

Role of Apoptosis in Erosive and Reticular Oral Lichen Planus Exhibiting Variable Epithelial Thickness

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Oral lichen planus (OLP) is a chronic inflammatory disease with different clinical types. Reticular and erosive forms are the most common. Although the cause of OLP remains speculative, many findings suggest auto-immune involvement, mediated by T lymphocytes against the basal keratinocytes. Inflammation, mechanical trauma or toxic agents can affect the epithelial homeostasia. Increased apoptosis may cause a decrease in epithelial thickness reflecting in the activity of the lesion. The objective of this study was to evaluate the occurrence of apoptosis and epithelial thickness in reticular and erosive forms of OLP. 15 samples of OLP each type (reticular and erosive) plus 10 of healthy mucosa were collected and processed. After morphometry, the apoptotic index and epithelial thickness were obtained. TUNEL and M30 CytoDEATH immunohistochemical assay were used to validate the morphologic criteria used. Apoptosis in the erosive OLP was significantly more intense than in the reticular type and both forms of OLP presented more apoptosis than the healthy oral mucosa. Healthy oral mucosa was thicker than both OLP forms and thicker in OLP reticular form than in the erosive one. The clinical differences between reticular and erosive forms of OLP are related to variations in epithelial thickness and in intensity of apoptosis.

Key Words: oral lichen planus, apoptosis, TUNEL, M30 CytoDEATH.

INTRODUCTION

Oral lichen planus (OLP) is a chronic inflammatory disease with unknown etiology (1), affecting approximately 2% of the population (2,3). OLP appears in different clinical forms (3) and Neville et al. (1) recognize essentially two types, the reticular and the erosive. The reticular lesions appear as a network of connecting and overlapping lines, papules or plaques (3). The erosive and ulcerative forms are more destructive forms and cause enormous oral discomfort (4), while reticular forms are associated with fewer symptoms and therefore might reflect an intermediate phase (5).

OLP is characterized histologically by epithelial basal cell destruction and a dense subepithelial lympho-

cytic infiltrate (6,7). Degenerating basal keratinocytes form colloid or "Civatte" bodies. The ultrastructure of colloid bodies suggests that they are apoptotic keratinocytes (2).

OLP pathogenesis seems to be mediated by CD4+ and CD8+ T lymphocytes. The basal layer disruption may be result of the cytotoxic effects of the T cells in variable distribution at the sub-epithelial inflammatory infiltrate (2,8,9). The presence of an intense inflammatory infiltrate of activated T lymphocytes releases cytokines, which are mediators to lymphocyte recruitment and retention as well as to the death of basal keratinocytes (5,10).

Cell death that occurs in a programmed form in physiological and pathological processes is known as

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apoptosis (11). It is an active process, requires energy and protein synthesis (12,13). Searle et al. (14) pointed out that apoptosis has two distinct phases: the nuclear condensation, cytoplasmic shrinkage and fragmentation, forming the apoptotic bodies, and phagocytosis and clearance of the fragments by adjacent cells.

Since the erosive type is a more destructive form of the disease when compared to the reticular type, and considering that the apoptosis can take part in the cells destruction of the cells of the basal layer, the aims of this study were to quantify the apoptotic index (AI) in the basal and parabasal layers of the epithelium in reticular and erosive types of OLP, as well as to measure the epithelial thickness, and to evaluate an eventual correlation between these findings.

MATERIAL AND METHODS

Patient Selection

This was a retrospective study. Oral mucosa biopsies were obtained from patients with diagnosis of OLP (reticular and erosive types) treated at the Stomatology Clinic of the Pontifical Catholic University of Minas Gerais (PUC/MG). Permission was obtained from all participants and the research proposal was approved by the local Research Ethics Committee.

Fifteen cases with a clinical diagnosis of reticular and erosive types of OLP were included in the study, together with 10 samples of healthy mucosa. As an inclusion criterion only inflamed areas of reticular and erosive OLP with no epithelial ulceration were considered. As a control group were used samples with no inflammation or acanthosis, confirmed histologically. Control samples were collected during the surgical procedures at PUC/MG's Clinic of Oral Surgery and Dental Implantology. Four-micrometers-thick paraffin-embedded sections were cut in all cases of OLP and healthy mucosa. Sections were stained with Shorr and a morphometric study under light microscope was conducted to determine the number of apoptotic cells. TUNEL reaction and M30 CytoDEATH immunohistochemistry were used to validate the morphological criteria employed for quantification of apoptosis.

Measurement of Epithelial Thickness

Epithelial thickness were obtained at a $\times 12$ mag-

nification (using a $\times 4$ objective lens) which allowed viewing the sections in their entire length, with basement membrane, basal, spinous, intermediated/granular and cornified layers. Average epithelial thickness in μm was estimated by measuring the epithelial area (A) in μm^2 from the basal membrane, above the inflammatory infiltrate and dividing this by the straight horizontal distance (B) in μm (5) (Fig. 1).

Parameters and Morphometric Evaluation

To establish the minimal representative number of microscopic fields *per* sample, apoptotic cells were counted in 50 fields (using a $\times 100$ objective lens) in a randomly chosen slide of each group as described by Moro et al. (15). Then, groups of 5, 10, 20, 30, 40 and 50 fields were taken randomly with reposition. Mean and coefficient of variation were calculated from each group. Coefficient of variation decreased as the sample size increased. The minimal representative number of 20 microscopic fields *per* samples was obtained when the increment in the number of fields did not result in considerable reduction in the respective coefficient of variation value. All morphometric evaluations were performed using specific software (Kontron KS300, v. 2.0; Kontron Elektronik, GmbH, Eching bei München, Germany).

Quantification of the Apoptotic Index (AI)

Quantification of the apoptotic cells, considered a morphological criterion to define apoptosis, as described by Wilson and Potten (16). Apoptotic and total cells were quantified using images of 20 fields obtained

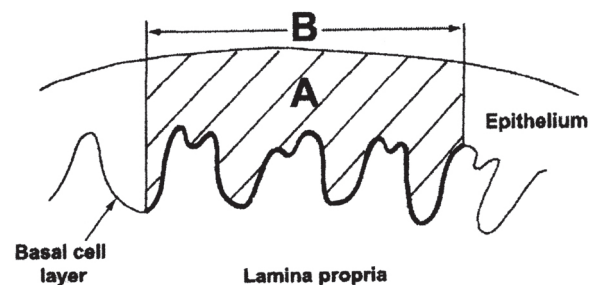


Figure 1. Schematic illustration showing measurements made to estimate epithelial thickness (Adapted from Karatsaidis et al.) (5).

with a $\times 100$ planachromatic objective from each slide. All morphometric evaluations were performed in a double blind assay by the same observer, in different days, in fields randomly chosen. Measurements were taken from epithelial areas above intense inflammatory infiltrate and free from ulceration. AI was obtained by the following equation: $AI = (\sum \text{apoptotic cells} / \sum \text{total cells}) \times 100$.

In Situ Detection of DNA Fragmentation

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) reaction was used for detect of in situ genome fragmentation and confirm apoptosis, using a commercial kit (TdT-FragEL DNA Fragmentation Detection Kit, Cat QIA33, Calbiochem, San Diego, CA, USA). Reactions were carried out as described by the manufacturer. Briefly, slides were incubated with 20 $\mu\text{g}/\text{mL}$ of proteinase K (Cat # P5568, Sigma, St. Louis, MO, USA) and endogenous peroxidase was quenched three times with 3% H_2O_2 in methanol. Terminal deoxynucleotidyl transferase (TdT) and deoxynucleotides were applied and the slides were placed in an humid atmosphere at 37°C for about 2-6 h. The reaction was stopped by a blocking buffer, and then the slides were treated with peroxidase streptavidin conjugate, placed in humid atmosphere at 37°C for 0.5-1 h. Finally, they were washed and treated with diaminobenzidin and counterstained with light green or Harris hematoxiline.

Immunohistochemistry of M30 CytoDEATH

The M30 CytoDEATH immunohistochemical assay used a mouse monoclonal antibody (M30clone) from a commercial kit (M30 CytoDEATH, Mouse monoclonal Ab, Clone M30, Cat 2 140 322, Roche, Nutley, NJ, USA). Before the Immunohistochemical reaction, the paraffin sections were incubated at 37°C overnight. Sections were deparaffinized, soaked in metanol/ H_2O_2 (3%) and immersed in boiling citric acid buffer. Sections treated with incubation buffer received 100 μL antibody working solution for 1 h in a humid chamber at 15-25°C. Slides were washed and covered with 100 μL of anti-mouse-Ig Biotin (1-2 $\mu\text{g}/\text{mL}$) for 30 min at 37°C in a humid chamber. Slides were re-washed and covered with 100 μL of Streptavidin-POD (0.5 U/ mL) for 30 min at 15-25°C in a humid chamber. Then,

slides received DAB until a clearly visible darkish color develops (1-5 min). Subsequently, the preparation was counterstained with Harries' hematoxylin.

Statistical Analysis

Results were presented as means \pm standard error after being submitted to a Kolmogorov-Smirnov test (for normal distribution) and to a Student's t test to detect eventual differences between samples. Values of $p < 0.05$ were considered as significant. GraphPad Prism 3.0 (GraphPad Inc., San Diego, CA, USA) software was used throughout in the statistical evaluation. Pearson's Correlation was used to evaluate an eventual link between apoptosis and epithelial thickness, using the BioEstat 4.0 software.

RESULTS

All cases of OLP showed a variety of spherical eosinophilic hyaline bodies, presenting nuclear condensation sometimes surrounded by clear halo in the basal and parabasal epithelial layers. These colloids or hyaline structures, so called "Civatte" bodies, result from the destruction of basal keratinocytes by apoptosis. Also, an intense inflammatory infiltrate was often found under the epithelium.

Sections processed by TUNEL technique showed a diffuse positive labeling (brownish dots in nuclei) of the basal and supra-basal keratinocytes indicating and confirming the occurrence of apoptosis, as previously detected by morphological criteria using Shorr staining (Fig. 2A and 2B). Sections processed by M30 cytoDEATH method also confirmed the occurrence of apoptosis as detected using Shorr staining, with a positive diffuse labeling (reddish brown dots in cytoplasm) within the basal and supra-basal keratinocytes (Fig. 3A and 3B).

Data referring to the AI and to epithelial thickness showed normal distribution by the Kolmogorov Smiroff test. Results of Student's t-test for both parameters showed statistically significant differences. Epithelial apoptosis was less intense in the control group (4.87 ± 0.17) than in the reticular group (26.85 ± 0.72), which presented even less intense apoptosis than the erosive group (57.27 ± 0.92), with $p < 0.0001$. On the other hand, epithelial thickness in the control group was greater (248.70 ± 25.24) than in the reticular group

(171.30 ± 11.21), which presented greater epithelial thickness than the erosive group (117.90 ± 7.13) ($p < 0.0001$) (Figs. 4 and 5). Pearson's correlation showed a highly negative value between the AI and the epithelial thickness ($r = -0.9803$) (Figs. 4 and 5).

DISCUSSION

Apoptosis seems to play a key role in several oral lesions. Loro et al. (17) reviewed the literature on the occurrence and role of apoptosis in oral diseases including viral infections, periodontal diseases, oral cavity cyst

and tumors, in addition to OLP. In the present study, a morphometric approach to evaluate apoptosis and epithelial thickness was used to examine lesions of reticular and erosive types of OLP in order to investigate the existence of a correlation between these factors. These two clinical forms of OLP were studied in the present work because the reticular type is the most common form while the erosive type not only causes great oral discomfort, but also reflects a more destructive phase of the disease. The main features in OLP injuries include the colloid bodies or "Civatte" bodies in the epithelial basal layer, which were ultrastructurally

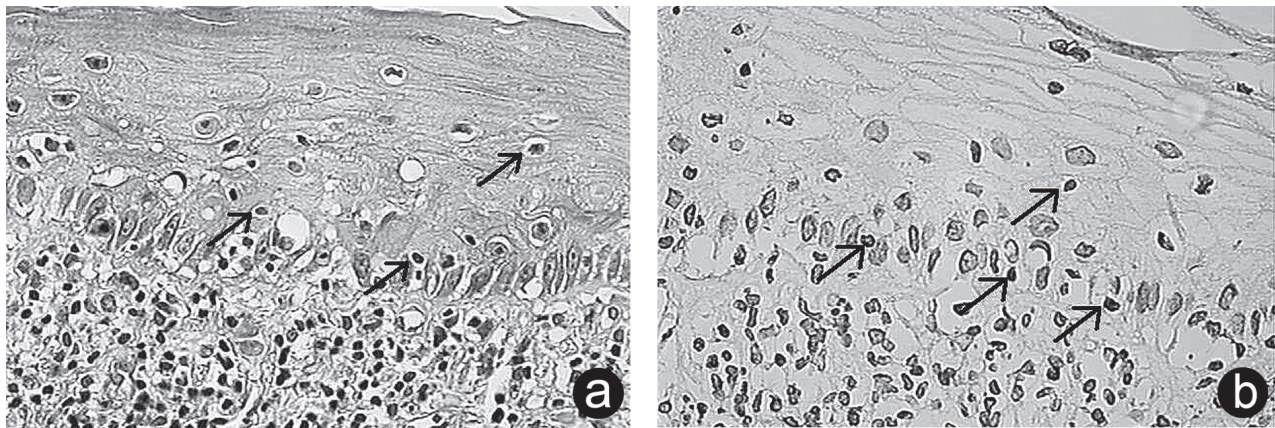


Figure 2. Photomicrographs of the erosive form of OLP. A= Shorr staining. Disruption of the epithelial basal layer with apoptotic cells surrounded by a clear halo, hyaline ("Civatte bodies"), and an intense subepithelial inflammatory infiltrate ($\times 40$ objective lens); B= TUNEL reaction. Positive labeling indicating the genome fragmentation (TUNEL +) in basal and parabasal keratinocytes ($\times 40$ objective lens).

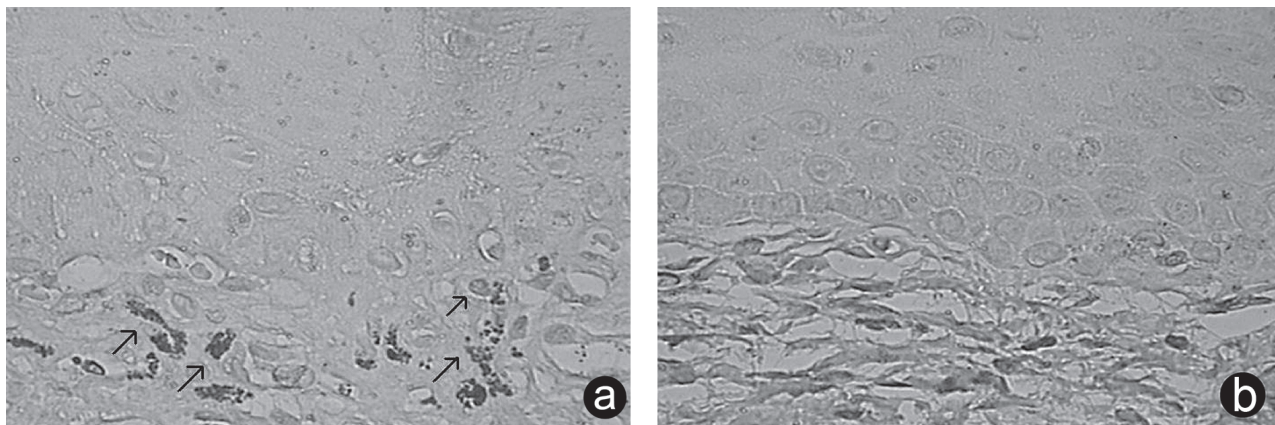


Figure 3. Photomicrographs of the reticular form of OLP. A= M30 CytoDeath immunohistochemistry. Positive labeling indicating caspase activity in the cytoplasm of basal and parabasal keratinocytes ($\times 40$ objective lens); B= Healthy control oral mucosa, M30 CytoDeath immunohistochemistry. No labeling whatsoever in basal and parabasal keratinocytes ($\times 40$ objective lens).

defined as keratinocytes in apoptosis (10). The inflammatory infiltrate seems to play an important role in the epidermal injury. Dekker et al. (10) and Bloor et al. (18) investigated the AI in epithelia in OLP and showed a significant increase of apoptosis in these injuries when compared with the healthy oral mucosa.

In this study, apoptosis was more intense in OLP lesions than in healthy (control) oral mucosa. Also, the erosive form of OLP presented more apoptosis than the reticular form. This is in agreement with Neppelberg et al. (19), who showed a larger number of apoptotic cells in OLP epithelia, when compared to the control oral mucosa. However, Karatsaides et al. (4) found a larger number of labeled cells by the TUNEL reaction in the basal and in spinous layers of the epithelium with OLP. These apoptotic labeled cells were interpreted as intraepithelial lymphocytes, since many of them also presented a double labeling to CD4 + lymphocytes or CD68 + macrophages. Also, in Karatsaides' et al. study (4), the number of apoptotic keratinocytes in the basal and spinous layers was similar between the OLP and the healthy oral mucosa.

Other authors also evaluated the expression of Caspase 3 in OLP. The proportion of Caspase 3 labeled (apoptotic) cells was significantly higher in all OLP forms when compared to the healthy (control) oral mucosa (20), indicating that the basal cells are the target of the lymphocyte attack (8).

Apoptosis seems to be involved in the OLP's cell

death, as demonstrated by the results of the present study, where epithelial basal layer destruction and "Civatte" bodies were evident (Fig. 2A). There are many controversies in this issue. Several authors (4,5,8) consider that epithelial apoptosis is not much important in OLP pathogenesis. They believe that those shrunken and condensed cells in the epithelium are not apoptotic epithelial cells but rather intra-epithelial lymphocytes. Anyway, it is likely the occurrence of epithelial cell death and resorption allowing the decrease of the epithelial thickness, a common feature in most aggressive types of OLP.

In order to validate the morphologic criteria used to determine the AI, TUNEL and M30 CytoDeath immunohistochemistry were used in this study. Most cells exhibiting morphology of apoptosis on Shorr staining also showed labeling with either M30 CytoDeath or TUNEL techniques. This work showed a higher frequency of apoptotic cells than other studies referring to epithelial apoptosis in OLP lesions (10,18). A possible explanation for the less expressive results in other studies could be the use of more intense and ulcerated lesions, which are not useful for accurately identifying and quantifying programmed cell death. Also, timing seems to be another important factor – therefore results from OLP in an initial stage could differ from those of OLP in an advanced stage. Any approach to clarify this issue must consider a window of time and solid inclusion and exclusion criteria. Only with that in mind one will be

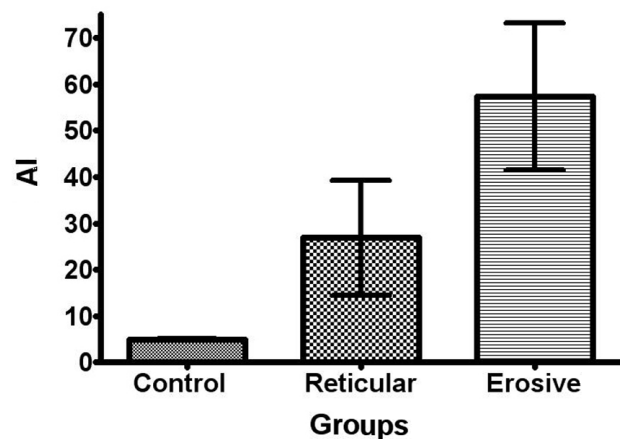


Figure 4. Graphic presentation of the distribution of apoptotic index in the control, reticular and erosive types of OLP ($p < 0.0001$).

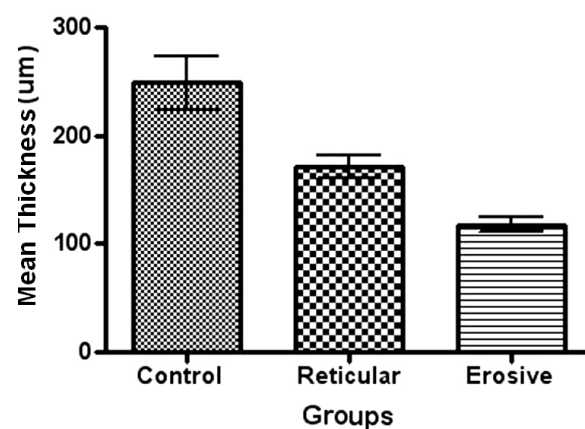


Figure 5. Graphic presentation of the epithelial thickness in the control, reticular and erosive types of OLP ($p < 0.0001$).

able to clearly conclude on the different moments and evolutive stages of the disease.

In this work, epithelial pathological apoptosis occurred only in the basal and parabasal layers because these layers are the most susceptible to aggressions by the subjacent lymphocytic inflammatory infiltrate. Also, apoptosis in spinous and/or corneal layers of the mucosa occurs physiologically as results of terminal differentiation of keratinocytes.

Erosive OLP had smaller epithelial thickness than reticular OLP, and both types of OLP had also thinner epithelia than the healthy control mucosa. This is in agreement with the findings of Karatsaides et al. (5), who obtained similar results in epithelial thickness in erosive and reticular OLP lesions. Decrease in epithelial thickness is a common OLP feature, however in this study, the epithelial thickness varied according to the clinical form and also to the intensity of apoptosis. Some patients with the reticular form of OLP presented an epithelial thickness within the normal range, but all patients with erosive type of OLP presented thinner epithelia. These clinical differences between the reticular and erosive forms are probably consequences of biological and histological variations.

A significant negative correlation between the epithelial thickness and intensity of apoptosis was detected in this study. The thinner the epithelia, the greater occurrence of apoptosis. Therefore the erosive type of OLP showed the thinnest epithelia and the highest AI. On the other hand, healthy control mucosa showed the thickest epithelia and the smallest AI. Alterations in cells of basal and parabasal epithelial layer, occurring as part of the OLP, are initiated by inflammatory cells and involve different cytokines and mediators that are capable to activate apoptosis in the basal keratinocytes (2,5). The mechanisms involved in between the inflammatory cell infiltrate and subepithelial and basal layer destruction are not yet well defined, but it is believed that a correlation exists between these two factors.

The results of this paper seem to indicate a role of apoptosis in the pathogenesis of those two forms of OLP. In fact, it suggests that apoptosis is responsible for the decrease in epithelial thickness. The thinner epithelia observed in the erosive form of OLP compared to the reticular form seems to indicate that the former corresponds to a more active stage of the disease, while the latter corresponds to a more quiescent phase (5). In this context, it is reasonable to believe that the apoptosis

may take part in this process, contributing to these differences.

RESUMO

O líquen plano oral (LPO) é uma doença inflamatória crônica com diferentes tipos clínicos. As mais comuns são as formas reticular e erosiva. Embora a causa do LPO permaneça no campo especulativo, muitos achados sugerem tratar-se de uma doença auto-imune, mediada por linfócitos T que têm como alvo os ceratinócitos basais. Inflamação, trauma mecânico ou agentes tóxicos podem afetar a homeostasia epitelial. O aumento da apoptose pode levar a uma diminuição da espessura epitelial e isto refletir na atividade da doença. O objetivo deste estudo foi avaliar a ocorrência de apoptose e a espessura epitelial nas formas reticular e erosiva de LPO. 15 amostras de LPO de cada tipo reticular e erosivo, além de 10 amostras de mucosa saudável foram coletadas e processadas. Depois da morfometria, o índice apoptótico (IA) e a espessura do epitélio foram obtidas. Reação de TUNEL e imunohistoquímica do M30 CytoDeath foram usadas para validação dos critérios morfológicos. A apoptose no LPO erosivo foi significativamente maior que no tipo reticular e ambas as formas de LPO apresentaram mais apoptose que a mucosa oral normal. A mucosa oral normal foi mais espessa que ambas as formas de LPO, sendo que, a forma reticular foi mais espessa que o tipo erosivo. As diferenças clínicas entre as formas reticular e erosiva de LPO têm relação com as variações na espessura epitelial e na intensidade da apoptose.

REFERENCES

1. Neville BW, Damm DD, Allen CM, Bouquot JE. Oral and maxillofacial pathology. 2nd ed. Philadelphia: WB Saunders; 2004.
2. Sugerman PB, Savage NW, Walsh LJ, Zhao ZZ, Zhou XJ, Khan A, et al. The pathogenesis of oral lichen planus. *Crit Rev Oral Biol Med* 2002;13:350-365.
3. Eisen D, Carrozzo M, Bagan Sebastian JV, Thongprasom K. Number V Oral lichen planus: clinical features and management. *Oral Dis* 2005;11:338-349.
4. Karatsaides A, Schreurs O, Axéll T, Helgeland K, Schenck K. Identity of TUNEL positive cells in the oral bucal epithelium of normal mucosa and lichen lesions. *J Oral Pathol Med* 2004;33:264-268.
5. Karatsaides A, Schreurs O, Helgeland K, Axéll T, Schenck K. Erythematous and reticular forms of oral lichen planus oral lichenoid reactions differ in pathological features related to disease activity. *J Oral Pathol Med* 2003;32:275-281.
6. Kim SG, Chae CH, Cho BO, Kim HN, Kim HJ, Kim LS, et al. Apoptosis of oral epithelial cells in oral lichen planus caused by up regulation of BMP-4. *J Oral Pathol Med* 2006;35:37-45.
7. Neppelberg E, Loro LL, Oijordsbakken G, Johannessen AC. Altered CD40 and E-cadherin expression - putative role in oral lichen planus. *J Oral Pathol Med* 2007;36:153-160.
8. Bascones C, Gonzalez-Moles MA, Esparza G, Gil-Montoya JA, Bascones A. Significance of liquefaction degeneration in oral lichen planus: a study of its relationship with apoptosis and cell cycle arrest markers. *Clin Exp Dermatol*

- 2007;32:556-563.
9. Eversole LR. Immunopathology of oral mucosal ulcerative, desquamative and bullous diseases: selective review of the literature. *Oral Surg Oral Med Oral Pathol* 1994;77:555-571.
 10. Dekker NP, Lozada-Nur F, Lagenaur LA, Macphail LA, Bloom CY, Regezi JA. Apoptosis associated marker in oral lichen planus. *J Oral Pathol Med* 1997;26:170-175.
 11. Kerr JFR, Wyllie AH, Curie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. *Br J Cancer* 1972;26:239-257.
 12. Bosman FT, Visser BC, Van Oeveren J. Apoptosis: pathophysiology of programmed cell death. *Path Res Pract* 1996;192:676-683.
 13. Wyllie AH. Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. *Cancer and Metast Rev* 1991;11:95-103.
 14. Searle J, Kerr JFR, Bishop CJ. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. *Pathol Annu* 1982;17:229-259.
 15. Moro L, Martins AS, Alves CM, Santos FGA, Nunes JES, Carneiro RA, Carvalho R, Vasconcelos AC. Apoptosis in canine distemper. *Arch Virol* 2003;148:153-164.
 16. Wilson JW, Potten CS. Morphological recognition of apoptotic cells. In: Studzinski GP (Editor). *Apoptosis - a practical approach*. Oxford: University Press; 1999; p. 19-40.
 17. Loro LL, Vintermyr OK, Johannessen C. Apoptosis in normal and disease oral tissues. *Oral Dis* 2005;11:274-287.
 18. Bloor BK, Malik FK. Quantitative assessment of apoptosis in oral lichen planus. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1999;88:187-195.
 19. Neppelberg E, Johannessen AC, Jonsson R. Apoptosis in oral lichen planus. *Eur J Oral Sci* 2001;109:361-364.
 20. Tobon-Arroyave SI, Villegas-Acosta FA, Ruiz-Restrepo SM, Vieco-Duran B, Restrepo-Misas M. Expression of caspase-3 and structural changes associated with apoptotic cell death of keratinocytes in oral lichen planus. *Oral Dis* 2004;10:173-178.

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