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Original Article

ANTI-INFLAMMATORY AND ANTI-ANGIOGENIC POTENTIAL OF CROMOLYN IN 7, 12-DIMETHYLBENZ (A) ANTHRACENE INDUCED HAMSTER BUCCAL POUCH CARCINOGENESIS

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ABSTRACT

Objectives: Cromolyn, a potent and safe anti-inflammatory drug, is used as a safe medication for the prophylactic treatment of asthma. The present study explores its anti-inflammatory and anti-angiogenic potential of cromolyn by analysing the expression pattern of inflammatory (NF-κB, COX-2, iNOS, IL-6 and IL-10) and angiogenic (VEGF) in 7, 12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis.

Methods: Topical application of 0.5% DMBA (three times a week) in the buccal pouches of hamsters resulted in well differentiated squamous cell carcinoma after 14 w.

Results: While tumor formation was observed in all the hamsters treated with DMBA alone, we noticed only precancerous lesions such as hyperkeratosis, hyperplasia, and mild dysplasia in DMBA+cromolyn treated hamsters. Furthermore, cromolyn prevented the dysregulation induced by DMBA on the expression pattern of inflammatory and angiogenic markers.

Conclusion: The present study thus concludes that the tumor preventive potential of cromolyn relies on its anti-inflammatory and anti-angiogenic potential in 7, 12-dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis.

Keywords: Cromolyn, Oral cancer, Inflammation, Angiogenesis.

INTRODUCTION

Oral carcinoma is one of the most predominant health problems worldwide, and it is the leading cause of cancer-related deaths in the developing countries including India. This disfiguring malignant cancer affects the overall life quality of the patients and imposes a significant burden to their families. Though oral cancer is an avoidable one, lack of awareness of oral cancer symptoms, late diagnosis and sometimes clinician's carelessness or mis prescription are responsible for the increased annual incidence of oral cancer. Since tobacco and alcohol abuse are documented as the major risk factors of oral cancer, avoiding or quitting, such habits may enormously decrease the incidence of oral cancer. Also, early diagnosis and treatment modalities at an early stage could definitely improve the life quality of the patients as well as overall 5 y survival outcomes [1, 2].

Golden Syrian hamsters are the preferred experimental model to study the biochemical, molecular or morphological aspects of oral carcinogenesis. This is due to the fact that hamsters possess a pocket like an anatomy in their mouth known as "buccal pouches", which can retain the topical application of carcinogens for a longer time [3]. 7,12-dimethylbenz(a)anthracene, the procarcinogen, elicits its tumorigenic behavior through its active metabolite, dihydro diol epoxide, upon metabolic activation in the host. Chronic inflammation, excessive generation of reactive oxygen species and extensive DNA damage are also documented as a responsible phenomenon for DMBA mediated oral carcinogenesis in experimental animals [4, 5].

Cromolyn is one of the most promising drugs used in the treatment of asthma. Cromolyn has been regarded as a potent anti-inflammatory agent and thus used for mast cell stabilization [6]. Extensive studies reported its anti-inflammatory, antidiabetic and antioxidant potential [7, 8]. *In vitro* cancer studies focused its antiapoptotic, anti-cell proliferative and anti-inflammatory efficacy using various cancer cell lines [9, 10]. However, there are no *in vivo* experimental studies to validate cromolyn efficacy on the expression pattern of inflammatory and angiogenic markers in DMBA induced oral carcinogenesis. The present study thus explores the anti-inflammatory and anti-angiogenic potential of cromolyn in 7, 12-dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

We purchased forty male golden Syrian hamsters from National Institute of Nutrition, Hyderabad, India. Animals were maintained in the Annamalai University Central Animal House, according to the ethical regulations issued by the Institutional Animal Ethics Committee (Register number 160/1999/CPCSEA). The animals were categorized into four groups, and the experimental protocol was followed as follows:

Group I: Vehicle treated control hamsters [liquid paraffin alone three times a week for 14 w]

Group II: DMBA alone treated hamsters [0.5% DMBA in liquid paraffin; three times a week, for 14 w]

Group III: DMBA+cromolyn treated hamsters [Cromolyn 80 mg/kg bw [11]; three times a week for 14 w; alternate days of DMBA treatment] Group IV: Cromolyn alone treated hamsters [80 mg/kg bw; three times a week for 14 w]

Cromolyn was administered along with a delivery agent (N-cyclohexanoyl-L-leucine) in order to increase its bioavailability in the experimental animals [12]. The cervical dislocation procedure was followed to sacrifice the experimental animals at the end of the experimental period.

Immunohistochemical analysis

The polylysine coated tissue sections were treated with corresponding primary antibodies (NF-κB and COX-2) after usual routine procedure carried out for immunohistochemical analysis. The slides were incubated at 4 °C for overnight. The slides containing the immune complex were further treated with horseradish peroxidase-conjugated secondary antibodies and incubated for 2h at room temperature. The immune reaction was visualized under the light microscope, the slides by treating with the substrate of horseradish peroxidase, diamino benzidine. The slides were counterstained with hematoxylin when the acceptable color intensity was noticed [13].

Western blotting

The proteins that are quantified in the tissue extracts of control and experimental animals are separated using SDS-PAGE electrophoresis and then transferred to PVDF membrane by electroblotting. The

membrane was probed with the primary antibodies (VEGF, iNOS, IL-6 and IL-10), followed by incubation with the horseradish peroxidase-conjugated secondary antibodies. The band formation was detected by treating with diamino benzidine and was then quantified densito-metrically. β-actin served as an internal control [14].

Statistical analysis

The densitometry analysis of molecular markers is expressed as mean±SD. The statistical significance between two groups was analyzed using One-way analysis of variance (ANOVA) followed by a Duncans multiple range test (DMRT). The two different groups were considered statistically significant if the p values were less than or equal to 0.05 between them.

RESULTS

The immune expression pattern of NF-κB and COX-2 in control and the experimental hamster is depicted in fig. 1. Over-expression of NF-κB and COX-2 was noticed in the buccal mucosa of hamsters treated with DMBA alone. Hamsters that received DMBA+cromolyn revealed expression pattern close to control hamsters.

Western blotting analysis for the expression pattern of VEGF, iNOS, IL-6 and IL-10 is shown in fig. 2 and fig. 3. We noticed an over-expression of VEGF, iNOS, IL-6 and IL-10 in hamsters treated with DMBA alone. Oral administration of cromolyn corrected the abnormalities in the above molecular markers expression pattern in DMBA treated hamsters.

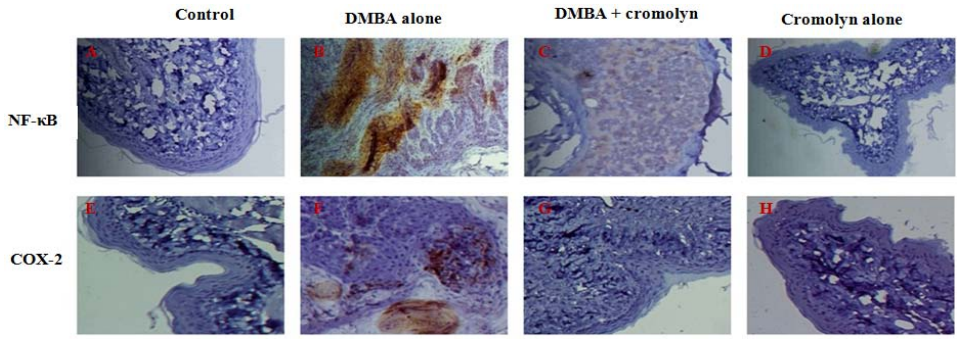
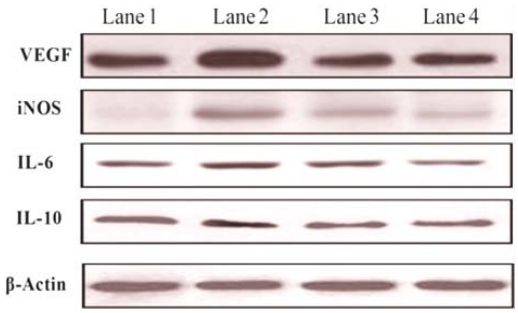


Fig. 1: Immuno expression pattern of NF-κB and COX-2 proteins observed in the buccal mucosa of control and experimental hamsters in each group, (A) NF-κB: A and D-Control and Cromolyn alone (an expression not detectable); B - DMBA alone (over expression); C-DMBA+Cromolyn (down-regulated). (B) COX-2: E and H-Control and Cromolyn alone (expression not detectable), F-DMBA alone (over expression), G-DMBA+Cromolyn (down-regulated)



Lane 1: Control, Lane 2: DMBA alone, Lane 3: DMBA+Cromolyn, Lane 4: Cromolyn alone
Fig. 2: Expression pattern of VEGF, iNOS, IL-6, IL-10 in the buccal pouch tissues of control and experimental animals

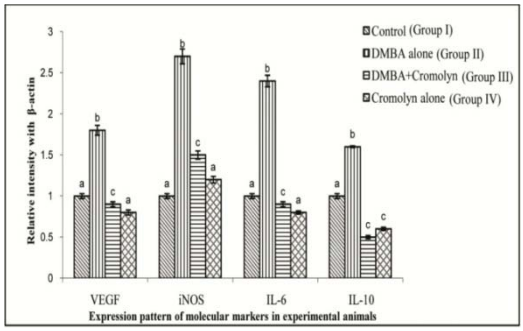


Fig. 3: Densitometric analysis of protein expression after normalization to β-actin in the buccal pouch tissues of control and experimental animals
Values are represented as the mean±SD (n=10 male golden Syrian hamsters). Common superscripts between two groups-not significant. Different superscripts between two groups-significant p<0.05

DISCUSSION

In the present study, hamsters that received the carcinogen alone (DMBA) developed oral tumors on their buccal pouches, and the incidence of the tumor was found to be 100%. Cromolyn administration to DMBA treated hamsters significantly suppressed the tumor formation, and the tumor incidence was 0%. However, we noticed mild dysplasia in the buccal pouches of hamsters treated with DMBA+cromolyn, which implies that cromolyn exhibited potent anticancer property by suppressing the tumor formation in DMBA, induced oral carcinogenesis.

Chronic inflammation and angiogenesis are recognized as a hallmark characteristic of malignant neoplasm [15]. In the present study, we have analyzed the expression pattern of inflammatory and angiogenic markers in hamsters treated with DMBA and hamsters treated with DMBA+cromolyn. NF-κB serves as a signal transcription factor for the expression of several classes of proteins, including genes involved in the inflammatory response. The activated NF-κB enters into the nucleus and binds to COX-2 promoters, thereby stimulating inflammatory response and carcinogenesis [16]. NF-κB plays a pivotal role in the transcriptional activation of angiogenesis factors, cytokines, anti-apoptotic proteins and cell adhesion molecules [17]. NF-κB plays a critical role in the genesis of various cancers, including oral cancer, if it is abnormally expressed [18]. NF-κB expression was found to be enhanced gradually from normal to precancerous lesions and to cancerous tissues [19]. Mounting and multiple studies showed over-expression of NF-κB in cancerous tissues [20]. While COX-2 exert its pathological effects in the early stages of carcinogenesis, NF-κB is involved in the late stages of carcinogenesis [21]. Sawhney *et al.* [22] reported that NF-κB over-expression occurred in the oral preneoplastic conditions and concluded that NF-κB over-expression was thus an early event in oral cancer. Wang *et al.* [23] found NF-κB activation as a major molecular mechanism in oral carcinogenesis. Our results corroborate these findings.

COX-2 plays a pivotal role in the formation of prostaglandins from arachidonic acid. Abnormal activities or expression of COX-2 could result in immunosuppression, apoptotic failure, and increase in angiogenesis [24]. COX-2 inhibits the genes involved in the process of

apoptosis and promotes the genes of cell proliferation [25]. While there was no expression of COX-2 reported in normal tissues, higher expression was reported in hyperplastic and neoplastic tissues [26]. COX-2 expression was significantly increased in leukoplakia, epithelial dysplasia and in oral carcinoma [27]. Wang *et al.* [28] pointed out that over-expression of COX-2 may impact poor prognosis in patients with oral carcinoma. Mounting preclinical data highlighted the over-expression of cyclooxygenase-2 in oral precancerous lesions and in malignant cancer [29, 30]. The present results confirm the previous findings. In the present study, cromolyn suppressed the over-expression of NF-κB and COX-2 in hamsters treated with DMBA. The results thus suggest that cromolyn might have explored potent anti-inflammatory efficacy during DMBA induced oral carcinogenesis.

Chronic inflammation and dysregulation in various intracellular signaling cascades such as angiogenesis were also documented as responsible factors in several DMBA induced experimental carcinogenesis. iNOS, a proinflammatory gene, is regulated by NF-κB, the major transcription factor, in eukaryotes. Inducible nitric oxide synthase (iNOS) mediates carcinogenesis via abundant generation of endogenous nitric oxide. Increase in endogenous nitric oxide (NO) could cause neoplastic transformation through extensive damage to DNA, inhibition of DNA repair and by negatively modulating p53 towards carcinogenesis [31, 32].

The precise effect of nitric oxide (NO) on apoptosis, invasion, metastasis and angiogenesis has also been reported [33]. iNOS over-expression was a common phenomenon in various cancers, including oral squamous cell carcinoma [34]. It has been pointed out that DMBA initiated skin carcinogenesis via over-expression of iNOS and COX-2 [35]. Over-expression if iNOS in the buccal mucosa of hamsters treated with DMBA alone. It has been suggested that agents that could be able to inhibit iNOS over-expression would emerge as a potent anticancer agent [36]. Cromolyn prevented the over expression of iNOS in the buccal mucosa of DMBA treated hamsters, which indicates its anti-inflammatory property during DMBA induced oral carcinogenesis.

Neovascularization is the major characteristic feature of tumor cells and is an essential phenomenon to supply nutrients and oxygen to the growing tumors. VEGF, a major endothelial cell-specific growth factor, is involved in several important biological functions including vascular permeability, migration of cells and angiogenesis [37]. VEGF serves as a potent mitogen for vascular endothelial cells to initiate the process of angiogenesis. VEGF initiates angiogenic sprouting by binding with tyrosine kinase receptors present on the endothelial cells. Oral cancer progression has been associated with hypoxia-induced VEGF expression [38]. Over-expression of VEGF has been reported in several cancers using biochemical, immuno histochemical and molecular procedures [39]. Over-expression of VEGF was correlated with the poor prognosis of various cancers, including oral carcinoma [40]. The present findings are in line with the previous observation. Oral administration of cromolyn at a dose of 80 mg/kg bw suppressed the expression of VEGF in the buccal mucosa of hamsters treated with DMBA. The results of the present study thus suggest that cromolyn has the potential to inhibit the process of angiogenesis during DMBA induced oral carcinogenesis.

Tumor cells develop a defense mechanism against the growth inhibitory potential of cytokines, which exist in the tumor microenvironment. IL-6 is a pleiotropic cytokine, which plays a crucial role in cell survival and proliferation, attenuation of the apoptotic signal and as a growth factor in various human tumors [41]. IL-6 exhibit diverse biological effects, which are however cell and tissue dependent. IL-6 increased cell migration in various cancers including oral cancer [42]. It has also been reported that IL-6 facilitated metastasis in several cancers [43]. Higher expression of IL-6 transcript was shown in oral cancer patients with lymph node metastasis [44]. Serum IL-6 concentrations were found to be higher in oral cancer patients as compared to healthy subjects [45]. IL-10, an inhibitory immune cytokine, is secreted by various cell types including normal and carcinoma cells [46]. Wei Sung *et al.* [47] reported that IL-10 produced by lung tumors stimulated tumor aggressiveness. It has also been pointed out that IL-10 has an immune regulating role in the lung tumor microenvironment [48]. IL-10 increased the abnormal proliferation of thyroid, melanoma and gastric cancer [49]. Previous studies pointed out the dual role of IL-10 in human cancer. While some studies claimed antitumor effect of IL-10 [50], a large number of *in vivo* experimental studies focused the tumor aggressive potential of IL-10 [51]. The variation could be attributed to the biopsy taken from the tumor site since non-tumor immune cells also secrete IL-10. Cromolyn administration to DMBA treated hamsters significantly reduced the expression of IL-6 and IL-10, as evidenced by western blot analysis, in the buccal mucosa. Our results suggest that cromolyn might have inhibited the release of IL-6 and IL-10 to suppress the tumor formation in the buccal mucosa of DMBA treated hamsters.

CONCLUSION

Phytochemicals modulate intracellular signaling cascades towards inhibition, suppression or prevention of carcinogenesis. In the present study, cromolyn suppressed the formation of tumors in the buccal mucosa of hamsters by modulating the inflammatory and angiogenic markers towards inhibition of carcinogenesis. To conclude, cromolyn might have inhibited oral tumors formation through its anti-inflammatory and anti-angiogenic potential. Further studies are in progress to assess the cromolyn efficacy on other molecular pathways that are related to oral carcinogenesis.

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CONFLICT OF INTERESTS

Declared None

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