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Effect of Dietary and Topical Celecoxib on Expression of bcl-2, bax, c-erb-B2 and Ki67 in Carcinogen-Induced Tongue Carcinoma in Rat

[1,2]M. Sohrabi, [3]F.A. Kalati, [4]S. Vatansever, [5]M.M. Abbasi, [1]L. Roshangar, [1,6]A.A. Khaki, [4]I.M. Tuglu, [4]I. Aydemir, [7]Y. Dustar, [8]Y. Javadzadeh and [1,9]J.S. Rad

[1]Department of Anatomical Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

[2]Department of Anatomical Sciences, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

[3]Department of Oral Disease, Faculty of Dentistry, Zahedan University of Medical Sciences, Zahedan, Iran

[4]Department of Histology-Embryology, Faculty of Medicine, Celal Bayar University of Medical Sciences, Manisa, Turkey

[5]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[6]National Management Center for Health (NPMC, University of Medical Sciences), Tabriz, Iran

[7]Department of Veterinary, Pathology College of Veterinary Medicine, Islamic Azad University (Branch of Tabriz University), Tabriz, Iran

[8]Department of Pharmaceutics, Faculty of Pharmacology, Tabriz University of Medical Sciences, Tabriz, Iran

[9]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran

Abstract: The aim of the study is to determine the effect of Celecoxib administration, dietary or topical, on expression of Ki-67, c-erb-B2, bcl-2 and bax genes in rat tongue by the immunohistochemistry methods and also tdt-mediated dupt-biotin nick end labeling assay in order to explore their role in malignant transformation and the proliferation rate, apoptosis rate in tongue squamous cell carcinoma. Effects of celecoxib on tongue carcinogenesis were investigated in 40 adult male Sprague Dawley 3-3.5 months rats initiated with 30 ppm 4-nitroquinoline-1-oxide. The immunohistochemical expression of Ki-67, bcl2, bax and c-erb-B2 were also examined for analysis of the effects of Celecoxib on tongue carcinogenesis. Differences among groups were statistically analyzed with one-way analysis of variance (SPSS-13, $p<0.05$). At week 8, the incidence of tongue precancer lesions was reduced by Celecoxib and there were significant differences in the average expression of Ki-67 ($p = 0.00$), c-erb-B2 ($p = 0.01$), bax ($p = 0.02$), bcl2 ($p = 0.02$) and also in TUNEL assay ($p = 0.00$). The results suggest probably that the level of c-erb-B2, bcl-2 and bax expression could show behavior of squamous cell carcinoma in initiation phase of developing carcinoma.

Key words: NSAIDs, oral cancer, chemoprevention

INTRODUCTION

Despite of advances, oral cancer remains a major problem. Non steroid anti inflammatory drugs may be chemo preventive agents (Sudbo *et al*., 2003). Cyclo oxygenase (Cox)-2 involved in carcinogenesis, with impact on cell proliferation, differentiation, apoptosis celecoxib, a potent NSAID, is a specific inhibitor of Cox-2 (Wang *et al*., 2002). In oral cancer development, the surviving transformed cells appear to have suppressed apoptosis and a high rate of proliferation (Resnicoff *et al*., 1995; Tomei and Cope, 1994). Diverse results regarding

Corresponding Author: Jafar Soleimani Rad, [a]Department of Anatomical Sciences, Faculty of Medicine and [b]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran Tel/Fax: +98-334 20 86

the over expression of c-erb-B2 proto-oncogene in oral cancer have been reported (Mann *et al*., 2005). Since, the topical use of drugs could have fewer side effects than the systemic use, the aim of study is determining efficacy of topical celecoxib on tongue carcinoma.

MATERIALS AND METHODS

This study was conducted in joint venture among Drug Applied Research Center and Department of Anatomical Science and Department of Pharmaceutics of Tabriz University of Medical Sciences, Tabriz, Iran in collaboration with Histology, Embryology Department of Celal Bayar Medical University, Manisa, Turkey during 7 June 2007 to 7 August 2008.

Animals: A total of 40 adult male Sprague Dawley rats 3-3.5 month old were used in the experiment. All rats were obtained from Tabriz Medical University animal-house and at first quarantined and acclimatized to laboratory conditions for 2 weeks. During the experiments, each rat was housed, in a metal cage, with hardwood chips for bedding, in an air-conditioned room with a 12 h light/ 12 h dark cycle. All animals' procedures complied with an approved TUMS animal care protocol and the Tabriz University of Medical Science Animal Care and use committee.

Chemicals: The chemicals, namely, 4-NQO and CCB were purchased from Sigma Inc. (Germany) and Aldrich Inc., (Germany), respectively. 4-NQO solution (30 ppm) was prepared trice a week by dissolving the carcinogen in distilled water and was given in light-opaque covered bottles. Rats were allowed access to the drinking water at all times during the treatment except 2-3 h after adhesive gels usage. Diets containing 2000 ppm CCB were also prepared trice a week. CCB powder was added to wet powdered basal diet and then dried in oven (50°C). Each rat received 2000 ppm CCB with daily food. Adhesive Gel was prepared by mixing the base plasty with oral paste including 2000 or 3000 ppm CCB. For base plasty preparation: liquid paraffin and LDPE (low density polyethylene) that were purchased from Petrochemical Industries Co., of Tabriz, were mixed with 9.5 to 0.5 proportion. For oral paste preparation: pectin, gelatine and sodium carboxy methyl seloluse 1/1/1 were mingled. Then oral paste added 2000 or 3000 ppm CCB, mixed with Base base plasty precisely. These adhesive gels were stored in plastic containers at 4°C until using.

Experimental procedure: The rats were randomly assigned to 1 of the 4 treatment groups. These groups include group 1, 30 ppm 4-NQO treatment; group 2, 30 ppm 4-NQO+2000 ppm dietary CCB treatment; group 3, 30 ppm 4-NQO+2000 ppm topical CCB treatment; group 4, 30 ppm 4-NQO+3000 ppm topical CCB treatment.

At the end of 8 weeks, animals were sacrified under ether anesthesia. Their whole tongues were incised and then oral part (2/3 anterior) longitudinally cut into 2 slices and each of them again cut into 2 halves. After fixation of specimens in 10% phosphate-buffered formalin for 48 h prepared for light, routinely embedded in paraffin and was sectioned serially. The serial 5 μm sections were stained with H and E, Ki67 (Code No. M7248/ Dako/Denmark), c-erb-B2 (Cat No. 115/DBS/CA, USA), bcl2 (Cat No. SC-7382/Santa Cruz Biotechnology/CA, USA), bax (Cat No. SC-7480/Santa Cruz Biotechnology/ CA, USA) and TUNEL (Cat No. 11 684 817 910) staining. Immunohistochemical staining was carried out according to factory instruction. The immunoreactive cells and also TUNEL positive cells appeared brown.

Evaluation of sections: Specimens qualified blindly by 1 histologist and 1 pathologist. Routinely stained (H and E) histological sections were evaluated by using a light microscope (Olympus BX40, Tokyo, Japan). Pathological changes including dysplasia, hyperplasia, hyperkeratosis, parakeratosis, tumor like cell and pearl body existence were determined in whole specimen and were scaled on the basis of WHO criteria: as intact, mild, moderate and severe (Pindborg *et al*., 1997). Immunohistochemically stained sections (for c-erb-B2, bcl2, bax) and also TUNEL stained sections, were evaluated by using a light microscope (Olympus BX40, Tokyo, Japan). Average number of positive cells were estimated through their counting randomly in 5 different fields with 400X power fields that included 100 cells (at least 500 cells in each specimen).

Statistical analysis: The difference between Means±SE for control and experimental groups were examined by using one-way Analysis of Variance (ANOVA) and or Chi-square test. Statistical difference of p-value at the level of 0.05 or less was considered significant.

RESULTS AND DISCUSSION

Histopathological studies: Microscopy revealed that different histopathological changes such as dyskeratosis, hyperkeratosis, parakeratosis, dysplasia, tumor like cells and pearl bodies were appeared in treated groups. These changes were maximum in group 1 and decreased by CCB

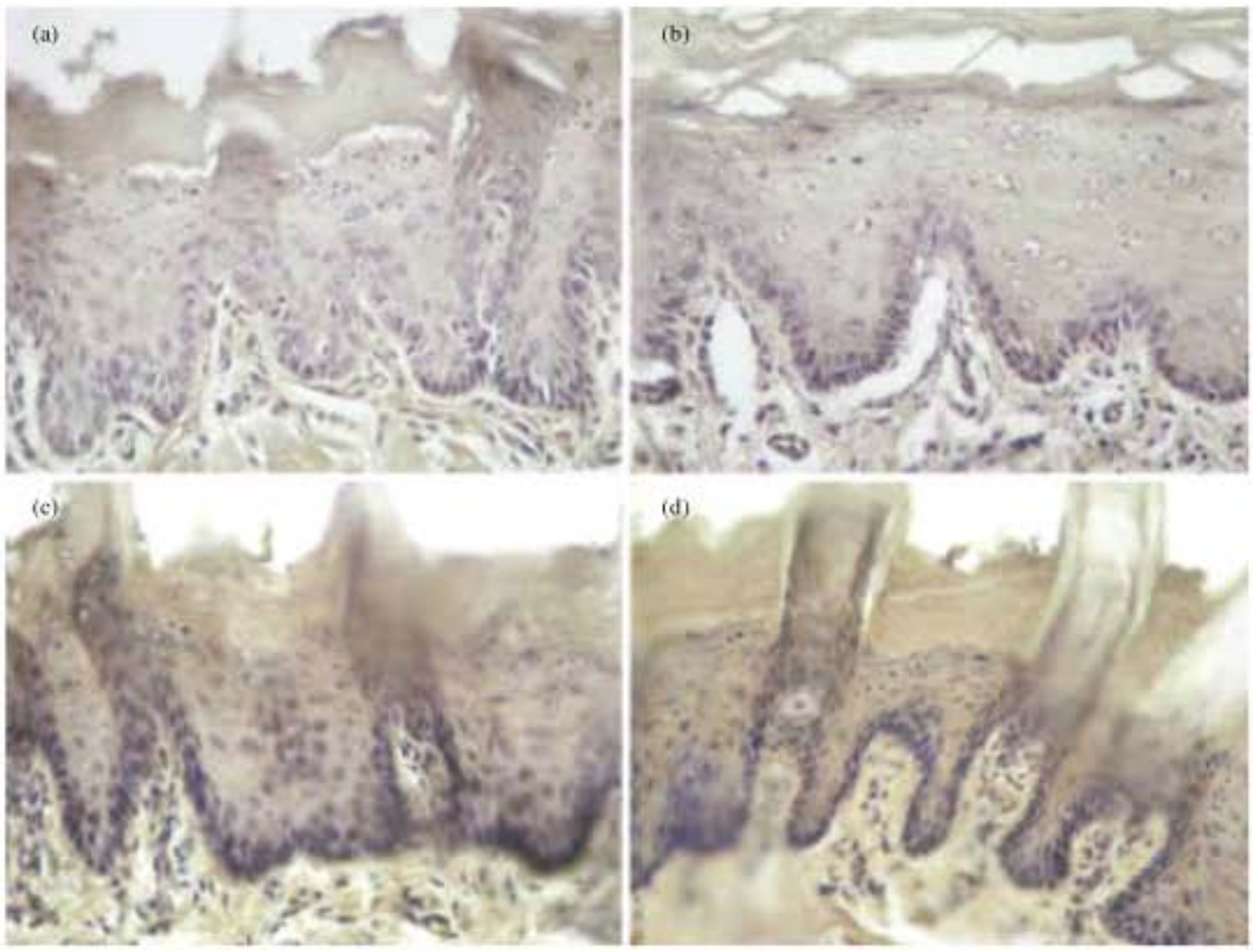

Fig. 1: Immunohistochemical staining for c-erb-B2 in different groups. Photomicrographs (400x) from tongue epithelium and underlying mucosa stained with immunohistochemical technique for c-erb-B2 in groups 1-4, (a-d)

Table 1: Different histopathological changes in treated groups

Group No.	Dys keratosis	Hyper keratosis	Para keratosis	Dysplasia	Tumor like cells	Pearl body
1	Mild	Severe	Mild-severe	Mild-severe	Moderate-severe	Mild-severe
2	Intact	Severe	Mild	Mild	Mild	Intact
3	Mild	Severe	Mild	Mild	Mild	Mild
4	Intact	Severe	Mild	Mild	Intact	Mild

treatment in groups 2-4. The changes were quantified as determining the percentages of affected cells and scaled as mild-severe according to their characteristics (Table 1).

Immunohistochemical studies: Histological sections stained with different immunohistochemical techniques are shown in Fig. 1-5. In different groups, c-erb-B2 positive cells (tumor cells) are shown in Fig. 1a-d. Ki67 positive cells (proliferative cells) are shown in Fig. 2a-d. Bcl2 positive cells (survival cells) are shown in Fig. 3a-d. Bax positive cells (apoptotic cells) are shown in Fig. 4a-d. TUNEL positive cells (apoptotic cells) are shown in Fig. 5a-d.

The results from immunohistochemical studies are shown in Table 2. As it is shown in Table 2, the percentages of c-erb-B2 positive cells were maximum in group 1 (received 4-NQO) and reached minimum in group 4 (received 4-NQO and topical CCB). Bcl2 and Ki-67 positive cells had the highest value in group 1 and the least value in group 4.

Adversely, bax and TUNEL positive cells were least in group 1 and maximum in group 4.

Table 2: Percentages of Ki-67, c-erb-B2, bcl2, bax and TUNEL positive cells in treated groups

	Group			
Staining	1	2	3	4
c-erb	14.0±33.43	7.7±26.5	9.2±22.83	5.7±16.8
bcl2	5.8±23.70	4.9±23.8	4.0±23.00	2.2±10.0
bax	8.6±14.90	3.6±17.0	9.0±18.50	13.9±27.8
Ki67	15.9±38.30	14.7±24.5	7.7±23.00	11.6±13.9
TUNEL	1.9±11.70	14.6±40.8	16.8±38.50	13.5±57.5

In the present study, the c-erb-B2 immunoreactivity was maximum in group 1, indicating that 4-NQO has acted as a carcinogen. In present study according to histopathological changes, it seems that there was a well differentiated tumor in animals that were taken 4NQO and the histological and cytological features were not very severe.

Many of the molecular alterations that cause abnormal biologic behavior of cancer cells are based on aberrations of cell cycle regulation. It would be necessary to discover more reliable and efficient markers to characterize the malignant transformation of oral epithelia. The Epidermal Growth Factor Receptor (EGFR), a product of the c-erb oncogene, is over expressed in the development of certain epithelial neoplasms early in the development of oral cancer. It is also over expressed in pre malignant oral leuko plakia. Furthermore, EGFR expression has been correlated with lesion severity and proliferative capacity (Shirasun *et al*., 1991). One study

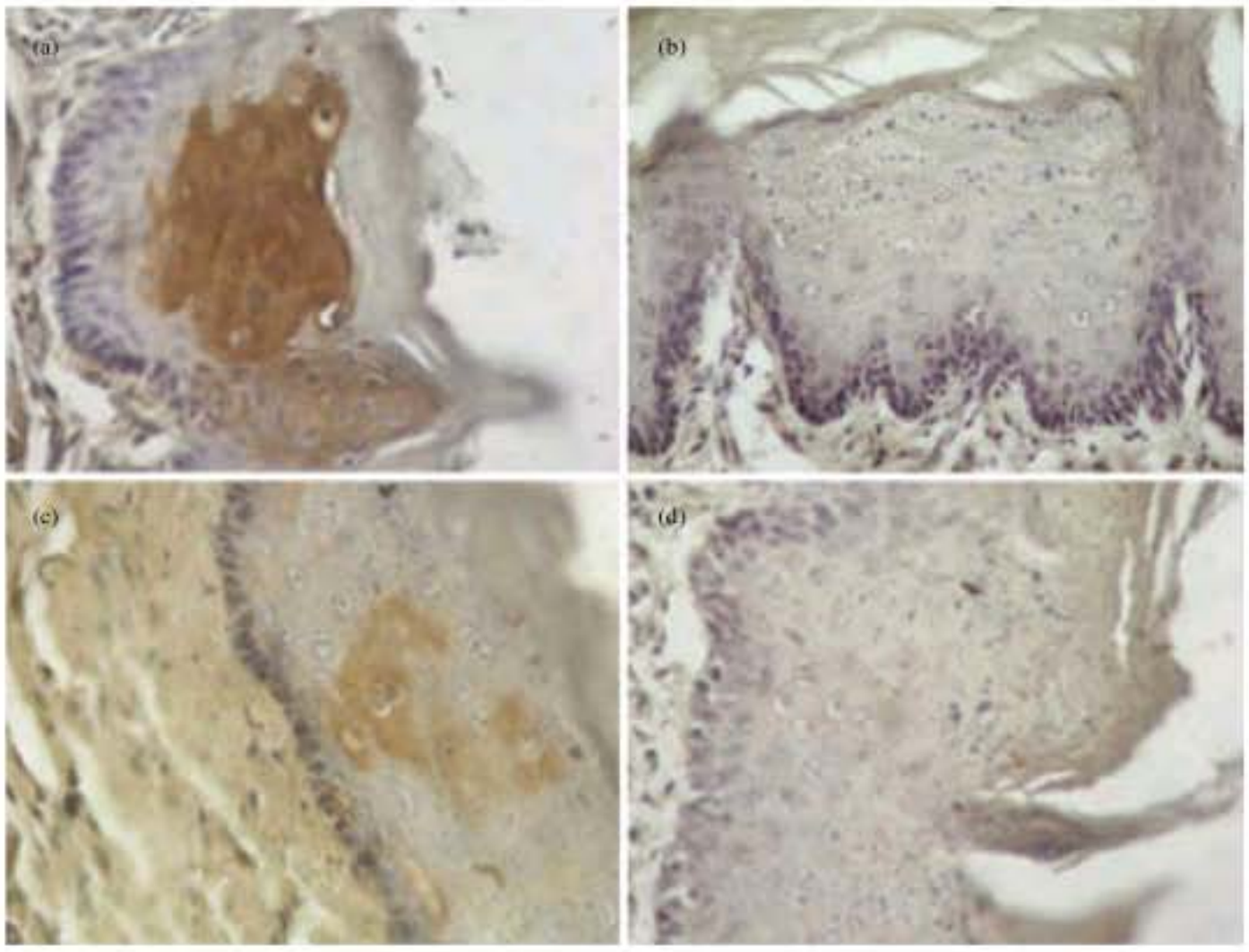

Fig. 2: Immunohistochemical staining for Ki-67 in different groups. Photomicrographs (400x) from tongue epithelium and underlying mucosa stained with immunohistochemical technique for Ki-67 in groups 1-4, (a-d)

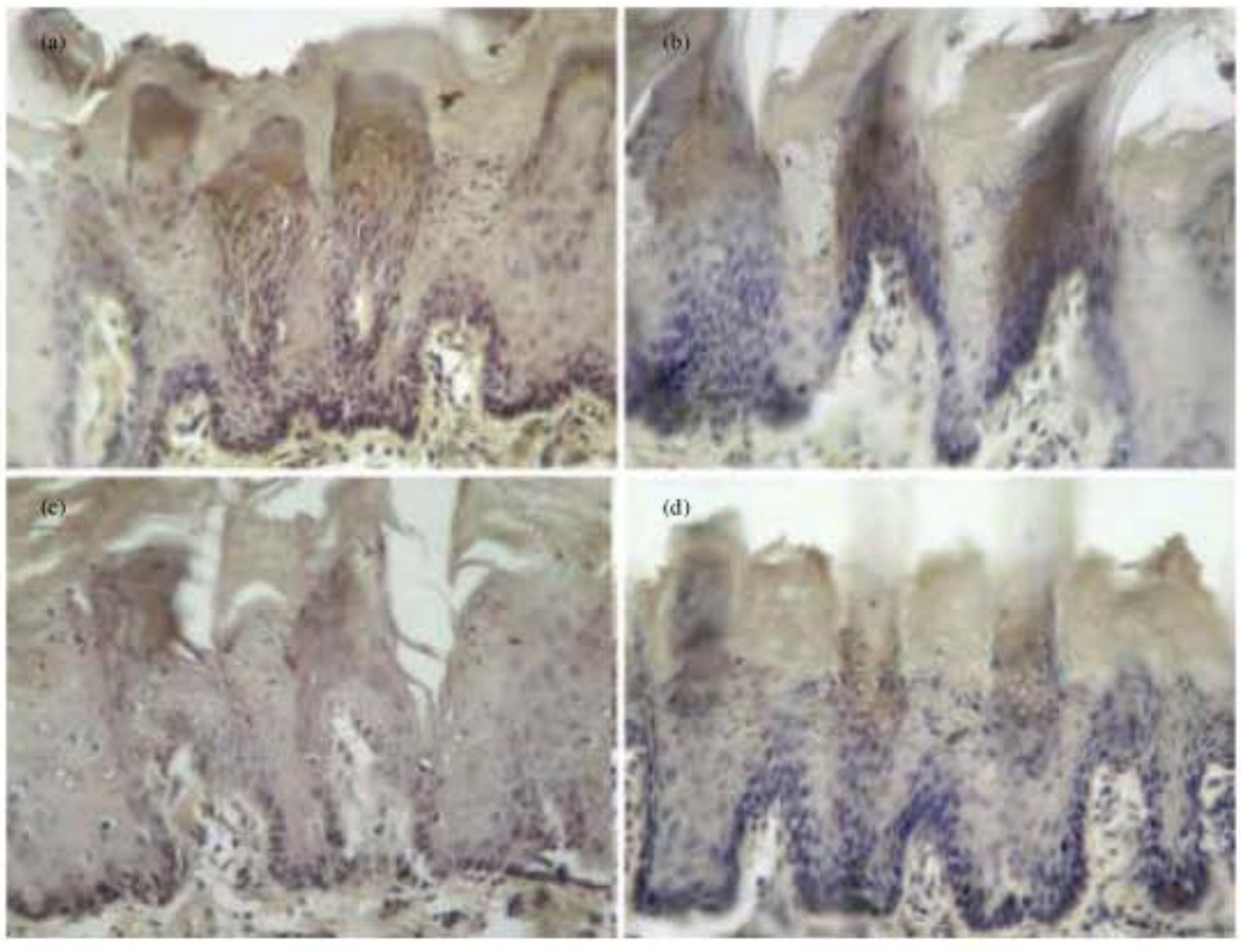

Fig. 3: Immunohistochemical staining for bcl2 in different groups. Photomicrographs (400x) from tongue epithelium and underlying mucosa stained with immunohistochemical technique for bcl2 in groups 1-4, (a-d)

concluded that changes in c-erb-B2 was associated with the proliferative and malignant phenotype (Gimenez-Conti and Slaga, 1993). Epidermal growth factor and the ligand of the EGFR induce COX-2, contributing to the increased levels of prostaglandin (PG) in premalignant and malignant cells in head and neck tumors and PG stimulates

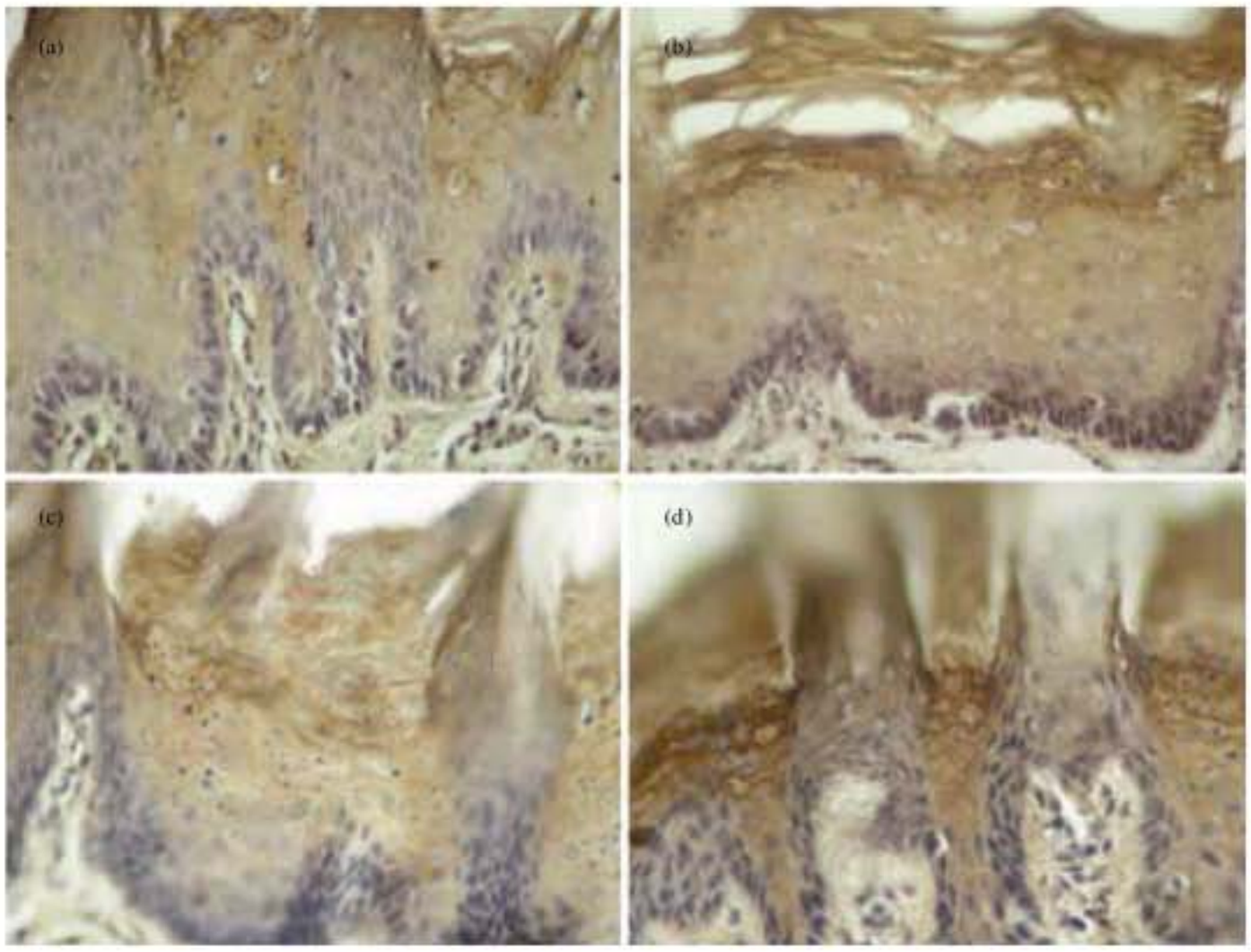

Fig. 4: Immunohistochemical staining for bax in different groups. Photomicrographs (400x) from tongue epithelium and underlying mucosa stained with immunohistochemical technique for bax in groups 1-4, (a-d)

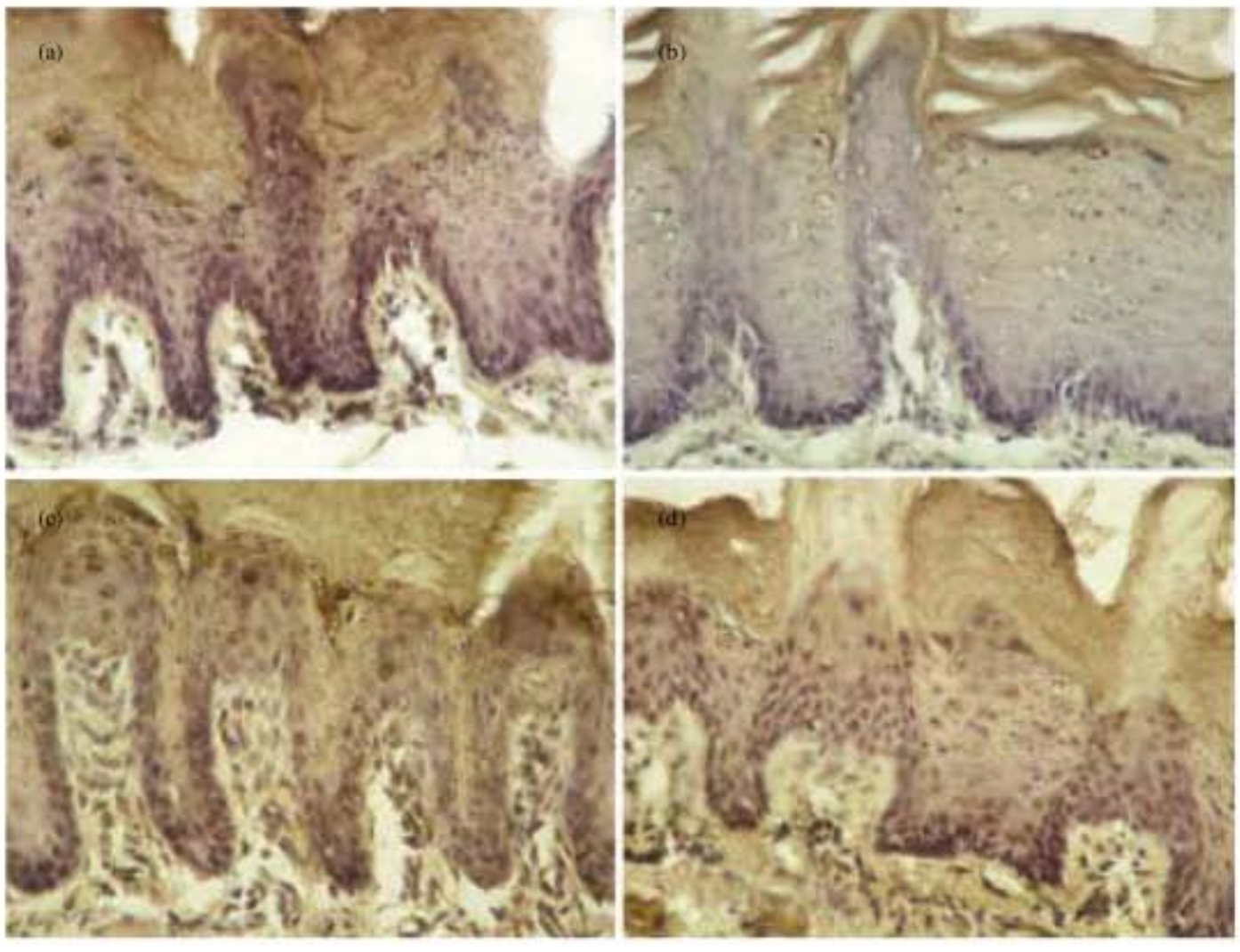

Fig. 5: Immunohistochemical staining for TUNEL in different groups. Photomicrographs (400x) from tongue epithelium and underlying mucosa stained with immunohistochemical technique for TUNEL in groups 1-4, (a-d)

epithelial cell proliferation and inhibits apoptosis (Slaughter *et al*., 1953). Epidermal growth factor receptor is over-expressed in the vast majority of oral pre malignant lesions and oral cancers and correlates with advanced stage and decreased survival. The lack of c-erb-B2 over expression was seen in many cancers, but was

controversial in OSCC from different reports. Studies of the c-erb-B2 oncoprotein have indicated that its expression is common in human head and neck cancer but does not seem to have a prognostic significance (Xie *et al*., 1999). The over expression of c-erb can be used as a marker in distinguishing normal oral tissue from OSCC.

It appears that our findings well correlate with the above reports. Other findings of this study were that bcl-2 and Ki-67 positive cells were frequent in group 1 and decreased with CCB treatment. The results indicate that 4-NQO as a carcinogen increased proliferation and suppressed apoptosis. These changes reversed by CCB treatments. It means that dietary as well as topical CCB treatment are effective chemopreventive. The above results accomplished by other series of findings showing that bax positive and TUNEL positive cells were less frequent in group 1 and increased by CCB treatment indicate that both dietary and topical CCB treatment could induce apoptosis. Furthermore, its pro apoptotic potential increases by increasing of treating dose. Present results were correlated with the findings of other researchers on expression of tumorogenic and apoptotic suppressor markers.

The dysregulation of the molecular events governing cell cycle control is emerging as a central theme of oral carcinogenesis. The cellular programs of proliferation, differentiation, senescence and apoptosis are intimately linked to the cell cycle regulatory machinery. Activated oncogenes and/or inactivated tumor suppressor genes through multiple checkpoints resulting in increased genomic instability. Inherently, the process of carcinogenesis selects against apoptosis to initiate, promote and erpetuate the malignant phenotype. Then apoptosis is arguably the most potent defense against cancer. Animal studies have provided a method for studying the multi step development of oral carcinoma. Targeting apoptosis pathways in premalignant cells, in which these pathways are still relatively intact, may be an effective method of cancer prevention. The BCL-2 (B-cell leukaemia 2) family proteins are the most important regulators of apoptosis. Oxidative stress appears to be associated with the modulation of bcl-2 family members in several cell systems (Odukoya and Shklar, 1982). During conditions of cell stress, the ratio of bcl-2 family members, proapoptotic (e.g., bax, bad and bak) to antiapoptotic (e.g., bcl-2 and bcl-XL) proteins increases. In various experimental systems, the antiapoptotic molecules bcl-2 has been found to interact with and bind to various tumor suppressors and signal regulatory molecules (e.g., apoptosis activating factor lapaf-11 ICED-41 and inhibit the intrinsic pathway (Yang and Korsmeyer, 1996). Many chemopreventive agents appear to target signaling intermediates in apoptosis-inducing pathways. Some NSAIDs are selective inhibitors of cyclooxygenase (Cox)-2. However, celecoxib can induce apoptosis through Cox-2-independent mechanisms. NSAIDs, induce apoptosis through the extrinsic pathway by increasing the expression of death ligands or death receptors but CCB could promote the production of reactive oxygen species, impair mitochondrial function (Chan *et al*., 1998) and trigger intrinsic effector mechanisms for cell degradation independent of the death receptor pathway in Jurkat T cells and induces apoptosis when bcl2 (also known as CED-9) is over expressed (Hsu *et al*., 2000). But, in one study to determine whether bcl2 over expression protects cells from celecoxib-induced apoptosis, there were essentially no differences in bcl-2 across the pre-cancer tissue compartments (Ananthanarayanan *et al*., 2004).

Some researchers used H460 cells transfected with an empty control vector (H460/V) and H460 cells transfected with a vector containing the gene for bcl2 (H460/Bcl2-6). Treatment with increasing concentrations of celecoxib did not alter the expression of endogenous (i.e., genomic) or exogenous (i.e., transfected) bcl2 protein in either cell line or CCB did not differentially alter survival of either of the 2 cell lines.

Thus, over expression of bcl2 did not appear to confer resistance to celecoxib (Jendrossek *et al*., 2003). In a study, no association between bcl-2 and the stage and grade of the tumour or patient survival was found (Atula *et al*., 1996). Researchers studied bcl-2 expression in SCC of the tongue and noted that the bcl-2 protein had a prognostic value: frequent expression of bcl-2 was associated with a decrease in the apoptotic index. Nevertheless, they did not find an association between the expression of bcl-2 and the prognosis of the patients with cancer of the tongue (Yao *et al*., 1999). Some researches indicated that the induction of bax or the inhibition of bcl-2 are commonly associated with apoptosis induction after exposure to certain chemopreventive agents (Jaeckel *et al*., 2001) bax is considered as a main effector of apoptosis. bax forms homodimers and also heterodimers with bcl-2. The function of the bax-bax dimer in active cell death is antagonized by bax-bcl-2 heterodimers. Thus, the ratio of bcl-2 and bax should control the susceptibility of cells to those stimuli that induce apoptotic cell death.bcl-2 and bax are involved in the progression of 4NQO-induced carcinoma [21]. An inverse relationship between bcl-2 and apoptosis has been reported in oral epithelial dysplasia. In a Norwegian study abundant bax expression was

related to a good prognosis in patients with cancer of the tongue. Furthermore, no correlation between bcl-2, bax or the bcl-2-to-bax ratio and the rate of apoptosis was observed (Jaeckel *et al*., 2001). It has been noted that the ratio between the apoptotic and mitotic indexes is higher in dysplasia than in carcinomas, which indicates that apoptosis is hidden because of the cell proliferation in invasive lesions (Hengartner, 2000). Over expression of COX-2 also increases the level of the antiapoptotic protein bcl-2 and may cause resistant to apoptosis in premalignant cells, resulting in the survival of damaged cells leading to tumorigenesis.

CONCLUSION

These results suggest that CCB, when given during the initiation and phase, exerts chemopreventive ability against 4-NQO-induced tongue tumorigenesis through increasing of apoptosis and suppressing of proliferation. Our results suggest that probably bcl-2 and bax expression are more reliable than the level of c-erb expression on prediction and showing the behavior of SCC in initiation phase of developing carcinoma.

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