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IMMUNOHISTOCHEMICAL EVALUATION OF BCL-2 AND KI-67 IN BUCCAL MUCOSA OF INDUCED-IMMUNOSUPPRESSED ALBINO RATS

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ABSTRACT

Introduction: Organ transplantation provides life-saving therapy for patients with end-organ disease. Tacrolimus is an immunosuppressive drug used to lower the risk of organ rejection. This study was performed to investigate the immunohistochemical expressions of Ki-67 (a proliferation associated antigen) and Bcl-2 (antiapoptotic protein) and their correlation with the increased risk of development of neoplasms in the buccal mucosa of tacrolimus-induced immunosuppressed albino rats.

Material & methods: Twenty adult male albino rats were divided into two equal groups. Group one treated with tacrolimus in a daily oral dose 0.5 mg/kg for 3 months. Group two served as negative control, received distilled water in comparable volume. Tissue samples were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin to evaluate histopathological finding. Other sections were used to reveal the immunohistochemical expression of Ki-67 and Bcl-2 antibodies through computerized image j analysis software. The expression of both markers was analyzed statistically through using Mann-Whitney test.

Results: Histopathological finding showed moderate dysplasia in tacrolimus-treated group. There was marked increase in normal mitotic figures, basal cell hyperplasia, loss of basal cell polarity, swirling of the spinous layer, prominent nucleoli, hyperchromatism, and altered N/C ratio. Immunohistochemical finding revealed marked increase in the nuclear expression of Ki-67 in basal cells and cytoplasmic expression of Bcl-2 in all layers of keratinocytes in tacrolimus-treated group. Statistical finding in tacrolimus – treated group showed significant difference in the expression of Ki-67 and highly significant difference in the expression of Bcl-2.

Conclusion: There is an obvious relation between prolonged systemic use of tacrolimus and development of premalignant lesions. The marked increase in Ki-67 and Bcl-2 expression may play a role in tumorgenesis.

Key words: Immunosuppresive – Tacrolimus- Buccal mucosa- Dysplasia, Ki-67, Bcl-2

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INTRODUCTION

All organ transplant patients' receive immunosuppressive drugs. Many oral side effects to the immunosuppressive medication as infections, gingival overgrowth, oral ulcerations, oral chronic graft-versus host disease (cGVHD) and risk of developing oral cancers were recorded.' Calcineurin inhibitors (CNIs), as tacrolimus and cyclosporine, are immunosuppressive drugs which are used to prevent rejection of transplanted organs. Calcineurin, a ubiquitous enzyme, plays an importance role in regulation of cellular processes. Tacrolimus, FK506, is a macrolide immunosuppressant produced by *Streptomyces Tsukubaensis*. It inhibits the phosphatase activity of calcineurin through binding to the intracellular FK-binding protein 12 kDa (FKBP12) immunophilins. The immunosuppressant/immunophilin complex binds to calcineurin and inhibits its activity. ' The nuclear factor of activated T cells (NFAT) is a transcription factor. If dephosphorylation occurs, it is translocated from cytoplasm to the nucleus and activates the transcription of several cytokines mandatory for initiating an immune response as interleukin (IL)-2 and interferon (IFN)-γ. Tacrolimus inhibit the action of NFAT that lead to a diminished inflammatory or reactive response.' It is 10-100 times more potent than cyclosporine through inhibition of mast cell and basophil mediators.

Proliferation is considered to be a fundamental biological process because of its role in growth and preservation of tissue homeostasis. The transition from normal oral epithelium to dysplasia and malignancy is represented by increase cell proliferation. Ki-67 nuclear protein is a good marker to measure the growth fraction of given cell population. It is present in all active phases of a cell cycle (G1, S, G2, and mitosis), but absent in resting phase (G0). The fraction of Ki-67 positive cells is correlated with the clinical course of a disease. Apoptosis means programmed cell death which regulated by several sets of genes through inducing pro-apoptotic members as Bax or inhibiting anti-apoptotic members as Bcl-2., B-cell lymphoma 2 (Bcl-2) is a proto-oncogene which has chromosomal translocations involving chromosomes 14 and 18 in follicular lymphomas. Bcl-2 product is a 26-kDa protein which is a component of the nuclear envelope, endoplasmic reticulum, and the outer mitochondrial membrane, encoded in chromosome 18q2.' Bcl-2 protein is one of the most prominent anti-apoptotic proteins which contribute to cancer development. Disturbances of proliferation and apoptosis are useful in characterizing tissue which histologically normal but at high-risk for neoplastic growth. Thus, according to the previous findings it would be valuable to investigate any histopathological changes in the buccal mucosa of tacrolimus-induced immunosuppressed albino rats and the expressions of Bcl-2 of and Ki-67 immunohistochemically.

MATERIAL AND METHODS

Drug preparation: The immune suppressive drug "Tacrolimus" was purchased from Novartis Company which supplied in tablets form with concentration 1mg /tab. These tablets were dissolved at distilled water to a concentration of 0.05 mg/ml.

Experimental Design: Twenty healthy adult male albino rats were obtained from laboratory animal colonies, Tudors Bilhars Institute, Imbaba, Egypt. The average body weight was 150 grams. The rats were caged in specially cages in the animal house of Faculty of Dentistry, Suez Canal University, Ismaillia. The rats were supplied with standard natural diet and tap water *adlibitum* throughout the whole experimental period which lasted for three months. They were maintained under good ventilation. The animals were divided into two equal groups, ten animals each as followed: **Group 1:** served as treated group which received Tacrolimus dissolved in distilled water in a daily

oral dose of 0.5 mg/kg body weight using a curved metallic oro-pharyngeal tube for 3 months. **Group 2:** served as control group which received distilled water in comparable volume to treated group with the same route of administration. At the end of the experiment, the rats were euthanized by cervical dislocation.

Tissue processing and staining: Tissue samples were fixed in 10% formalin solution, embedded in paraffin, sectioned into 5μm at the central region of each specimen to obtain maximum standardization. These sections were mounted and stained with hematoxylin and eosin for light microscopic examination to evaluate its histopathological presentation, then photographed by E-330 Olympus tdigital camera. Grading of epithelial dysplasia was carried out according to Banoczy and Sciba and modified by El-Dakhakhny et al.

For immunohistochemical (IHC) evaluation, 5μm sections were cut and mounted on positively-charged slides. The immunostaining were performed using rabbit polycolonal Mouse antibody to Ki-67, purchased from Thermo Fisher Scientific, Cat. No. RB-1510 with brown nuclear expression. The other rabbit polycolonal Mouse antibody to bcl-2 purchased from Gene Tex International Corporation, Cat. No. GTX100064 with brown cytoplasmic expression. The steps of IHC were followed according to manufacturer's instructions.

Digital image analysis: Image analyzer computer system (image J / Fiji 1.46) was used. The area of the screen was measured by digitizing the slides under 400X objective magnification. Computerized image analysis software was used to count the number of immunopositive cells as well as the number of the remaining unstained ones. The fraction of the positive cells for each marker was calculated.

Statistical analysis: Microsoft excel 2013 was used for data entry and the statistical package for social science (SPSS) version 24 was used for data analysis. Simple descriptive statistics (median ± standard deviation) used for summary of skewed quantitative data and frequencies used for qualitative data. Mann-Whitney test was used to compare non-normally distributed quantitative data. Differences were considered statistically significant when $P<0.05$.

RESULTS

Microscopical features in rat's buccal mucosa of the control group showed structural integrity of keratinized stratified squamous epithelium. The basement membrane showed few or short folding with normal underlying lamina propria. (***figure 1-a***). In tacrolimus treated group, the surface epithelium showed focal areas of hyperplasia, hyperkeratinization, and broad shaped rete ridges with areas of variable degrees of dysplasia as basal cell hyperplasia, loss of basal cell polarity, marked increase of normal mitotic figures, swirling of the spinous layer (***figure 1- b)*** prominent nucleoli, hyperchromatism, and altered N/C ratio (***figure 1-c,d***). Other areas revealed marked atrophy in surface epithelium with intense inflammatory cells in the connective tissue (***figure 1-e***), mucous salivary glands in submucosa layer showed cystic formation in the acini in addition to atrophy of the duct lining (***figure 1-f***).

For (IHC) evaluation, Ki-67 expression (brown nuclear staining) in normal control tissue appeared only in few areas in the basal cell layers of epithelium (***figure 2-a*****),** while samples treated with tacrolimus revealed heavy expression of Ki-67 in basal and para-basal layers of epithelium (***figure 2-b***). Bcl-2 expression (brown cytoplasmic staining) in normal tissue appeared localized to the basal and para-basal cell layers of epithelium (***figure 2-c*****),** while samples treated with tacrolimus revealed heavy expression of Bcl-2 in all layers of keratinocytes(***figure 2-d***). Tacrolimus group exhibited obviously higher expression of Bcl-2 positive cells in the connective tissue rather than Ki-67 positive cells.

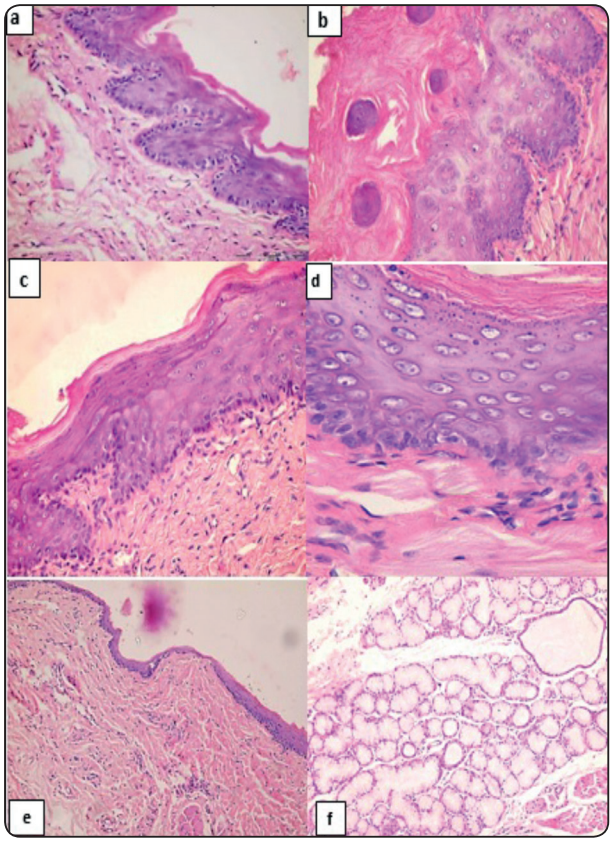

Fig. 1: Photomicrograph showing: a) normal keratinized stratified squamous epithelium with normal underlying lamina propria (H&E 200x). Buccal mucosa of tacrolimus treated group showing: b) swirling pattern of the spinous layer, hyperkeratinization, broad rete ridges and prominent nucleoli (H&E 400x). c, d) loss of basal cell polarity, basal cell hyperplasia, altered nuclear cytoplasmic ratio, with marked increase of normal mitotic figures. The lamina propria has moderate inflammatory cells (H&E 400x- 1000x). e) marked atrophy in surface epithelium with inflammatory cells in the connective tissue stroma (H&E 200x). f) cystic transformation in the mucous acinin of buccal salivary glands in addition to duct atrophy with stagnation of the secretions (H&E 400x).

For statistical results evaluation (**table 1**): Regarding Ki-67, there was a statistical significant difference (P-value =0.003) between tacrolimus-treated group [65.292 ± 116.500] and control group [0.040 ± 0.045]. Regarding Bcl-2, there was a high statistical significant difference (P-value <0.001) between tacrolimus-treated group [90.998 ± 47.750] and control group [17.569 ± 2.986]. ***Figure 3***

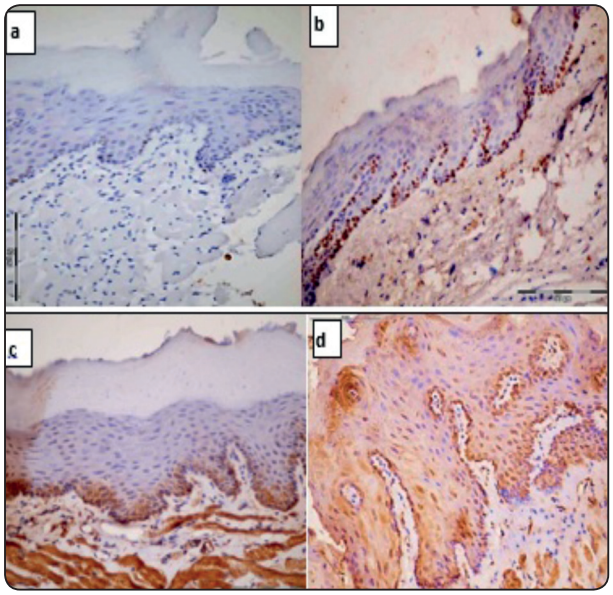

Figure 2: Photomicrograph showing: a) no or scattered nuclear expression of Ki-67 in the control group. b) heavy nuclear expression of Ki-67 limited to basal and parabasal layers in the tacrolimus - traeted group. c) showing heavy cytoplasmic expression of Bcl-2 limited to basal and parabasal layers in the control group . d) showing heavy cytoplasmic expression of Bcl-2 in all layers of keratinocytes in the tacrolimus - traeted group.

TABLE (1) Mann-Whitney test

Group	N	Median ± (SD)	P value
KI control	10	0.040 ± 0.045	0.003
KI treated	10	65.292 ± 116.500	
Bcl-2 control	10	17.569 ± 2.986	<0.001
Bcl-2 treated	10	90.998 ± 47.750	

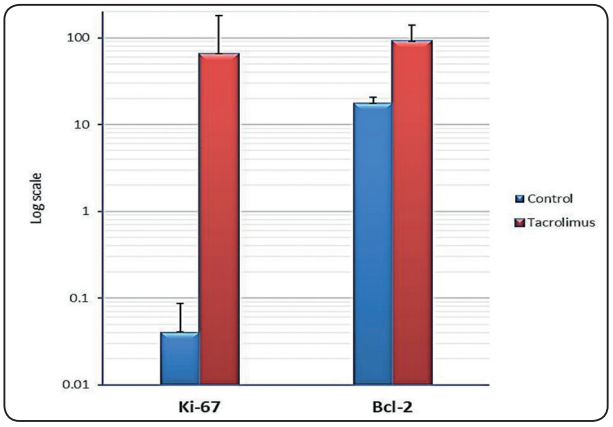

Fig. (3) Showing the median score of Ki-67 and Bcl-2 expression in control (blue bar) and tacrolimus-treated (red bar) group

DISCUSSION

Oral mucosa is lined by stratified squamous epithelium (SSE), this stratification is due to cell proliferation and sequential differentiation. Oral epithelial dysplasia is an alteration in the cellular maturation and proliferative activity of the supra-basal layers that helps to establish a more objective diagnosis. The microscopically results of the present study in tacrolimus-treated group showed moderate signs of dysplasia as epithelial hyperplasia, hyperkeratinization, broad drop shaped rete ridges, basal cell hyperplasia, loss of basal cell polarity, marked increase of normal mitotic figures, swirling of the spinous layer, prominent nucleoli, hyperchromatism, and altered N/C ratio. Other areas revealed marked atrophy in surface epithelium with intense inflammatory cells in the connective tissue stroma.

Calcineurin inhibitors (CIs) are considered one of the potential mechanisms of carcinogenesis due to their direct effect on keratinocytes. It leads to inhibition of DNA repair and evading from apoptosis in healthy human epidermal keratinocytes. Systemic administration of CI is highly associated with increase rate of lymphomas, melanomas and non-melanoma skin cancers due to systemic absorption. The long-term use of immunosuppressive drugs puts the patients at risk to develop cancers. After organ and bone marrow transplantation, the incidence of epithelial dysplasia, squamous cell carcinomas, basal cell carcinomas, Kaposi's sarcoma has been reported to be 10% after 10 years, 40% after 20 years post-transplant. After hematopoietic stem cell transplantation, the incidences of secondary solid tumors are increase over time as squamous cell carcinomas. After renal transplantation, the most frequent oral cancer is lip cancer, making up to 1.5-8% of all *de novo* neoplasms. Other study demonstrated that verrucous hyperplasia, dysplasias, ulcerations, crusting, papillary lesions, and invasive carcinomas were appeared after allogenic hematopoietic stem cell transplantation. Secondary oral cancers are associated with higher rates of recurrence and poorer prognosis after organ transplantation.

In this study, the samples of normal mucosa showed mild positive staining with Ki-67 in the basal layers of epithelium because of physiological proliferative activity. The immunohistochemical expression of Ki-67 in tacrolimus-treated group revealed heavy expression of Ki-67 in basal and para-basal layers of epithelium. This result was online with other study revealed the significant correlation between normal oral epithelium and its progression to neoplasia. The increase of these antigens is useful biomarkers of malignant transformation in oral precancerous lesions. Ki-67 positivity increased according to the proliferative activity and degree of epithelial dysplasia. In oral squamous cell carcinoma, ki-67 positivity were located in the periphery of the tumor nests due to marked mitosis than the central areas which became highly differentiated with increase the ability to keratinize. Other study supported that Ki-67 expression is a predictive marker for oral potentially malignant diseases and prognostic tool used in grading of oral epithelial dysplasia. Maximum difference was observed between low risk and high risk groups between basal and supra-basal layers when compared to control group. The increased proliferation in premalignant oral epithelium is likely related to increase oncogenic events and the risk of developing multiple tumors.

In the current study, the immunohistochemical expression of Bcl-2 in control group showed heavy expression limited to basal cell layer of epithelium. This result was in agreement with Abou Elkhier et al who detected Bcl-2 as scattered positive cells in the basal cells of normal gingival epithelium. This result reflected the progenitor cell role of basal cells, which require evasion from apoptosis to ensure survival of the entire epithelium. Its absence

in the supra-basal layers indicated that Bcl-2 is not required during completion of the differentiation process. The immunohistochemical expression of Bcl-2 in tacrolimus-treated group in the present study revealed heavy expression in all layers of keratinocytes. This result was in consistent with Ter Harmsel et al who documented that the significant increase of Bcl-2 protein in the higher degrees of cervical intraepithelial dysplasia which indicate high proliferative capacity of epithelium. It is one of fundamental importance in the development of cervical carcinoma through protection of epithelial cells from apoptosis. Other study confirmed that impairment of apoptosis is a critical step in tumor development. Another in vitro study caused a significant decrease in apoptosis through the intrinsic and extrinsic pathways. This study suggested that the evading from apoptosis might play a marked effect than the increase in cell proliferation. Bcl-2 prevents apoptosis through inhibition of cytochrome c translocation and prevention of caspase activation that lead to downstream events in apoptosis. Juneja et al suggested that any alterations in the expression of Bcl-2 protein, creating a favorable environment for malignant transformation and genetic aberrations.

Overall, the tacrolimus group in this study exhibited obviously higher expression of Bcl-2 positive cells rather than Ki-67 positive cells. This result in consistent with Batista et al who observed higher expression of Bcl-2 in cyclosporine - treated group suggested that the gingival overgrowth was due to inhibition of keratinocytes apoptosis rather than increase proliferation. Bulut et al was on line with this suggestion using TUNEL technique revealed lower rate of apoptosis in the group had gingival overgrowth when treated with cyclosporine.

Analysis of Ki-67 and Bcl-2 in the connective tissue of tacrolimus treated group in this study revealed a much smaller number of immunopositive spindle-shaped cells. In agreement with these results, Bcl-2 expression was restricted to the gingival epithelium in nifedipine-induced gingival overgrowth in an animal model. Furthermore, some authors reported the absence of Ki-67 staining in fibroblasts of the lamina propria in samples of gingival overgrowth induced by calcium channel blockers. Similarly, in a study on cyclosporine-induced gingival overgrowth, it was observed that gingival fibroblasts were negative for proliferating cell nuclear antigen (PCNA).

The findings of this study suggested that immunosuppressed patients under tacrolimus may develop neoplasms. This is on line with Ananthanarayanan et al who reported that disturbances of proliferation and apoptosis are fundamental events in early carcinogenesis. It may be useful in characterizing tissue that is histologically normal but at high-risk for neoplastic growth.

CONCLUSION

There is an obvious relation between prolonged systemic use of tacrolimus and development of premalignant lesions. The marked increase in Ki-67 and Bcl-2 expression may play a role in tumorgenesis. These markers allow better understanding of the biological behavior of molecules that control cell proliferation and cell survival.

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