Original Article

Opiorphin Level in Unstimulated Whole Saliva of Burning Mouth Syndrome Patients

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Background: Burning mouth syndrome (BMS) is characterized by intense burning sensation of the tongue or other regions of the oral mucosa with no known medical or dental cause. BMS is poorly understood condition which requires multidisciplinary management. Opiorphin is a natural antinociceptor, previously identified from human saliva and demonstrated analgesic properties. It has been reported to involve in pain pathways and could potentially be a biomarker for different types of physiological disorders.

Objective: Because of its abundance in the oral cavity, we aimed to measure the level of opiorphin in unstimulated whole saliva (UWS) of patients with BMS.

Materials and Methods: Demographic data, history taking, oral examination and UWS samples were obtained from 20 BMS patients before starting the drug treatment. Opiorphin levels were measured with a quantitative assay using commercially immunoenzymatic competitive ELISA kit (cat No. E1779h, Wuhan, China) and compared to those of healthy control.

***Results*:** UWS of 20 BMS patients (3 males and 17 females, average age 60.2 years old) were collected. The average concentration of opiorphin in UWS of BMS patients was 1.25±0.79 ng/ml. The result demonstrated that opiorphin in BMS patients' UWS was significantly lower, compared to 4.16±0.82 ng/ml in the healthy control group (n = 3, 3 females, average age 50.6 years old).

***Conclusion*:** Our results indicate that the decreased levels of opiorphin in UWS of patients with BMS may be associated with the inhibition of analgesic property of encephalin, thereby causing chronic pain. To dates, knowledge on opiorphin's involvement in pain pathways has been controversial. Further study remains significant to understand the mechanistic role of opiorphin in chronic orofacial pain.

Keywords: Burning mouth syndrome, Salivary opiorphin, Natural nociceptor

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Burning mouth syndrome (BMS) is a chronic painful intraoral neuropathic pain characterized by discomfort or pain of the mouth, mostly affecting anterior part of tongue, hard palate, lips and oral mucosa bilaterally, with no known medical or dental cause. The pain is constant but can be varied in severity throughout the day, and often described as being hot, searing and burning pain. Pain is usually aggravated by the consumption of hot or spicy foods and some BMS sufferers may report taste disturbances, dry mouth and other similar problems. The diagnostic criteria of BMS are included in both the International Association for the Study of Pain (IASP) and the International Headache Society (IHS) classification systems. The International Association for the Study of Pain (IASP) defines BMS as "a chronic condition characterized by a burning sensation of the oral mucosa for which no cause can be found"[1]. The International headache society (IHS) defines BMS as "an intraoral burning sensation or dysesthesia, recurring daily for more than 2 hours per day over more than 3 months, without clinically evident causative lesions"[2]. Both diagnostic criteria state the need for constant burning pain, normal appearance of the oral mucosa and exclusion of any local/systemic diseases. BMS usually affects post-menopausal women aged between 40 and 60 years and the prevalence in the general population is 3.7% (1.6% men and 5.5% women)[3].

The etiology of BMS is not fully understood and the onset of pain is sometimes associated with systemic factors such as diabetes, nutritional deficiencies and psychological disorders, as well as local causes including: contact mucositis, oral candida infections, side effects of medication used, history of lingual nerve trauma, endocrine disorders, are considered possible mechanisms[4]. BMS has been classified into primary or idiopathic BMS, and secondary BMS with the primary BMS is of unknown etiology and is a diagnosis of exclusion. Secondary BMS is diagnosed in the presence of systemic factors as shown in hematological investigations[5]. As a result, they can include many causative

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factors in heterogeneous patients and because of the lack of accurate biomarkers and little knowledge of pathophysiology, diagnosis and managing this group of patients can be challenging. This constant burning pain severely affects the patients psychologically with high reported rates of anxiety, muscular tension and depression in the BMS patients and lower socialization scores, compared to controls[3,6]. Moreover, Cancerphobia, a type of anxiety disorder, was more frequently seen in patients with BMS than in those with other types of orofacial pain[7]. The management of BMS, like other chronic pain conditions, is difficult as several groups of medications are being used with minimal analgesic effect. Evidence based treatments include cognitive behavioral therapy (CBT) and current pharmacological treatments such as tricyclic antidepressants are the main stay[8]. But this group of drug is not always suitable for elderly patients with systemic diseases of whom this condition is mostly associated with.

Opiorphin is an endogenous pentapeptide isolated in 2006 from human saliva[9]. It was described as a mature product of the proline-rich, lacrimal 1 (PROL1) protein. It was further demonstrated to inhibit enkephalin-inactivating ectopeptidases, human neutral ecto-endopeptidase (hNEP), and human ectoaminopeptidase (hAP-N), resulting in prolonged activity of enkephalins and pain alleviation. Previous studies on rats showed that it suppresses pain sensation for both chemical-induced and acute mechanical pain as efficiently as morphine but without causing drug tolerance and with fewer side effects of morphine[9,10]. It was also previously observed that central administration of opiorphin induced an antidepressant-like effect by activation of μ and δ opioid receptors[11-13]. Apart from saliva, opiorphin has been detected in plasma, cerebrospinal fluid, urine, tears, semen, and breast milk[10]. Opiorphin may be a quantifiable biomarker for chronic pain. Because of its abundance in the oral cavity, it might play an important role in orofacial pain conditions, such as burning mouth syndrome (BMS).

Recent case-control study in BMS patients demonstrated higher quantities of salivary opiorphin in BMS patients using validated LC-MS/MS method[14]. Another study quantified opiorphin levels in fluids of BMS patients and showed the lack of significative difference in salivary opiorphin levels between idiopathic BMS and controls using competitive-ELISA immunoassay but found higher blood opiorphin levels which may reflect a systemic dysregulation in idiopathic BMS[15].

The aim of the study was to investigate levels of salivary opiorphin in patients with BMS in unstimulated whole saliva (UWS) and to compare with the healthy control group.

Materials and Methods

The present study was approved by the Ethics Committee in Human Research, Khon Kaen University with the reference number HE562173. The study followed the ethical principles of Helsinki Declaration and Good Clinical Practice (ICH GCP). All subjects provided written informed consent before inclusion and had their anonymity respected throughout the course of the study.

Diagnostic, inclusion and exclusion criteria

BMS patients age between 25 and 80 years old were diagnosed by criteria of the International Classification of Headache Disorders (ICHDII-2004)[16] and included in the study before starting any treatments. The authors included BMS patients with all complains of oral pain in tongue and/or oral mucosa associated or not with xerostomia or dysgeusia, pain present continuously for at least 6 months with normal mucosa, numerical rating scale of pain >4, no cigarette smoking and no prior history of psychological treatment. Control group included healthy subjects between 25 and 80 years old without orofacial pain from oral lesions or odontogenic origins, no cigarette smoking and no others medication taken within a month before the study. Thorough history taking and oral examination were taken in all participants. Patients with diabetes mellitus, iron deficiency, immunodeficiency, parafunctional habit and allergic reaction from acrylic denture were excluded in both groups.

Salivary sample collection

The authors collected unstimulated whole saliva (UWS) after participants sat at rest for at least 5 min, rinsed their mouth with water 2 times, no food or drink taken 1 hr prior to saliva collection. Once ready, participants were asked to sit and hold a 50 ml centrifuge tube while spitting whole saliva for 10 mins or until 3 to 5 ml of saliva were achieved. Centrifuge tubes were placed in an ice container when immediately transported to laboratory. Then saliva samples were vortexed for 1 min before centrifuged at 2,300 g for 10 mins. Supernatant was aliquot into 1 m/tube and stored at -80°C until measuring opiorphin concentration using enzyme linked immunosorbent assay (ELISA). Total protein concentration in each sample was determined by the Bradford protein assay.

Opiorphin level detection

Human Opiorphin ELISA kit (cat No. E1779h, Wuhan, China) was used for quantification of opiorphin levels in UWS based on the competitive binding enzyme immunoassay technique with detection range 0.156 to 10 ng/ml. Opiorphin standard was prepared according to the instruction by reconstituting the standard with 1.0 ml of sample diluent to produce a stock solution of 10 ng/ml. Let the standard sit for 15 min with gentle agitation prior to making serial dilutions of 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0 ng/ml. The undiluted standard served as high standard concentration (10 ng/ml) and the sample diluent served as the zero concentration (0 ng/ml). Prepared wash buffer by diluting 30 ml of wash buffer concentration with deionized water to prepare 750 ml of wash buffer. The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to opiorphin. Then 50 μl of standard, blank, or sample were added per well and immediately added 50 μl of detection A working solution to each well. During the

reaction, in the sample or standard competes with a fixed amount of biotin-labeled for sites on a pre-coated Monoclonal antibody specific to opiorphin. Covered with the plate sealer. Gently tapped the plate to ensure thorough mixing. Incubated for 1 hour at 37°C. Aspirated each well and washed, repeated the process three times for a total of three washes. Washed by filling each well with wash Buffer (approximately 400 µl) using multi-channel pipette. Excess conjugate and unbound sample or standard were washed from the plate. After the last wash, removed any remaining wash Buffer by aspirating. Inverted the plate and blotted it against clean paper towels. 100 µl of detection reagent B working solution (Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated by covering with a new Plate sealer. Incubated for 45 minutes at 37°C. Repeated the aspiration/wash process for five times as conducted in step 3. Added 90 µl of TMB substrate solution to each well. Covered with a new plate sealer. Incubated within 15 to 30 minutes at 37°C. Protected from light. Added 50 µl of stop solution (sulphuric acid solution) to each well to terminate the enzyme-substrate reaction. Determination of the optical density of each well at once was measured spectrophotometrically at a wavelength of 450 nm ±2 nm. The concentration of in the samples is then determined by comparing the OD of the samples to the standard curve.

Results

UWS of 20 BMS patients (3 males and 17 females, average age 60.2 years old) and healthy controls (n = 3, 3 females, average age 50.7 years old) were included in the present study. BMS patients presented with various ranges of burning pain duration (11.4±9.6 mo) and degree of burning sensation (NRS 5.75±2.1) before the first dental visit. The authors performed psychological assessment on stress, anxiety and depression using Thai Stress test and Thai Hospital Anxiety and Depression test. The scores for stress, anxiety and depression were 17.4, 8.9 and 5.6, respectively. Patients reported spicy, hot food, salty taste and GERD are most common aggravating factors whereas stress and anxiety and poor sleep were associated factors that mostly were found (Table 1).

Burning pain were mostly located at the tip of tongue more than the other areas of the oral cavity, whereas buccal mucosa, lips and hard palate are equally burning sensationareas. Gingiva is the least frequent area reported by the patients (Table 2). BMS patients always present with underlying systemic diseases as shown in Table 3. BMS with diabetes mellitus and hypertension are most common. Pharmacotherapy was initiated for BMS patients after saliva collection as shown in Table 4. The average concentration of opiorphin in UWS of BMS patients was 1.25±0.79 ng/ml whereas opiorphin in the healthy control group was 4.16±0.82 ng/ml (Figure 1).

Discussion

Opiorphin is an endogenous pentapeptide isolated in 2006 from human saliva, since then it has been described in several studies as a physiological pain suppressant and a mood-related modulator[9,10]. The present study aimed to investigate the level of opiorphin in patients suffering from BMS and compare it with healthy control subjects. The present study showed a significant decrease in opiorphin level in BMS patients, in contrast with the previous studies by Salaric et al (2017) who demonstrated higher quantities of salivary opiorphin in BMS patients[14] and by Boucher et al, 2017 who has reported no difference between the BMS patients and healthy individuals[15]. The differences in our findings may arise from the differences in saliva collection, handling and technique used to quantify salivary opiorphin concentrations.

Table 1. Demographic data of BMS patients and healthy controls

	BMS patients	Healthy controls
Age (mean ± SD) (year)	60.2±10.5	50.7±16.1
Gender		
Female	17	3
Male	3	-
Duration of burning pain (mean ± SD) (mo)	11.4±9.6	-
Pain intensity (mean ± SD) (NRS)*	5.75±2.1	-
Stress score (Thai stress test)**	17.4±8.9	-
Anxiety score (Thai HAD test)***	8.9±4.6	-
Depression score (Thai HAD test)***	5.6±3.5	-
Aggravating factors	Spicy and hot food Salty taste GERD	-
Associated factors	Stress and Anxiety Poor sleep	-

* NRS = numerical rating scale
** Thai stress test [score 0 to 5 = 1 less than normal, 0 to 17 = 2 normal, 18 to 25 = 3 slightly more than normal, 26 to 29 = 4 moderately more than normal, 30 to 60 = 5 highly more than normal]
*** Thai HAD test (Thai Hospital Anxiety Depression test) [score 0 to 7 = normal, 8 to 16 = borderline abnormal, 11 to 21 = abnormal]

Table 2. Areas of burning sensation in BMS patients (n = 20)

Areas	BMS patients n (% of disease)
Buccal mucosa	10 (50%)
Tips of Tongue	19 (95%)
Lips	10 (50%)
Hard palate	10 (50%)
Gingiva	3 (15%)

Table 3. Systemic diseases in BMS patients (n = 20)

Systemic diseases	BMS patients n (% of disease)
Diabetes mellitus	4 (20%)
Hypertension	4 (20%)
Arthritis	3 (15%)
Anemia	1 (5%)
GERD	3 (15%)
Allergy	1 (5%)
Heart disease	2 (10%)
Liver disease	1 (5%)
Thyroid disease	1 (5%)
Psychological disease	1 (5%)
None	7 (35%)

Table 4. Pharmacotherapy for BMS treatment (n = 20)

Pharmacotherapy	BMS patients n (% of treatment)
Calcium channel blockers	9 (45%)
Benzodiazepine derivatives	9 (45%)
Tricyclic antidepressant	9 (45%)
Topical analgesic application	5 (25%)
Topical capsaicin application	1 (5%)
Multivitamin and supplement	9 (45%)
Topical antifungal application	2 (10%)
Antifungal medication	5 (25%)
Chlorhexidine mouth wash	1 (5%)
None	1 (5%)

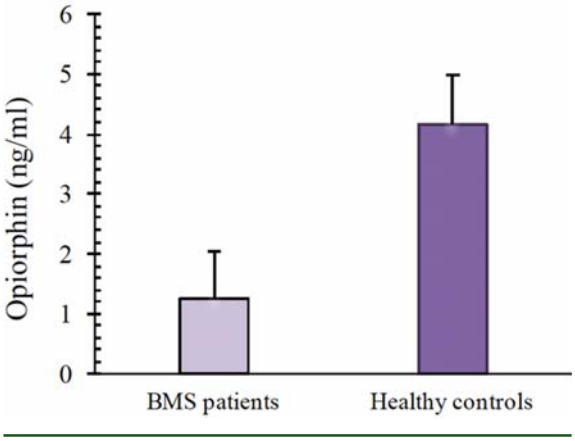

Figure. 1 Opiorphin concentration in human saliva compared between BMS patients (n = 20) and healthy controls (n = 3).

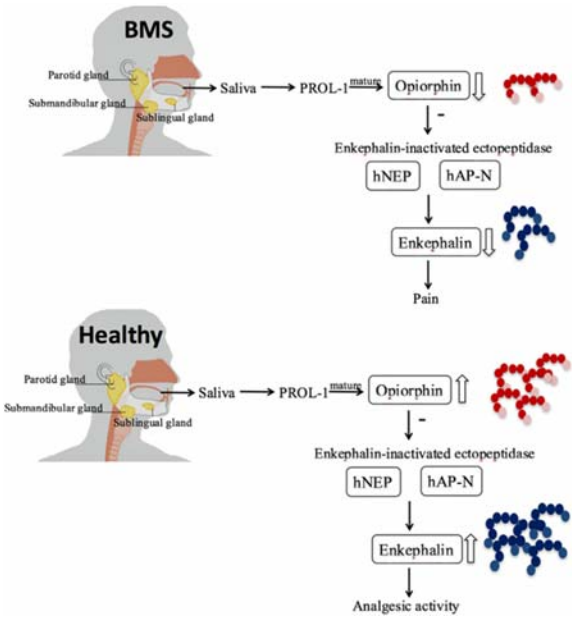

Figure. 2 Proposed mechanism of opiorphin related to enkephalin levels between healthy and BMS individuals.

As opposed to the previous studies on salivary opiorphin in BMS patients, lower opiorphin levels in BMS found in our study may play an important role in the neural mechanisms leading to onset of BMS, due to loss of an inherent pain controlling mechanism. As opiorphin is known as a mature product of proline-rich, lacrimal (PROL1). These results can be further implicated mechanistically than if there is abundant opiorphin in human saliva; there will be more opiorphin inhibits, enkephalin-inactivating ectopeptidases (enzymes that inactivate enkephalin) *i.e.* hNEP and hAP-N, resulting in more available enkephalin to play an analgesic activity. This phenomenon can be found in healthy individuals. However, if there is insufficient salivary opiorphin, there will be a lack of opiorphin inhibits enkephalin-inactivating ectopeptidases. Therefore, enkephalin is digested and less available in saliva to control pain in oral mucosa. This can be implied with burning mouth syndrome patients as shown in Figure 2.

The role of salivary opiorphin has further been demonstrated in other orofacial pain conditions. Ozdogan et al (2019) investigate the change, before and after treatment, in salivary opiorphin concentrations in dental pain related to symptomatic irreversible pulpitis, and symptomatic apical periodontitis and they found a strong correlation between the pre-treatment pain levels and the saliva opiorphin concentrations[17]. Prevalence of xerostomia or dry mouth in BMS patients is high and can be found up to over 60% of patients suffering from this condition[18] and this can further explain the direct local role of salivary opiorphin in the mechanism of BMS.

The main weaknesses of the present study were the low number of BMS subjects and the differences in collection times of salivary samples as patients may not have similar appointment times in our clinic. Moreover, only

unstimulated, whole saliva from patients was collected; therefore further study should be done to investigate opiorphin levels from stimulated whole saliva.

Conclusion

Our results indicate that the decreased levels of opiorphin in UWS of patients with BMS may be associated with the inhibition of analgesic property of enkephalin due to the reverse inhibition of enkephalin-inactivating ectopeptidases, human neutral ecto-endopeptidase (hNEP), and human ectoaminopeptidase (hAP-N), thereby causing chronic pain. To date, knowledge of opiorphin's involvement in pain pathways has been controversial. Further study remains significant to understand the mechanistic role of opiorphin in chronic orofacial pain.

What is already known on this topic?

Opiorphin can be found in saliva and can be a potential biomarker to nociceptive mechanism of pain.

What this study adds?

Salivary opiorphin may be implicated in pain regulation within the oral cavity.

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Potential conflicts of interest

The authors declare no conflicts of interest.

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