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EFFECT OF OXYGENATED WATER AS A NEW TREATMENT MODALITY ON EXPERIMENTALLY INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS

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ABSTRACT

Abstract: The aim of the present study was directed to investigate the effect of oxygenated water as a new treatment modality on experimentally induced hamster buccal pouch (HBP) carcinogenesis. **Material and methods**: Eighty-five Syrian male hamsters, five weeks old, weighing 80-120g. The experimental animals were divided into three groups, GI (negative control): 5 animals left untreated. GII {Dimethylbenz[a]anthracene (DMBA) treated group}: 30 animals were painted with 0.5% DMBA in paraffin oil using 3 times a week for 4 weeks (GIIA), 8 weeks (GIIB) and12 weeks (GIIC). GIII (oxygenated water treated group): 45 animals, following each period of DMBA-treatment, were treated in the normal drinking (free access) in addition to once daily obligatory by oral tube for 5 weeks which divided into 3 subgroups GIIIA, GIIIB and GIIIC. **Results**: Gross observation and histopathological findings revealed a-GI: normal stratified squamous epithelium b- GIIA, GIIIA and GIIIB: mild epithelial dysplasia c: GIIB: moderate and severe epithelial dysplasia d: GIIC and GIIIC: well and moderately differentiated SCC. Immunohistochemical results revealed variable observations among the treated groups (GII and GIII) compared to that observed in group GI. **Conclusion**: Oxygenated water is considered as a promising treatment agent in preventing of induced HBP carcinogenesis (epithelial dysplasia & invasive carcinoma).

KEYWORDS: HBP carcinoma, oxygenated water, hypoxia.

INTRODUCTION

Head and neck cancer is the sixth most prevalent cancers accounting for approximately 600,000 new cases annually worldwide [(1)]. Oral squamous cell carcinoma (OSCC) is the major subtype of head and neck cancer and accounts for two-thirds of the cases occurring in least developed countries[(2)] and about 4.500 people will be diagnosed with oral cancer every year in Egypt[(3)]. OSCC can be presented as a "natural history", which originates from non-aberrant keratinocytes which are chronically exposed to a stimulus that breaks its homeostasis, following an epithelial hyperplasia, dysplasia in different degrees, carcinoma in situ and an invasive carcinoma leading to the generation of distant metastases [(4)], Oral carcinogenesis is a highly complex multifocal process that takes place when squamous epithelium is affected by several genetic alterations [(5)] Probably oral carcinogenesis starts with the transformation of a limited number of normal keratinocytes via cytogenetic changes and epigenetic processes that modify the progression of the cell cycle, DNA repair mechanisms, cell differentiation and apoptosis, which may be caused by random mutation, exposure to a variety of biological factors, carcinogens or errors in the DNA repair process, resulting in an unstable keratinocyte into a precancerization field[(6)]. The hamster cheek pouch carcinogenesis model, using treatments with the carcinogen 7, 12 Dimethylbenz[a]anthracene

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(DMBA) is well documented. It has been found that, dysplastic lesions of hamster closely mimic the human oral tumor, both histologically and at molecular level[7]. Unfortunately, most available therapies suffer from significant limitations. Surgical excision is greatly restricted by the complicated anatomical nature of the head and neck area and vital structures, hindering the ability to adequately remove the entire neoplasm. Radio- and chemo therapy has a high failure rate for advanced tumors, where radio-toxicity limits the given doses in one full course treatment. Hypoxia is a common phenomenon in the solid tumor due to rapid proliferation of cancer cells and/or insufficient blood supply[8]. The hypoxic tumor cells, which induces the expression of more than one hundred genes related to angiogenesis, invasion, metastasis, and resistance to conventional treatments such as chemotherapy and radiotherapy[9]. There are numerous well-accepted indications for oxygen therapy including carbon monoxide poisoning, compromised skin grafts, acute traumatic wounds, chronic non healing diabetic ulcers, crush injuries, burns, gas gangrene, and compartment syndrome[10-14]. The oral administration of oxygen-enhanced water was found to be a feasible technique to increase the oxygen supply to tumors[15]. Normal tap water contains approximately 5–7 mg/l dissolved oxygen, and fresh fountain water contains 10–12 mg/l dissolved oxygen. Oxygenation of tap water can increase the concentration of dissolved oxygen from 30–120 mg/l. It has been considered that drinking oxygenated water improves oxygen availability, which may increase vitality and improve immune functions[16]. Hence, the main target of the present study was to assess effect of oxygenated water as a new treatment modality on DMBA induced HBP carcinoma. The assessment was based on the gross observation, histological tumor tissue changes and immunohistochemical 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 model for OSCC induction utilizing DMBA as chemical carcinogen. Then, oxygen treatment started in the normal drinking (free access) in addition to once daily obligatory by oral tube, were employed. After that, various investigations: hematoxylin and eosin (H&E) stain and immunohistochemical staining utilizing Bcl-2 and Bax antibodies, were done.

Animals and materials:

Eighty Syrian male hamsters five weeks old, weighing 80-120g were obtained from the animal house, Cairo University (Cairo, Egypt). The experimental animals were housed in standard cages with sawdust bedding under controlled environmental conditions of humidity (30-40%), temperature (20 ± 2°C), and light (12-h light/12-h dark). All experimental animals were supplied with standard diet and water ad libitum. DMBA (0.5%) was obtained from Sigma-aldrich company, dissolved in paraffin oil. Oxygenated water was prepared according to the following equation: $2H2O2+MnO2 \rightarrow 2H2O+O2\uparrow+MnO2\downarrow$A: Regarding partial conversion: 2 g of MnO_2 were added to 20 ml of H_2O_2 and the reaction was left for 15 min then the mixture was filtrated on filter paper. 2 ml of the filtrated mixture was added to 58 ml of distilled water and mixed well and then left to be given obligatory via oral tube. B: Regarding complete conversion: 20 g of MnO_2 were added to 50 ml H_2O_2 then to 950 ml water the reaction was left for 15 min to complete the conversion then the mixture was filtrated on filter paper then left as a free access for normal drinking in all treatment periods. The dose and the duration of the treatment were determined based on pilot studies carried out in the laboratory of Pharmacology and Toxicology Department, Faculty of Pharmacy (Boys, Cairo) Al-Azhar University as there were no previous studies performed

on oxygenated water regarding it is anticancer effect against OSCC before. The amount of dissolved oxygen in the water was determined before the start of the experiments in the National Research Center, Cairo, Egypt.

Experimental design:

The experimental animals were divided into three groups. GI (negative control): 5 hamsters, not treated and served as negative controls.. GII: (DMBA treated group): 30 hamsters, the rights HPB 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 3 subgroups, A-GIIA: (10 hamsters) served as positive control for 4 weeks. B-GIIB: (10 hamsters) served as positive controls for 8 weeks. C-GIIC: (10 hamsters) served as positive control for 12 weeks. GIII (oxygenated water treated group): 45 animals, following DMBA-treatment, oxygen treatment started in the form of normal drinking (free access) in addition to once daily obligatory by oral tube, the animals were randomly divided into the following 3 subgroups, A-GIIIA: (15 hamsters) DMBA treated group for 4 weeks followed by oxygen treatment for 5 weeks. B-GIIIB: (15 hamsters) DMBA treated group for 8 weeks followed by oxygen treatment for 5 weeks. C- GIIIC: (15 hamsters) DMBA treated group for 12 weeks followed by oxygen treatment for 5 weeks.

Investigations:

After termination of the experiment, the animals were euthanized, the cheek pouches were 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 stain 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% H_2O_2 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 (Bax & Bcl-2) in a dilution of 1:100 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 (Sigma, USA) 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 antibody 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) [17] .

RESULTS

The gross observation results of HBP mucosa of GI were pink in color with smooth surface with no observable abnormalities (Fig.1A). In GIIA, HBP mucosa showed whitish membrane which coming out with DMBA application leaving thick erythematous and hemorrhagic area (Fig.1B). In GIIB, HBP mucosa showed multiple exophytic nodules of variable size. The nodules were surrounded with ulcerative and bleeding areas (Fig.1C). In GIIC, HBP mucosa showed multiple exophytic masses of variable size surrounded with areas of ulceration and bleeding. Depilating of all animals was an observable remark (Fig.1D). In GIIIA, HBP mucosa showed absence of erosion or erythematous areas (Fig.1E). In GIIIB, HBP mucosa showed small size nodule and the animals appeared healthy (Fig.1F). In GIIIC, HBP mucosa showed exophytic masses of variable sizes surrounded with area of ulceration and bleeding and the animals showed health improvement (Fig.1G).

Histopathological and immunohistochemical results: The tissue sections of HBP mucosa of experimental groups showed variable results. In GI, histological sections, using H&E stain, revealed normal HBP mucosa, composed of thin stratified squamous epithelium, consists of two to four layers of squamous cells exhibiting slight keratinization (i.e.; one layer of basal cells and one, two or three layers of spinous and thin keratinized cells with lacking rete ridges. Sub-epithelial connective tissue (C.T), muscular layer and areolar layer were seen (Fig.2A). The IHC staining using Bcl-2 antibody exhibited weak positive expression (mean = 6.7) which limited to basal and suprabasal layers (Fig.2B) while the Bax expression showed moderate positive expression (mean = 46.02) which present throughout the epithelial layers (Fig.2C). In GIIA, histological sections, using H&E stain, all animals exhibited mild epithelial dysplasia and hyperkeratosis. Mild dysplasia was characterized by changes in the epithelium such as basilar crowding and hyperplasia, cellular disorganization, and maturational disturbances not extending more than one-third of the epithelial thickness with little interruption of the keratin layer (Fig.2D). The IHC staining using Bcl-2 exhibited weak positive cytoplasmic expression (mean = 17.4) which present in basal and suprabasal epithelial layers (Fig.2E) while the Bax expression showed moderate positive cytoplasmic expression (mean= 34.89) throughout the epithelial layers (Fig.2F). In GIIB, histological sections, using H&E stain, 8 animals exhibited moderate epithelial dysplasia and 2 animals exhibited severe epithelial dysplasia. Severe dysplasia included the above parameters extending beyond one-half of the epithelial thickness but not affecting the entire of the epithelium. Additional features included frequent mitotic figures, cellular pleomorphism, nuclear atypia, and some early disturbance of the keratin layer with drop shaped rete pigs (Fig.2G). The IHC staining using Bcl-2 moderate positive cytoplasmic expression (mean=42.267) throughout the epithelial layers (Fig. 2H) while the Bax expression showed weak positive cytoplasmic expression (mean = 23.58) throughout the epithelial layers (Fig.2I). In GIIC, histological sections, using H&E stain, 5 animals exhibited well differentiated SCC and 5 animals exhibited moderately differentiated SCC. Dysplastic features in multiple areas, destruction of basement membrane, and prominent true invasion with formation of various forms of epithelial nests (Fig.2J). The IHC staining using Bcl-2 exhibited strong positive cytoplasmic expression (mean=63.07) throughout the tumor cells (Fig.2K) while Bax exhibited weak positive cytoplasmic expression (mean=12.47) throughout the tumor cells positive cytoplasmic expression throughout the tumor cells (Fig.2L). In GIIIA, histological sections, using H&E stain, 3 animals exhibited mild epithelial dysplasia and 12 animals exhibited hyperkeratosis to normal (Fig.3A). The IHC staining using Bcl-2 exhibit weak positive cytoplasmic expres-

sion (mean =10.6) throughout the epithelial layers (Fig.3B) while the Bax expression showed moderate positive cytoplasmic expression (mean = 41.84) throughout the epithelial layers (Fig.3C). In GIIIB, histological sections, using H&E stain, 6 animals exhibited mild epithelial dysplasia and 9 animals exhibited hyperkeratosis to normal (Fig.3D). The IHC staining using Bcl-2 exhibited moderate positive cytoplasmic expression (mean =31.02) throughout the epithelial layers (Fig.3E) while the Bax expression showed moderate positive cytoplasmic expression (mean = 39.15) throughout the epithelial layers (Fig.3F). In GIIIC, histological sections, using H&E stain, all animals exhibited well differentiated SCC (Fig.3G). The IHC staining using Bcl-2 exhibited strong positive cytoplasmic expression (mean = 61.25) throughout the tumor cells (Fig.3H) while the Bax expression showed weak positive cytoplasmic expression (mean = 23.63) throughout the tumor cells (Fig.3I).

Statistical analysis results of Bcl-2 & Bax expression were obtained by comparing the area % of Bcl-2 & Bax expression in the groups used. Statistical analysis results revealed that , In regard to expression of Bcl-2, group I had recorded the lowest mean area percentage (6.7%), while GIIC had the highest mean area percentage (63.07%) while in regard to expression of Bax, GIIC had recorded the lowest mean area percentage (12.47%), while group I had the highest mean area percentage (46.02%). Comparing the DMBA treated groups (GII) with group I(normal), there was highly significant difference between group I and GIIA regarding Bcl-2, the P value recorded 0.001 (< 0.01) while Bax shown significant difference between the same groups, the P value recorded 0.034 (< 0.05).There was highly significant difference between group I and group II (GIIB and GIIC) regarding Bcl-2 and Bax; the P value recorded 0.001 (< 0.01) Chart (1). Comparing the oxygenated water treated groups (GIII) with group I(normal), there was highly significant difference between group I and GIIIA regarding Bcl-2, the P value recorded 0.005(< 0.01) while Bax showed non-significant difference between the same groups, the P value recorded 0.441 (> 0.05).There was a highly significant difference between group I and GIIIB regarding Bcl-2, the P value recorded 0.001 (< 0.01) while Bax shown non-significant difference between the same groups, the P value recorded 0.196.There was highly significant difference between group I and GIIIC regarding Bcl-2 and Bax, the P value recorded 0.001 (< 0.01) (Chart 2).

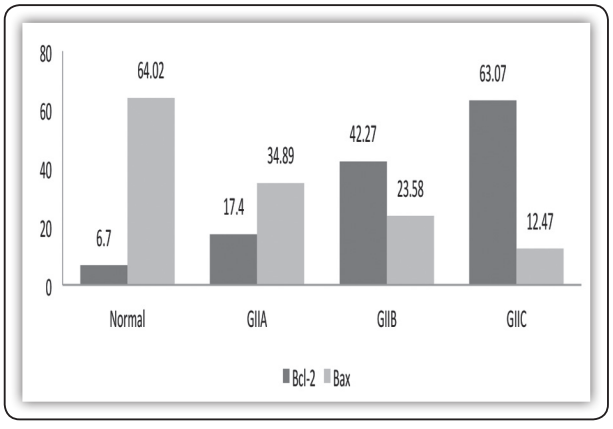

Chart (1): Bar chart representing mean area % results of Bcl-2 & Bax between normal and GII.

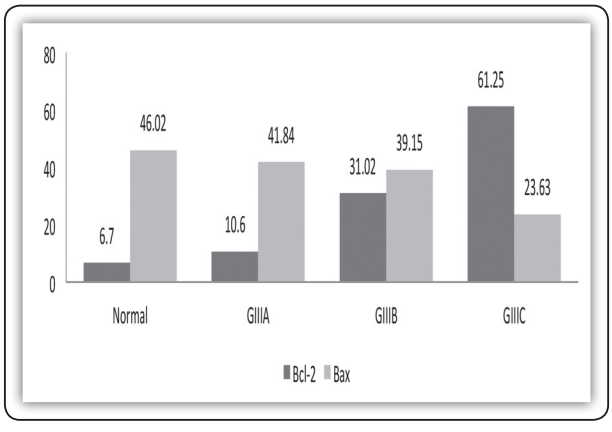

Chart (2): Bar chart representing mean area % results Bcl-2 & Bax between normal and GIII.

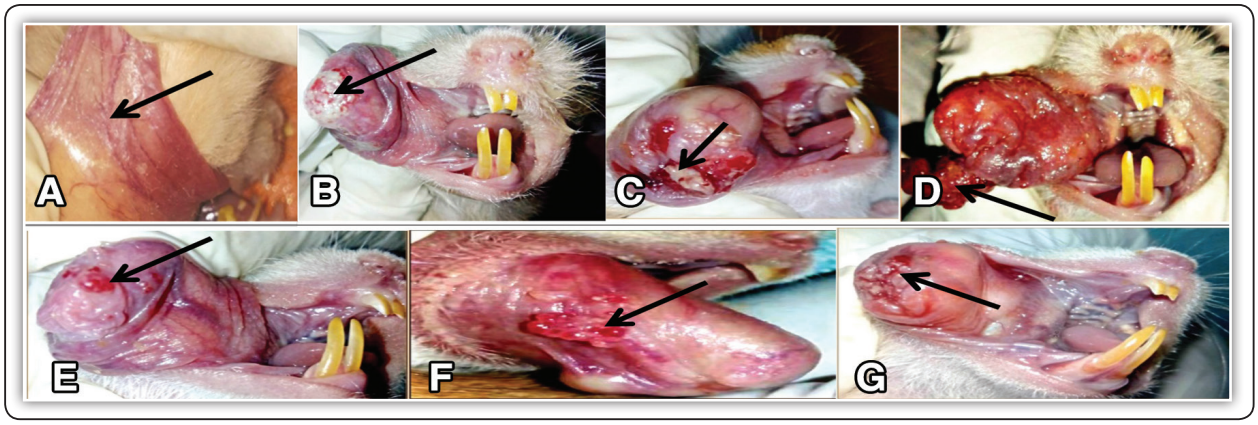

Fig.1(A): HBP of GI showing normal buccal pouch mucosa which appeared pink in color with smooth surface (arrow). Fig.1(B): HBP of GIIA showing thick whitish membrane speckled with erythematous areas (arrow). Fig.1(C): HBP of GIIB showing multiple exophytic nodules surrounded with bleeding ulcerative areas (arrow). Fig.1(D): HBP of GIIC showing multiple exophytic masses surrounded with ulcerative and bleeding areas (arrow). Fig.1(E): HBP of GIIIA showing small tiny elevation with absence of ulceration and bleeding (arrow). Fig.1(F): HBP of GIIIB showing small sized nodule with absence of ulceration and bleeding (arrow). Fig.1(G): HBP of GIIIC showing exophytic masses with areas of ulceration and bleeding.

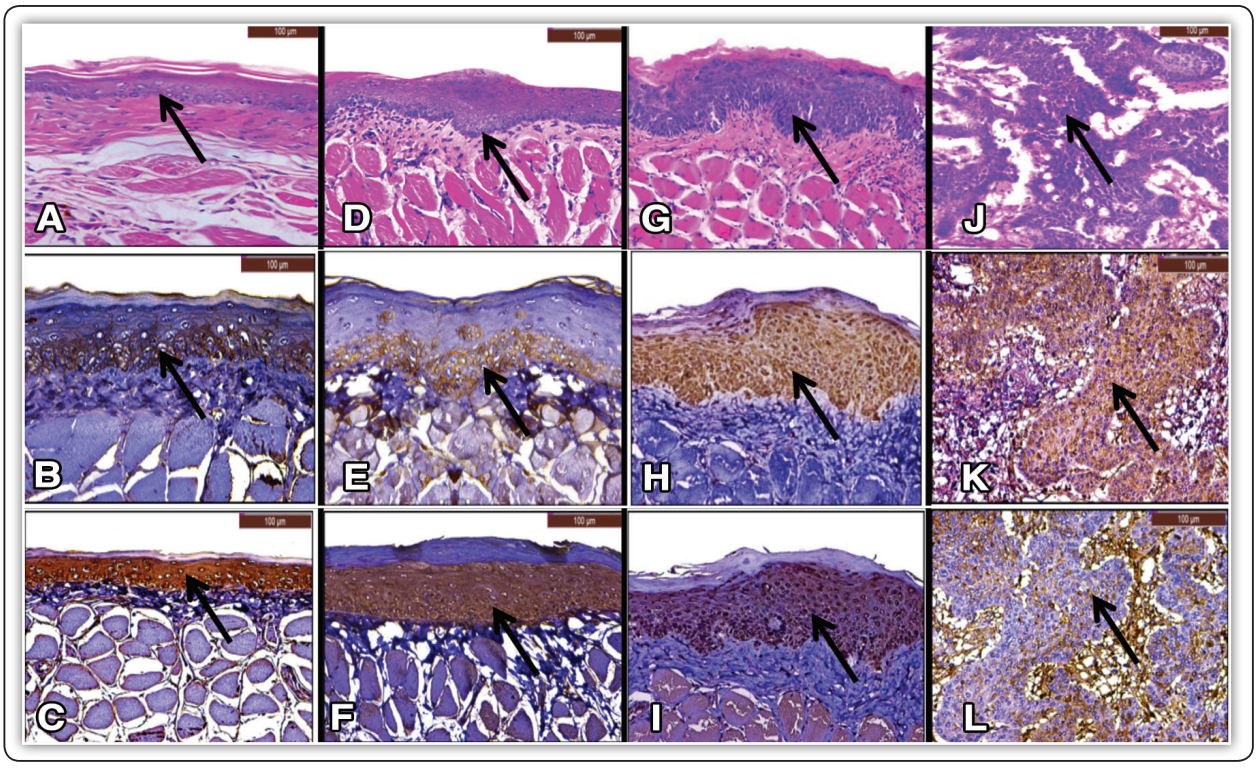

Fig.2 (A): H&E stain of GI showing keratinized stratified squamous epithelium with flattened rete ridges, sub-epithelial connective tissue layer and muscular layer (arrow). Fig.2(B): IHC expression of Bcl-2 showing positive cytoplasmic expression in basal and suprabasal epithelial layers (arrow). Fig.2(C): IHC expression of Bax showing positive cytoplasmic expression throughout the epithelial layers (arrow). Fig.2(D): H&E stain of GIIA of HBP mucosa showing mild epithelial dysplasia (arrow). Fig.2(E): IHC expression of Bcl-2 showing positive cytoplasmic expression in basal and suprabasal layers (arrow). Fig.2(F): IHC expression of Bax showing positive cytoplasmic expression throughout the epithelial layers (arrow). Fig.2(G): H&E stain of GIIB of HBP mucosa showing severe epithelial dysplasia (arrow). Fig.2(H): IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the epithelial layers (arrow). Fig.2(I): IHC expression of Bax showing positive cytoplasmic expression throughout the epithelial layers (arrow). Fig.2(J): H&E stain of GIIC of HBP mucosa showing moderately differentiated SCC (arrow). Fig.2(K): IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the tumor cells (arrow). Fig.2(L): IHC expression of Bax showing positive cytoplasmic expression throughout the tumor cells (arrow).

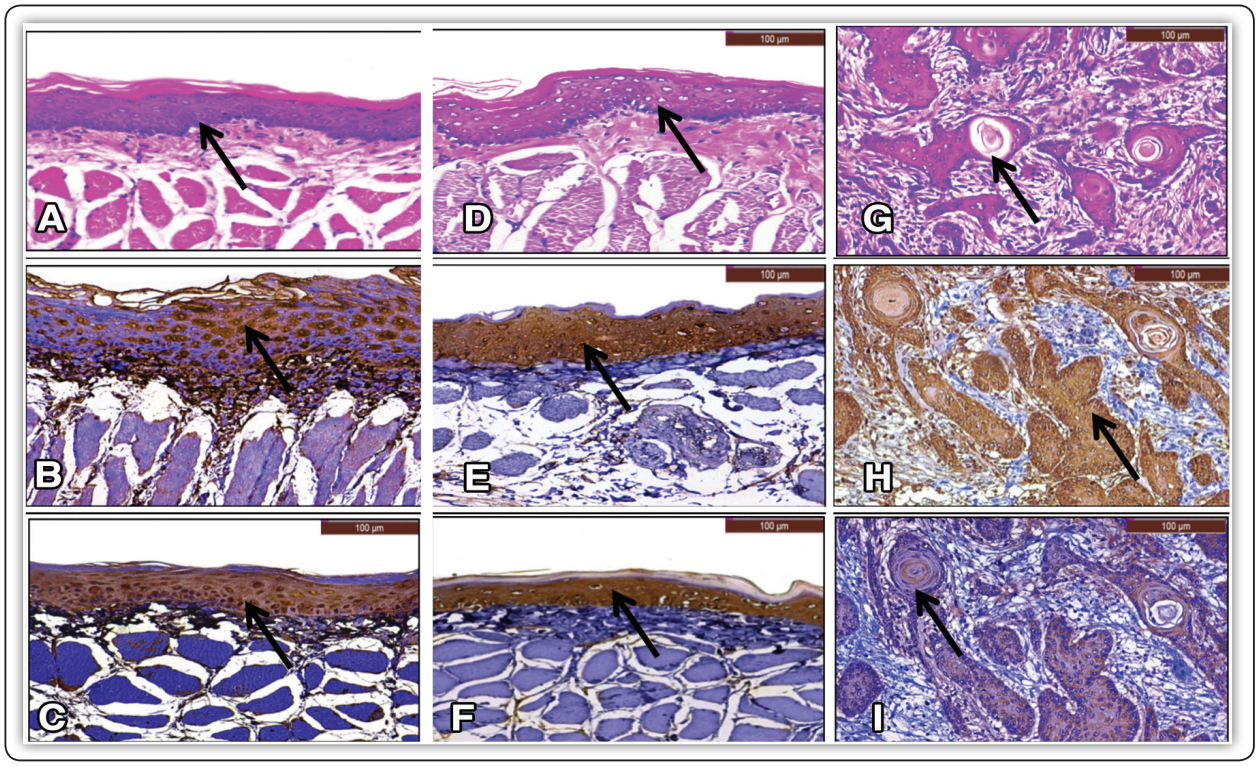

Fig.3 (A): H&E stain of GIIIA of HBP mucosa showing mild epithelial dysplasia and hyperkeratosis (arrow). Fig.3(B): IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the epithelial layers (arrow). Fig.3(C): IHC expression of Bax showing positive cytoplasmic expression throughout the epithelial layers (arrow). Fig.3(D): H&E stain of GIIIB of HBP mucosa showing moderate epithelial dysplasia (arrow). Fig.3(E): IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the epithelial layers (arrow). Fig.3(F): IHC expression of Bax showing positive cytoplasmic expression throughout the epithelial layers (arrow). Fig.3(G): H&E stain of GIIIC of HBP mucosa showing well differentiated SCC (arrow). Fig.3(H): IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the tumor cells (arrow). Fig.3(I): IHC expression of Bax showing positive cytoplasmic expression throughout the tumor cells (arrow).

DISCUSSION

Oral carcinogenesis has been widely experimented, aiming to develop either biomarkers for early diagnosis or effective treatment. HBP model is effective because of a close similarity to human oral carcinogenesis at both histological and molecular level. Moreover, DMBA was chosen as the chemical carcinogen, because it plays the same etiological role in hamster SCC as do alcohol and tobacco in human OSCC. To our knowledge, in English literatures, this was the first study to evaluate the effect of oxygenated water as a new treatment modality on DMBA induced HBP carcinogenesis. The results of gross observations, H&E stain and immunohistochemical staining utilizing Bcl-2 and Bax antibodies, revealed variable observations.

In the present study, group I (normal group), the gross observation showed no observable changes. The HBP mucosa appeared normal, with smooth surface. After scarification of the animals of this group, the buccal pouch length was about 5cm for all hamsters. This finding reflected on H&E staining which showed thin keratinized stratified squamous epithelium. Subepithelial C.T formed of small amount of sporadic fibrocytes and blood vessels which present between the epithelium and the muscular layer. Bruna et al. [18] reported the same finding. In the current study, group I (normal group), the immunohis-

tochemical results of Bcl-2 showed weak positive expression (mean = 6.7) which limited to basal and suprabasal layers while the Bax expression showed moderate positive expression (mean = 46.02) which present throughout the epithelial layers. The finding of this study was in agreement of some researches[19-22]. Karthikeyan and Morgn [23] . stated that, under normal conditions, Bcl-2/Bax ratio determines the fate of cell survival or cell death, through the regulation of the release of Cyt C from the mitochondria. This result may be due to that; Bcl-2 participates in the control of the terminal differentiation of keratinocytes by protecting their stem cells from apoptosis.

In the present study, GIIA (DMBA treated group at 4 weeks), gross observation revealed whitish membrane, hemorrhagic areas, buccal pouch retracted and accompanied by an engrossment of the mucosa. This finding reflected on H&E staining in which, the epithelium showed hyperkeratosis and mild epithelial dysplasia restricted to basal and suprabasal layers, the underlying C.T infiltrated by abundant inflammatory cells. These results are in agreement with some studies[18, 24, 25] . This finding may be explained by the occurrence of chronic inflammation as a result of DMBA application for 4 weeks. Tumor promoter-induced secretion of proinflammatory molecules by keratinocytes results in the recruitment of inflammatory cells, e.g., leukocytes, lymphocytes and macrophages, into the dermis. These activated cells produce growth factors, cytokines and chemokines that promote cell proliferation, matrix remodeling, angiogenesis and suppression of adaptive immunity, all of which promote tumor growth. In addition, activated inflammatory cells produce ROS and nitric oxide resulting in oxidative stress, which has been shown to be associated with tumor promotion[26].In this study, GIIA (DMBA treated group at 4 weeks), The Bcl-2 immunohistochemical results revealed weak positive cytoplasmic expression (mean = 17.4) which present in basal and suprabasal epithelial layers, while the Bax expression showed moderate positive cytoplasmic expression (mean= 34.89) throughout the epithelial layers. These results are in agreement with those of some studies[22, 27] . This result may be attributed to the dysplastic changes occur which change the Bcl-2/Bax ratio, this means increase the proliferation and decrease apoptosis.

In the current study, GIIB (DMBA treated group at 8 weeks), gross observation revealed multiple exophytic nodules of variable size. This finding reflected on H&E staining which showed features of moderate to severe epithelial dysplasia but not invading the subepithelial C.T. This result is in agreement with those of some studies[18, 24, 28] .This finding may be attributed to generation of ROS during DMBA application. The toxic metabolites of DMBA, including ROS, are capable of binding to adenine residues of DNA causing chromosomal damage[29]. This increase of ROS may induce cell proliferation and cause oxidative damage to lipids, proteins, and DNA, provoking oncogenic transformation, increased metabolic activity, and mitochondrial dysfunction. This mitochondrial dysfunction may induce a low coupling efficiency of the mitochondrial electron chain, increasing electron leakage and leading to enhanced ROS formation. The resulting oxidative stress may cause further damage to both mitochondrial DNA (mtDNA) and the respiratory chain, amplifying the ROS generation[30] .In this study, GIIB (DMBA treated group at 8 weeks), the Bcl-2 immunohistochemical results revealed moderate positive cytoplasmic expression (mean=42.267) throughout the epithelial layers while the Bax expression showed weak positive cytoplasmic expression (mean = 23.58) throughout the epithelial layers. Nishimura.[27] confirm these findings. These results may be referred to; the increased supra-basal cell positivity of Bcl-2 than normal which may also be correlated to the dysplastic changes and basal cell degeneration along with its

risk of carcinoma development which supported by the increase the Bcl-2/Bax ratio. The increased expression of Bcl-2 makes the removal of genetically modified cells difficult, favoring the accumulation of new mutations, which can result in the appearance of cells with malignant phenotype. Bcl-2 has the capacity of interrupting the apoptosis process both in the initial and final phases because this protein stabilizes the potential of the mitochondria membrane when forming heterodimers with Bax[31].

In the present study, GIIC (DMBA treated group at 12 weeks), gross observation revealed multiple exophytic masses of variable size surrounded with areas of ulceration, bleeding with the buccal pouch contracted, stiffs and features of necrosis. This finding reflected on H&E staining which showed features of well and moderate differentiated SCC in which the epithelium showed abundant cellular and nuclear pleomorphism, the stratification was lost, frequent mitotic figures, hyperchormatism, disturbance of the keratin layer with drop shaped rete pigs and basement membrane was discontinuous, the inflammatory cells were abundant. This results in consistence with that shown by some researches[18, 32, 33] . These findings may be referred to higher level of intracellular ROS during DMBA application which may be attributed to repeated exposures to tumor promoters create a chronic inflammatory state with a sustained release of ROS, which results in chronic oxidative stress. Free radicals and non-radical ROS such as H_2O_2 released by phagocytic cells can cause damage, such as DNA strand breaks, mutations, sister chromatid exchanges, protein modifications and lipid peroxidation, to adjacent epithelial cells. In addition, protein modifications induced by free radicals/ROS can affect DNA repair capacity, transcriptional regulation, apoptosis, metabolism and cell signaling.[26] The results of Ebihara et al.[28] do not match with the present study as they stated that, at approximately 8–12 weeks, moderate and severe dysplasia was observed. At approximately 14–16 weeks CIS was seen. At approximately 18–20 weeks, SCC was observed. These discrepancies may be explained with that, they used different types of fixation and DMBA solvent than that used in this study. In current study, GIIC (DMBA treated group at 12 weeks), the Bcl-2 immunohistochemical results revealed strong positive cytoplasmic expression (mean=63.07) throughout the tumor cells. These results are in agreement with those of some studies[21, 23, 27, 34] . Over expression of anti-apoptotic Bcl-2 may contribute to that, Bcl-2 prolong cell survival through inhibiting apoptosis by prevent the release of Cyt C from mitochondria and may promote tumor development. In disagreement with the present study, Ribeiro *et al*.[22]stated that, following 12 weeks of carcinogen administration, there are no changes in the expression level of Bcl-2, but the distribution of Bcl-2 is altered, being restricted to the superficial layers of the epithelium. These differences in results may be attributed to that; they used different type of carcinogen material and different kind of animals. In the present study, GIIC (DMBA treated group at 12 weeks), The Bax immunohistochemical results revealed weak positive cytoplasmic expression (mean=12.47) throughout the tumor cells but the expression was in lower level than normal epithelium and at 4, 8 weeks. These results are in agreement with those of some researches[20, 21, 35, 36]. The decreased expression of Bax in the cancerous tissues may be due to increase reduction of apoptotic cell death as well as accelerated their growth.

There was highly statistically significant differences between the expression of Bcl-2 and Bax in oral epithelial dysplasia group II (GIIA & GIIB) and OSCC (GIIC) were observed (the P value recorded 0.001) which mean, the increased expression of Bcl-2 and decreased expression of Bax in OSCC as compared to oral epithelial dysplasia may be an evidence of the disease progression of oral epithelial dysplasia to OSCC, as the results of this study

suggest alterations in expression of Bcl-2 family proteins, creating a favorable environment for malignant transformation. The findings from this study showed that, inhibition of apoptosis is a frequent event in oral carcinogenesis. The Bcl-2 family of proteins appears to be involved in regulating the terminal differentiation of keratinocytes. The down regulation of Bcl-2 expression and up regulation of Bax was concomitant with terminal differentiation. Over expression of antiapoptotic protein Bcl-2 and down expression of proapoptotic protein Bax, protects the tumor cells from undergoing apoptosis, thus facilitating their survival.

In the present study, GIIIA (oxygenated water treated group), gross observation showed somewhat improvement in the clinical condition of hamsters in comparing to GIIA (DMBA treated group) in which, there are no erosion or erythematous areas. These findings conflicted on H&E staining in which only 3 animals exhibited mild epithelial dysplasia and 12 animals exhibited hyperkeratosis to normal. In this study, GIIIA (oxygenated water treated group), The Bcl-2 immunohistochemical results revealed weak positive cytoplasmic expression (mean =10.6) throughout the epithelial layers while the Bax expression showed moderate positive cytoplasmic expression (mean = 41.84) throughout the epithelial layers. These findings are in line with Nafarzadeh et al. [36], stated that, The more distribution of the Bax than Bcl-2 may be consistent with the concept that, Bax regulates apoptosis by forming heterodimers with Bcl-2. These findings indicate that oxygenated water caused improvement of apoptosis through decrease the chance of tissue hypoxia and maintains the normal oxygenation level in the diseased tissues. There was highly significant difference between the expression of Bcl-2 in GIIA and GIIIA were observed (the P value recorded 0.001). However, the Bax expression showed non-significant difference between the same subgroups observed (the P value recorded 0.062) which mean, the significant decreased expression of Bcl-2 and slightly increased expression of Bax in GIIIA as compared to GIIA may be explained by the disease began to regress to normal condition, as a results of eliminating the hypoxia occurred by carcinogenic material through oxygenated water.

In the current study, GIIIB (oxygenated water treated group), gross observation showed somewhat improvement in the clinical condition of hamsters in compare to GIIB (DMBA treated group) in which, there are decrease in number and size of the nodular elevations, ulcerative and bleeding areas. These findings conflicted on H&E staining in which 6 animals exhibited mild epithelial dysplasia and 9 animals exhibited hyperkeratosis to normal. In this study, GIIIB (oxygenated water treated group), The Bcl-2 immunohistochemical results revealed moderate positive cytoplasmic expression (mean =31.02) throughout the epithelial layers while the Bax expression showed moderate positive cytoplasmic expression (mean = 39.15) throughout the epithelial layers. This can be explained by what was reported by Greijer and Van der Wall [37], indicated that during hypoxia, antiapoptotic proteins can be over expressed, whereas the proapoptotic protein can be down regulated. Thus, when the cells treated with a strong apoptosis inducer, become more close to apoptosis. In the current study, the oxygenated water act as antioxidant that work to relieve oxidative stress and back to the normal state of the cell. In the current study, there was highly significant difference between the expression of Bcl-2 and Bax in GIIB and GIIIB were observed (the P value recorded 0.002 and 0.001 respectively) which mean, the decreased expression of Bcl-2 and increased expression of Bax in GIIIB as compared to GIIB may be an evidence of the disease regression to normal condition, as a results of improving oxygenation level and decrease the hypoxia through oxygenated water.

In the present study, GIIIC (oxygenated water treated group), gross showed almost the same clinical condition of hamsters in compare to GIIC (DMBA treated group) in which, there are exophytic masses of variable size surrounded with area of ulceration and bleeding .These findings conflicted on H&E staining in which all animals exhibited well differentiated SCC. In this study, GIIIC (oxygenated water treated group), the Bcl-2 immunohistochemical results revealed strong positive cytoplasmic expression (mean = 61.25) throughout the tumor cells while the Bax expression showed weak positive cytoplasmic expression (mean = 23.63) throughout the tumor cells. There was non-significant difference between the expression of Bcl-2 in GIIC and GIIIC were observed (the P value recorded 0.749). However, the Bax expression showed highly significant difference between the same subgroups(the P value recorded 0.001) which mean, the slightly decreased expression of Bcl-2 and increased expression of Bax in GIIIC and GIIC may be due to the advanced stage of the disease or toxicity by oxygen free radicals. The decrease in improvements of animals of GIIIC in compare to GIIIA and GIIIB may be attributed to toxicity that may happen due to the presence of few traces of H_2O_2 in the administrated oxygenated water due to incomplete conversion of H_2O_2 by the catalyst (MnO2).

Accumulated evidence suggests that the proteins of the Bcl-2 gene family may interact with each other as homodimers and heterodimers, and that their relative proportions regulate the process of apoptosis [39] .Therefore, the current study analyzed the possible correlation between two of these proteins in determining clinical outcome.

Interestingly, this study found that the ratio of Bcl-2/Bax expression appeared to be the best variable in predicting disease specific survival in OSCC in which, there was a statistically significant negative (reverse) correlation between Bcl-2 and Bax (r= -0.785) (p-value= 0.000).This means that an increase in one variable is associated with decrease in the other variable and vice versa. Animals with a high Bcl-2/Bax expression ratio had a significantly poorer prognosis than those with a low Bcl-2/Bax ratio. Chen et al.[40] confirmed these results. These findings are consistent also with certain tumors at other sites, such as gastric carcinoma[41]and neuroendocrine lung carcinoma[42] . In the study done by Xie et al.[38] stated that, the Bcl-2/ Bax expression ratio was the strongest independent prognostic parameter more than apoptotic index, individual Bax and Bcl-2 expression.

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