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EFFECT OF OXYGENATED WATER AS A NEW CHEMOPREVENTIVE MODALITY IN EXPERIMENTALLY INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS

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ABSTRACT

The aim of the present study was directed to investigate the effect of oxygenated water as a new chemopreventive modality in experimentally induced hamster buccal pouch (HBP) carcinogenesis. **Material and methods**: sixty one golden Syrian male hamsters five weeks old, weighting 80-120 gs were used as experimental animals and divided into three group$_{(s)}$ ($G_{(S)}$) (G1, G2 and G3): G1 (negative control): 5 animals were left untreated. G2: (DMBA painting-HBP group): 14 animals, their right buccal pouches were painted with 0.5% DMBA in paraffin oil 3 times a week. G3 (oxygenated water chemoprevention group): 42 animals, were subdivided according to the methods of receiving oxygen into 3 equal models each contained 14 animals: G3A which received oxygen in the free access (in water), G3B which received oxygen once daily obligatory 1 ml of the (by oral tube) in addition to the free access and G3C which received oxygen twice daily obligatory 1 ml in addition to the free access. At 6 and 14 weeks, 7 (half) animals of G2 and of each model of the G3 (G3A, G3B and G3C) were sacrificed, and the buccal mucosa was excised. **Results**: Gross observations revealed variable features in the treated groups (G2 and G3) compared to that observed in group G1ranging from normal and smooth surface to fungating tumor masses of large sizes. Histopathological findings revealed variations among the treated groups ranging from normal epithelial layers to epithelial dysplasia to squamous cell carcinoma with invading nests and pearls. Immunohistochemical (IHC) results, regarding Bax expression, revealed variability in the area percentage throughout the groups used. At 6 weeks, area percentages of G2, G3A, G3B and G3C were (28.58 %, 32.9 %, 37.86% and 41.27%) respectively, and at 14 weeks were (10.38 %, 14.64 %, 27.19 % and 15.59 %) respectively. Bcl2 expressions also had variability in the area percentage throughout the same groups at 6 weeks were (31.77 %, 27.36 %, 16.9 % and 8.8 %) respectively, while at 14 weeks they were (68.3 %, 54.8 %, 39.8 % and 51.74 %) respectively. **Conclusion**: Oxygenated water is considered as a promising chemopreventive agent in prevention of induced HBP carcinogenesis (epithelial dysplasia & invasive carcinoma) and prove beneficial role in improving the outcome by modulating apoptosis and proliferation throughout the process of carcinogenesis as visualized by Bax and Bcl2.

KEYWORDS: HBP carcinoma, oxygenated water, hypoxia.

INTRODUCTION

Oral carcinogenesis is a highly complex multifocal process that takes place when squamous epithelium is affected by several genetic alterations [(1)]. Dysplastic changes are one of the sings of premalignancy which can be observed in histopathologic view especially in epithelium. Recognition of such changes is vital in preventing carcinoma changes [(2,3)]. Cancer is usually initiated as a result of the stepwise accumulation of genetic and epigenetic changes in the epithelial compartment. However, increasing evidence indicated that the tumor microenvironment can dictate aberrant tissue function and play a critical role in the subsequent development of more advanced and refractory malignancies [(4)]. Induction of oral squamous cell carcinoma (OSCC) can be successfully done by 7, 12-dimethylbenz[a]anthracene (DMBA) in hamster buccal pouch (HBP).

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It has been found that induced OSCC closely mimics with that of human on morphological, histological and biochemical aspects as well as at molecular level [5]. Hypoxia was shown to promote angiogenesis and distant metastasis processes that add to the challenge of managing hypoxic solid tumors [6]. Therefore, low oxygenation status and intensified angiogenesis are equally linked to treatment failure in head and neck cancer [7] .The identification of reliable factors which can determine a best or worst prognosis is a continuous challenge since the overall mortality rate for OSCC has remained unchanged at approximately 50% [8-10] .Oxygen therapy has, for centuries, been used to improve or cure disorders involving hypoxia and ischemia, by enhancing the amount of dissolved oxygen in the plasma and thereby increasing oxygen delivery to the tissue [11] .Oxygen therapy was found to be, possibly through stimulation of angiogenesis, the most effective available therapy for severe and chronic radiation injury to the alimentary tract [12] . The peroral administration of oxygen-enhanced water was found to be a feasible technique to increase the oxygen supply to tumors. Oxygen-enhanced water also called oral oxygen therapy (OOT) and the effect of OOT in the therapy of head and neck carcinomas has been measured by pO2- histography [13]. In this regard, the present study was carried out to investigate the effect of oxygenated water as a new chemopreventive modality in induced HBP carcinogenesis. The assessment was based on the gross observation, histological tumor tissue changes and immunohistochemical (IHC) examination utilizing antibodies against Bcl-2 and Bax proteins.

MATERIAL AND METHODS

Sixty one golden Syrian male hamsters five weeks old, weighting 80-120g_s were obtained from the animal house, Cairo University (Cairo, Egypt).The hamsters were used as model for OSCC induction utilizing 7, 12 DMBA (Sigma-aldrich company) (0.5% in paraffin oil) as chemical carcinogen. Oxygen was used in the form of oxygenated water and given as a chemopreventive agent in different methods. Oxygenated water was prepared according to the following equation: $\mathbf{2H_2O_2+MnO_2 \rightarrow 2H_2O+O_2\uparrow+MnO_2\downarrow}$.

Regarding partial conversion

2 g_s of MnO_2 were added to 20 ml_s of H_2O_2 and the reaction was left for 15 min then the mixture was filtrated on filter paper. 2 ml_s of the filtrated mixture was added to 58 ml_s of distilled water and mixed well and then left to be given obligatory via oral tube.

Regarding complete conversion

20 g_s of MnO_2 were added to 50 ml_s H_2O_2 then to 950 ml_s 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 throughout the experiment. 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 as an anticancer agent before. The amount of dissolved oxygen in the water was determined before the beginning of the experiments in the National Research Center, 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.

Experimental design

The experimental animals were divided into three $group_{(s)}$ ($G_{(S)}$). G1 (negative control): 5 hamsters, not treated and served as negative controls. G2: (DMBA painting-HBP group): 14 hamsters, the

right HPB_s were painted with 0.5% DMBA (Sigma Aldrich) in paraffin using a number 4 camel hair brush three times a week. G3 (oxygenated water chemoprevention group): 42 animals, were subdivided according to the methods of receiving oxygen into 3 models (G3A, G3B and G3C) each contained 14 animals: G3A which received oxygen in the free access (in water), G3B which received oxygen once daily obligatory 1 ml of the mixture (by oral tube) in addition to the free access and G3C which received oxygen twice daily (6-8 hours intervals) obligatory each one 1 ml (by oral tube) in addition the free access. At 6 and 14 weeks, 7 (half) animals of G2 and of each model of the G3 (G3A, G3B and G3C) were sacrificed, and the buccal mucosa was excised.

Investigations

After termination of the experiment and recording all gross observations and alterations that may happened throughout the experiment, the animals were sacrificed by cervical dislocation, 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 and then statistical analysis based on these examinations was done.

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

For immunohistochemical examination:

Other tissue sections were cut at 4μm and put on positive charged slides for the application of standard labeled streptavidin- biotin method to apply each antibody used separately (Bcl-2 and Bax antibodies). Paraffin sections were cut and mounted on positive charged glass slides. Each section was carried into two similar sections one for Bax and one for Bcl-2.The sections were deparaffinized in xylene and rehydrated through graded ethanol (100 %, 95 % and 70 %) each run for 5 minutes. Slides were washed in distilled water then in phosphate buffered saline (PBS), each for 5 minutes. Endogenous peroxidase activity was blocked using 3% solution of hydrogen peroxide (H_2O_2) in methanol for 30 minutes at room temperature. Slides were then washed in PBS. Slides were then immersed in plastic jars containing 200 ml of 10 M citrate buffer (pH 6) (ready to use from DAKO). The jars were put in microwave at maximum power at 100°C for 3 intervals, each one 5 minutes. Slides were left at room temperature to coal gradually. Slides were then washed in distilled water followed by PBS for 5 minutes. Tissue sections were received one or two drops of the primary antibodies (Bax or Bcl-2) in a dilution of 1:100 and incubated in a humid chamber at room temperature overnight. Slides were then washed in distilled water, followed by PBS for 5 minutes. Biotinylated secondary antibody was added and incubated at room temperature for 30 minutes. Tissue sections were then washed in PBS for 5 minutes. One or two drops of peroxidase-labeled streptavidin were applied for 30 minutes at room temperature then washed in PBS. The tissue sections were received DAB for 2-4 minutes to develop color, followed by putting in distilled water. Tissue sections were counterstained using Mayer's hematoxylin for one minute and then washed in tap water. The slides were placed in two changes of 95% alcohol followed by two changes of absolute alcohol, each for 3 minutes then mounted with DPX and covered with plastic covers in order to be examined. 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 each

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).[14]

RESULTS

The gross observation results **(Table 1)**: HBP mucosae of G1 were pink in color with smooth surface and no observable abnormalities **(Fig.1)**. At 6 weeks: in G2, HBP mucosae showed multiple small nodules, area of ulceration and bleeding **(Fig.2)**, in G3A, HBP mucosae showed few small nodules with bleeding **(Fig.3)**, in G3B, HBP mucosae showed some areas of ulceration and bleeding **(Fig.4)**, in G3C, HBP mucosae showed absence of observable changes **(Fig.5)**. At 14 weeks: in G2, HBP mucosae showed variable sized nodular elevations and fungating tumor masses **(Fig.6)**, in G3A, HBP mucosae showed nodular elevations with tumor masses **(Fig.7)**, in G3B, HBP mucosae showed noticeable small sized tumor growth **(Fig.8)**, in G3C, HBP mucosae showed small nodule **(Fig.9)**.

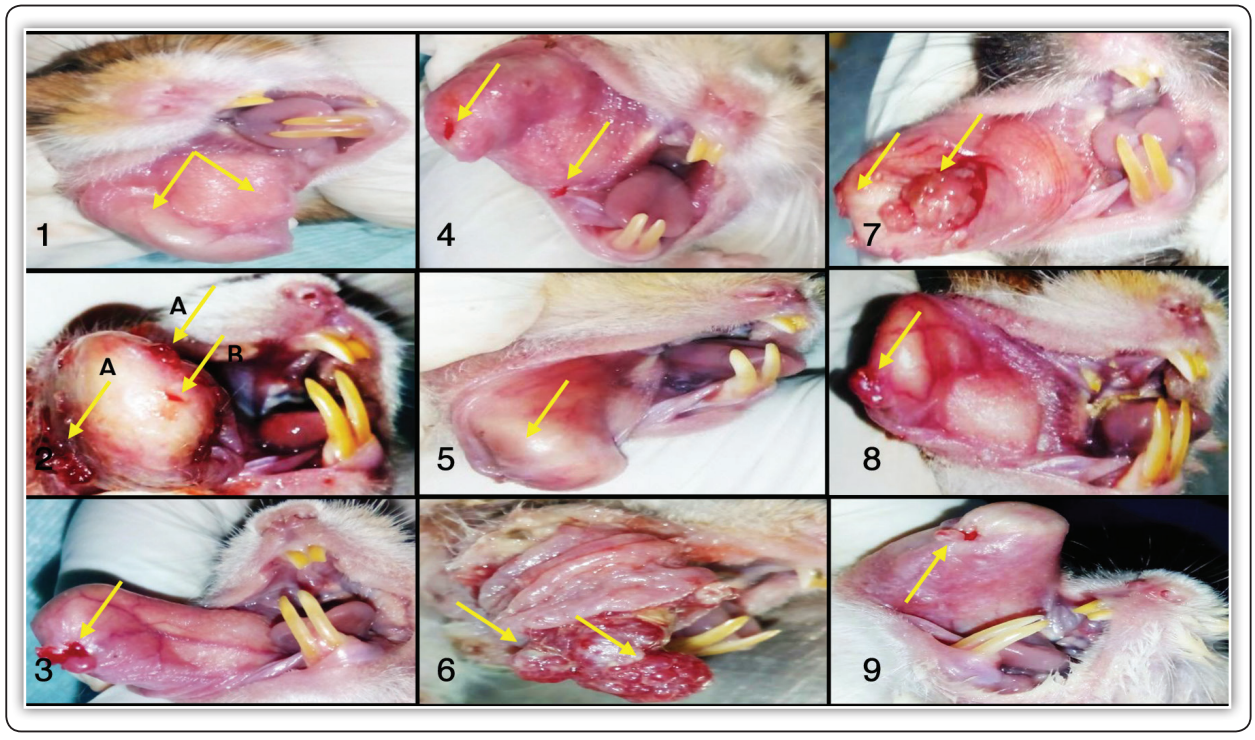

Table.1 (Fig.1): HBP of GI showing normal buccal pouch mucosa which appeared pink in color with smooth surface (arrows). Fig.2: HBP of G2 animal at 6 weeks showing multiple small nodules (arrow A), area of ulceration and bleeding (arrow B). Fig.3: HBP of G3A animal at 6 weeks showing few small nodules with bleeding (arrow). Fig.4: HBP of G3B animal at 6 weeks showing some areas of ulceration and bleeding (arrows).Fig.5: HBP of G3C animal at 6 weeks showing absence of observable changes (arrow). Fig.6: HBP of G2 animal at 14 weeks showing variable sized nodular elevations and fungating tumor masses (arrows). Fig.7: HBP of G3A animal at 14 weeks showing nodular elevations with tumor masses (arrows). Fig.8: HBP of G3B animal of at 14 weeks showing noticeable small sized tumor growth (arrow). Fig.9: HBP of G3C animal at 14 weeks showing small nodule (arrow).

Histopathological and immunohistochemical results (Table 2):

In G1, histological sections, using H&E stain, revealed normal HBP mucosae, composed of thin stratified squamous epithelium, consists of two to four layers of squamous cells exhibiting slight keratinization one layer of basal cells and one, two or three layers of spinous and thin keratinized cells with lacking rete ridges. Subepithelial connective tissue (C.T), muscular layer and areolar layer were seen **(Fig.1)**. The IHC staining using Bax showed moderate (45.3%) positive expression which present throughout the epithelial layers **(Fig.2)** while Bcl-2 expression exhibited weak (6.72%) positive expression which limited to basal and suprabasal layers **(Fig.3)**. At 6 weeks: regarding G2, histological sections, using H&E stain, revealed that the overlying epithelium has obvious, hyperplasia, hyperkeratosis and severe dysplastic features in most epithelial layers in multiple areas including: loss of adhesion, hyperchromatism and abnormal mitosis with intact basement membrane **(Fig.4)**. IHC staining using Bax showed moderate (28.58 %) positive cytoplasmic expression throughout the dysplastic epithelial layers **(Fig.5)**, similarly Bcl-2 expression exhibited moderate (31.77 %) positive cytoplasmic expression throughout the dysplastic epithelial layers **(Fig.6)**. Regarding G3A at 6 weeks, histological sections, using H&E stain, revealed that the overlying epithelium in most animals of G3A (6 of 7) specimens has hyperplasia, hyperkeratosis and moderate to severe dysplastic features in most epithelial layers in multiple areas including: loss of adhesion and abnormal mitosis with intact basement membrane **(Fig.7)** while only one specimen appeared normal almost the same as G1. IHC staining using Bax showed moderate (32.9 %) positive cytoplasmic expression throughout the dysplastic epithelial layers **(Fig.8)**, similarly Bcl-2 expression exhibited moderate (27.36 %) positive cytoplasmic expression throughout the dysplastic epithelial layers **(Fig.9)**. Regarding G3B at 6 weeks, histological sections, using H&E stain, revealed that the overlying epithelium in most animals of (6 of 7) specimens have hyperplasia, hyperkeratosis and mild to moderate dysplastic features in few epithelial layers in some areas including: loss of adhesion and pleomorphism **(Fig.10)** while only one specimen appeared normal. IHC staining using Bax showed moderate (37.86%) positive cytoplasmic expression throughout the epithelial layers **(Fig.11)**, while Bcl-2 expression exhibited mild (16.9 %) positive cytoplasmic expression throughout the epithelial layers **(Fig.12)**. Regarding G3C at 6 weeks, histological sections, using H&E stain, revealed that the overlying epithelium in specimens of 3 animals appeared normal and few areas in the remaining 4 specimens have mild dysplastic features; hyperplasia and hyperkeratosis which appeared limited to few layers of the whole epithelial thickness **(Fig.13)**. IHC staining using Bax showed moderate (41.27 %) positive cytoplasmic expression throughout the epithelial layers **(Fig.14)**, while Bcl-2 expression exhibited mild (8.8 %) positive cytoplasmic expression throughout the epithelial layers **(Fig.15)**.

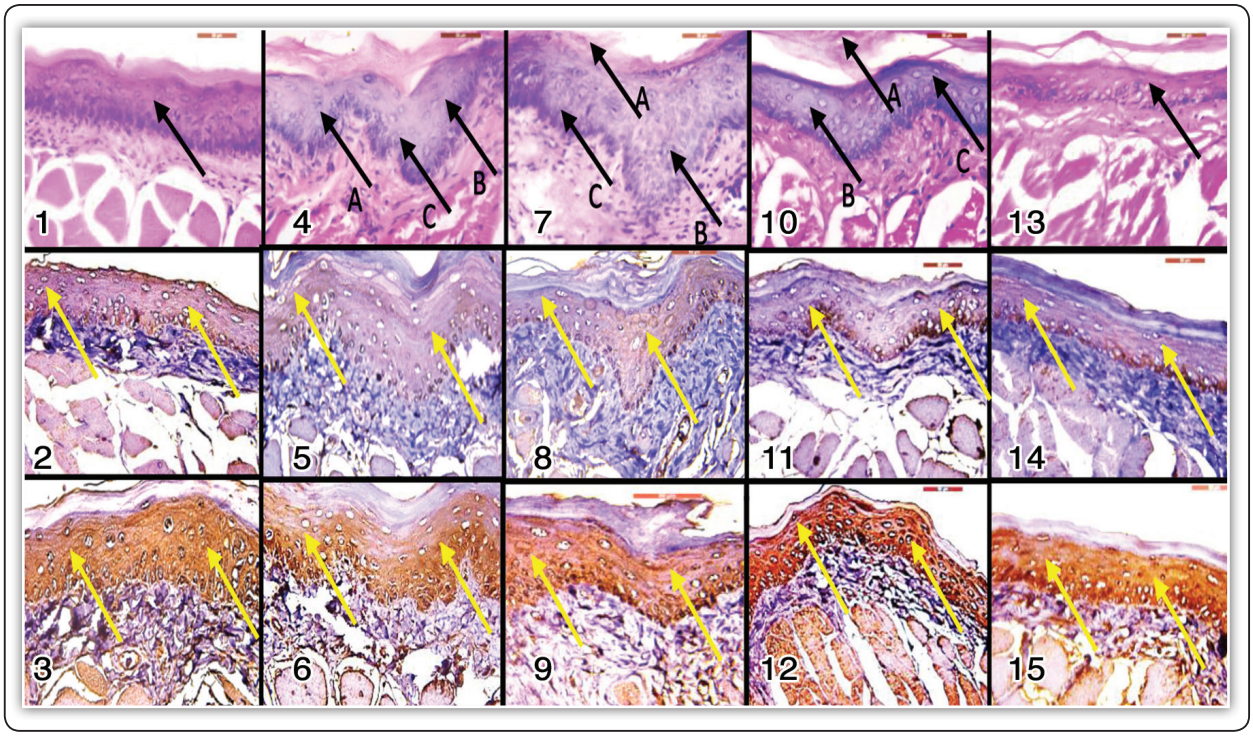

Table.2 (Fig.1): H&E stain of G1 showing: keratinized stratified squamous epithelium (arrow). Fig.2: IHC expression of Bax showing positive cytoplasmic expression throughout the epithelial layers (arrows). Fig.3: IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the epithelial layers (arrows). Fig.4: H&E stain of G2 at 6 weeks showing epithelial dysplastic features including: abnormal mitosis (arrow A), hyperchromatism (arrow B) and loss of adhesion (arrow C). Fig.5: IHC expression of Bax showing positive cytoplasmic expression throughout the dysplastic epithelial layers (arrows). Fig.6: IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the dysplastic epithelial layers (arrows). Fig.7: H&E stain of G3A at 6 weeks showing: hyperkeratosis (arrow A) and epithelial dysplastic features including: loss of adhesion (arrow B) and abnormal mitosis (arrow C). Fig.8: IHC expression of Bax showing positive cytoplasmic expression throughout the dysplastic epithelial layers (arrows). Fig.9: IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the dysplastic epithelial layers (arrows). Fig.10: H&E stain of G3B at 6 weeks showing: hyperkeratosis (arrow A) and epithelial dysplastic features including loss of adhesion (arrow B) and pleomorphism (arrow C). Fig.11: IHC expression of Bax showing positive of cytoplasmic expression throughout the epithelial layers (arrows). Fig.12: IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the epithelial layers (arrows). Fig.13: H&E stain of G3C at 6 weeks showing almost normal epithelial layers (arrow). Fig.14: IHC expression of Bax showing positive cytoplasmic expression throughout the epithelial layers (arrows). Fig.15: IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the epithelial layers (arrows).

At 14 weeks **(Table 3)**: regarding G2, histological sections, using H&E stain, revealed that that the overlying epithelium has obvious dysplastic features in multiple areas with evidence of prominent true invasion. Appearance of various squamous cell carcinoma (SCC) nests: well differentiated as well as moderately differentiated were observed in the underlying C.T. **(Fig.1)**. IHC staining using Bax showed mild (10.38 %) positive cytoplasmic expression throughout the invading nests **(Fig.2)**, while Bcl-2 expression exhibited strong (68.3 %) positive cytoplasmic expression throughout the invading nests **(Fig.3)**. Regarding G3A at 14 weeks: histological sections, using H&E stain, revealed that the overlying epithelium in most animals of G3A (5 of 7) specimens has obvious dysplastic features in multiple areas with evidence of prominent true invasion with formation of epithelial nests. Well differentiated SCC nests were observed in the underlying C.T. **(Fig.4)**, while other 2 specimens exhibited severe dysplastic features without true invasion. IHC staining using Bax showed mild (14.64 %) positive cytoplasmic expression throughout the invading nests **(Fig.5)**, while Bcl-2 expres-

sion exhibited strong (54.8 %) positive cytoplasmic expression throughout the invading nests **(Fig.6)**. Regarding G3B at 14 weeks, histological sections, using H&E stain, revealed that the overlying epithelium of most animals of G3B (5 of 7) specimens has severe dysplastic features and carcinoma in situ that the dysplastic features include the whole thickness of epithelial layers including pleomorphism, abnormal mitosis and loss of adhesion, also hyperplasia and hyperkeratosis can obviously be seen without true invasion **(Fig.7)**, while only 2 specimens exhibited invasive SCC. IHC staining using Bax showed moderate (27.19 %) positive cytoplasmic expression throughout the dysplastic epithelial layers **(Fig.8)**, similarly Bcl-2 expression exhibited moderate (39.8 %) positive cytoplasmic expression throughout the dysplastic epithelial layers **(Fig.9)**. Regarding G3C at 14 weeks, histological sections, using H&E stain, revealed that the overlying epithelium in 4 specimens had developed SCC with prominent invading nests, while the other 3 specimens had severe dysplastic features, carcinoma in situ and early invasive SCC **(Fig.10)**. IHC staining using Bax showed mild (15.59 %) positive cytoplasmic expression throughout the epithelial layers **(Fig.11)**, while Bcl-2 expression exhibited strong (51.74 %) positive cytoplasmic expression throughout the epithelial layers **(Fig.12)**.

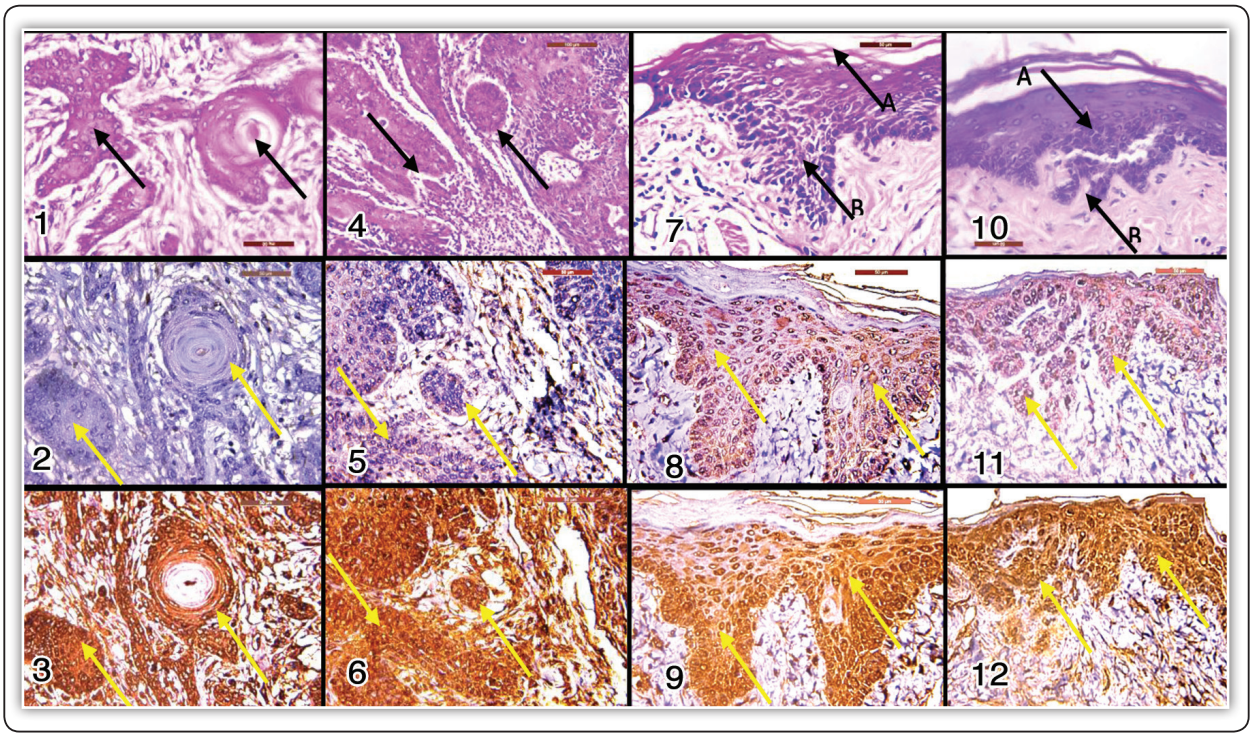

Table.3 (Fig.1): H&E stain of G2 at 14 weeks showing epithelial nests with keratin pearls invading the C.T. (arrows). Fig.2: IHC expression of Bax showing positive cytoplasmic expression throughout the invasive nests (arrows). Fig.3: IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the invasive nests (arrows). Fig.4: H&E stain of G3A at 14 weeks showing epithelial nests invading the C.T. (arrows). Fig.5: IHC expression of Bax showing positive cytoplasmic expression throughout the epithelial layers and invasive nests (arrows). Fig.6: IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the epithelial layers and invasive nests (arrows). Fig.7: H&E stain of G3B at 14 weeks showing hyperkeratosis, hyperplasia (arrow A), and epithelial dysplastic features (arrow B). Fig.8: IHC expression of Bax showing positive cytoplasmic expression throughout the dysplastic epithelial layers (arrows). Fig.9: IHC expression of Bcl-2 showing positive cytoplasmic expression throughout the dysplastic epithelial layers (arrows). Fig.10: H&E stain of G3C at 14 weeks showing areas of epithelial dysplasia (arrow A) with early invasion of epithelial cells to the C.T. (arrow B). Fig.11: IHC expression of Bax showing positive cytoplasmic expression throughout the dysplastic epithelial layers reach to negative in the early invasive nests (arrows). Fig.12: IHC expression of Bcl-2 showing strong positive cytoplasmic expression throughout the dysplastic epithelial layers and early invasive nests (arrows).

Statistical analysis results of Bcl-2 & Bax expression were obtained by comparing the area % between the groups used. Statistical analysis results were revealed that , in regard to expression of Bax at 6 weeks, G1 had recorded the highest mean area percentage (45.3%), while G2 had recorded the lowest mean area percentage (25.58%) and the comparison revealed that there was high significant difference between G1 and G2 where P value was (0.009), there was significant difference between G1 and G3A where P value was (0.044), there was no significant difference between G1 and G3B where P value was (0.220), there was no significant difference between G1 and G3C where P value was (0.528) **(Chart 1)**. While in regard to expression of Bcl-2 at 6 weeks, G2 had recorded the highest mean area percentage (31.77%), while G1 had recorded the lowest mean area percentage (6.72%) and the comparison revealed that there was high significant difference between G1 and G2 and between G1 and G3A where P value was (<0.001), there was high significant difference between G1 and G3B where P value was (0.000) and there was no significant difference between G1 and G3C where P value was (0.061) **(Chart 2)**.

In regard to expression of Bax at 14 weeks, G1 had recorded the highest mean area percentage (45.3%), while G2 had recorded the lowest mean area percentage (10.38%) and the comparison revealed that there was high significant difference between G1 and G2, G1and G3A, G1 and G3B and between G1 and G3C respectively where P value was (<0.001) **(Chart 3)**. While in regard to expression of Bcl-2 at 14 weeks, G2 had recorded the highest mean area percentage (68.3%), while G1 had recorded the lowest mean area percentage (6.72%) and the comparison revealed that there was high significant difference between G1 and G2 and between G1 and G3A where P value was (<0.001), there was high significant difference between G1 and G3B where P value was (0.000), and there was high significant difference between G1 and G3C where P value was (<0.001) **(Chart 4)**.

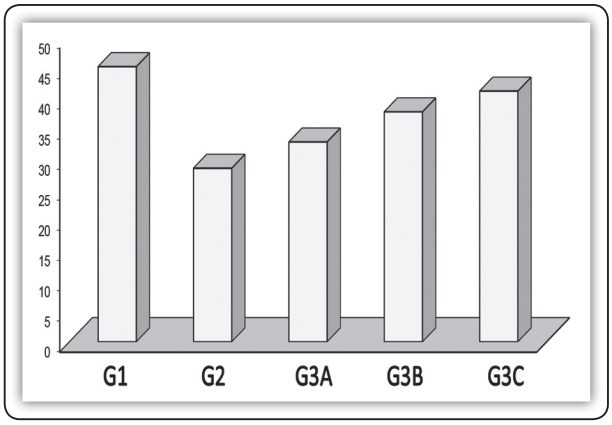

Chart 1 : Bar chart representing mean area % results of Bax expressions in the groups used at 6 weeks.

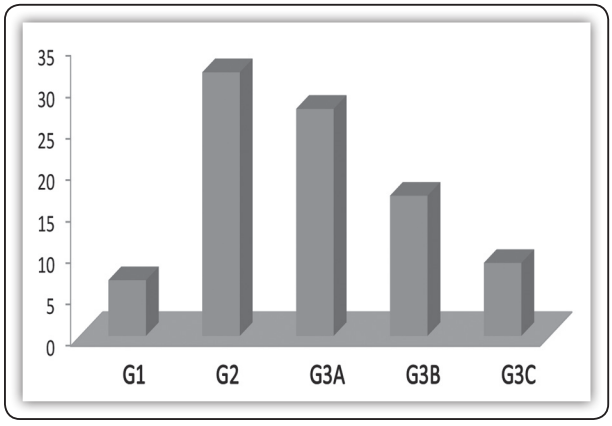

Chart 2: Bar chart representing mean area % results of Bcl-2 expressions in the groups used at 6 weeks.

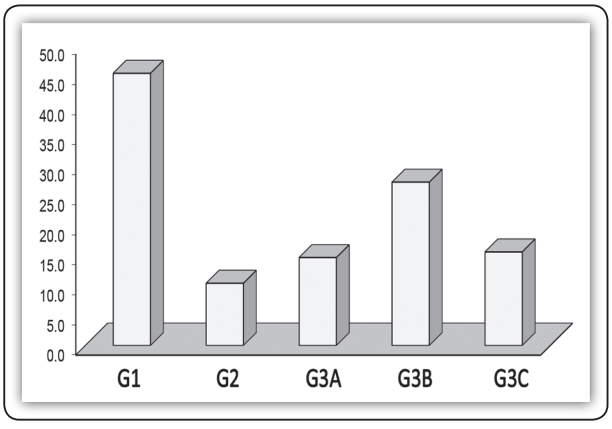

Chart 3: Bar chart representing mean area % results of Bax expressions in the groups used at 14 weeks.

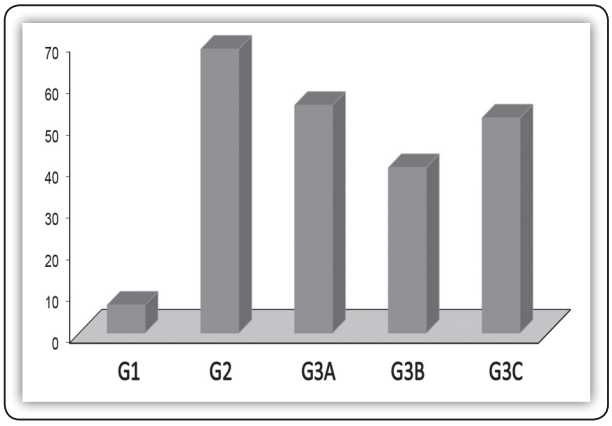

Chart 4: Bar chart representing mean area % results of Bcl-2 expressions in the groups used at 14weeks regarding.

DISCUSSION

Oral carcinogenesis is a highly complex process which takes place when squamous epithelium is affected by multiple genetic and environmental alterations. The challenge now directed not only toward the treatment, but also toward early detection or even toward preventing the progression of this process using new chemopreventive modalities. In the present study, the results of the effect of oxygenated water as a new chemopreventive modality in experimentally induced HBP carcinogenesis revealed variable alterations. The results of H&E stain revealed that topical application of DMBA to the HBP for 6 weeks induced severe epithelial dysplasia and for 14 weeks induced invasive SCC. In the DMBA treated animals, the development of dysplastic features as well as tumor cell invasion were realized. These results are in agreement with those of other investigators [5,15,16]. The current results with those of other studies [5,17-19] supported the concept that DMBA induced HBP carcinoma appeared to go through the same changes as in human. Many of the structural alterations observed in carcinogen-treated HBP mucosa, both at gross and the light microscopic observations closely resemble those observed during the course of human oral cancer development. 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 [20,21]. The present work supports that HBP carcinogenesis model is one of the most well characterized animal systems to analyze the stepwise evolution of OSCC. The gross observation in G1 (control: untreated animals) showed no observable gross changes. HBP mucosa appeared normal, with smooth surface. After sacrificing, the buccal pouches length was about 5cm for all hamsters. Other studies reported almost the same findings [22-24]. H&E stain showed that the mucosa is composed of a thin stratified squamous epithelium exhibiting slight keratinization with lacking rete ridges. Sub-epithelial C.T and muscular layer were seen.[25,26] The weak (6.72 %) positive cytoplasmic expression of Bcl-2, in the basal and suprabasal epithelial layers, is in agreement with those of other investigators [22,24,27] .The moderate (45.3 %) cytoplasmic expression of Bax, in the superficial epithelial layer, is in agreement with those of other investigators [27,28] .These investigations 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 Cytochrome 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, gross observations of G2 (DMBA treated group), at 6 weeks, revealed multiple small nodules, areas of ulceration and bleeding while at 14 weeks HBP mucosa revealed variable sized nodules and fungating tumor masses, eroded and ulcerative areas with hard or rubbery consistency on palpation. These are in consistence with that shown by other investigators [26,29,30]. These results were confirmed by H&E stain. The results at 6 weeks revealed severe epithelial dysplasia while at 14 weeks revealed SCC. These results are in agreement with those of other investigators [21-24]. The dysplastic effect caused by DMBA at 6 weeks and the carcinogenic effect at 14 weeks were attributed to chronic inflammation that led to generation of reactive oxy-

gen species (ROS) during DMBA application. The toxic metabolites of DMBA, including ROS are capable of binding to adenine residues of DNA causing chromosomal damage. The increase of ROS by time 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 (medina) and the respiratory chain, amplifying the ROS generation [22,31-33]. In the present study, the Bcl-2 immunohistochemical results at 6 weeks revealed moderate (31.77 %) positive cytoplasmic expression while at 14 weeks revealed strong (68.3 %) positive cytoplasmic expression. Similarly Bax immunostaining results at 6 weeks revealed moderate (28.58 %) positive cytoplasmic expression while at 14 weeks revealed weak (10.38 %) positive cytoplasmic expression. Contrarily in invasive epithelial nests, Bax immunostaining revealed negative expression. These results are in agreement with those of other investigators [22,24,27,28,30,31,34] .These results were attributed to the important role of Bcl-2 in promoting cell survival and inhibition of apoptosis, whereas Bax is involved in induction of apoptosis and both Bcl-2 and Bax together regulate the mitochondrial transmembrane passage of cytochrome C which in turn activates caspase protein, and so the effect of DMBA led to disturbance in Bcl2/Bax ratio which determine the cell survival or death [35] .

Hypoxia is a non-physiological level of oxygen tension, a phenomenon common in a majority of malignant tumors and in response tumor-hypoxia leads to advanced but dysfunctional vascularization [36] .Intratumorally hypoxia has been identified as an adverse indicator for patient prognosis independent of clinical stage at diagnosis in meantime cancer cells respond to decreased oxygen availability by increasing the activity of the hypoxia-inducible factors, HIF-1 and HIF-2 [37] .Based on other oxygen therapy like hyper baric oxygen therapy (HBOT), ozone and hydrogen peroxide, the current study showed the effect of oxygenated water as a new chemopreventive modality on DMBA induced HBP carcinogenesis. The results of G3, at 6 weeks, revealed variable observations for each model. Gross observation of G3A model revealed decrease in distribution and size of the nodules, ulcerative and bleeding areas compared to G2 at the same period. Consistently H&E stain revealed moderate to severe dysplastic features in multiple areas, hyperplasia and hyperkeratosis in 6 of 7 HBP mucosae while the last HBP mucosa appeared with normal features. Gross observations of G3B model revealed marked improvements, but only 2 of 7 HBP mucosa had some areas of ulceration, bleeding and whitish color with slight elevations while 5 animals appeared without observable abnormalities. H&E stain revealed that, the overlying epithelium in most HBP mucosae (6 of 7) has mild to moderate dysplastic features in some areas; hyperplasia and hyperkeratosis while only the last HBP mucosa appeared with normal features. The best results at 6 weeks appeared in G3C model in which the gross observations of HBP mucosa revealed few irregularities and whitish in color in 2 of 7 HBP mucosae while the remaining (5 HBP mucosae) appeared normal without observable abnormalities compared to other groups G2, G3A and G3B. H&E stain revealed that the overlying epithelium in 3 specimens appeared normal and few areas in the remaining (4 specimens) showed mild dysplastic features; limited hyperplasia and hyperkeratosis.

The improvements in G3 which shown in gross observations and H&E results were clear and these results are in agreement with those have been observed by other investigators.[38-40] Immunohistochemical staining utilizing Bcl-2 and Bax antibodies in G3 at 6 weeks also revealed different results in each model of this group. In G3A model, Bcl-2

revealed moderate (27.36 %) positive cytoplasmic expression while in G3B model, Bcl-2 exhibited weak (16.9 %) positive cytoplasmic expression and in G3C model revealed weak (8.8%) positive cytoplasmic expressions almost looks like G1. Bax expression in G3A revealed moderate (32.9%) positive cytoplasmic expression; similarly it revealed moderate (37.86 %) positive cytoplasmic expression in G3B and moderate (41.27%) positive cytoplasmic expression in G3C which recorded the best expressions of Bax compared to other groups. The results of the present study with these results with those of the aforementioned findings by other investigators indicated that, increasing tissue oxygenation causes induction of apoptosis in the tissue. This can be explained by that was reported by Piret et al., [41] who indicated that, hypoxia cause overexpression of activator protein 1 (AP-1) gene that possesses antiapoptotic activity. Oxygen may prove beneficial in improving the outcome by modulating apoptosis and proliferation. These results are concomitant with those observed by other investigators [42-44]. This may be explained by that was reported by Nikfarjam et al., [43] who indicated that, apoptosis is an energy-requiring process and thus it may not be as efficient in a hypoxic environment. During oxygen therapy, tissue oxygenation is increased to levels that are sufficient to support tissues independent of hemoglobin, providing sufficient oxygen for the cells to generate enough adenosine triphosphate (ATP) for apoptosis to occur through the possible role of the caspase-3 dependent cell death pathway and subsequent Bcl-2/Bax ratio.

In the current study, the improvements of HBP mucosa, in gross observations and H&E stains in addition to Bax and Bcl-2 expressions of G3 compared to G2 were attributed to the positive effect of oxygen. The differences in results among G3 models were attributed to the form or dose of the administrated oxygen. The significant difference in improvements appeared in G3B compared to G3A was attributed to the obligatory administration of oxygenated water in G3B which make sure that the drug arrives daily to the treated animals by specific amount in contrast to G3A which depends on the animal's desire to drink and differs in both the amount and intervals from animal to another. Increasing the amount of oxygenated water in G3C results revealed much improvements compare to G3B which may be due to decrease the chance of tissue hypoxia and maintain the normal tissue oxygenation level. The present study revealed, also, discrepancies results throughout G3 models at 14 weeks. Gross observation of G3A model revealed improvements in 3 of 7 animals (the nodular swelling appeared less in distribution with reduction of tumor masses) compared to G2. H&E stain confirmed these improvements and revealed that, the changes are limited to the epithelial layers in the form of severe epithelial dysplasia while the basement membrane appeared intact with no evidence of true invasion or nest formation. The gross observations of other 4 animals of G3A revealed almost the same gross observations of G2 and these results were confirmed by H&E stain which indicated that, the overlying epithelium has obvious dysplastic features, in multiple areas, and provide evidence of prominent true invasion with formation of epithelial nests with destruction of the basement membrane.

Regarding the gross observations, the results of G3B revealed noticeable reduction in distribution of tumor growth with regression in the tumor size in 5 of 7 animals compared to that seen in G2 and G3A. Subsequently, H&E stain of these specimens indicated that, the overlying epithelium has severe dysplastic features without true invasion. Contrarily, the remaining 2 animals showed tumor masses similar to those in G2 and G3A and by H&E stain these specimens exhibited squamous cell carcinoma almost the same as G3A model and G2. The gross observations results of G3C revealed only 3 of 7 animals exhibited improvements (the nodular swelling appeared less in distribution with reduction of tumor masses) compared to G2. H&E stain of HBP mucosa shows severe dysplastic features with appearance of carcinoma in situ or even early invasive

squamous cell carcinoma in some areas. The gross observations of the other 4 animals were similar to G2 in which there were obvious nodules and fungating tumor masses, eroded and ulcerative areas with hard or rubbery consistency on palpation. H&E stain revealed that, the epithelium of these specimens has prominent invading nests of SCC in the underlying C.T. These results indicated that, G3 animals recorded better results compared to G2 and the improvements appeared in the gross observations confirmed by H&E stain. These results are concomitant with those reported by other studies [39-41] and explained by Piret et al. [41]. Immunohistochemical staining utilizing Bcl-2 and Bax antibodies in G3 at 14 weeks also revealed different results in each model of this group. Bcl-2 revealed strong (54.8 %) positive cytoplasmic expression in G3A followed by G3C which revealed strong (51.74 %) positive cytoplasmic expression and then G3B which revealed moderate (39.8 %). Bax expressions revealed weak (14.64 %) positive cytoplasmic expression in G3A, weak (15.59 %) positive cytoplasmic expression in G3C and moderate (27.19 %) positive cytoplasmic expressions in G3B.

The improvements appeared in animals of G3 compared to G2 at 14 week may be attributed to the increase of tissue oxygenation which stimulate apoptotic process by inhibition of Bcl-2 and stimulation of Bax expressions. These results indicated that oxygen may prove beneficial in improving the outcome by modulating apoptosis and proliferation. Oxygenated water technique was used for delivering oxygen to cancer cells and diminishing the hypoxia inside the tumor cells. These results are concomitant with those observed by other investigations [42-44]. Contrarily the decrease in improvements of animals of model G3C compared to G3B even of the increase in the amount of the obligatory administrated drug 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).

CONCLUSIONS

From the results of the present study the following conclusions could be drawn: Oxygenated water is considered as a promising chemotherapeutic agent in prevention of induced HBP carcinogenesis (epithelial dysplasia & invasive carcinoma) and the technique used for preparation of oxygenated water (oxygen enriched water) is favorably effective. Increasing the daily amount of oxygenated water led to more favorable results in prevention dysplasia. Increasing the daily amount of oxygenated water up to specific dose led to favorable results in preventing SCC and vice versa. Finally oxygenated water proved beneficial role in improving the outcome by modulating apoptosis and proliferation throughout the process of carcinogenesis.

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