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Chemopreventive Effects of Curcumin Against 7,12-Dimethylbenz[a]anthracene-Induced Hamster Buccal Pouch Carcinogenesis.

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Abstract

BACKGROUND: Chemoprevention is an important means that has a high potential to reverse, prevent or suppress the development of cancer in initial stages or the development of pre-cancerous cells to the cancer stage.

OBJECTIVE: This study aimed to investigate the potential protective effect of curcumin as a chemopreventive agent against induced oral squamous cell carcinoma in Syrian hamster.

MATERIALS AND METHODS: In this experimental study, forty male Syrian hamsters were divided equally into two main groups. The carcinogenic DMBA was only applied to the control group (g1 = 20 hamsters) by topical painting in the buccal pouch, as for the experimental group (g2 = 20 hamsters) curcumin was administered orally (80 mg\kg) concurrently with the DMBA. The animals were sacrificed at consecutive periods (after 2, 6, 10 and 14 weeks), then the histopathological changes in the buccal pouches were studied by H&E staining, and the VEGF expression during the previous sacrifice periods was studied. The Kruskal–Wallis test was used to study the significance of differences in VEGF expression between the two groups at the level of confidence 95%.

RESULTS: P-value = 0.116>0.05 - 0.005<0.05 - 0.007<0.05 - 0.006<0.05, in 2, 6, 10 and 14 weeks respectively. Thus, the statistically significant differences in VEGF expression were found in the second, third and fourth sacrifice period between group 1 and group 2.

CONCLUSION: Curcumin had chemopreventive effects against DMBA-induced buccal pouch OSCC through the modulating effect on the abnormal expression of VEGF.

Keywords: oral squamous cell carcinoma; curcumin; chemoprevention.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is defined according to the World Health Organization (WHO) as an invasive carcinoma with varying degrees of squamous differentiation and with a tendency to metastasize to lymph nodes in early stages. It arises from the oral mucosal epithelium, and is the most common oral cancers. It constitutes more than ninety Percent of the malignancies that affect it [1].

OSCC arises from the accumulation of many mutations that affect the cell life cycle, cellular proliferation, and angiogenesis [2, 3]. Angiogenesis is defined as the process of creating new blood vessels from pre-existing vasculature [4, 5]. Angiogenesis occurs physiologically in many conditions such as pregnancy, development, and wound healing. As for angiogenesis disorders, they are involved in many diseases, such as chronic inflammation, cardiovascular diseases, and carcinogenesis [2, 3]. Angiogenesis is an essential part of solid tumors evolution larger than 2mm in diameter, and the vascular

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endothelial growth factor (VEGF) is the main factor in this process which make the detection of the role of this factor becomes a topic of high importance [6].

Currently, most cancers are being treated and may be cured, but it is common to encounter poor therapeutic response and recurrence despite great progress in surgery, radiotherapy and chemotherapy [7, 8]. Chemotherapy has suffered from many problems, the most important of which is the non-selectivity, as it attacks both healthy and cancerous cells, so it has a highly toxic effect. In addition to noting the phenomenon of multi-drug resistance (MDR) in cancer cells at times, which makes it ineffective. In addition to the possibility of creating new mutations in the DNA, may lead to the formation of new cases of cancer [9]. Therefore, the low efficacy and high toxicity of chemotherapy led to resort to another type of treatment that includes what is called Nutraceutical, which combines Nutrition and Pharmaceutical and is defined as a substance isolated or purified from plants, that has proven effective in the treatment or chemoprevention of cancer [10, 11]. Chemoprevention has been described as the use of agents that allow suppression, regression, or delay the progression of carcinogenesis [12, 13].

The current chemopreventive agents have low side effects and low toxicity, and most of these agents are plant extracts that are divided into two categories: (i) blocking agents that prevent the initiation step by inhibiting the activation of carcinogens, (ii) suppressing agents that inhibit the cancerous cell proliferation during the evolution of carcinogenesis. However, there is a distinct class of chemopreventive agents including curcumin (from the turmeric plant) belong to both classes because they represent multiple mechanisms of action [14].

Turmeric is an herbaceous plant with a yellow rhizome that belongs to the ginger family, native to southern India. It is known to contain compounds that have an important effect on public health, the most important and effective of which is curcumin. Curcumin, is a polyphenolic compound found in the turmeric plant (Curcuma longa) with the chemical formula C21H20O6 [15-17].

The decrease in cancer incidence, especially colon cancer, was noted in India, where turmeric is widely used as a spice. Researchers have linked turmeric intake to this decrease in cancerous cases number. This prompted them to investigate the effectiveness of its active component "curcumin" through in-vitro, in-vivo, and clinical studies. Indeed, this compound has proven highly effective in curbing carcinogenesis in addition to its safety, as high doses of it can reach 12 g per day without leaving any toxic side effects [18-20].

Curcumin has been studied extensively over the past years, with most studies focusing on its anti-inflammatory and anti-cancer effects. Shanmugam et al. reviewed most of the experimental studies conducted on curcumin to confirm its efficacy as a safe treatment for cancer patients. It was concluded that this substance has supportive efficacy for most types of cancer, including multiple myeloma, pancreatic, colon, and lung cancer [21].

Curcumin plays a crucial role in cancer prevention by inhibiting many mechanisms that can cause the initiation of cancer cell formation. The most important of these mechanisms is the inhibition of the nuclear factor NF-κB pathway, which is responsible for inflammation. in addition, curcumin is curbing reactive oxygen species (ROS), which is one of the most important factors causing mutations and forming abnormal cells with a high ability to divide abnormally [19, 22].

Furthermore, curcumin has been described as an important anti-angiogenic agent, this phenomenon could be attributed to VEGF and angiopoietin 1 and 2 inhibition, and also by inhibition of KDR/ Flk-1 (VEGF receptor-2) [23, 24]. Moreover, curcumin inhibits angiogenesis through downregulation of the expression of COX-2 and Hypoxia-inducible factor 1 (HIF-1), which in turn leads to inhibition of the VEGF expression, suggesting that the curcumin possesses antiangiogenic effects, which warrants further

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investigation as a chemopreventive agent [25-27].

Some studies have demonstrated the ability of curcumin to inhibit abnormal cell division and induce apoptosis in different types of cancer cells [28]. Among the anti-cancer effects is its ability to inhibit the pathway of cyclooxygenase (COX-2) and lipoxygenase (LOX), which in turn also helps to inhibit cell proliferation [29].

Curcumin has been confirmed to be active in several head and neck squamous cell carcinoma cell lines (HNSCC), including CAL27 and CCL23 (laryngeal) and UMSCC14A (oral) by affecting mainly the NF-κB signaling pathway through decreasing its expression, in addition, inhibiting its nuclear localization resulting in sequestering the NF-κB in the cytoplasm [30, 22] and through suppressing the expression of several proteins regulated by NF-κB including interleukin-6 and 8, matrix metalloproteinase-9 (MMP-9), cyclin D1, c-Myc, cyclooxygenase-2 (COX-2) and Bcl-XL [31-33].

The exact mechanism of action of curcumin is still under research and study, for example, in 2009, Manoharan et al. [34] induced oral squamous cell carcinoma in the buccal pouches of hamsters that were administered orally (80 mg\kg) concurrently with the DMBA carcinogen, and the results of this experimental study indicated an effective preventive role of curcumin in delaying the occurrence of epithelial dysplasia in the buccal pouch mucosa, due to its antioxidant potential in addition to its effect on the detoxification process of carcinogens. Saleh et al. [35] concluded that curcumin alone has an important chemopreventive role, and it was also confirmed that this protective effect of curcumin increases when combined with green tea extract.

This study aimed to investigate the potential protective effect of curcumin against OSCC induced in the buccal pouch of Syrian golden hamsters by evaluating VEGF expression levels.

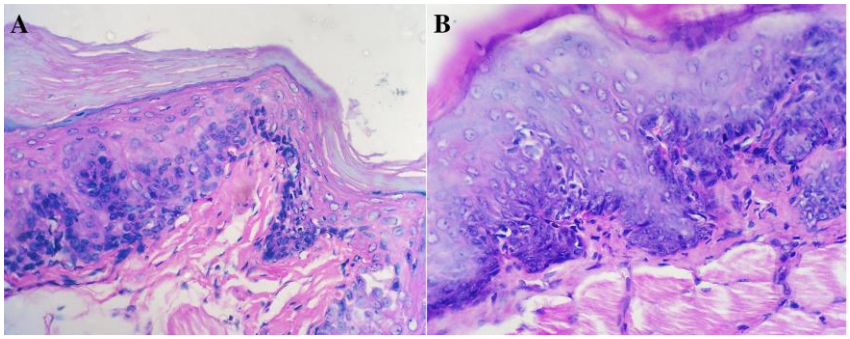

Figure 1: Histopathological photomicrograph shows the mild dysplasia after 6 weeks of DMBA in group 1 (A), and after 6 weeks of DMBA + Curcumin in group 2 (B). H&E staining, magnification 400.

MATERIALS AND METHODS

- Animals:

An approval was gained from the Ethics Committee at Damascus university to conduct this study (Approval ref: 3574/2019).

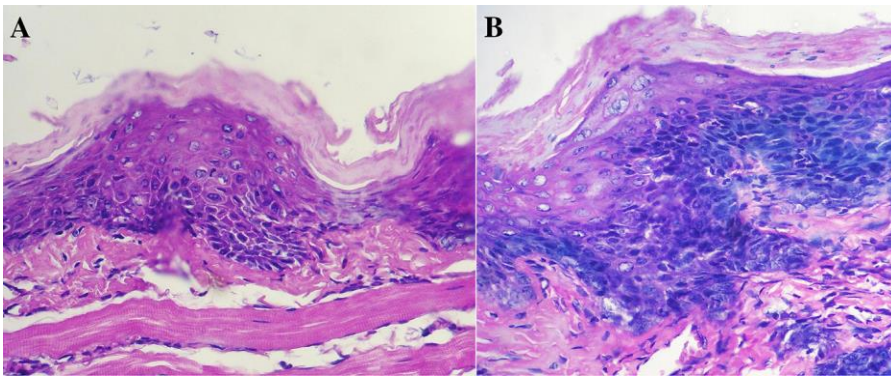

Figure 2: Histopathological photomicrograph shows the moderate dysplasia after 10 weeks of DMBA in group 1 (A), and after 10 weeks of DMBA + Curcumin in group 2 (B). H&E staining, magnification 400.

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This study was conducted on Syrian golden hamsters (Mesocricetus auratus) at department of oral histology and pathology, Damascus university.

Inclusion criteria: healthy male hamsters, aged about 7-10 weeks old, weight about 90-110 g, no anatomical deformities and were quarantined previously for adaptation purposes (for one week prior to the start of this study).

Afterwards, all hamsters that fulfilled the inclusion criteria (40 hamsters) were divided equally into two main groups: the first main group, the control group, included 20 hamsters to which only the carcinogenic DMBA was topically applied (g1 DMBA alone), while in the second main group, the experimental group, the curcumin was administered orally (80 mg\kg) concurrently with the DMBA (g2 Curcumin + DMBA).

- 7,12-Dimethylbenz[a]anthracene (DMBA):

DMBA was obtained from Sigma-Aldrich chemical company. (St. Louis, MO, USA). This chemical substance can bind to DNA and cause mutations that play a role in malignant transformation. OSCC was induced in a buccal pouch through topical application of 0.5% DMBA dissolved in mineral oil three times a week for fourteen weeks by a paintbrush (size 4). DMBA was applied only on the left buccal pouch to ensure the ability of the hamster to eat and store its food using the right one, which helps to preserve the life of the hamster and prevent it from dying because of nutritional deficiency [36, 37].

- Curcumin:

The curcumin powder (Sigma-Aldrich Co. St. Louis, MO, USA) was administered orally (80 mg of body weight) concurrently with the DMBA (3 times per week) by a 1 cm3 syringe after dissolving it in 1 ml of almond oil according to the recommended dose to hamsters to avoid toxic effects [38].

- Experimental design:

Each main group was divided according to the sacrifice time into four sub-groups, each of which included five hamsters. The first main group (g1 DMBA alone) was sub-divided into four groups: five hamsters were sacrificed after two weeks, five hamsters after 6 weeks, five hamsters after 10 weeks, and five hamsters after 14 weeks. And the same was applied on the second group (g2 Curcumin + DMBA). The potential protective effect of curcumin against the development of OSCC induced during these consecutive periods was investigated by evaluation of VEGF expression.

The hamsters were sacrificed according to ethical procedures by injecting sodium pentobarbital (180 mg/kg body weight) by using a 26-G syringe and then the buccal pouches were removed unilaterally for each hamster's side which was exposed to the DMBA or DMBA + curcumin.

- Immunohistochemical evaluation (IHC):

Immunohistochemical detection of VEGF was carried out by using Bio SB, Inc. CO. detection system. The VEGF antibody (rabbit monoclonal antibody) was used, with

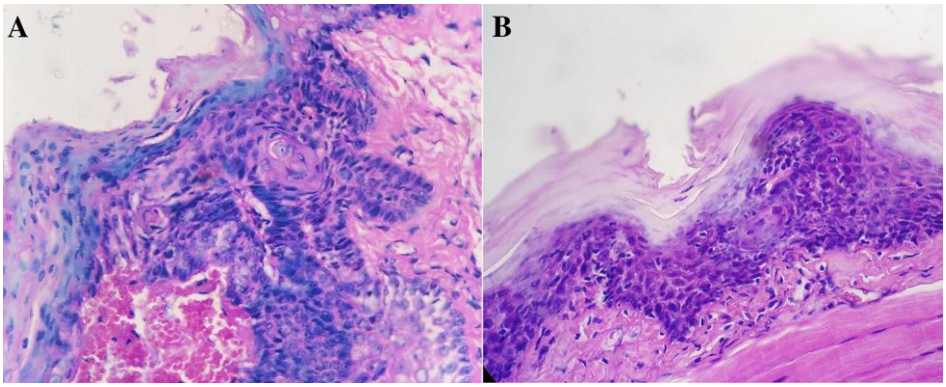

Figure 3: Histopathological photomicrograph shows the severe dysplasia after 10 and 14 weeks of DMBA in group 1 (A), and after 14 weeks of DMBA + Curcumin in group 2 (B). H&E staining, magnification 400.

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brown cytoplasmic\membranous expression. All steps of IHC staining were followed according to the manufacturer's instructions. The staining evaluation was done in epithelial cells of the buccal pouch mucosa by using a light microscope (OLYMPUS –Nikon CX21). Any epithelial cell that exhibited brown cytoplasmic\membranous immunopositivity to VEGF was considered positive.

The staining score of VEGF was evaluated by Allred's modified semi-quantitative immunoreactive score as following [39]:

Quantitative assessment (percentage %): was graded as following: (1) <25% of positive cells, (2) 25%–50% of positive cells, (3) 50%–75% of positive cells, and (4) >75% of positive cells. no counts were done in areas of necrosis.

Qualitative assessment (staining intensity): was rated as following: (0) no staining, (1) weak staining, (2) moderate staining, and (3) strong staining.

The final score was obtained by adding qualitative and quantitative indices as following: (0) negative, (1–2–3) weak, (4–5) moderate, and (6–7) strong.

- ❖ Statistical analysis:

SPSS software (v.23; IBM, Armonk, New York) was used to analyze the data obtained statistically and G*power software was used to calculate the sample size. The normality distribution was checked by using the Kolmogorov-Smirnov test. The Kruskal–Wallis test was used to study the significance of differences in VEGF expression between the two groups.

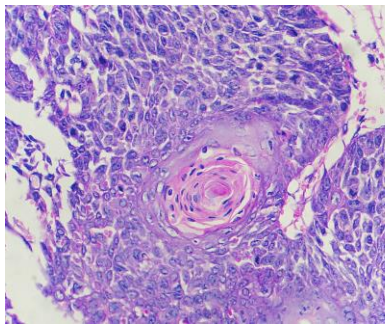

Figure 4: Histopathological photomicrograph shows the invasive OSCC after 14 weeks of DMBA in group 1. H&E staining, magnification 400.

RESULTS:

All developmental sequential stages of well-differentiated OSCC were observed in the control group (g1 DMBA alone), starting from hyperplasia of the buccal pouch epithelium, passing through mild and moderate dysplasia, to severe dysplasia and up to the well-differentiated invasive carcinoma, while in the experimental group (g2 Curcumin + DMBA), the progression of OSCC had stopped at the severe dysplasia after the fourteenth week of the experiment where no cases of carcinoma were observed in the last week of the experiment (14th week) (Figures 1,2,3,4).

The Kruskal–Wallis test was used to analyze and compare the results of both groups in order to evaluate the effectiveness of curcumin as a chemopreventive agent. The Kruskal–Wallis test indicated that the p-value in the second, third, and fourth sacrifice period (6-10-14 weeks) was smaller than 0.05 (p-value = 0.005 - 0.007 – 0.006, respectively), and therefore there were statistically significant differences between both groups at the confidence level of 95%. While there were no statistically

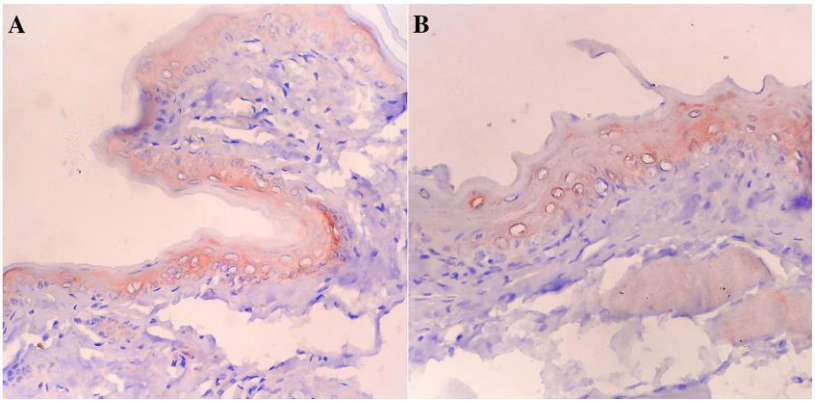

Figure 5: Histopathological photomicrograph shows the weak expression of VEGF: (A) group 1, (B) group 2. magnification 400.

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significant differences in the first sacrifice periods (two weeks) because the p-value = 0.116 > 0.05 (Table 1), (Figures 5,6,7).

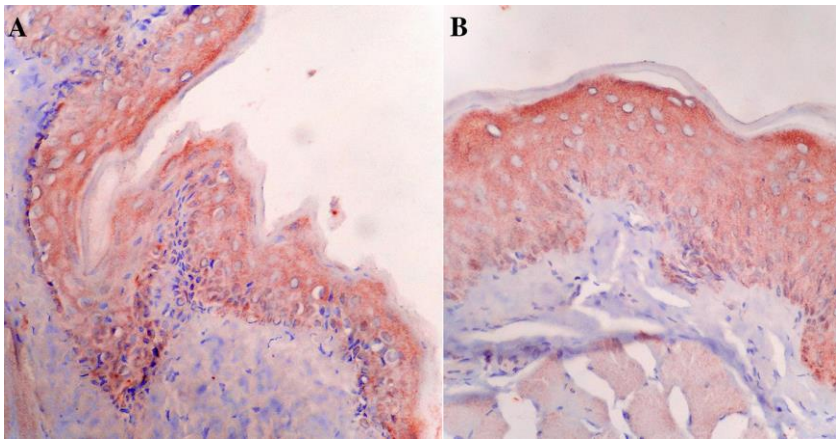

Figure 6: Histopathological photomicrograph shows the moderate expression of VEGF: (A) group 1, (B) group 2. magnification 400.

DISCUSSION

The better practical approach to decrease the morbidity of cancer is delaying the process of carcinogenesis by using chemopreventive agents. This necessitates that compounds extracted from natural sources must be critically evaluated for chemoprevention [20].

This study focused on the potential protective potential of curcumin (diferuloylmethane) in reducing the development of OSCC induced in the buccal pouch mucosa of Syrian hamsters by DMBA, as this substance produces "dihydrodiol epoxide" a compound that mediates the carcinogenesis through chronic inflammation, overproduction of ROS, activation of protooncogenes, inactivation of tumor suppressor genes, induction of extensive DNA damage, reduction of DNA damage repair, and reduced ability to induce apoptosis [37].

The process of oral carcinogenesis induced by the application of DMBA is very similar histologically and morphologically to human OSCC [36, 40]. In this study, it was noted the similarity between signs of dysplasia in the buccal pouch of hamsters and the pathological changes that occur in the human oral mucosa, and this was consistent with several previous studies [41-43]. DMBA was also chosen as a carcinogen in the animal models because it has similar etiological effects as alcohol and tobacco do in human oral cancer therefore, the carcinogenesis in the animal models induced by DMBA can be used as an ideal model for studying the chemoprevention [37,40].

The poor solubility and low oral bioavailability of curcumin are common impediments to its clinical benefit despite its multidirectional pharmacological properties. However, studies have shown that stability increases in acidic environments such as the stomach [44]. Curcumin at a dose of 80 mg/kg was used in this study in line with most papers in the context [38, 45, 46, 34], but on the other hand, some papers showed that although low concentrations of curcumin stimulate

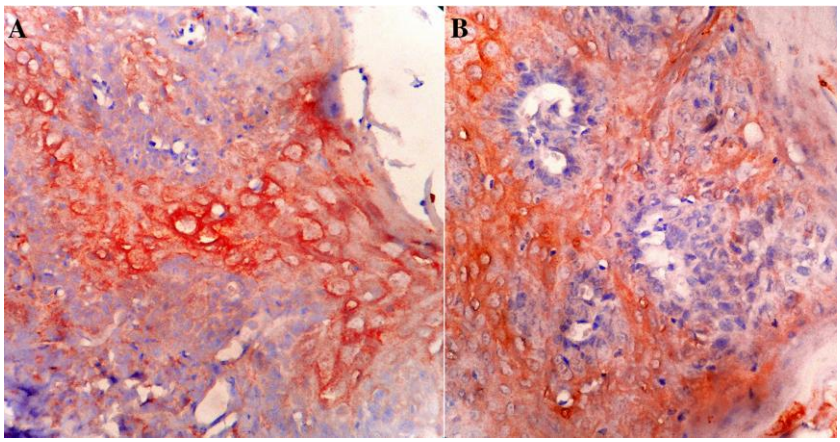

Figure 7: Histopathological photomicrograph shows the strong expression of VEGF: (A) group 1, (B) group 2. magnification 400.

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its antioxidant effects, the higher concentrations can increase cellular levels of ROS [47].

statistically significant, while they were not in the first sacrifice period, and this indicates that the chemopreventive effect of curcumin + DMBA was observed, while it did not have any positive role within two weeks of the application of the DMBA.

	Group	N	Mean	Std. Deviation	Mean Rank	Chi-Square	p-value	
2 weeks	g1 DMBA alone	5	2.4	.548	10	4.31	0.116>0.05	NS
	g2 Curcumin + DMBA	5	2	.0	7			
6 weeks	g1 DMBA alone	5	4.6	.548	13	10.75	0.005<0.05	*
	g2 Curcumin + DMBA	5	2.2	.447	5			
10 weeks	g1 DMBA alone	5	6.6	.447	13	9.99	0.007<0.05	*
	g2 Curcumin + DMBA	5	3.6	.894	5.1			
14 weeks	g1 DMBA alone	5	7	.0	13	10.18	0.006<0.05	*
	g2 Curcumin + DMBA	5	4.4	1.14	5.2			

Table 1: Results of the Kruskal–Wallis test to study the significance of differences in VEGF expression between the two groups. NS = not significant, * = significant, Std. Deviation = standard deviation, g1 = group 1, g2 = group 2,

Referring to the results of the Kruskal–Wallis test, it was found that the differences between both groups in each of the second, third, and fourth sacrifice periods were

was greatest in most of the experiment periods, especially at the end of the fourteenth week when no incidence of OSCC in the g2 Curcumin

These findings were consistent with Manoharan et al. [34]. Manoharan had studied the protective role of curcumin and piperine against OSCC induced by DMBA in the buccal pouch

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in hamsters and It was concluded that feeding on curcumin at a dose of 80 mg/kg had played an important chemopreventive efficacy in delaying the occurrence of signs of dysplasia in the buccal pouch due to its antilipidperoxidative potency as well as its modulating effect on the detoxification process of carcinogens.

This study was also agreed with Balakrishnan et al. [45]. It was demonstrated by Balakrishnan that the curcumin at a dose of 80 mg/kg and ferulic acid had a modulating effect on the abnormal expression of p53 and bcl-2 proteins induced by DMBA in the buccal pouch mucosa of Syrian hamsters.

These results were in line with Saleh et al. [35], as this study concluded that curcumin alone has an important chemopreventive role, and it was also confirmed that this protective effect of curcumin increases when it is shared with green tea.

Whereas the difference was made with Maulina et al. [38], which did not prove the protective efficacy of curcumin despite the use of a similar dose (80 mg/kg). This difference can be explained through several points, the first of which was the difference in the animal model, where male white Sprague - Dawley rats were used, and the second was the difference in the protocol of applying the DMBA, Maulina scratched the buccal mucosa of the rat by using a 27-G syringe that contained DMBA, while in this study, the application was done through topical painting in the buccal pouch, and the third and most important point was the short period of administration of curcumin, which amounted to only about four weeks, while the period of administration in the current study lasted fourteen weeks concurrently with the DMBA.

Last but not least, in regards to the results of the current study, further investigations are required to better understand the mechanisms of the chemopreventive effects of curcumin.

CONCLUSION:

Based on the results of this study, it can be concluded that the oral administration of curcumin at a dose of 80 mg/kg had chemopreventive effects against DMBA-induced buccal pouch OSCC through the modulating effect on the abnormal expression of VEGF.

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