

## Original Article

# Analysis of the immunoexpression of Ki-67 and Bcl-2 in the pericoronal tissues of impacted teeth, dentigerous cysts and gingiva using software image analysis

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## ABSTRACT

**Background:** Pericoronal tissue is the soft tissue located between the bony crypt and the impacted tooth, which is histologically composed of fibrous connective tissue and is usually lined by the reduced enamel epithelium. Increased epithelial cell proliferation in the pericoronal tissue is associated with pathological changes such as development of an odontogenic cyst or an epithelial odontogenic tumor. This study is an attempt to evaluate and compare the proliferative index in the epithelium surrounding the impacted third molar teeth, dentigerous cysts, and gingiva.

**Materials and Methods:** A case control study on pericoronal tissues and dentigerous cysts was carried out using immunomarkers. Forty pericoronal tissues were obtained from asymptomatic impacted third molars with pericoronal radiolucency less than 2.5 mm. Samples of 20 dentigerous cysts and normal gingiva were also included. Routine hematoxylin and eosin and immunostaining for Ki-67, a cell proliferation marker and Bcl-2, an anti apoptotic protein were performed on sections of pericoronal tissues, dentigerous cysts, and gingival tissues. The percentage of Ki-67-positive cells and Bcl-2 positive areas was found using the DigiPro™ version 4.0 Image analysis software. Bcl-2 immunopositivity and Ki-67-Li were analyzed using the Chi-square test and paired t-test. P-values of less than .05 were considered to indicate statistical significance.

**Results:** The immunohistochemical analysis revealed overexpression of Bcl-2 in the pericoronal tissues with squamous metaplasia, which was comparable to the dentigerous cyst. Ki-67 Li of the pericoronal tissue with squamous metaplasia was equal to the proliferative index of the dentigerous cyst. The expression of Ki-67 Li and inflammatory cells were highly significant ( $P < 0.0001$ ).

**Conclusions:** The results of this study indicate that the pericoronal tissues of asymptomatic impacted third molars may be actively proliferating and normal pericoronal radiolucency cannot serve as an indicator for the differentiation potential.

**Key Words:** Apoptosis, Bcl-2, dentigerous cyst, impacted third molar, Ki-67 Li, pericoronal tissue

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## INTRODUCTION

The pericoronal tissue or dental follicle is defined as the soft tissue located between the bony crypt

and the impacted tooth and comprises of one of the components of tooth germ. Radiographically; it appears as a thin; semicircular radiolucent object, less than 2.5 mm in width.<sup>[1]</sup> Histologically; it is associated with unerupted or impacted teeth, and shows fibrous connective tissue with remnants of reduced enamel epithelium.<sup>[2,3]</sup> Inflammatory changes in the pericoronal tissue upregulate the cell turnover of epithelial components and can lead to an increase in the thickness of the epithelium.<sup>[4]</sup> This epithelium of the pericoronal tissues can give rise to several pathologies such as odontogenic cysts

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(e.g., dentigerous cyst) and odontogenic epithelial neoplasms (e.g., ameloblastoma).<sup>[3]</sup> The inflammatory and cystic pathologies have been greater in incidence as compared to neoplastic ones.<sup>[3,5]</sup>

The growth rate of tissues is determined by proliferative activity and cell death. Ki-67 is a widely used cellular proliferation marker and is a non-histone protein that is expressed in all stages of the cell cycle, except for G0.<sup>[6]</sup> It has been reported that Ki-67 overexpression is an early marker of disease progression in the oral mucosa and its expression is estimated to be the percentage of the tumor cells positively stained by the antibody, with nuclear staining being the most common criterion of positivity. Ki-67 antibodies have been used widely for the estimation of the growth fraction of clinical samples of human neoplasms and of normal cells.<sup>[7]</sup> Molecular and functional characterization of Ki-67 is necessary for a greater understanding of the role of the antigen in the cell cycle of normal and neoplastic cells. The *Bcl-2* proto-oncogene was first discovered in B-cell lymphomas, exhibiting the t (14:18); q (32:31) chromosomal aberration, and is the prototype of cell death regulatory genes.<sup>[8]</sup> It is a gene product; the Bcl-2 protein is associated with an outer mitochondrial membrane and is located on the endoplasmic reticulum and nuclear envelope and blocks a distal step in an evolutionary conserved pathway of apoptosis. Its abnormal expression, usually in terms of overexpression, contributes to the expansion of the damaged cell clone, by preventing cell turnover due to programmed cell death, leading to cellular immortalization.

Previous studies have reported cyst-like epithelium in 2-60% of the dental follicular tissues.<sup>[9]</sup> Histopathological changes that occur in different stages of dental follicle development may lead to the formation of odontogenic cysts and tumors. The expression of Bcl-2 and Ki-67 in some epithelial cells neighboring the basement membrane indicates sites of cellular proliferation. According to Razavi, *et al.*, a higher percentage of Ki-67 and Bcl-2 confirms the aggressive behavior of neoplastic lesions like ameloblastomas.<sup>[10]</sup> Therefore, detection of Bcl-2 and Ki-67 is considered a reliable marker of cellular proliferation and aggressiveness.

The aim of the present study was to objectively analyze the immunohistochemical expression of Ki-67 and Bcl-2 in the pericoronal tissues and dentigerous cyst, as well as the gingiva, using the DigiPro™

version 4.0 Image Analysis Software. (LaboAmerica Inc). Excess amounts of these markers could indicate a developing pathology at the third molar sites before the disease became radiographically evident and could suggest the need for constant follow-up and management of asymptomatic impacted third molars.

## MATERIALS AND METHODS

Forty pericoronal tissues were obtained from 40 patients; 27 male and 13 female, within the age range of 20 to 30 years (mean: 26.7 years). There were three cases in the maxilla and 37 cases in mandible. Radiologically, pericoronal tissues appeared as symmetric, semicircular, radiolucent lesions, less than 2.5 mm, around the crowns of the impacted third molars. The exclusion criteria included a history or sign of infection; enlarged tissues surrounding the impacted third molars or pericoronal tissues not lined by the epithelium. Approval for the study was obtained from the local ethics committee and informed consent was obtained from all participating patients.

In contrast, 20 dentigerous cysts showing radiolucency associated with completely impacted teeth and a pericoronal radiolucent width of more than 2.5 mm in all cases, were included. Archival samples of 20 normal gingival tissues were also included in the study.

All operations were carried out under local anesthesia by conventional third molar surgery. The pericoronal tissues of the impacted teeth were carefully removed, placed immediately in 10% buffered formalin, and processed to paraffin wax. Sections (5 µm thick) were cut from each block containing the pericoronal tissue and stained with hematoxylin and eosin (H and E) for routine histological examination. All specimens were examined by two pathologists. Only those pericoronal tissues showing the presence of fibrous connective tissue with epithelium were included. Histologically, the epithelial components were divided into two groups: Normal pericoronal tissue (lined by reduced enamel epithelium (REE) and pericoronal tissue with squamous metaplasia (all the cases lined by stratified squamous epithelium (SSE) and/or proliferative epithelium). The grades of the subepithelium inflammatory change were classified into none-to-mild and moderate-to-severe.

### Immunohistochemical method

Immunohistochemical analysis was performed using the Biogenex (USA), QD400 60K Super Sensitive™ Polymer-HRP IHC Detection System. The

paraffin-embedded tissues were cut into 4- $\mu$ m thick sections and taken onto poly-L-Lysine (Biogenex; USA.) adhesive-coated microslides. The sections were brought to water after de-paraffinization, and quenching of the endogenous peroxidase activity was achieved by adding the peroxide block over the slide for 15 minutes in a moist chamber. The slides were then incubated with a power block for 15 minutes to minimize the non-specific binding of antibodies to the charged sites. An antigen-retrieval procedure was performed by placing the sections in a citrate buffer working solution in the microwave oven, with power 450, five times, for three minutes each. After bringing the section to room temperature; they were briefly rinsed in phosphate buffered saline (PBS) and treated with 10% goat serum at room temperature for 30 minutes, to block any non-specific antigenic sites. The sections were then incubated with a primary antibody. The following primary antibodies were used: Monoclonal anti-Ki-67 (clone-BGX-297) at a 1:100 dilution (Biogenex; USA) and monoclonal anti-Bcl-2 (Bcl-2 2/100) ((Biogenex; USA) at a 1:50 dilution. For negative control; the primary antibody was substituted by non-immune serum of a mouse (HK119-5M). The slides were incubated with the Super Enhancer in a moist chamber for 25 minutes. Following a PBS wash for three minutes, thrice, the sections were incubated with biotinylated secondary antibody at 1:200 dilutions at room temperature, for 30 minutes (Biogenex; USA). Following a PBS wash for five minutes, thrice, the sections were incubated with streptavidin-peroxidase conjugate (Biogenex; USA); at a concentration of 1:200 for 30 minutes, and detected using 0.05% 3,3-diaminobenzidine tetra-hydrochloride (DAB) (Dakocytomation; Glostrup; Denmark). The slides were counterstained with Mayer's hematoxylin, subsequent to which, the sections were dehydrated, cleared, and mounted with DPX and cover slipped. A known positive control (lymph node sections for Bcl-2 and papilloma for Ki-67) was run with every immunostaining to confirm the immunoactivity of the antibodies;

### Evaluation

Immunostaining for Bcl-2 and Ki-67 was evaluated as per Edamatsu M *et al.*<sup>[4]</sup> using a Digi 3 microscope with a DigiPro™ version 4.0 Image analysis software. (LaboAmerica Inc.). A brown colored cytoplasmic reaction (regardless of the intensity) was considered to indicate immunopositivity for Bcl-2. The proliferative fractions of epithelial cells

in the epithelial components of the pericoronal tissues, lining epithelium of the dentigerous cysts, and epithelium of the gingiva, were calculated using an image analyzer. Digital images were prepared for analyses using DigiPro™ version 4.0 Image Analysis Software (LaboAmerica Inc.), which is a window image processing program. In each image; the epithelium to be analyzed was selected and separated from the rest of the field using an image brush tool. Ki-67 positive cells (brown stain) were counted using the magic wand in five randomly selected fields. The positive cells in the analyzed field were automatically counted and the proliferative index was expressed as a ratio of the cell nucleus positivity for Ki-67 per number of cells counted in the five fields, which ranged from 300 to 500. The results were expressed for each case as labeling indices for Ki-67 immunostaining (Ki-67-Li).

### Statistical analysis

Bcl-2 immunopositivity and Ki-67-Li were analyzed using the Chi-square test and paired t-test. *P* values of less than 0.05 were considered to indicate the statistical significance.

## RESULTS

Out of 40 pericoronal tissues included in the study, 27.5% were diagnosed as normal, as they were lined by reduced enamel epithelium; 72.5% of the pericoronal tissues showed squamous metaplasia, in the form of stratified squamous epithelium and/or proliferative epithelium [Figure 1] and 60% of the cases showed none-to-mild grade inflammation, while 40% revealed moderate-to-severe inflammation.

### Bcl-2 immunoexpression

Moderate-to-strong cytoplasmic staining for Bcl-2 was detected throughout the epithelial cells in 70% of the pericoronal tissues and 80% dentigerous cyst [Figure 2]. Expression of Bcl-2 was 45.45% in reduced enamel epithelium and 79.31% pericoronal tissue showing squamous metaplasia with and without proliferating rete processes [Table 1]. The positive ratio of Bcl-2 in pericoronal tissues with squamous metaplasia was slightly lower than that in dentigerous cyst but was not statistically significant.

### Ki-67 labeling index

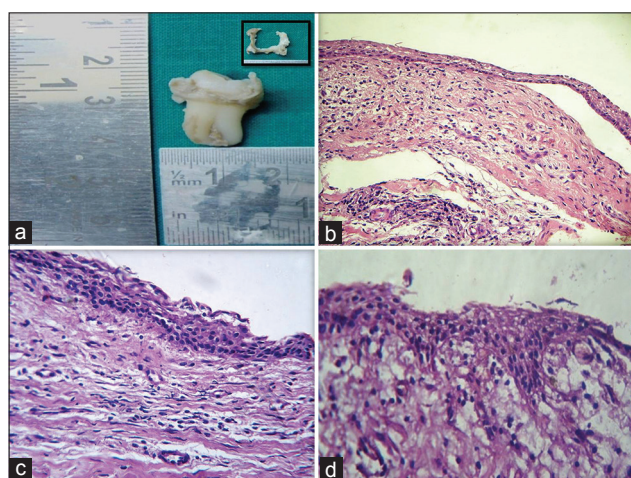
Ki-67-labeling index was statistically significantly higher ( $19.8715 \pm 1.45$ ) in pericoronal tissue showing squamous metaplasia than in reduced enamel epithelium ( $P < 0.05$ ) [Table 2, Figure 3].



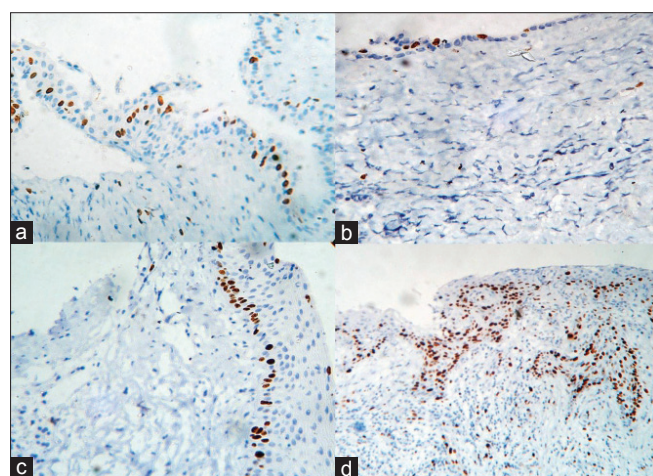
Immunohistochemical analysis for Ki-67 labeling index in normal gingiva was about  $3.006 \pm 0.76$  which when compared with the mean Ki-67 labeling index of the pericoronal tissue was not statistically higher ( $P$  value  $> 0.05$ ). The mean Ki-67-labeling index ranged  $19.8715 \pm 1.45$  in pericoronal tissues with squamous metaplasia and  $23.819 \pm 2.647$  in dentigerous cyst which was statistically non significant [Table 2, Figure 3].

### Comparison of the inflammatory changes

The mean Ki-67-labeling in pericoronal tissues with moderate to severe inflammation was significantly higher than that in pericoronal tissues with no or slight inflammation ( $P < 0.001$ ) [Figure 4].



**Figure 1:** Gross specimen of the pericoronal tissue (a) Photomicrographs showing reduced enamel epithelium ( $\times 10$ ) (b) squamous metaplasia ( $\times 20$ ) (c) squamous metaplasia (epithelium with proliferative rete processes) (d) (H and E original magnification  $\times 40$ )



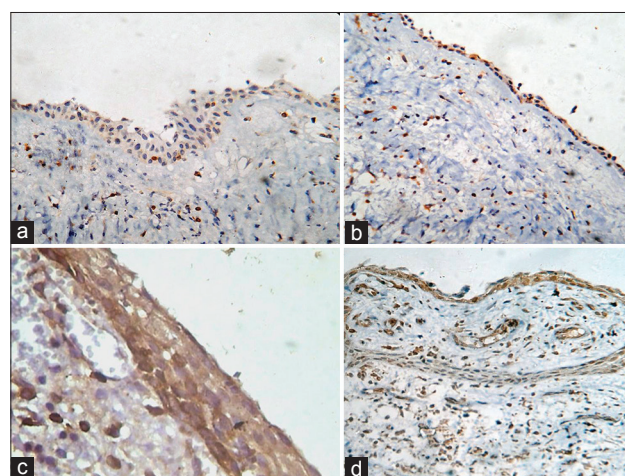
**Figure 3:** Immunohistochemical nuclear staining of Ki-67 in a dentigerous cyst (a) ( $\times 20$ ) REE (b) ( $\times 10$ ) squamous metaplasia of pericoronal tissue (c) ( $\times 40$ ) (d) ( $\times 20$ )

The Ki-67 labeling index of the none-to-mild inflammatory changes was around  $12.08 \pm 4.8$

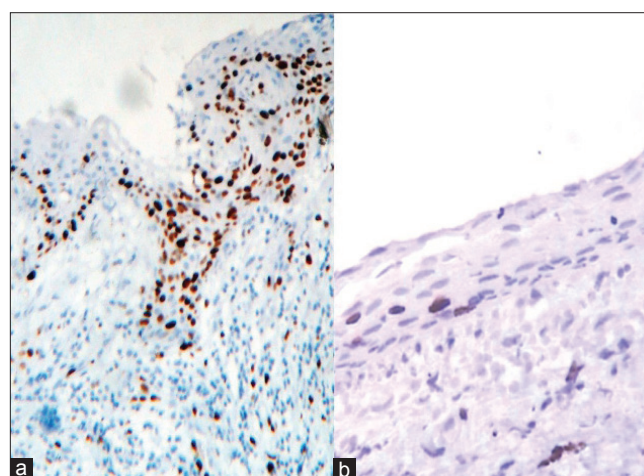
**Table 1: Correlation between the histopathological and immunohistochemical findings in the dental follicles**

Groups	No of samples	Bcl-2 positivity	Ki-67 Li
Normal pericoronal tissue	11	45.55%	7.15 $\pm$ 1.67
PT Squamous metaplasia	29	79.31%	19.87 $\pm$ 1.45
Dentigerous cyst	20	80%	23.81 $\pm$ 2.64
Normal gingiva	20	40%	3.006 $\pm$ 0.76
Grades of inflammation in dental follicles			
None-to-slight	24	66.6%	12.08 $\pm$ 4.8
Moderate-to-severe	16	68.75%	20.58 $\pm$ 3.8

PT: Pericoronal tissue; Grades of inflammation: Ki-67Li:  $< 0.0001$ ; Bcl-2:  $> 0.8796$  (NS)



**Figure 2:** Immunohistochemical cytoplasmic staining of Bcl-2 in a dentigerous cyst (a) ( $\times 20$ ) REE (b) ( $\times 10$ ) squamous metaplasia (c) (X40) (d) (Immunostaining; original magnification  $\times 10$ )



**Figure 4:** Ki-67-Li in the pericoronal tissue in moderate-to-severe inflammation (a) and pericoronal tissue with slight inflammation (b) (Immunostaining; original magnification  $\times 40$ )

**Table 2: Comparison of P values between immunostaining of Ki-67 Li and Bcl-2 positivity in different groups**

Groups	Value of 't statistic for Ki-67 labeling index	Ki-67 Li P values	Bcl-2 positivity P values
Normal DF vs. PT squamous metaplasia	2.900323099	<0.05	0.0001
Normal DF vs. normal gingiva	0.957197379	>0.05	0.2837
PT with squamous metaplasia vs. normal gingiva	2.938677066	<0.05	0.0001
Normal PT vs. PT squamous metaplasia	1.88550806	<0.05	0.0001
Normal PT vs. dentigerous cyst	2.579644014	<0.05	0.0001
PT with squamous metaplasia vs. dentigerous cyst	0.237633767	>0.05	0.5000

PT: Pericoronal tissue

and of the moderate-to-severe inflammatory changes was  $20.58 \pm 3.65$ , which was statistically significant [Table 1, Figure 4]. On the other hand there seems to be no effect of inflammation on Bcl-2 immunopositivity in all the tissues.

## DISCUSSION

Controversy exists over the prophylactic removal of asymptomatic impacted teeth. Totally impacted third molars were seen in around 30% of the cases.<sup>[11]</sup> A National Institutes of Health Consensus Development Conference on the Removal of Third Molars, in 1979, developed criteria for the treatment of impacted teeth once pathosis had developed; but no consensus was reached on the question of asymptomatic impacted teeth.<sup>[12]</sup> The presence of pericoronal tissue adjacent to impacted teeth would occasionally lead to the development of cysts and tumors. However, the mechanisms underlying these changes had not yet been explained, but could reflect an imbalance between cell proliferation and apoptosis. In the present study; we analyzed the proliferation state of the pericoronal tissue by assessing the distribution of Ki-67 Li and Bcl-2 immunopositivity and comparing it with a dentigerous cyst, to ascertain if pericoronal tissues could be predictors of progression to odontogenic cysts and tumors.

Out of 40 pericoronal tissues included in the study, 11 cases (37.5%) were diagnosed as normal, as they were lined by reduced enamel epithelium and 29 cases (72.5%) of pericoronal tissues showed

squamous metaplasia, which were in the form of stratified squamous epithelium and proliferating epithelium. This finding was consistent with that of the previous studies, which reported 34 to 70% squamous metaplasia in the lining of the pericoronal tissue associated with impacted third molars, surrounded by pericoronal radiolucency of less than 2.5 mm.<sup>[2,4,13-16]</sup>

Cyst formation, like the development of neoplasms, involved a dysregulation of balance between cell proliferation and cell death.<sup>[17]</sup> Our results demonstrated that overexpression of Bcl-2 was found in the epithelial cells of the pericoronal tissues, with squamous metaplasia, as well as a dentigerous cyst, suggesting that anti-apoptotic reaction was suppressed in both.<sup>[4]</sup> Furthermore, this apoptosis inhibitory agent might function as a pathogenetic factor in pericoronal tissue with squamous metaplasia and could lead to the formation of a cyst. Although the role played by apoptosis in the pathogenesis of cysts in the jaws was not extensively investigated; the apoptotic outcome of the cell was largely determined by the interactions among the Bcl-2 family members.<sup>[18]</sup> Bcl-2 protects cells, by blocking post-mitotic differentiation from apoptosis, maintained a stem cell pool.<sup>[19]</sup> In the present study, Bcl-2 were detected in some basal cells of gingival stratified squamous epithelium, but statistical significance was not noted between the expression of Bcl-2 in the gingival and pericoronal tissue. Therefore, our results suggested that overexpression of Bcl-2 helped in maintaining a fixed thickness of the lining epithelium in cooperation with cellular proliferation. This would explain why pericoronal tissues with squamous metaplasia could be considered to be pathological. On the contrary, Edamatsu, *et al.* (2005)<sup>[4]</sup> conducted a study to clarify the possible role of apoptosis-related factors in pericoronal odontogenic tissues and dentigerous cysts associated with impacted third molars of the mandible and found Bcl-2 expression to be lower in the pericoronal tissue than that of the dentigerous cyst. This difference could be attributed to many factors, such as, difference in staining methodology and also the fact that both completely as well partially impacted third molars were included in their study.

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase; the antigen can be exclusively detected within the nucleus; whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the



Ki-67 protein is present during all active phases of the cell cycle [G(1); S; G(2); and mitosis]; but is absent from resting cells [G(0)]; makes it an excellent marker for determining the so-called growth fraction of a given cell population. Therefore; detection of Ki-67 is considered a reliable marker of cellular proliferation.<sup>[20]</sup>

In the present study; positive nuclear staining of Ki-67 was automatically measured under the same magnifications and light settings; providing an accurate objective and quantitative analysis of Ki-67 immunoexpression. Using the computer-based image analysis; we were able to detect even scant immunoreactivity and obtain accurate calculations of positive nuclear staining.

The results of the study revealed that the Ki-67 labeling index (Ki-67 Li) was significantly higher in dentigerous cyst when compared with pericoronal tissues. On the contrary, Ki-67 Li of the gingiva was lower than that of the pericoronal tissue. However; Ki-67 Li of the pericoronal tissue with squamous metaplasia showed no statistical significance to the proliferative index of the dentigerous cyst, suggesting greater proliferative activity in the pericoronal tissue. This greater proliferative activity in the pericoronal tissues appears to indicate an epithelial lining with intrinsic growth potential. The predominant Ki-67 activity and Bcl-2 expression in the pericoronal tissue with squamous metaplasia, together with increased epidermal growth factor receptor (EGFR)<sup>[21]</sup> and p63<sup>[22]</sup> expression shown in the previous studies, suggest a unique proliferative and differentiation process in the pericoronal tissue, with squamous metaplasia. Proliferating cell nuclear antigen (PCNA) uptake in the pericoronal tissue of the third molar was studied by Adelsperger, who concluded that PCNA uptake in the cystic specimen of the pericoronal tissue was more than the normal tissue, suggesting that the squamous metaplasia could lead to cyst formation.<sup>[2]</sup>

Cabbar F *et al.*<sup>[14]</sup> studied 59 dental follicles and reported 55.9% squamous metaplasia, 15.3% glandular epithelium, and 33.9% inflammation in the dental follicle; expression of both proliferation markers in the basal epithelial cells, mucous, and squamous epithelium; and inflammatory cells were found to be statistically significant in their study. On the basis of these observations they concluded that pericoronal tissues of the asymptomatic third molars were actively proliferating, and impacted teeth may be prophylactically removed.

However; in our study 40% of the cases showed inflammatory changes indicating a potential prepathological stage and the Ki-67 Li increased with increasing inflammatory changes. In the presence of stimulation, the response of the epithelial lining of the pericoronal tissue could be faster, suggesting that the initiation of the most common odontogenic lesion, such as the dentigerous cyst, was related to the proliferation of the lining of the pericoronal tissue. This was consistent with other studies in the literature. Damante, *et al.*<sup>[23]</sup> observed about 36.1% inflammation in the follicles they examined. It was also suggested that the pericoronal tissue proliferated in response to inflammation and could result in cystic formation. It would also seem that the inflammation observed in the mesenchymal components of the pericoronal tissue upregulated the cell turnover of the odontogenic epithelium and led to proliferation. Cellular kinetics of the pericoronal tissue or its pathological change into the cyst might also depend on the mode of inflammatory changes.

Both proliferative activity and anti-apoptotic activity in the pericoronal tissues with squamous metaplasia were greater than those in the pericoronal tissues with reduced enamel epithelium. These results also indicated that proliferative activity and anti-apoptotic activity in the lining of the epithelium of the pericoronal tissue had different characteristics from those in the normal stratified squamous epithelium of the gingiva. The cellular kinetics of our data might suggest an increased growth potential of the cells or turnover of the lining epithelium, which might also be related to the grade of inflammatory changes.<sup>[20]</sup>

Finally; it can be concluded that asymptomatic pericoronal tissue associated with impacted teeth shows a high rate of squamous metaplasia, and the expression of Ki-67 and Bcl-2 is affected by the structure of the lining epithelium and degree of inflammatory change. Therefore, the pericoronal tissue of the asymptomatic third molar may be actively proliferating and because of the changes seen in the epithelial lining of the pericoronal tissue in our study, we recommend careful histological examination and further immunochemical investigations.

## CONCLUSION

In conclusion, the finding of the study implies that the absence of radiographic features is not necessary to reflect absence of the disease. Expression of the

anti-apoptotic and proliferative markers is most likely modulated by the morphological characteristics of the epithelial components as well as inflammatory changes. The results obtained and the inferences of our study can contribute toward a better understanding of the mechanism underlying the development of odontogenic cysts. However; study on larger samples of the pericoronal tissue, with other immunomarkers, such as Bcl-2 and its other family members like Bax, Bad, Bab, p53, PCNA, ssDNA, EGF, and the *MIB1* gene may provide accurate assessment of the cell surveillance and cellular kinetics of the epithelium of the pericoronal tissue.

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