions affecting the lining of the oral cavity. Although the aetiology of all of them is not clearly known and there is no specific treatment, various studies have focussed on their aetiopathogenesis and treatment.

The oral cavity is an environment in which antimicrobial peptides are important contributors to maintaining the balance between health and disease. There is a lot of salivary antimicrobial peptides, part of the host innate immune response, the β-defensin expressed in the epithelium, the α-defensins expressed in neutrophils, and the cathelicidin, LL-37, expressed in both epithelium and neutrophils. Antimicrobial peptides in the oral cavity are expressed in Table 1 (1).

Defensins have been predicted to play a major role in innate antimicrobial host defence. They are present in oral tissues, salivary glands, salivary secretions, gingival cravicular fluid, and are ideally positioned to trigger adaptive immune responses in the oral nasal cavity. There are two main subgroups, the alpha- and beta- defensins, which differ in their cysteine motifs, but share a similar secondary structure and are both rich in cationic residues (2). These defensins generally occur in the areas with infection or inflammation. The beta-defensin family of antimicrobial peptides may contribute to oral defences but until recently has received little attention (3−5).

This study was carried out to investigate the presence of human beta-defensin-1 (hβD-1) and human beta-defensin-2 (hβD-2) in the saliva of patients with various types of oral inflammation pre-treatment and post-treatment and to determine whether the salivary hβD-1 and hβD-2 concentrations can be used as a marker of inflammation in patients with inflammatory diseases affecting the oral cavity. To our knowledge, no study has investigated patients with oral

Table 1:   Antimicrobial peptides expressed in the oral cavity [1]

Antimicrobial peptide	Site of expression	Role/comments
HNP 1−4 (α defensins)	Neutrophils (azurophilic granules) Gingival sulcus Sites of inflammation	Antibacterial, antifungal and antiviral. Functional levels in GCF Expression defective in Morbus Kostmann syndrome (congenital neutropenia associated with periodontal disease)
LL−37	Neutrophils Gingival sulcus Saliva	Primarily antibacterial Expression is defective in Morbus Kostmann syndrome
β-Defensins hβD−1 hβD−2 hβD−3	Suprabasal layers of stratified epithelium; hβD-3 mRNA is widely expressed but peptide localization is not known	Antibacterial, antifungal, and antiviral. Part of the protective barrier function of epithelium. Secreted, may be associated with cell or mucosal surface. Also found in salivary glands and saliva
Histatin	Saliva (parotid and submandibular)	Antifungal. Histidine-rich group of peptides. Histatin 5 (24 amino acids) is most active. Antifungal action requires metabolic activity
Adrenomedullin	Epithelium	Antibacterial, mitogenic, vasodilator, inducible peptide

lichen planus (OLP), Behçet's disease (BD) and recurrent apthous stomatitis (RAS) using saliva.

SUBJECTS AND METHODS
Patients and saliva sampling
The saliva samples were collected from a group of 30 patients (13 men, 17 women) with 10 cases of OLP (age range, 23−61 years), 10 cases of BD (age range, 19−55 years) and 10 cases of RAS (age range, 16−50 years). The control group consisted of 10 healthy controls (age range, 20−65 years). All patients presenting at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Selcuk University, were enrolled.

Whole saliva samples were taken from the patients before and after treatment. The post-treatment samples were obtained after resolution of all signs and symptoms. Sterilized medical sample containers (polystyrene, 25 mL) were used for the collection of the stimulated saliva samples. Saliva secretion was stimulated through the use of citric acid. The saliva sample was similarly obtained from each control subject; each saliva sample was immediately adjusted with citric acid to a pH of 3.0 and stored at -20 °C until assayed. The saliva samples at -20 °C were allowed to reach room temperature immediately before use and centrifuged at 15.000 rpm for five minutes.

Reagents
All chemicals and solvents used in the experiments were of either analytical or high-performance liquid chromatography (HPLC) grade, and the solutions and solvents were used after the filtration. Ultra high purity water was generated by using a Milli-Q Integral Water Purification System and solvents were filtered through 0.2 μm membrane filters for use in liquid chromatography. Defensin hβD-1 and hβD-2 standards were purchased from Sigma-Aldrich (Chemie GmbH, Germany) and kept at approximately -20 °C until they were analysed.

Chromatographic procedure for determination of hβD-1 and hβD-2 levels in saliva samples
Agilent 1100 series HPLC system was used for the isolation and determination of hβD-1 and hβD-2 defensins. The system consisted of a G1311A model quaternary pump, a G1314A model variable wavelength UV detector, and a 7725i model Rheodyne manual injector system with 20 μL loop. The solutions and solvents were degassed with a G1379A model degasser. The data were collected and recorded by using a Chemstation 2001 data processor. A 10 mL aliquot of the supernatant was assayed by C18-AM column (4.6 mm x 250 mm, nacalai tesque), and a UV detector set at 225 nm. High-performance liquid chromatography was performed under gradient conditions using solvent A (0.021% trifluoroacetic acid, 80% acetonitrile) and solvent B (0.025% trifluoroacetic acid) at a flow rate of 0.25

mL min^{-1}. The solvent gradient went from 100% A to 100% B over a time period of 60 minutes. Candidate peaks for hβD-1 and hβD-2 were selected by comparing the absorbance profiles of the β defensin standards and quantification of hβD-1 and hβD-2 defensins was performed using the peak areas. Each assay sample was analysed in triplicate.

Isolation and purification of hβD-1 and hβD-2
A C18-AM column (10 x 250 mm, nacalai tesque) was used to isolate hβD-1 and hβD-2 from saliva. High-performance liquid chromatography was performed for 20 minutes by the linear concentration gradient method (flow rate, 2.3 mL per minute) through use of the same solvents used for the analysis (A and B), and hβD-1 and hβD-2 were collected according to a previous study (6). This fraction underwent HPLC under the same conditions a second time to purify the hβD-1 and hβD-2.

Measurement of molecular weight
The chromatogram of standard hβD-1 and hβD-2 (Sigma-Aldrich Chemie, Germany) and extracts from the saliva of the patients were compared. The molecular weight of the hβD-1 and hβD-2 was measured by means of a triple-stage quadruple mass spectrometer with an electrospray interface.

Measurement of hβD-1 and hβD-2
Standard solutions of β defensins were prepared for use prior to an HPLC analysis. The concentrations of standard solutions used for plotting of calibration curves ranged from 1.67−200 μg mL^{-1} and 3.13−100 μg mL^{-1} for hβD-1 and hβD-2 defensins, respectively. The calibration curves are linear over a wide range ($R^2 = 0.9998$ for hβD-1, $R^2 = 0.996$ for hβD-2). For both hβD-1 and hβD-2, the calibration curves were linear up to the highest concentration tested.

All saliva samples were subjected to HPLC analysis and the peak area was used in order to quantify hβD-1 and hβD-2 defensins. Besides, recovery of these defensins was determined for the overall assay by adding known amounts of standards to whole saliva samples: 88−96% recovery was found with a mean value of 92% in six determinations.

Statistical analysis
A probability level of 0.05 was accepted as the maximum value for statistical significance. Data are presented as mean ± SD. The SPSS statistical package (version 15.0; SPSS) was used to analyse all data.

RESULTS
Identification of hβD-1 and hβD-2 in saliva of patients with oral mucosal diseases
Figures 1 and 2 show the HPLC chromatograms obtained from a patient with OLP before and after treatment and from a healthy control subject. The retention time of hβD-1 and
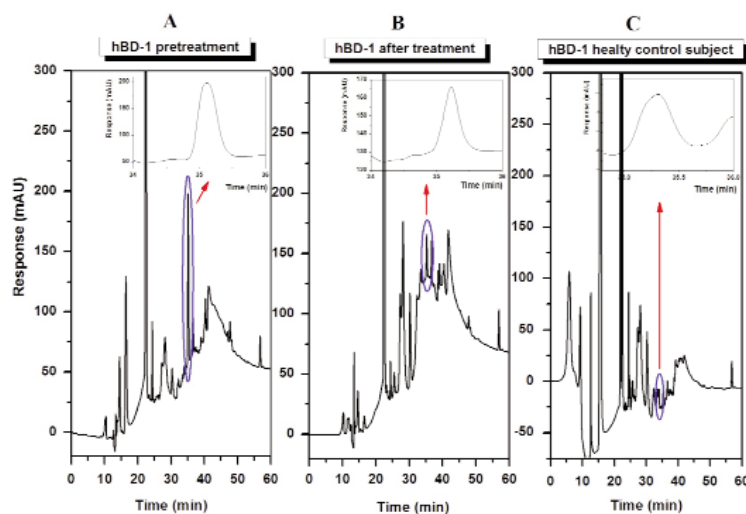
Fig. 1:   Reversed-phase high performance liquid chromatography (RP-HPLC) chromatograms for human beta-defensin-1 (hβD-1) defensin obtained from a patient with oral lichen planus (OLP) and from a healthy control subject. (A) patient with OLP before treatment, (B) patient with OLP after treatment, (C) healthy control subject.

Fig. 2:   Reversed-phase high performance liquid chromatography (RP-HPLC) chromatograms for human beta-defensin-2 (hβD-2) defensin obtained from a patient with oral lichen planus (OLP) and from a healthy control subject. (A) patient with OLP before treatment, (B) patient with OLP after treatment, (C) healthy control subject.
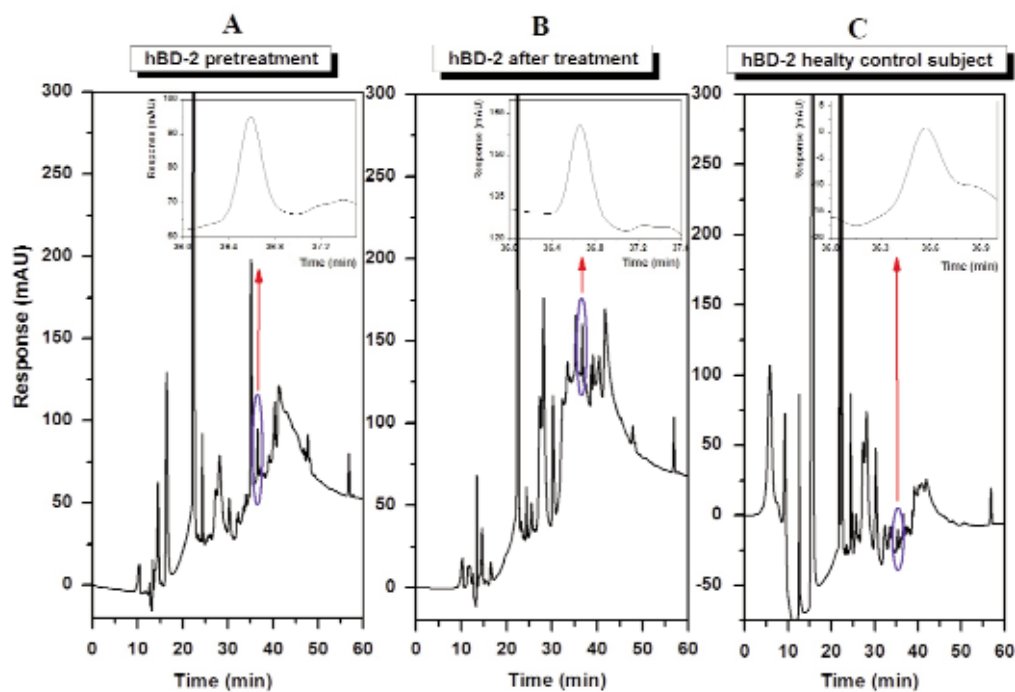
hβD-2 peaks was about 35.2 and 36.6 minutes, respectively. While the peak areas of hβD-1 and hβD-2 compounds for pre-treatment subjects were observed significantly as expected (Figs. 1a and 2a), the peak areas obtained after treatment and for the healthy control subjects were very small or unappreciable (Figs. 1b, 1c, 2b and 2c). The chromatograms for sample extracted from the saliva of the patients had a retention time of 35.3 and 36.6 minutes, identical to those of standard hβD-1 and hβD-2. Although equal amounts of both peptides were present in each case, lower signals were obtained for hβD-2 than for the same amount of hβD-1. The purity of the peptides was verified by mass spectroscopy and the molecular masses of hβD-1 and hβD-2 were found to be 3928.6 and 4328.06 Da, correspondingly (data not shown).

Measurement of hβD-1 and hβD-2 levels in saliva
The quantification of hβD-1 and hβD-2 defensins was performed for saliva samples taken from patients with OLP before and after treatment. The calibration curves plotted for hβD-1 and hβD-2 defensins (Fig. 3) showed high linearity with a wide concentration range of 1.67−200 µg mL^{-1} and 3.13−100 µg mL^{-1} for hβD-1 and hβD-2 defensins. Statistical data for the calibration curve are also given in Table 2. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.62 µg mL^{-1} and 5.39 µg mL^{-1} for hβD-1 and 0.94 µg mL^{-1} and 3.13 µg mL^{-1} for hβD-2. Beta-defensin concentrations in saliva of patients pre- and post-treatment were calculated using calibration curve. Table 3 indicates the gender and age of the subjects and also the calculated concentration levels of hβD-1 and hβD-2 for pre- and post-treatment. An important decrease in beta-defensin concentrations was observed for all patients after treatment (Figs. 4 and 5). The mean concentrations of hβD-1 and hβD-2 defensins for OLP were 94.81 µg mL^{-1} and 48.89 µg mL^{-1} for pre-treatment, 10.34 µg mL^{-1} and 4.34 µg mL^{-1} for post-treatment ($p < 0.05$, Table 4). These results verify that there is a meaningful relation between oral inflammation and beta-defensin levels in saliva samples.
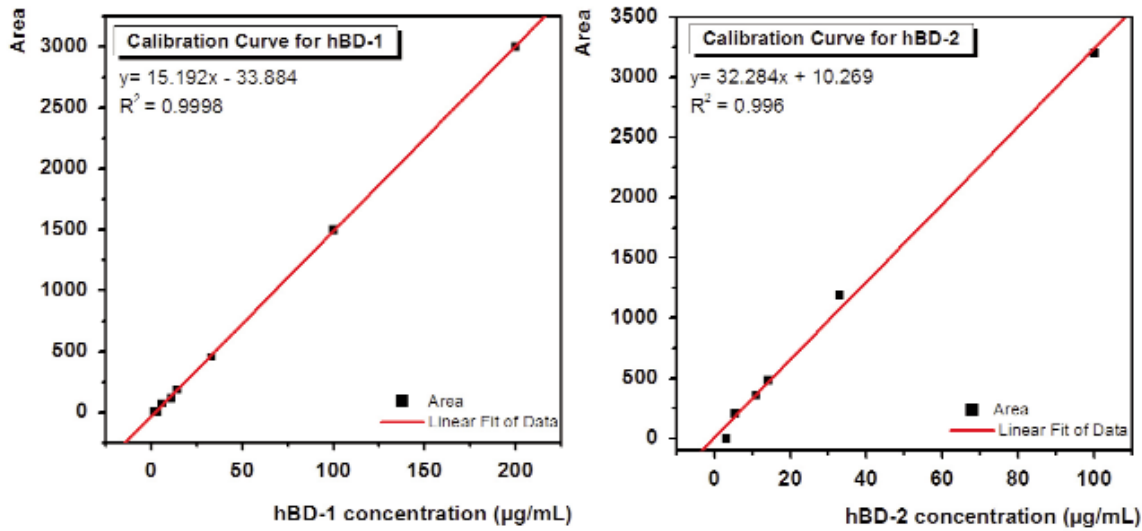

Fig. 3:   Calibration curve plotted for human beta-defensin-1 and -2 (hβD-1 and hβD-2) defensins.

Table 2:   Statistical data for calibration curves

		Intercept	Slope
hβD-1	**Value**	-33.88409	15.19227
	Error	6.34644	0.07913
hβD-2	**Value**	10.26867	32.28369
	Error	44.63865	1.02177

Table 3:   Human beta-defensin-1 and -2 (hβD-1 and hβD-2) defensin levels in saliva of patients with oral
            lichen planus, Behçet disease and recurrent apthous stomatitis

	Patient No	Age (year)	Sex	hβD-1 (µg mL^{-1})		hβD-2 (µg mL^{-1})	
				Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Oral lichen planus	1	24	F	56.86	7.76	38.42	3.06
	2	40	F	175.20	13.17	62.05	7.46
	3	23	F	69.44	8.48	49.75	4.23
	4	61	M	152.23	18.71	70.19	2.86
	5	32	F	73.89	5.53	54.57	2.89
	6	24	M	129.68	12.46	49.20	6.38
	7	59	M	38.21	4.95	16.07	1.90
	8	52	F	94.52	12.28	47.00	4.83
	9	32	M	60.76	9.72	33.12	5.02
	10	31	M	97.35	10.34	68.53	4.78
Behçet	1	55	M	136.12	12.09	80.02	4.31
	2	35	F	84.64	8.57	49.95	2.96
	3	42	M	59.91	6.34	33.91	1.98
	4	26	F	125.25	9.45	86.35	6.74
	5	19	M	161.53	12.27	91.23	7.23
	6	29	F	76.70	5.92	39.67	4.31
	7	36	F	52.73	12.63	26.45	1.74
	8	41	F	78.01	9.05	52.89	2.23
	9	29	M	61.62	4.46	41.07	5.68
	10	38	F	147.23	13.98	102.84	6.40
Recurrent apthous stomatitis	1	28	F	38.27	4.17	17.54	0.97
	2	33	F	61.59	8.20	45.86	2.57
	3	36	F	80.02	5.70	52.75	6.03
	4	50	M	112.36	9.06	79.22	7.32
	5	38	F	145.81	13.17	92.04	6.54
	6	16	M	98.12	7.11	68.63	5.90
	7	48	F	57.07	11.73	28.00	2.37
	8	18	M	45.47	6.03	25.19	1.86
	9	28	F	74.55	8.80	49.75	3.82
	10	36	F	95.55	9.12	52.08	5.09

Table 4:   The mean concentration of human beta-defensin-1 and -2 (hβD-1 and hβD-2) in the saliva of patients with oral
            inflammation

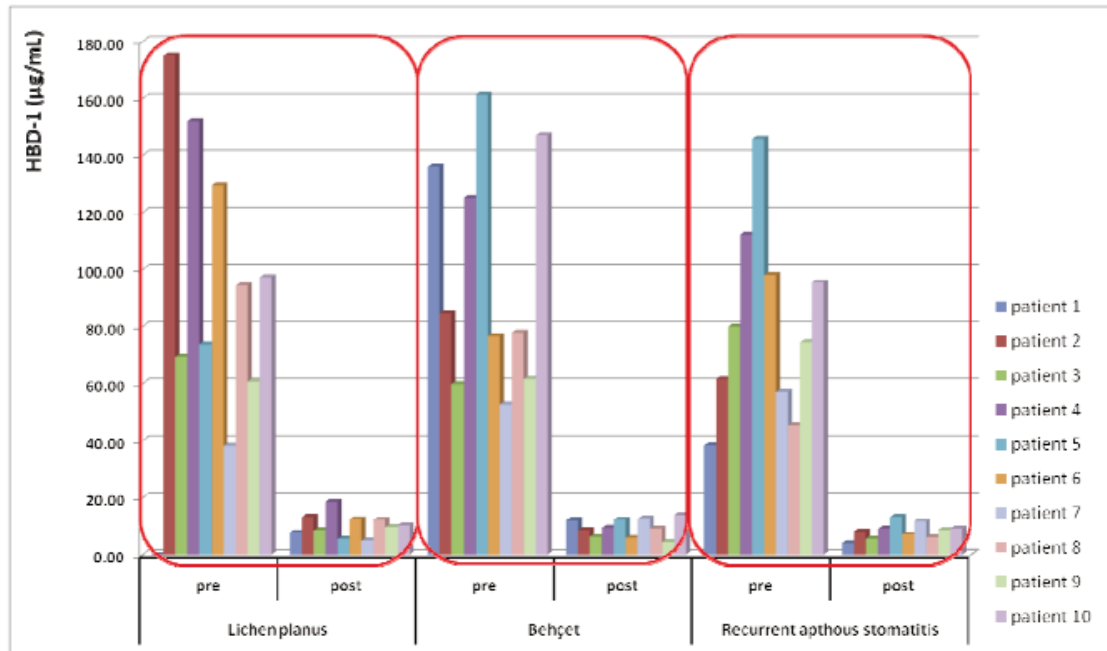| Type of disease	Number (n)	hβD-1, µg mL^{-1} (mean $\pm \frac{ts}{\sqrt{N}}$)*		hβD-2, µg mL^{-1} (mean $\pm \frac{ts}{\sqrt{N}}$)*	
		Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
Oral lichen planus	10	94.81 ± 44.55	10.34 ± 4.07	48.89 ± 16.62	4.34 ± 1.72
Behçet	10	98.37 ± 40.15	9.47 ± 3.22	60.43 ± 27.15	4.35 ± 2.08
Recurrent apthous stomatitis	10	80.88 ± 32.94	8.30 ± 2.74	51.11 ± 23.89	4.24 ± 2.21
Control	10	2.25 ± 0.94	1.97 ± 0.13	0.98 ± 0.10	0.25 ± 0.06

* 95% confidence interval

Fig. 4: Illustration of human beta-defensin-1 (hβD-1) defensin levels for samples with oral mucosal diseases.
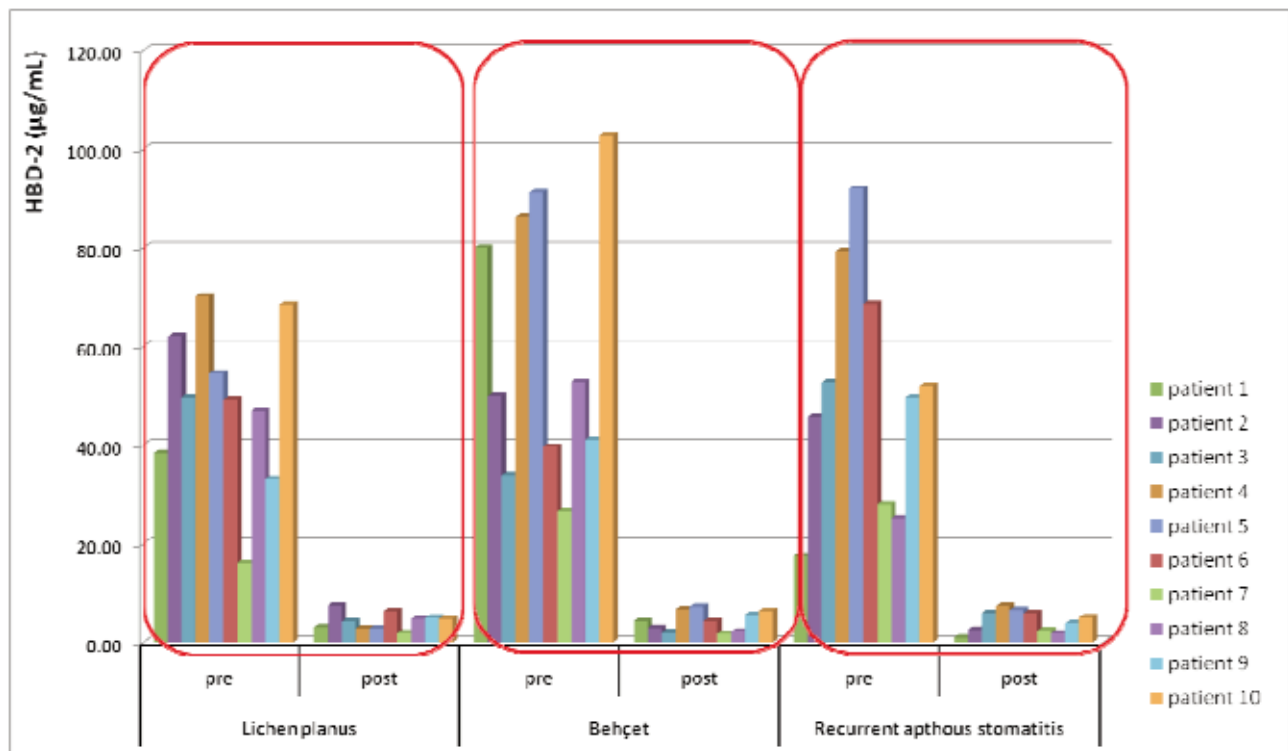

Fig. 5: Illustration of human beta-defensin-2 (hβD-2) defensin levels for samples with oral mucosal diseases.

DISCUSSION

Saliva, by rinsing and delivering antimicrobial peptides and proteins to the oral epithelium, contributes greatly to its protection. Saliva contains several types of antimicrobial peptides and proteins including peroxidases, lactoferrin, lysozyme, histatins, phospholipase and calprotectin that mediate the innate immune response (7). Another group of antimicrobial peptides called defensins has been detected in saliva and many of these peptides have been studied in extraoral sites (8, 9). Characterization of antimicrobial factors in the oral cavity may permit new insights into oral defence mechanisms and the pathogenesis of disease (10). For example, inactivation of these factors could lead to increased microbial colonization or invasion of the oral soft tissues, increasing the risk for candidiasis and periodontal disease. Additionally, they may play a role in preventing viral infections (11). The beta-defensin family of antimicrobial peptides may contribute to oral defensins but until recently has received little attention (3−5). Bensch *et al* (12) discovered hβD-1 in 1995 and hβD-2 was discovered by Harder *et al* in 1997 (13). In the oral cavity, Krisanaprakornkit *et al* described hβD-1 for the first time in 1998 (4). The widespread expression of beta-defensins in oral tissues suggests that they contribute to host defences in the oral cavity (14). Large-scale, population-based screening studies have identified the most common oral lesions as candidiasis, recurrent herpes labialis, recurrent aphthous stomatitis, mucocele, fibroma, mandibular and palatal tori, pyogenic granuloma, erythema migrans, hairy tongue, lichen planus, and leukoplakia (15, 16). Behçet`s disease is a multisystem inflammatory disorder dominated clinically by recurrent oral and genital ulceration, uveitis and erythema nodosum. It runs a chronic course, with unpredictable exacerbations and remissions whose frequency and severity may diminish with time. Behçet`s disease typically arises in young adults, although childhood-onset BD has also been reported. The disease can affect both genders and has a worldwide distribution, although it is more prevalent in countries of the ancient Silk Route. The cause of BD remains unknown, although an autoimmune reaction triggered by an infectious agent in a genetically predisposed individual has been suggested (17). It was observed that in the case of RAS patients, the lesions were only found in the oral region, whilst in patients with BD and lichen planus, this phenomenon was found on other sites of the body. Saliva is an ultra filtrate of blood and is normally viewed as a medium to moisten and soften food and to keep the mouth moist (18). The role of defensins in saliva is as yet unclear; an increase in the number of neutrophils in the blood could result in increased levels in the saliva. The presence of antimicrobial substances such as the defensins in saliva suggests some important roles that saliva may play in maintaining general oral health and as a first front against infection. Kucukkolbasi *et al* detected a significant positive correlation in the concentration of human neutrophil peptide-1 (HNP-1) in the saliva of patients with especially BD and

RAS as well as OLP (6). So, the salivary HNP-1 concentration can be used clinically as a marker of inflammation in these patients. These results open a new field of interest in the analysis of a biological fluid that is not commonly used in clinical chemistry. However, to our knowledge, no study has investigated hβD-1, hβD-2 in the saliva of patients with OLP, BD and RAS. Mathews *et al* found that both human β-defensin peptides were readily detected in saliva; a conservative estimate for the concentrations of hβD-1 and hβD-2 in saliva is 150 ng mL^{-1} (14). We detected both human β-defensin peptides in saliva and a conservative estimate for the concentrations of hβD-1 and hβD-2 in saliva was also around 150 ng mL^{-1}. Like Mathews *et al*, we speculate that these concentrations may be sufficient to be microbicidal for some organisms, especially considering that they may act synergistically with other microbicidal factors present in saliva (14). These results suggest that epithelial β-defensin may play an important role in the mucosal defensins of the mouth. The results shown in this paper indicate that the concentration level of hβD-1 and hβD-2 was significantly higher in the saliva of patients with BD, RAS and OLP before treatment than in healthy subjects (Figs. 4 and 5). However, six months after treatment, the concentrations in these patients decreased to the levels seen in the healthy subjects.

It is important to also understand the involvement of antimicrobial peptides in other mucosal disorders. In oral mucosal diseases, β-defensin expression may reveal clues on disease progression and prognosis. The development of new peptide antimicrobial agents for therapeutic use in the oral environment is an important area for future investigation and testing. Peptide antimicrobial agents may augment the innate defences of individuals at high risk for oral infection because of compromised immune status (1).

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