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THERAPEUTIC POTENTIAL OF CARVACROL ON EXPERIMENTALLY INDUCED HAMSTER BUCCAL POUCH EPITHELIAL DYSPLASIA

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ABSTRACT

Abstract: The aim of the present study was directed to investigate the therapeutic potential of carvacrol on experimentally induced hamster buccal pouch epithelial dysplasia. **Material and methods**: Seventy golden Syrian male hamsters, five weeks old, weighting 80-120g were divided into three groups($G_{(s)}$) GI, GII and GIII. GI (negative control): 10 animals were left untreated. GII: dimethylbenz(a)anthracene (DMBA) induced group): 30 animals were divided into 2 subgroups; GIIA and GIIB. GIIA was painted with 0.5% DMBA in paraffin oil 3 times a week for 8 weeks.GII B was painted with 0.5% DMBA in paraffin oil 3 times a week for 14 weeks. GIII: (carvacrol treated group): 30 animals were divided into 2 subgroups; GIIIA and GIIIB. GIIIA was painted with 0.5% DMBA in paraffin oil 3 times a week for 8 weeks followed by oral administration of carvacrol (15 mg/kg) 3 times a weeks for 6 weeks. GIII B was painted with 0.5% DMBA in paraffin oil 3 times a week for 14 weeks followed by oral administration of carvacrol (15 mg/kg) 3 times a weeks for 6 weeks. **Results**: Gross observation revealed variation in reduction of the tumor size in the GIII compared to that observed in GII. Histopathological findings in GII revealed variations ranged from moderate dysplasia to well differentiated squamous cell carcinoma while in GIII ranged from mild dysplasia to well differentiated squamous cell carcinoma. Immunohistochemical results revealed that there was highly significant difference between GIIA and GIIIA regarding to Bcl-2, the p value recorded 0.001 (< 0.01) and Bax also showed highly significant difference between the same groups the p value recorded 0.001 (< 0.01). There was highly significant difference between GIIB and GIIIB regarding to Bcl-2, the p value recorded 0.001 (< 0.01) and Bax also showed highly significant difference between the same groups the p value recorded 0.001 (< 0.01). **Conclusion:** Carvacrol is a novel approach for the treatment of DMBA induced hamster buccal pouch epithelial dysplasia.

KEYWORDS: HBP carcinogenesis, carvacrol, apoptosis.

INTRODUCTION

Oral cancer is commonly preceded by premalignant lesions and conditions grouped under common terminology as potentially malignant disorders[1]. The precursor lesions that precede oral cancer are defined as an altered epithelium that shows a variety of cytological and architectural changes that have been traditionally brought under the common denominator oral epithelial dysplasia (OED) with an increased likelihood for progression to oral squamous cell carcinoma (OSCC)[2]. One of the best oral cancer prevention strategies is to treat the oral cancer at its precancerous stage to prevent its further malignant transformation[3].

The potent polycyclic aromatic hydrocarbon, 7,12 dimethylbenz(a)anthracene (DMBA), is commonly utilized asa carcinogen to induce tumors in the buccal pouches of golden Syrian hamsters[4]. DMBA can cause neoplasm by inducing severe inflammation and dysplasia in the hamster buccal pouches (HBP) in addition to causing extensive oxidative damage to deoxyribonucleic acid (DNA)[5]. Accumulated evidences pointed out that DMBA induced experimental oral carcinogenesis in the HBP produces premalignant and malignant changes that resemble premalignancy and malignancy of human oral mucosa[6]. DMBA induced HBP carcinogenesis is therefore preferred to study the tumor preventive potential of natural products and their bioactive constituents[7].

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Extensive studies highlighted the chemopreventive effects of diverse natural products[5,8]. Though several mechanisms were pointed out for the chemopreventive potential of the natural products, the pro-apoptotic properties were documented as a major mechanism[9]. Apoptosis, programmed cell death, plays a crucial role in the removal of unwanted and damaged cells from the body; B cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) have crucial role in the process of apoptosis. Deregulations in the expression of these markers are associated with abnormal proliferation[6].

Carvacrol (5-isopropyl-2-methyl phenol) is an ingredient of the essential oil obtained from origanumhirtum, wild bergamot, pepperwort, and several other essential oils that possesses antioxidant and antimicrobial activities and a particular aroma which makes it an attractive component for certain types of foods[10]. Literature reports have demonstrated that carvacrol possesses multiple biological activities including analgesic, antimicrobial, anti-inflammatory, antioxidant, and antiangiogenic properties[11]. As regard to anticancer effect, carvacrol was reported to have an anti-proliferative effect on lung, breast, and colon cancer cell lines[12–14]. Furthermore it has been shown that carvacrol treatment of OSCC cell lines causes cell growth inhibition and promotes apoptosis[15].

The main objective for the present study was directed to assessthe therapeutic potential of carvacrol on experimentally induced HBP epithelial dysplasia. The evaluation of the effect of such treatment was based on the histological tumor tissue changes and immunohistochemical (IHC) examination utilizing Bcl-2 and Bax antibodies.

MATERIAL AND METHODS

The experimental animals used in the current study were golden Syrian hamsters. They were used as a model for carcinoma induction utilizing DMBA as chemical carcinogen. Then, carvacrol treatment by oral administration, was employed. After that, various investigations including hematoxylin and eosin (H&E) stain and Immunohistochemical (IHC) staining utilizing Bcl-2 and Bax were done.

Animals:

Seventy golden Syrian male hamsters, five weeks old and weighting 80-120g, were obtained from the animal house, Cairo University (Cairo, Egypt). Hamsters were housed in show box cages at the experimental animal unit at the Faculty of Pharmacy, Boys, Cairo, AL-Azhar University (Cairo, Egypt). The controlled environment was maintained under standard conditions. The hamsters were fed and watered ad –libitum.

Material used:

DMBA (0.5%) was obtained from Sigma-Aldrich company, dissolved in paraffin oil. Carvacrol was obtained from Sigma-Aldrich company and dissolved in corn oil and administered orally (15 mg/kg bw).

Experimental design:

The experimental animals were divided into three groups ($G_{(s)}$). GI (negative control): 10 hamsters not treated and served as negative controls. GII: (DMBA induced group): 30 hamsters, the right HPBs were painted with 0.5% DMBA (Sigma Aldrich) in paraffin using a number 4 camel hair brush three times a week. Then, the animals were randomly divided into the following 2 subgroups, GIIA: (15 hamsters) served as positive controls for 8 weeks, GIIB: (15 hamsters) served as positive controls for 14 weeks. GIII (carvacrol treated group): 30animals, following DMBA-painting, carvacrol treatment started, the animals were randomly divided into the following 2 subgroups, GIIIA: (15 hamsters) the animals in this group were painted DMBA for 8 weeks followed by carvacrol treatment for 6 weeks, GIIIB: (15 hamsters) the animals in this group were painted DMBAfor 14 weeks followed by carvacrol treatment for 6 weeks.

Investigations:

After termination of the experiment, the animals were anaesthetizedby ether inhalation, and then sacrificed. After that, the cheek pouches excised and fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin blocks for preparation in order to be examined histologically and immunohistochemically.

For histological examination, the fixed specimens were dehydrated in an ascending ethanol series, embedded in paraffin wax to form paraffin blocks. Tissue sections of 4μm thickness on rotary microtome were cut, mounted on slides, processed, and stained with H&E for light microscopic examination.

For immunohistochemical examination, other tissue sections were cut for the application of standard labeled streptavidin- biotin method to demonstrate the expression of Bcl-2 and Bax antibodies. The paraffin embedded tissue sections were dewaxed and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H2O2 in methanol for 10 min. The antigen retrieval was achieved by adding citrate buffer solution (pH 6.0) and put in microwave for 3 intervals, 5 minutes each at 95°C, followed by washing with phosphate buffered saline (PBS). The tissue sections were then received one or two drops of the primary antibodies (Bcl-2 and Bax) in a dilution of 1:125and 1:100 respectively in Tris buffer solution and incubated in a humid chamber at room temperature overnight at 4°C. After washing with PBS, biotinylated secondary antibody was added and incubated for 30 min at room temperature. After rinsing with PBS, tissue sections were received diaminobenzidine (DAB) (Sigma, USA) which was applied for 2-4 minutes to develop color. When acceptable colour intensity was reached, the slides were washed, counter stained with haematoxylin and covered with a mounting medium. The immunostained sections were examined using light microscope to assess the prevalence of positive cases and the localization of immmunostaining within the tissues. In addition, image analysis computer system was used to assess area percentage of positive cells of the immunostaining. This was done in the Oral and Dental Pathology Department, Faculty of Dental Medicine, Boys, Cairo, Al-Azhar University. The degree of positive staining for the antibodies used was evaluated by a well-established semi-quantitative scoring on a scale range from negative to strong positive staining as follow: Strong staining (more than 50% stained), moderate staining (between 25 and 50% stained), weak staining (between 5 and 25% stained), and negative (less than 5% stained)[16].

RESULTS

The gross observation results of HBP mucosa of GI were pink in color with smooth surface and no observable abnormalities (Fig.1A). In GIIA, HBP mucosa showed white ruffled mucosal surface, erythematous areas and/or development of small nodules (Fig.1B). In GIIB, HBP mucosa showed multiple exophytic masses of variable sizes surrounded with area of ulceration and bleeding (Fig.1C). Debilitation of all animals was also observed. In GIIIA, HBP mucosa showed tiny elevations with no erythema (Fig.1D). In GIIIB, HBP mucosa showed multiple exophytic masses of variable sizes. The animals showed health improvement and reduction of inflammatory manifestation (Fig.1E).

Histopathological and immunohistochemical results: the tissue sections of HBP mucosa of the experimental groups showed variable results in regard to the histopathological and IHC results. In GI, the histological sections, using H&E stain, revealed normal HBP mucosa composed of keratinized stratified squamous epithelium

with no rete pegs, subepithelial connective tissue and muscular layers (Fig.2A).The IHC staining with anti Bcl-2 antibody showed mild positive expression (6.7%) and only identified in the basal and suprabasal cell layers (Fig.2B). In contrast, IHC staining with anti Bax antibody showed moderate positive expression (46.02%) in all layers of the epithelium. Both markers were detected in the cytoplasm with a granular pattern (Fig.2C). In GIIA, histological sections, using H&E stain indicated that 4 animals exhibit moderate epithelial dysplasia and 11animals exhibit severe epithelial dysplasia. The overlying epithelium displayed moderate epithelial dysplasia that is characterized by architectural abnormalities as disordered maturation, loss of polarity, basilar hyperplasia and acanthosis which causes elongation and broadening of rete ridges and cellular abnormalities as frequent mitotic figures, cellular pleomorphism, nuclear atypia and hyperchromatism. In moderate epithelial dysplasia, the previous criteria don't extend beyond the middle third of the epithelium. In severe dysplasia, the above parameters extended beyond one-half of the epithelial thickness but not affecting the entirety of the epithelium (Fig.2D). In both conditions no invasion of the underlying connective tissue has occurred. The IHC staining with anti Bcl-2 antibody showed moderate positive expression (39.41%) in all layers of the epithelium (Fig.2E). The IHC staining with anti Bax antibody showed mild positive expression (21.20%) in all layers of the epithelium (Fig.2F). In GII B, H&E stain indicated that 15 animals exhibit well differentiated SCC which is characterized by the presence of keratin pearls. Tumor cells showed pleomorphism, hyperchromatism and altered nuclear/cytoplasmic ratio. Papillary projections of Para keratinized squamous epithelium into the underlying connective tissue were also seen. Dysplastic features in multiple areas, destruction of basement membrane, and prominent true invasion with formation of various forms of epithelial nests (Fig.2G).The IHC staining with anti-Bcl-2 antibody showed strong positive expression (59.94%) in all of the epithelial nests (Fig.2H). The IHC staining with anti-Bax antibody showed mild positive expression (12.86%) in all of the epithelial nests (Fig.2I). In GIIIA, histological sections, using H&E stain indicated that, 10 animals exhibited moderate epithelial dysplasia and 5 animals exhibited mild dysplasia (Fig.2J). The IHC with anti-Bcl-2 antibody showed mild expression (11.35%) in all layers of the epithelium (Fig.2K). The IHC with anti-Bax antibody showed strong expression (52.02) in all layers of the epithelium (Fig.2L). In GIII BH&E stain showed that all animals exhibit well differentiated SCC (Fig.2M). The IHC with anti-Bcl-2 antibody showed moderate positive staining (40.04%) in all epithelial nests (Fig.2N). The IHC with anti-Bax antibody showed moderate positive staining (26.45%) in the epithelial nests (Fig.2O).

Statistical analysis results of Bcl-2 and Bax expression were obtained by comparing the area % of Bcl-2 and Bax expression among the groups in the present study. There was highly significant difference between GI and GII A regarding to Bcl-2, the p value recorded 0.001 (< 0.01) and Bax also shown highly significant difference between the same groups. There was highly significant difference between group I and GII B regarding to Bcl-2 and Bax, the p value recorded 0.001 (< 0.01) as shown in (chart 1). There was highly significant difference between GI and GIII A regarding to Bcl-2, the p value recorded 0.003 (< 0.01) while Bax showed non-significant difference between the same groups the p value recorded 0.186 (> 0.05). Also there was highly significant difference between GI and GIII B regarding to Bcl-2, the p value recorded 0.001 (< 0.01) and Bax also showed highly significant difference between the same groups the p value recorded 0.001 (< 0.01) as shown in (chart 2).There was highly significant difference in group II between

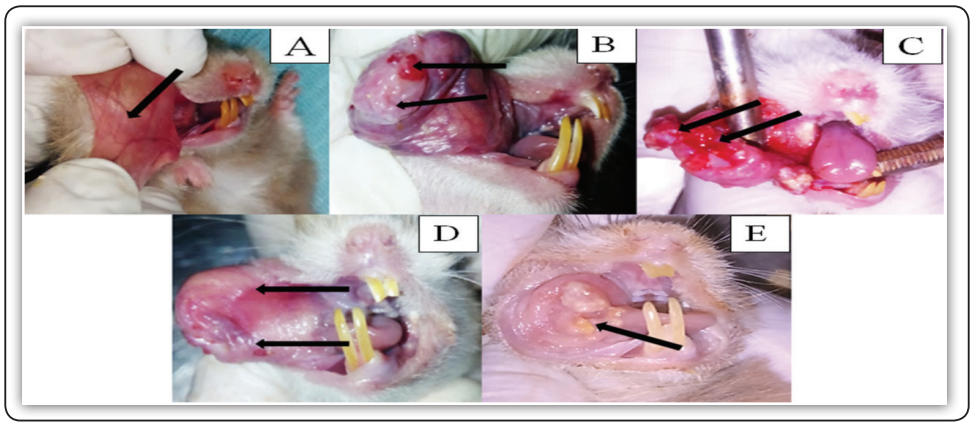

Fig.1 (A): HBP of GI showing normal buccal pouch mucosa which appeared pink in color with smooth surface.(arrow).Fig.1(B): HBP of GII A showed white ruffled mucosal surface, erythematous areas and development of small nodules (arrows). Fig.1(C): HBP of GIIB showing multiple exophytic nodules surrounded with bleeding and ulcerative areas (arrow). Fig.1(D): HBP of GIII A showing tiny elevations surrounded with normal mucosa (arrow). Fig.1(E): HBP of GIIIB showing multiple exophytic masses of variable size (arrow).

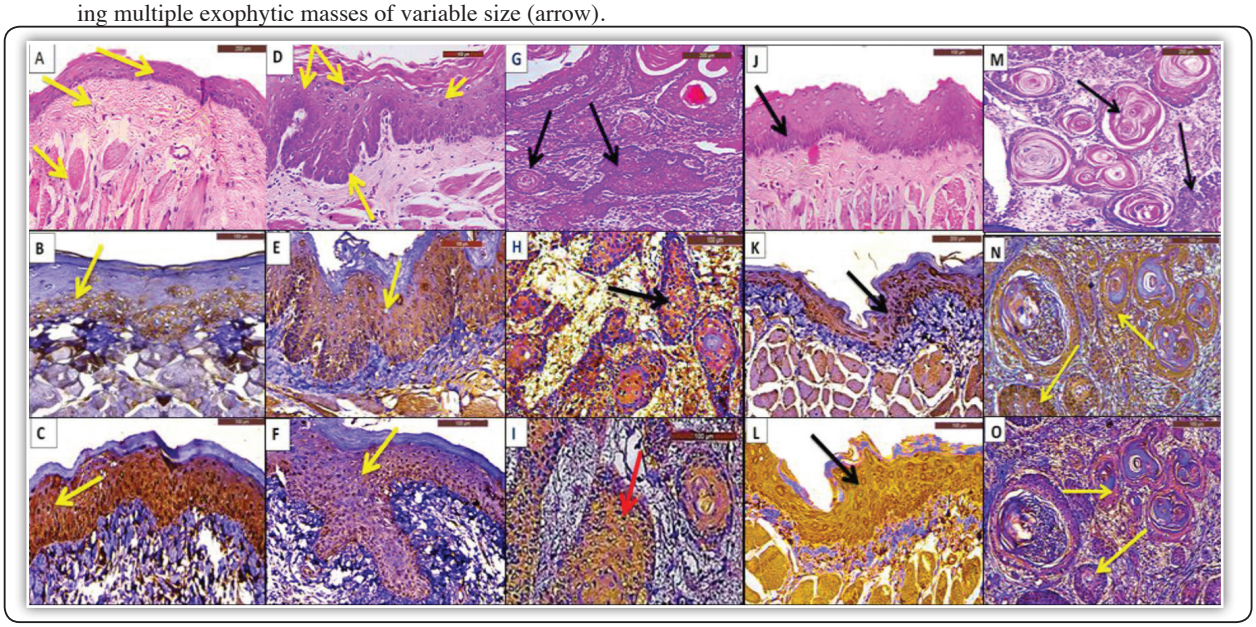

Fig.2(A): GI HBP mucosa showing thin keratinized stratified squamous epithelium with no rete ridges, sub epithelial connective tissue layer and muscular layer(arrows).Fig.2(B): GI HBP mucosa showing positive cytoplasmic expression of Bcl-2 in basal and suprabasal epithelial layers (arrow). Fig.2(C): GI HBP mucosa showing positive cytoplasmic expression of Bax in all epithelial layers (arrow). (Fig.2D): GII A HBP mucosa showingsever dysplasia. (Fig.2E): GII A HBP mucosa showing positive cytoplasmic expression of Bcl-2 in all epithelial layers (arrow). (Fig. 2F): GII A HBP mucosa showing positive cytoplasmic expression of Bax in all epithelial layers (arrow). (Fig. 2G): GII B HBP mucosa showing well differentiated SCC which revealed epithelial islands and keratin pearls (arrows) invading the connective tissue. (Fig. 2H): GII B HBP mucosa showing positive cytoplasmic expression of Bcl-2 in all epithelial nests (arrow). (Fig. 2I): GII B HBP mucosa showing positive cytoplasmic expression of Bax in all epithelial nests (arrow). (Fig. 2J): GIII A HBP mucosa showing mild dysplasia which revealed basilar hyperplasia (arrow). (Fig. 2K): GIII A HBP mucosa showing positive cytoplasmic expression of Bcl-2 in all epithelial layers (arrow). (Fig. 2L): GIII A HBP mucosa showing positive cytoplasmic expression of Bax in all epithelial layers (arrow). (Fig. 2M): GIII B HBP mucosa showing well differentiated SCC which revealed epithelial islands and keratin pearls (arrows) invading the connective tissue. (Fig. 2N):GIII B HBP mucosa showing positive cytoplasmic expression of Bcl-2 in all epithelial nests (arrows). (Fig. 2O): GIII B HBP mucosa showing positive cytoplasmic expression of Bax in the epithelial nests (arrows).

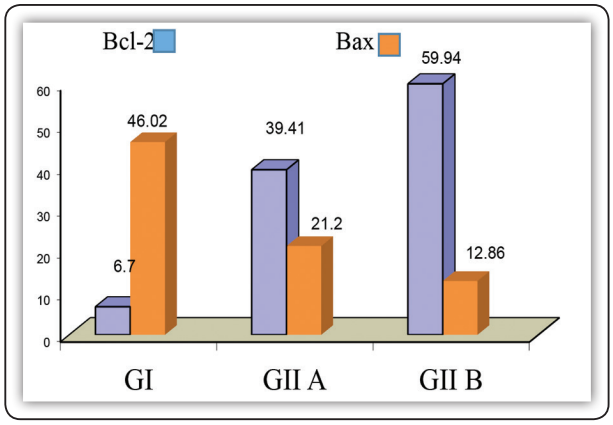

Chart (1): Bar chart representing mean area % values of Bcl-2&Bax between GI and G II (A &B).

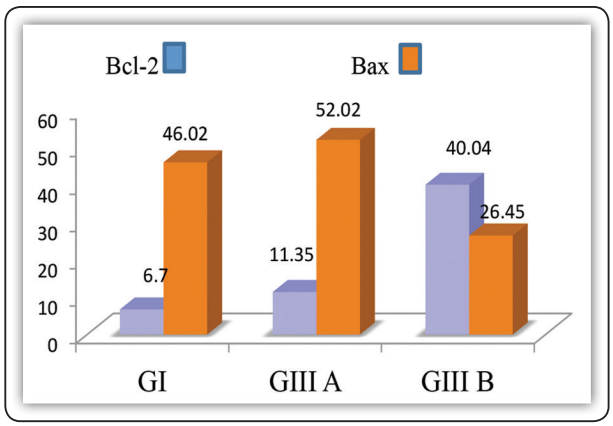

Chart (2): Bar chart representing mean area % values of Bcl-2&Bax between GI and G III (A &B).

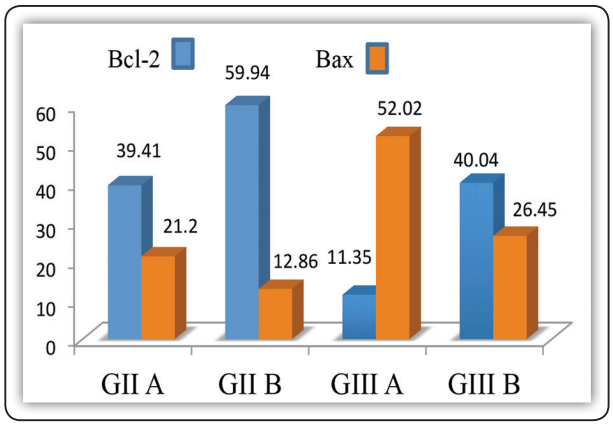

Chart (3): Bar chart representing mean area % values of Bcl-2&Bax between GII (A &B) and G III (A &B).

sub groups A and B regarding to Bcl-2, the p value recorded 0.001 (< 0.01) and Bax also showed highly significant difference between the same groups the p value recorded 0.001 (< 0.01).There was highly significant difference in GIII between subgroups A and B regarding to Bcl-2 expression, the p value recorded 0.001 (< 0.01) and Bax also showed highly significant difference between the same groups the p value recorded 0.001 (< 0.01). There was highly significant difference between GII A and GIII A regarding to Bcl-2, the P value recorded 0.001 (< 0.01) and Bax also showed highly significant difference between the same groups the P value recorded 0.001 (< 0.01) as shown in chart (3). There was highly significant difference between GII B and GIII B regarding to Bcl-2, the p value recorded 0.001 (< 0.01) and Bax also showed highly significant difference between the same groups the P value recorded 0.001 (< 0.01) as shown in chart (3).

DISCUSSION

Globally, therapeutic approaches to cancer include chemotherapy, radiation therapy and surgery-which are frequently associated with severe side effects[17]. Furthermore, treatment strategies for OSCC are diverse due to the unpredictable behavior of the cancer, local invasion, frequent regional lymph node metastases and a relative resistance to chemotherapeutic drugs leading to an unpredictable prognosis. The consensus is that the reversal of precancerous lesions or protection from malignant transformation would have a great impact on the prevention and treatment of OSCC[18].

Traditional treatment for oral precancers is total surgical excision that always leads to scar formation for a large precancerous lesion. Thus, in recent years major research has been focused on components isolated from herbs and plants which have been considered for being nontoxic and for the prevention and treatment of certain types of cancer.

Extensive studies highlighted the chemopreventive effects of diverse natural products[5,19,20]. Though several mechanisms were pointed out for the chemopreventive potential of the natural products, the pro-apoptotic properties were documented as one of the major mechanism[9].

The HBP oral carcinogenesis model is the best known animal system that closely correlates with sequential common events involved in the development of human oral premalignant and malignant lesions[7]. The dysplastic lesions developed in Syrian golden hamster mimic the etiology (DMBA may be present in cigarette smoke), the progression and the response to chemopreventive agents of human OED[21]. In addition, hamster tumors mimic many biochemical and molecular markers that are expressed in human oral cancer[22].

In the present study, the chemotherapeutic agent has been administered after carcinogen administration. Some previous studies have used the same protocol[8,23] . Xu et al (2010)[24] studied the role of the Chinese herbal medicine Xianhuayin on the reversal of premalignant mucosal lesions in the HBP and showed that after application of DMBA for 8 weeks variable degree of epithelial dysplasia was observed under light microscopy. Then, after treatment with Xianhuayin, simple hyperplasia was mainly observed in a high proportion, a difference with statistical significance ($p < 0.05$)[24].

The present study demonstrated for the first time, the therapeutic efficacy of carvacrol against HBP carcinogenesis based on induction of apoptosis, and modulation of their target genes associated with it. According to some previous researches on carvacrol, one of its mechanisms of cancer chemoprevention is induction of apoptosis of cancer cells[14,25,26].

In order to prove the theory apoptosis-related markers (Bax and Bcl-2) were examined[23]. In the current study, the duration of DMBA application and time of sacrifice were carefully planned according to the previous literatures, at 8 weeks for the animals of GII A inorder to achieve development of epithelial dysplasia, but not invasive SCC, at a rate of 100%[7,27] and at 14 weeks for the animals of GII B in order to achieve development of invasive OSCC at a rate of 100%[7,27,28]. In the present study, the gross observation findings in untreated control group represented by GI showed no abnormalitiesof the HBP. Buccal pouch mucosa was pink in color with smooth surface with no observable abnormalities.

These results are in line with other studies[23,29]. The HBP epithelium consists of a thin, regular, keratinized stratified squamous epithelium, the epithelium-connective tissue junction being relatively flat. These observations are similar to other investigators[18,30]. The IHC staining in GI with anti- Bcl-2 monoclonal antibody was mild, cytoplasmic and only identified in the basal and suprabasal cell layers. In contrast, staining with anti- Bax antibody was moderate, cytoplasmic and seen in all layers of the epithelium. These results are in agreement with those of other investigators[17,31].

The present results obtained for normal epithelium agree with the suggestion of Kummoona et al (2008)[32] that Bcl-2 plays a role in the control of terminal differentiation of keratinocytes through the protection of basal cells of the proliferative compartment against apoptosis, thus guaranteeing structural epithelial integrity. Under normal conditions, epithelial cells migrate upward through the layers of the epidermis and a high apoptosis rate and loss of expression of the antiapoptotic protein Bcl-2 are observed during this process. After migration to the surface the cells accumulate keratin. Negative Bcl-2 expression in the remaining epithelial layers concomitant with terminal cell differentiation (keratinization)[33,34]. Bax a proapoptotic protein, is present in viable cells to increase the susceptibility of apoptosis of unwanted cells and induces apoptosis in reaction to genotoxicstress so protects the cells from neoplastic transformation.[17] Under normal conditions p53 stimulates the up-regulation

of Bax and down regulation of Bcl-2 to remove the unwanted cells from the host[31]. The current results corroborate these findings.

In the current study, the gross observation findings of GII A revealed white ruffled mucosal surface, development of small nodules and or erythematous areas. These results are almost the same with that shown by other investigators[35,36]. Histopathological results of GIIA showed moderate to severe epithelial dysplasia. The changes in histopathological status of this group and their timing were similar to other studies[28,35–37]. These observations are due to DMBA carcinogenic effects which include formation of DNA adducts, induction of chronic inflammation, over production of ROS and oxidative DNA damage thereby leads toneoplastic transformation[5]. In GII A the immunostaining with anti-Bcl-2 monoclonal antibody was moderate, seen in all layers of the epithelium and show highly significant increased expression than normal group, while the immunostaining with anti- Bax monoclonal antibody was mild, seen in all layers of the epithelium and show highly significant lower expression than normal group. The Bcl-2/Bax ratio is increased. These results are in agreement with some of other studies[19,38–42]. Bcl-2 protein, the gene product of Bcl-2 protooncogene, is an antiapoptotic protein which extends the survival of genetically damaged cells as well as facilitates neoplastic transformation. Bax induces apoptosis in reaction to genotoxic stress and thus protects the cells from neoplastic transformation. Therefore, the significant overexpression of Bcl-2 and down-regulation of Bax protein suggests that buccal tissues from hamsters painted with DMBA alone escaped from the apoptotic cascade[19]. Overexpression of Bcl-2 has also been observed in precancerous lesions of the colorectum, stomach, and esophagus and this suggests that Bcl-2 may be associated with early oncogenesis in these organs[43].

Few other studies conducted on oral tissues, have reported sporadic Bcl-2 expression or lack of expression in oral dysplasias[44,45]. Also in disagreement with the present results, Sousa et al[46] and Shailaja et al[2] who found that Bax increase in dysplasia than in normal epithelium. These discrepancies may be explained with that, they used samples from human tissue which have different etiologic factors, and different immune reaction. In addition the contradictory results of both markers expressionmay result from diverse antibody clones which are sources for various interpretations of protein expression.

In the present study, the gross observation of the right HBP mucosa of GII B showed multiple exophytic masses of variable size surrounded with area of ulceration and bleeding. Furthermore, animals are debilitated. These results are almost the same with that shown by other investigators used the same protocol[19,29]. There was a significant decrease in the food intake due to the occurrence of tumors in the buccal mucosa, resultant in reduced weight gain in DMBA alone painted hamsters. Moreover, increased progression of the tumor leads to growth rate inhibition.[17]. Histopathological results of GII B showed that animals exhibited well differentiated OSCC. These observations are in consistence with other studies[7,47]. The Bcl-2 IHC staining results revealed strong positive cytoplasmic expression throughout the epithelial layers and the expression was highly significant higher in level than GI and GII A. These results are in agreement with those of some studies[17,19,20,29]. Literature data regarding the expression of Bcl-2 during the progression of OSCC are controversial. Some investigators reported only weak or no expression of this protein.[44,45] The contradictory results of Bcl-2 expressionmay result from differences in antigen retrieval procedure and diverse antibody clones are sources for various interpretations of protein expression.[43] The Bax IHC staining results revealed mild positive cytoplasmic expression throughout the epithelial layers but the expression was highly significant lower in level than normal epithelium and at 8 weeks. These results are

in agreement with those of some studies[17,19,20,29]. The increase in the expression of Bcl-2/Bax ratio suggests its deregulation of apoptotic machinery of various cancers including oral cancer[5,48,49].

In the current study, GIII A, gross observation showed improvement in the clinical condition of hamsters if compared toGII A in which, there were no erosion or erythematous areas and the nodules decreased in size to tiny elevations. These findings reflected on H&E staining in which 10 animals exhibited moderate epithelial dysplasia and 5 animals exhibited mild epithelial dysplasia.

In this study, GIII A, The Bcl-2 IHC staining results revealed mild cytoplasmic expression throughout the epithelial layers but the expression was in lower level than GII A while the Bax expression showed positive cytoplasmic expression throughout the epithelial layers but the expression was in higher level than GII A. The Bcl-2/Bax ratio is decreased.

It was observed that apoptosis is induced following administration of carvacrol to animals painted with DMBA as revealed by the down regulation of Bcl-2 protein expression and up regulation of Bax. Therefore, the therapeutic effect of carvacrol on DMBA- painted animals is possibly mediated via suppressing Bcl-2 expression and stimulating the expression of Bax.It has been reported that cell growth inhibition by apoptosis and restoring the levels of Bcl-2 and Bax is a frequent effect of carvacrol. Carvacrol induced apoptosis in colon cancer and metastatic breast cancer[14,50]. Furthermore, it has been shown that carvacrol treatment of oral SCC cell lines causes cell growth inhibition and promotes apoptosis[15].

In the present study, the administration of carvacrol to GIII B for 6 weeks after 14 weeks of DMBA painting has led to some degree of improvement in general state of animals and reduction of inflammatory manifestations around the lesions but there is no apparent change in the pattern and distribution of exophytic nodules. The H&E stain exhibited well differentiated SCC. Sivaranjani et al (2016)[51] studied the chemopreventive effect of carvacrol on DMH induced experimental colon carcinogenesis and found that carvacrol supplementation to rats administered DMH improved weight gain and growth rate. The present study corroborates these findings. The reduction of inflammatory manifestation can be elucidated by the anti-inflammatory action of carvacrol reported in other studies[52,53]. In the present study the Bcl-2 IHC staining results in GIIIB revealed moderate positive cytoplasmic expression throughout the epithelial layers but the expression was highly significant lower in level than GIIB while Bax expression showed moderate positive cytoplasmic expression throughout the epithelial layers but the expression was highly significant higher in level than GIIB. This means that carvacrol treatment improved the Bcl-2/Bax ratio in comparison to corresponding control group. Hence lowered ratio of Bcl-2/Bax, indicate increased signaling of the cells toward apoptotic death, the result therefore, indicated that carvacrol induced apoptotic death in this treatment group.

The current results suggested that apoptosis is present in OSCC, but other mechanisms of cell growth outcome those of cell-death. There were some previous studies which strengthens these findings in that they reported that carvacrol has antiproliferative effect in human metastatic breast cancer cell line (MDA-MB 231)[50] and also carvacrol treated MCF-7 cells[13]. The present study indicated that there was a statistically significant reverse correlation between area % of Bcl-2 and area % of Bax expression. This means that an increase in one variable is associated in decrease in the other and vice versa. Shailaja et al[2] are in disagreement with this observation. They reported that, although Bax and Bcl-2 are strongly associated in apoptosis, no correlation between these proteins was observed in this study and explained this finding by the existence of different mechanisms of apoptosis regulation.

CONCLUSION

Carvacrol is a novel approach for the treatment of DMBA induced HBP epithelial dysplasia. In addition, oral administration of carvacrol to DMBA induced HBP carcinoma significantly induced apoptosis as evidenced by increased Bax and decreased Bcl-2.

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