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Increased melatonin in oral mucosal tissue of oral lichen planus (OLP) patients: A possible link between melatonin and its role in oral mucosal inflammation

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ABSTRACT

Objective: The existence of extra-pineal melatonin has been observed in various tissues. No prior studies of melatonin in human oral mucosal tissue under the condition of chronic inflammation have been reported. The aim of this study was to investigate the presence of melatonin in oral mucosal tissue of patients with oral lichen planus (OLP) which was considered as a chronic inflammatory immune-mediated disease causing oral mucosal damage and ulcerations.
Materials and methods: Sections from formalin-fixed and paraffin-embedded oral mucosal tissue of OLP patients (n = 30), and control subjects (n = 30) were used in this study. Immunohistochemical staining was performed and the semiquantitative scoring system was used to assess the levels of arylalkylamine-*N*-acetyltransferase (AANAT: a rate-limiting enzyme in the biosynthesis pathway of melatonin), melatonin, and melatonin receptor 1 (MT1) in oral mucosa of OLP patients and normal oral mucosa of control subjects.
Results: AANAT, melatonin, and MT1were detected in oral mucosal tissue of OLP patients and control subjects. Immunostaining scores of AANAT, melatonin, and MT1 in oral mucosal tissue of OLP patients were significantly higher than those in control subjects ($p = 0.002$, $p < 0.001$, and $p = 0.031$, respectively).
Conclusions: Increased levels of AANAT, melatonin, and MT1 in the inflamed oral mucosal tissue of OLP patients imply that chronic inflammation may induce the local biosynthesis of melatonin via AANAT, and may enhance the action of melatonin via MT1.

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is mainly produced by the pineal gland, and considered as the major chronobiotic hormone (Hardeland, 2008; Johnston & Skene, 2015). The biosynthesis of pineal derived melatonin is regulated by the suprachiasmatic nuclei-driven sympathetic innervation of the pineal gland, leading to the activation of arylalkylamine-*N*-acetyltransferase (AANAT) which is a rate-limiting enzyme in the melatonin synthesis pathway (Klein, 2007). Melatonin has two major mechanisms of actions: receptor-mediated and receptor-independent pathways (Reiter, Tan, & Galano, 2014). There are two membrane-bound G-protein related melatonin receptors: MT1 and MT2 (Dubocovich, & Markowska, 2005). Melatonin also binds other molecules such as quinone reductase 2 (Calamini, Santarsiero, Boutin, & Mesecar, 2008), and nuclear receptors (Smirnov, 2001). Besides its influence on circadian regulation, melatonin has other biological functions such as anti-oxidant activity (Tan, Manchester, Esteban-Zubero, Zhou, & Reiter, 2015); anti-inflammatory activity (Mauriz, Collado, Veneroso, Reiter, & Gonzalez-

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Gallego, 2013); and immunomodulation (Carrillo-Vico, Lardone, Alvarez-Sanchez, Rodriguez-Rodriguez, & Guerrero, 2013). The role of melatonin in the innate and acquired immune response were addressed in several review articles (Cardinali, Esquifino, Srinivasan, & Pandi-Perumal, 2008; Calvo, Gonzalez-Yanes, & Maldonado, 2013). It was reported that melatonin and its related molecules were produced by activated immune cells in the inflamed tissue (Markus, Cecon, & Pires-Lapa, 2013).

In addition to the pineal gland, the existence of melatonin-synthesizing enzymes, melatonin, and melatonin receptors has been demonstrated in many tissues (for a review, see Acuna-Castroviejo et al., 2014). It was suggested that extra-pineal melatonin might help protecting cells from oxidative and inflammatory damage (Acuna-Castroviejo et al., 2014). Information on melatonin and its receptors in human oral tissue is limited. Melatonin and melatonin-synthesizing enzymes were observed in epithelial cells of striated ducts of submandibular glands, whereas MT1 was undetected (Shimozuma et al., 2011). It was reported that MT1 was detected in ameloblasts and odontoblasts of dental tooth germs (Kumasaka et al., 2010). No prior studies of melatonin in human oral mucosal tissue under the condition of chronic inflammation have been reported.

Oral lichen planus (OLP) is a chronic inflammatory immune-mediated disease of oral mucosal tissue, and is usually found in middle-aged adults with more female predilection (Alrashdan, Cirillo, & McCullough, 2016). Clinical characteristics of OLP consist of redness and white lines on oral mucosa, called Wickham striae (Scully & Carrozzo, 2008). The manifestation of OLP can be classified as reticular, papular, plaque, atrophic, erosive, and bullous (Gupta & Jawanda, 2015). The lesions with atrophic and erosive types usually cause mucosal damage and oral ulcerations which interfere patients' quality of life (Rana, Kanatas, Herzberg, Gellrich, & Rana, 2015; Tadakamadla, Kumar, & Johnson, 2015). Histopathological findings of OLP usually demonstrate intense subepithelial infiltration of T-lymphocytes, degeneration of basal keratinocytes, and epithelial basement membrane disruption (Gupta & Jawanda, 2015; Scully & Carrozzo, 2008). OLP has been considered as a potentially malignant disorder associated with an increased risk for oral cancer (Gandolfo et al., 2004; Warnakulasuriya, Johnson, & van der Waal, 2007; van der Waal, 2009). The exact etiology of OLP is not fully understood. Most studies concerning the pathogenesis of OLP have focused on the immunological aspects (Chaiyarit et al., 1999; Kurago, 2016; Payeras, Cherubini, Figueiredo, & Salum, 2013; Sugerman et al., 2002). Accumulated data support the involvement of cell-mediated immune dysfunction in the development of OLP (Firth et al., 2015; Wang, Zhou, Fu, Wang, & Zhou, 2015; Zhang et al., 2015). However, data of melatonin in oral mucosa from OLP patients have never been reported. Therefore, we hypothesized that chronic inflammation might affect the production of melatonin in the inflamed oral mucosal tissue of OLP patients. The present study was conducted to test the hypothesis by comparing the levels of AANAT, melatonin, and MT1 in the inflamed oral mucosal tissue from OLP patients with those in the normal oral mucosa of control subjects, using an immunohistochemical method.

2. Materials and methods

2.1. Selection of oral mucosal tissue specimens

This study was approved for the use of tissues from human subjects by the institutional human ethics committee of Khon Kaen University (HE572236). Thirty formalin-fixed and paraffin-embedded biopsy specimens from OLP patients and 30 normal oral mucosal (NOM) specimens from control subjects were obtained from the archives of Oral Pathology Division, Faculty of Dentistry, Khon Kaen University. The inclusion criteria to select tissue biopsy specimens were based on OLP patients's recorded data as follows: (1) oral lesions clinically demonstrated redness and white lines defined as Wickham striae; (2) OLP patients had no history of systemic diseases, smoking cigarettes, receiving medications or being pregnant; (3) selected biopsy specimens of OLP patients were histopathologically confirmed by oral pathologists. The histopathological criteria of OLP were defined as: lymphocytic infiltration in the subepithelial layer and degeneration of basal epithelial cells. The exclusion criteria were as follows: (1) OLP patients had history of systemic diseases, cigarettes smoking, receiving medications, or being pregnant; (2) OLP patients received topical or systemic steroid for treatment of OLP in the past 3 months. Systemic diseases were defined as infectious or non-infectious diseases which were reported in the patient's dental chart such as heart diseases, hematologic diseases, lung diseases, GI tract diseases, liver disease, kidney diseases, and endocrine disorders. Medications were defined as any drugs that the patient had taken according to his or her systemic diseases reported in the patient's dental chart.

According to the ethical limitations in collecting normal tissue samples with the same sites of surgical biopsy and with age and gender matching to those in the OLP group, tissue biopsy specimens in the control group were alternatively obtained from retromolar area of the surgical procedure of tooth extraction from healthy individuals. The subjects had no history of systemic diseases, cigarettes smoking, receiving medications or being pregnant. Selected tissue biopsy specimens in the control group were clinically and histopathologically diagnosed as normal oral mucosa. Demographic and clinical data of OLP patients and control subjects were summarized in Table 1.

2.2. Immunohistochemistry

The sections were cut with 5 micrometer thickness from each biopsy specimen, placed on glass slides, and left in hot air oven at 60 °C for 24 h. The sections were deparaffinized in xylene, rehydrated through graded alcohols. Microwave-based antigen retrieval was done in 1 mmol/L sodium citrate buffer at pH of 6.0 for 10 min. The sections were left at room temperature for 10 min, and washed with phosphate buffered saline (PBS) twice for 5 min. Endogenous peroxidase activity was quenched by Peroxo-Block™

Table 1
Demographic and clinical data of OLP patients and control subjects.

	OLP patients	Control subjects
Gender (cases)		
male	4	10
female	26	20
Age* (years)		
mean ± SD	55.67 ± 9.62	22.67 ± 7.08
range	34–86	18–57
Biopsy sites (cases)		
buccal mucosa	26	–
gingival mucosa	2	–
labial mucosa	2	–
retromolar area of buccal mucosa	–	30
Clinical diagnoses (cases)		
reticular OLP	1	–
atrophic OLP	15	–
erosive OLP	14	–
normal oral mucosa with impacted teeth	–	30

* Significant differences in age between OLP patients and control subjects were observed ($p = 0.001$).

(Invitrogen, Life Technologies Ltd, Paisley, UK). Blocking of nonspecific antibody binding with Protein Block serum-free (DAKO, Carpinteria, CA, USA) was performed for 2 h at room temperature. Polyclonal antibodies for human melatonin (Thermo Scientific, Rock ford, IL, USA), AANAT (Santa Cruz, California, USA), and melatonin receptor 1 (MT1) (Abnova, Taipei, Taiwan) were used in this study. The serial dilutions of primary antibodies were tested and the optimal dilutions were as follows: 1:1000 for anti-melatonin antibody; 1:1200 for anti-AANAT antibody; and 1:400 for anti-MT1antibody. Forty microliter of each primary antibody solution was added on each slide, and kept at 4 °C overnight. Immuno-detection system was based on horseradish peroxidase (HRP) labeled polymer which was conjugated with secondary antibodies (DAKO EnVision+ System-HRP labeled polymer anti-rabbit). A substrate chromogen: 3,3'diaminobenzidine (DAB) (DAKO) was used. The sections were counterstained with hematoxylin, dehydrated, cleared and mounted. Tissue specimens from human pineal glands were used as a positive control. A negative control was achieved by neglecting primary antibodies and substituting with antibody diluent (DAKO).

2.3. Morphometric analysis

Immunohistochemically stained cells were evaluated by using a light microscope with 200× magnifications. The whole area of oral epithelial layers in each specimen was selected for analysis of positively-stained cells. The immunostaining data of positive control was provided in the Supplementary section. Distribution of positively stained cells was evaluated visually by scanning the slide systematically. The semi-quantitative scoring system was used to assess distribution of positively stained cells as follows: no positively stained epithelial cells = 0; <25% positively stained epithelial cells = 1; 25% to <50% positively stained epithelial cells = 2; 50% to <75% positively stained epithelial cells = 3; ≥75% positively stained epithelial cells = 4. All specimens were assessed, and immunostaining scores were done twice with an interval of 2 weeks by 3 examiners (KL, WW, and VV) independently. The final

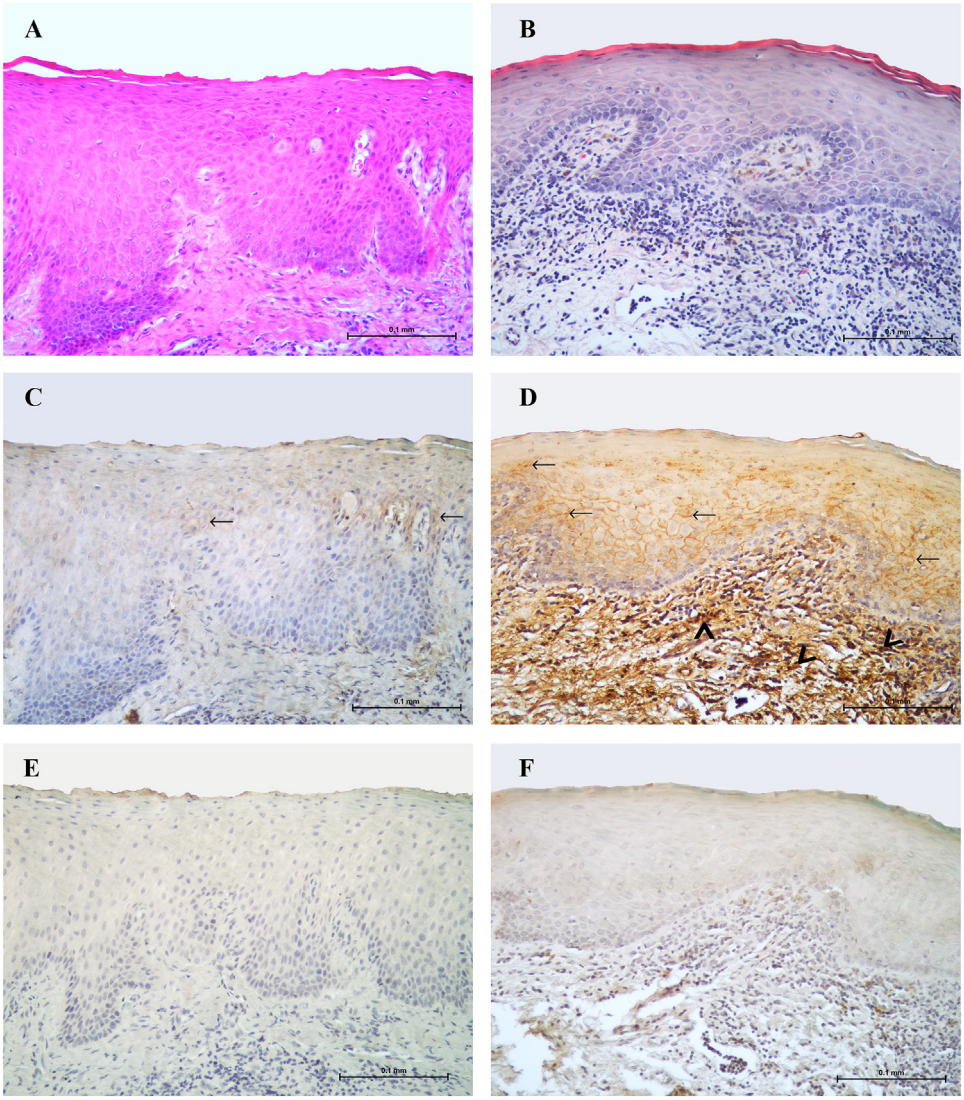

Fig. 1. Photomicrographs of representative samples for immunohistochemical staining with antibody against Arylalkylamine-*N*-acetyltransferase (AANAT). 1A and 1B demonstrate hematoxylin and esosin (H&E) staining of normal oral mucosa (NOM), and oral lichen planus (OLP). 1C and 1D demonstrate the presence of AANAT in NOM, and OLP. A stronger AANAT immunoreactivity is observed in oral epithelium (arrows) and connective tissue (arrowheads) of OLP as compared with NOM. 1E and 1F represent negative controls of NOM, and OLP.

immunostaining score for each tissue sample was derived from agreement of at least two-thirds of examiners.

2.4. Statistical analysis

The data were analyzed by SPSS program (version 20.0). Agreement levels, inter and intra observations, in the judgment of immunostaining scores were analyzed by the kappa statistics. After analysis of normality and Variance of the investigated data, Mann–Whitney *U* test was used to compare the immunostaining scores of AANAT, melatonin, and MT1between NOM and OLP. To control the effect of age, multivariable analyses were performed according to the characteristics of our investigated data as follows. Proportional odds logistic regression was used to assess the independent effect of the two investigated groups on the immunostaining scores of AANAT, and MT1. Binary logistic regression was used to determine the association between the two investigated groups and the immunostaining scores of melatonin. Significance was established at a P-value < 0.05.

3. Results

3.1. Agreement levels in the judgment of immunostaining scores

The range of agreement on immunostaining scores by three examiners was 0.73–0.92 for inter-observer reliability, and 0.75–0.90 for intra-observer reliability.

3.2. Assessment of immunohistochemical staining of AANAT, melatonin, and MT1

The distribution of AANAT in biopsy specimens from NOM (Fig. 1C) and OLP (Fig. 1D) was detected in oral epithelium and connective tissue. The presence of AANAT was also observed in inflammatory cells located in connective tissue of OLP. In NOM and OLP, there was apparent variability in immunostaining scores of AANAT from low to high (Table 2). Immunostaining scores of AANAT were significantly higher in OLP as compared with NOM (Mann–Whitney *U* test; p = 0.002). Due to a marked difference in age between OLP patients and control subjects, multivariable analysis after adjusting for age was performed and the results confirmed that immunostaining scores of AANAT in the OLP group were significantly higher than those in the control group [odds ratio (OR) = 7.21; 95% confidence interval (CI) = 1.13–46.05, and p = 0.037].

The distribution of melatonin was observed mainly in oral epithelium of NOM (Fig. 2C), whereas melatonin was detected in oral epithelium and connective tissue of OLP (Fig. 2D). Melatonin was also demonstrated in inflammatory cells located in connective tissue of OLP. Most specimens from OLP demonstrated high immunostaining scores, whereas specimens from NOM demonstrated low and moderate immunostaining scores (Table 2). Immunostaining scores of melatonin were significantly higher in OLP as compared with NOM (Mann–Whitney *U* test; $p < 0.001$). According to heavily skewed immunostaining data of melatonin, these data were dichotomized into two categories: one category was defined as <50% positively stained epithelial cells, and the other category was defined as ≥50% positively stained epithelial cells. Multivariable analysis after adjusting for age demonstrated that levels of melatonin in the OLP group were significantly higher than those in the control group [OR = 28.84; 95% CI = 1.18–702.04, and p = 0.039].

The distribution of MT1 was detected in oral epithelium of NOM (Fig. 3C), whereas MT1 was observed in oral epithelium and connective tissue of OLP (Fig. 3D). MT1was also detected in inflammatory cells located in connective tissue of OLP. There was variability in immunostaining scores of MT1 from low to high in OLP specimens, whereas low immunostaining scores was demonstrated in most NOM specimens (Table 2). Immunostaining scores of MT1 were significantly higher in OLP as compared with NOM (Mann–Whitney *U* test; p = 0.031). Multivariable analysis after adjusting for age demonstrated that levels of MT1 in the OLP group were significantly higher than those in the control group [OR = 9.92; 95% CI = 1.58–62.19, and p = 0.014].

According our investigated data shown in Table 1, statistical analyses confirmed that there was no significant difference in gender between OLP patients and control subjects. No significant gender-related differences in immunostaining scores of AANAT, melatonin, and MT1 between male and female subjects were observed. In addition, no significant differences in immunostaining scores of AANAT, melatonin, and MT1 between atrophic and erosive types of OLP patients were observed.

Table 2
Immunostaining scores of Arylalkylamine-N-acetyltransferase (AANAT), melatonin (MLT), and melatonin receptor 1 (MT1) in oral epithelial cells of normal oral mucosa (NOM) and oral lichen planus (OLP).

Immunostaining scores[a]	AANAT*		MLT**		MT1***	
	NOM (n = 30)	OLP (n = 30)	NOM (n = 30)	OLP (n = 30)	NOM (n = 30)	OLP (n = 30)
0	0	0	0	0	0	2
1	6	3	0	0	15	8
2	7	4	13	2	13	8
3	17	11	17	7	2	4
4	0	12	0	21	0	8

[a] Immunostaining scores were graded as follows: no positively stained epithelial cells = 0; <25% positively stained epithelial cells = 1; 25% to <50% positively stained epithelial cells = 2; 50% to <75% positively stained epithelial cells = 3; ≥75% positively stained epithelial cells = 4.

* Immunostaining scores of AANAT are significantly increased in OLP as compared with NOM (p = 0.002).

** Immunostaining scores of MLT are significantly increased in OLP as compared with NOM ($p < 0.001$).

*** Immunostaining scores of MT1 are significantly increased in OLP as compared with NOM (p = 0.031).

4. Discussion

Extra-pineal melatonin, melatonin-synthesizing enzymes, and melatonin receptors have been observed in various tissues (Acuna-Castroviejo et al., 2014). In agreement with other published studies (Kumasaka et al., 2010; Shimozuma et al., 2011), our findings confirmed the presence of AANAT, melatonin, and MT1 in human oral mucosa. Melatonin produced in extra-pineal tissues may act in autocrine, paracrine, and/or intracrine manner (Luchetti et al., 2010), and has a variety of functions depending on organ localization and physiological context (Soderquist, Hellstrom, & Cunningham, 2015). Melatonin has been investigated in several chronic inflammatory diseases such as multiple sclerosis (Farez et al., 2015); systemic lupus erythematosus (Medrano-Campillo et al., 2015); rheumatoid arthritis (Afkhamizadeh, Sahebari, & Seyyed-Hoseini, 2014); and inflammatory bowel diseases (Park et al., 2015). We demonstrated that AANAT, melatonin, and MT1 in oral mucosa of OLP patients were significantly increased as compared with those in control subjects. It should be noted that there was a marked difference in age between OLP patients and control subjects which might affect the interpretation of these observed data. Previous studies demonstrated age-related alterations of melatonin levels in serum (Pang et al., 1990; Waldhauser et al., 1988). However, no prior studies have investigated the effects of age on the expression of AANAT, melatonin, and MT1 in human oral mucosa. To address this issue, we conducted multivariable

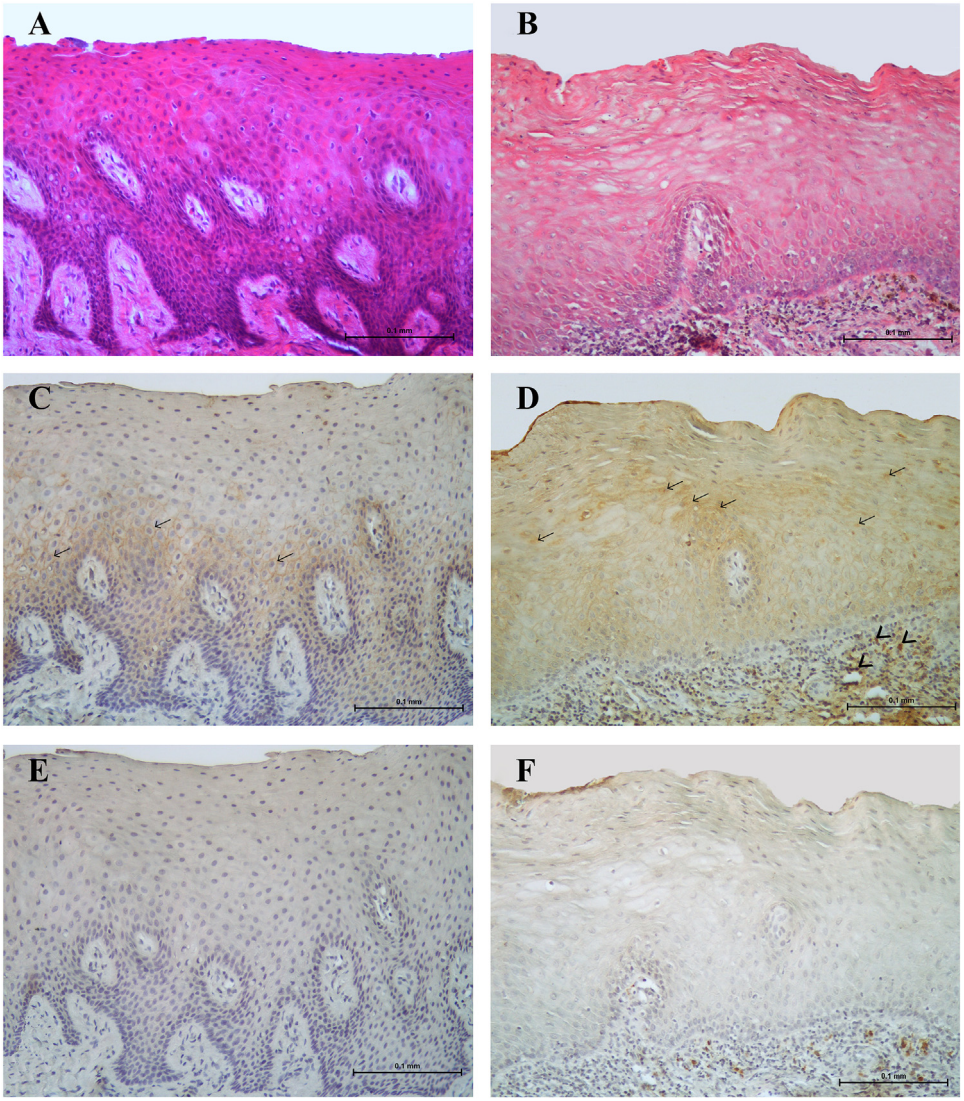

Fig. 2. Photomicrographs of representative samples for immunohistochemical staining with antibody against melatonin. 2A and 2B demonstrate H&E staining of NOM and OLP. 2C and 2D demonstrate the presence of melatonin in NOM and OLP. A stronger melatonin immunoreactivity is observed in oral pithelium (arrows) and connective tissue (arrowheads) of OLP as compared with NOM. 2E and 2F represent negative controls of NOM, and OLP.

analyses controlling for age and confirmed a significant increase in the levels of AANAT, melatonin, and MT1 in oral mucosal epithelium and connective tissue of OLP patients. Thus, regardless of age-related limitation, our observations support the hypothesis that chronic inflammation in the oral mucosal tissue of OLP patients may induce the local biosynthesis of melatonin via AANAT, and may enhance the action of melatonin via MT1. However, other clinical data of OLP patients including duration, severity, and pathological conditions such as stress and sleeping disorders should be concerned for the local biosynthesis of melatonin in oral mucosa.

According to our findings, increased levels of AANAT, melatonin and MT1 in oral epithelial cells of OLP patients may reflect the cytoprotective role through its anti-oxidative and anti-inflammatory properties (Luchetti et al., 2010). This speculation is based on our previous study in OLP lesions demonstrating an increase in 8-oxodG and 8-nitroquanine known as biomarkers for oxidative and nitrative DNA damage (Chaiyarit et al., 2005). In addition, other studies reported that the levels of NF-kB and pro-inflammatory cytokines were increased in the inflamed oral mucosal tissue of OLP patients (Lu et al., 2014; Thongprasom, Dhanuthai, Saridee-chaigul, Chaiyarit, & Chaimusig, 2006; Zhou et al., 2009). Taking these findings into account, increased melatonin may protect oral epithelial cells from oxidative, nitrative, and inflammatory damage by its anti-oxidative and anti-inflammatory properties. Further studies are needed to clarify melatonin's cytoprotective functions in oral mucosa. It was demonstrated that melatonin decreased epithelial paracellular permeability (Sommansson, Nylander, & Sjoblom, 2013). Based on these findings, it is possible that changes in production of melatonin and its receptors during chronic inflammation may affect oral epithelial cell permeability and cell integrity, contributing to various manifestations of OLP. However, further studies on melatonin in the context of cell permeability and clinical manifestations of OLP are needed to verify this speculation.

It was suggested that production of melatonin by activated immune cells at the site of an inflamed lesion might enhance phagocytosis and reduce inflammatory reactions (Markus et al., 2013). According to our observations, infiltrated lymphocytes and other immune cells in the connective tissues of OLP patients could be a source of the local melatonin biosynthesis. Melatonin

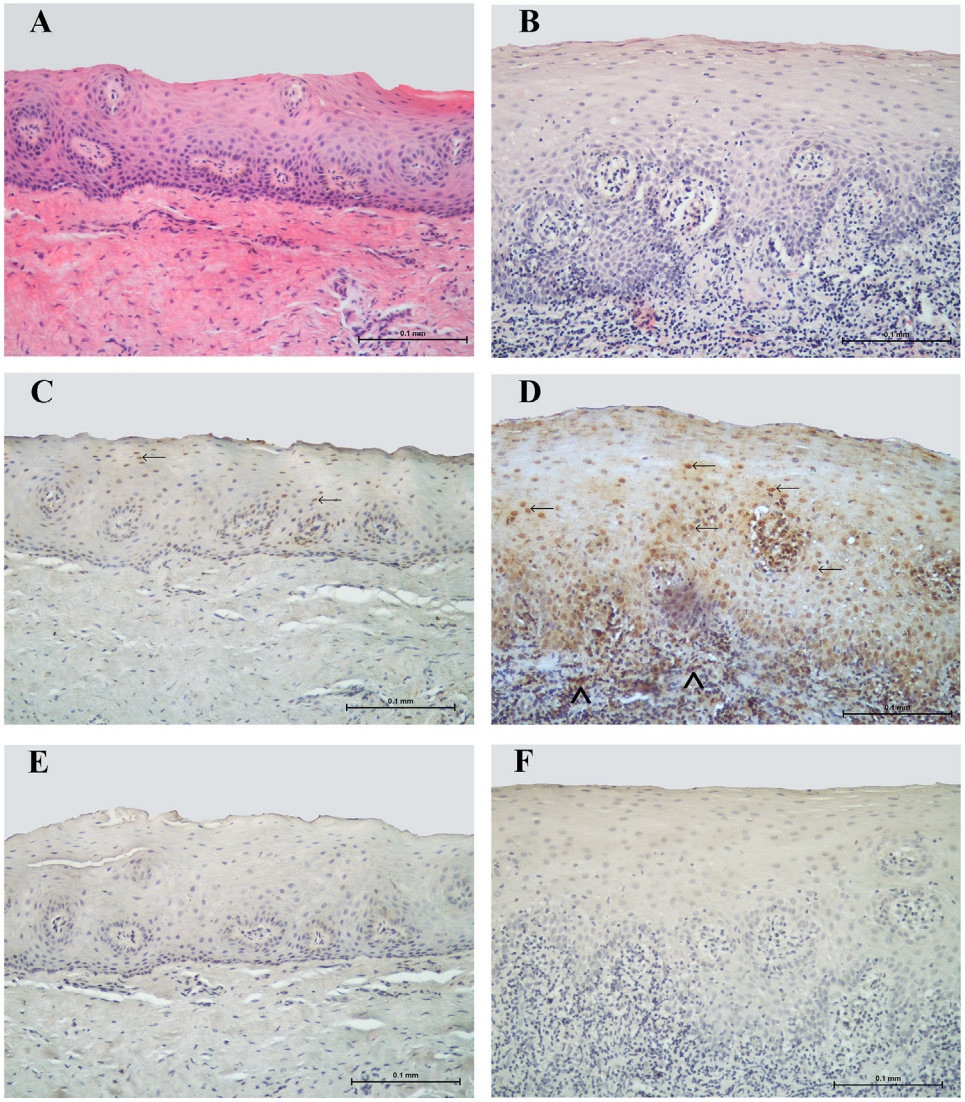

Fig. 3. Photomicrographs of representative samples for immunohistochemical staining with antibody against melatonin receptor 1(MT1). 3A and 3B demonstrate H&E staining of NOM and OLP. 3C and 3D demonstrate the presence of MT1 in NOM and OLP. A stronger MT1 immunoreactivity is observed in oral pithelium (arrows) and connective tissue (arrowheads) of OLP as compared with NOM. 3E and 3F represent negative controls of NOM, and OLP.

produced by lymphocytes was involved in modulation of the IL-2/IL-2 receptor system (Carrillo-Vico et al., 2005). The production of melatonin in macrophages was induced by NF-kB signaling molecules through activation of AANAT (Muxel et al., 2012). It remains unclear whether increased melatonin expression in inflamed oral mucosa is associated with its role in immunoregulation. Therefore, further clarification of melatonin's immunoregulatory functions in oral mucosa is needed. Moreover, expression of AANAT, melatonin, and MT1 in other pathological conditions of oral mucosa should also be evaluated to confirm the specific role of melatonin in association with chronic inflammation.

In conclusion, the present study confirmed the presence of melatonin in human oral mucosa. Increased levels of AANAT, melatonin, and MT1 were observed in the inflamed oral mucosal tissue of OLP patients. These results suggest that chronic inflammation may induce the local biosynthesis of melatonin via AANAT, and may enhance the action of melatonin via MT1in the inflamed oral mucosal tissue of OLP patients. In addition, the present study provides new information that encourages upcoming research projects on biological functions of melatonin in response to chronic inflammation in human oral mucosa.

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Conflicts of interest statement

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2017.02.007.

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