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Research Article

Coumarin modulates apoptotic, cell proliferative, inflammatory and angiogenic markers in favor of tumor inhibition during 7,12-dimethylbenz[a]anthracene-induced hamster buccal pouch carcinogenesis

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ABSTRACT

Oral carcinoma, the fifth most frequent aggressive epithelial tumor, accounts for 40-50% of all cancers in developing countries, especially in India. The aim of the present study was to assess the modulating effect of coumarin on the immunohistochemical expression pattern of apoptotic, inflammatory, angiogenic and cell proliferative markers during DMBA-induced oral carcinogenesis. Oral squamous cell carcinoma was developed in the buccal pouches of hamsters using topical application of 0.5% 7,12-dimethylbenz[a]anthracene (DMBA) three times a week for 14 weeks. The expression patterns of molecular markers were studied using immunohistochemistry (p53, Bcl-2, Bax, VEGF and PCNA), RT-PCR (cyclin D1 and NF-κB) and ELISA reader (COX-2 and c-Fos). Deregulation in the expression pattern of apoptotic (p53, Bcl-2 and Bax), cell-proliferatives (c-Fos, PCNA and cyclin D1), inflammatory (COX-2 and NF-κB) and angiogenic (VEGF) markers were noticed in the buccal mucosa of hamsters treated with DMBA alone. Oral administration of coumarin at a dose of 100 mg/kg bw protected the deregulation of above mentioned markers in hamsters treated with DMBA. The present study thus concludes that the apoptotic, anti-cell proliferatives, anti-inflammatory and anti-angiogenic potential of coumarin might have played a possible role in the prevention of oral tumors during DMBA-induced hamster buccal pouch carcinogenesis.

Keywords: Apoptosis; angiogenesis; cell proliferation; inflammation; DMBA; oral cancer.

INTRODUCTION

Carcinogenesis proceeds through the step-wise manner, starting with neoplastic transformation of individual cells followed by rapid and abnormal growth of the transformed cells. Oral cancer, the fifth most frequent cancer worldwide, includes malignancies of the mouth, tongue, pharynx and other sites in the oral cavity (Scully and Bagan, 2009). Oral cancer affects 500,000 new cases every year worldwide. Its incidence is rapidly increasing in most of the developing countries; especially in India, Pakistan, Bangladesh and Sri Lanka due to wide-spread habits of tobacco smoking, chewing and alcohol consumption (Lee et al., 2012). Oral cancer accounts for 40-50% of all cancers in India (Scully and Bagan, 2009). Despite recent improvement in therapeutic strategies and management of oral cancer, the five year survival rate of these cancers has not changed significantly due to delay in diagnosis. Even after surgical or combined treatment, the oral cancer patients lead poor life quality due to disfigurement and dysfunction.

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7, 12-dimethylbenz(a)anthracene (DMBA), a potent pro-carcinogen, needs metabolic activation to become an ultimate carcinogen. It mediates carcinogenesis in a step-wise and sequential manner from hyperplasia to dysplasia and from dysplasia to carcinoma. The pre-malignant and malignant lesions induced by DMBA are histologically and morphologically similar to that of human oral cancer (Nagini, 2009). Repeated DMBA exposure to oral mucosa may cause persistent inflammation, which may induce neoplastic transformation by causing DNA damage and inciting tissue reparative proliferation. DMBA is thus commonly employed to induce oral carcinogenesis as well as to study the anti-cancer potential of natural products and synthetic entities (Manoharan et al., 2012). The site accessibility and macroscopic observations of preneoplastic lesions offer oral cavity as an excellent target for chemoprevention research and to evaluate disease progression. Investigation of ideal biochemical and molecular markers during various phases of carcinogenesis could lead to valuable diagnostic techniques for cancer prevention. In recent years, numerous biochemical and molecular markers have been investigated in oral neoplasms (Balakrishnan et al., 2010; Manoharan et al., 2011).

Apoptosis, also known as programmed cell death, removes the unwanted and damaged cells from the

body. Apoptosis is co-ordinated and regulated by a net work of genes and is the major target in the investigation of new anticancer therapies. The expression pattern of proteins involved in apoptosis and cell proliferation could be used as markers to assess neoplastic transformation (Sousa et al., 2009). p53, the best characterized tumor suppressor gene, plays a crucial role in triggering apoptosis. p53 plays a pivotal role in maintaining the genomic integrity of higher eukaryotic organism. p53 over expression and loss of function has been reported in many types of cancers including oral cancer (Heah et al., 2011; Manoharan et al., 2011). Bcl-2, an anti apoptotic protein, is involved in the suppression of cell death rather than stimulating cell proliferation. The extent of Bcl-2 expression is highly variable and its expression was reported in various oral lesions including epithelial dysplasia and carcinoma (Manjunatha et al., 2011). Oral cancer development and progression are associated to a decrease in the expression of Bax, a proapoptotic protein.

PCNA, a 36KDa nuclear protein synthesized during the S-phase within the cell cycle, is regarded as a biomarker of cell proliferation. PCNA plays an important role in DNA synthesis, DNA repair, cell cycle progression and cell proliferation. Over expression of PCNA was reported in several cancers including oral cancer (Watanabe et al., 2010). Cyclin D1 is involved in the G1 to S phase cell cycle progression. Deregulation of cyclin D1 is a common phenomenon of the neoplastic phenotype and has been implicated in the pathogenesis of cancer. Altered cyclin expression could play an important role in the progression of preneoplastic and neoplastic lesions (Biliran et al., 2005). c-Fos, one of a component of the activator protein-1, regulates transcription of several genes implicated in cell proliferation, differentiation and apoptosis. It has been pointed out that c-Fos is a major nuclear target for signal transduction pathways involved in the regulation of cell-growth, differentiation and transformation (Sachdev et al., 2008).

Chronic inflammation causes genomic abnormalities and eventually contributing to carcinogenesis. Repeated inflammatory insult could lead to neoplastic transformation. COX is involved in the biosynthesis of prostaglandins from arachidonic acid. The activity of COX-2 is usually induced in response to tumor promoters, growth factors and inflammatory reactions. COX-2 mediates carcinogenesis by inhibiting apoptosis and enhancing angiogenesis as well as increasing epithelial cell adhesion to the extracellular matrix. Over expression of COX-2 was reported in a large number of tumor cells including lung, stomach and oral tumor cells (Koch et al., 2011; Mendes et al., 2009). Nuclear factor kappa B (NF-κB) plays a pivotal role in inflammatory responses and is activated by several stimuli including free radicals and UV-radiation (Ali and Sultana, 2012).

Angiogenesis, acquisition of new capillaries from pre-existing blood vessels, takes place both under normal conditions (wound healing) and pathological conditions (diabetes, arthritis and cancer). Solid tumors beyond the size of 1-2 mm meet their adequate oxygen and nutritional demand through the process of angiogenesis. Angiogenesis plays a crucial role for the initiation and progression of carcinogenesis. Tumor angiogenesis depends on angiogenic factors forming a vascular network. VEGF is one of the best characterized angiogenic factors that plays a pivotal role for the regulation of angiogenesis during physiological and pathological conditions. Over expression of VEGF has been demonstrated in various solid tumors including oral carcinoma (Manoharan et al., 2011; Son et al., 2009).

Coumarin (1,2-Benzopyrone), an oxygen heterocycle, can occur either free or combined with the sugar glucose. Coumarin has vanilla like flavor and is found in several plants, including tonka beans, lavender, licorice, strawberries, apricots, cherries, cinnamon, and sweet clover. Coumarin possesses blood-thinning, antifungicidal and anti-tumor activities (Khan et al., 2004; Mueller, 2004). Although coumarin has a potential liver and kidney toxin and its use as a food additive is heavily restricted, it is perfectly safe to eat foods, which naturally contain the compound. Coumarins have been found to have multi-biological activities such as anti-HIV, anti-tumor, anti-hypertension, anti-arrhythmia and anti-osteoporosis activities (Khan et al., 2004; Murat Bilgin et al., 2011). Extensive studies demonstrated coumarin as a potential antioxidant and suggested that the antioxidant activity is due to its ability to scavenge free radicals and to chelate metal ions (Khan et al., 2004; Lacy and O'Kennedy, 2004; Rajarajeswari and Pari, 2011). Previous studies from our laboratory demonstrated the antigenotoxic potential of coumarin and protective effect on cell surface glycoconjugates abnormalities during DMBA-induced hamster buccal pouch carcinogenesis (Baskaran et al., 2011a, b). The present study demonstrates the modulating effect of coumarin on the expression pattern of biomarkers of apoptotic (p53, Bcl-2 and Bax), cell proliferative (c-fos, PCNA and cyclin D1), angiogenic (VEGF) and inflammatory (NF-κB and COX-2) during DMBA-induced hamster buccal pouch carcinogenesis.

MATERIALS AND METHODS

Animals

Male golden Syrian hamsters, aged 8–10 weeks, weighing 80–120 g, were purchased from the National Institute of Nutrition, Hyderabad, India and were maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages and provided with a standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) and water *ad libitum*. The animals were maintained under controlled conditions of temperature (27±2°C) and humidity (55±5%) with a 12 h light/dark cycle.

Chemicals

The carcinogen, 7,12-dimethylbenz(a)anthracene and coumarin was obtained from Sigma-Aldrich Chemical Pvt. Ltd., Bangalore, India. PCNA, Bcl-2, Bax, VEGF and p53 primary antibodies were purchased from Dako, Carprinteria, CA, USA. Power Block^{TM} reagent and secondary antibody conjugated with horseradish peroxidase were purchased from BioGenex, San Ramon, CA, USA. C-fos ELISA kit was purchased from Uscn Life Science Inc. Wuhan, China. COX activity assay kit was purchased from Cayman Chemical Co., USA. Trizol reagent was purchased from Invitrogen, CA, USA. cDNA reverse transcriptase kit and SYBR green fluorophore assay reagents were purchased from Applied Biosystems, Foster City, CA. Oligo nucleotide primers were purchased from Bangalore Genei.

Experimental design

The institutional animal ethics committee (Register number 160/1999/CPCSEA), Annamalai University, Annamalainagar, India, approved the experimental design (Proposal No.731, dated 02.09.2010). The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with Indian National Law on animal care and use. A total number of 40 hamsters were randomized into four groups of ten hamsters in each. Group I hamsters served as control and were painted with liquid paraffin alone three times a week for 14 weeks on their left buccal pouches. Groups II and III hamsters were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on their left buccal pouches. Group III hamsters were orally given coumarin at a dose of 100mg/kg bw/day, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the end of the experiment. Group IV hamsters received oral administration of coumarin 100mg/kg bw/day alone throughout the experimental period. The experiment was terminated at the end of 16th weeks and all hamsters were sacrificed by cervical dislocation.

Immunohistochemical staining

Paraffin embedded tissue sections were dewaxed and rehydrated through grade ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H2O2 in methanol for 10 minutes. The antigen retrieval was achieved by microwave in citrate buffer solution (2.1 g citric acid/L D.H2O; 0.37g EDTA/L D.H2O; 0.2g Trypsin) (pH 6.0) for 10 minutes, followed by washing step with Tris-buffered saline (8g NaCl; 0.605g Tris) (pH 7.6). The tissue section was then incubated with power BlockTM reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent, for 15 minutes at room temperature to block non-specific binding sites. The tissue sections were then incubated with the respective primary antibody (p53, Bcl-2, Bax, PCNA and VEGF - Dako, Carprinteria, CA, USA) overnight at 4°C. The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase (BioGenex, San Ramon, CA, USA) for 30 minutes at room temperature. After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3,3´-diamminobenzidine, the substrate of horseradish peroxidase. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium. Each slide was microscopically analyzed and enumerated the percentage of the positively stained cells semi-quantitatively. The percentage of positive cells was scored according to the method of Nakagawa et al. (1994) as follows: 3+ = strong staining, more than 50% of cells were stained; 2+ = moderate staining, between 20 and 50% of cells were stained; 1+ = week staining, between 1 and 20% of cells were stained; 0 = negative, less than 1% of cell staining.

Estimation of COX-2 and c-fos activities by Enzyme-linked immunosorbent assay [ELISA]

c-fos and COX-2 activities were assayed in the buccal mucosa using ELISA kit according to the manufacturer's instructions. In c-fos assay, the buccal mucosa tissues were homogenized in 1X PBS and the supernatant obtained was added to the microtiter plate wells precoated with biotin-conjugated antibody preparation specific to c-fos. Then, Avidin conjugated Horseradish peroxidase (HRP) followed by TMB substrate was added to each well. The enzyme – substrate reaction was terminated by the addition of a sulphuric acid and the color change was measured at 450 nm in a microtiter plate reader. The peroxidase activity of COX-2 activity was assayed colorimetrically by monitoring the appearance of oxidized N, N, N', N' – tetramethyl– P - phenylenediamine (TMPD) at 590 nm in a microtiter plate reader.

Expression of NF-κB and Cyclin D1 using Real-Time PCR

Total RNA from the buccal mucosa was extracted with Trizol reagent. The RNA integrity and concentration were determined by electrophoresis on agarose gel and nanodrop analysis at 260 nm. Isolated total RNA (1µg) was reverse transcribed to cDNA with random primers from the High cDNA Reverse Transcriptase Kit. cDNA was amplified in duplicates using a thermal cycler (9700 HT RT – PCR, Applied Biosystem, UK) for the expression of NF-κB, Cyclin D1 and β-actin with SYBR green fluorophore following the manufacturers recommended amplification procedure. List of primers used for Real-time PCR analysis was given in Table 1. The relative quantification of target gene expression was determined using the comparative CT method. The ΔCt was calculated as the difference between the average Ct values of the endogenous control (β-actin) from the average Ct value of test gene. The ΔΔCt was determined by subtracting the ΔCt of the control from

Table 1: List of Primers Used for Real-time PCR Analysis

Genes	Primers	Sequences
NFκB	forward reverse	5′-ATGGACGATCTGTTTCCCCT-3′ 5′- CGGTTTACTCGGCAGATCTT-3′
Cyclin D1	forward reverse	5'-CGGAGGACAACAAACAGATC-3'; 5'-GGGTGTGCAAGCCAGGTCCA-3'
β-actin	forward reverse	5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3′ 5'-AGCAGCCGTGGCCATC TCTTGCTCGAAGTC-3'

Table 2: Incidence of oral neoplasm in control and experimental hamsters in each group (n=10)

Parameter	Group I Control	Group II DMBA alone	Group III DMBA + coumarin	Group VI Coumarin alone
Tumour incidence (oral squamous cell carcinoma)	0	100%	0	0
Total number of tumours/animals	0	29/10	0	0
Tumour volume (mm^3)/ animals	0	321.72 ± 15.39	0	0
Tumour burden (mm^3)/ animals	0	932.98 ± 44.63	0	0

Tumour volume was measured using the formula, $v = (4/3)\pi [D_1/2][D_2/2][D_3/2]$ where D1, D2 and D3 are the three diameters (mm^3) of the tumour. Tumour burden was calculated by multiplying tumor volume and the number of tumors/animal.

the ΔCt of the test sample. Relative expression of the target gene was calculated by the formula, $2^{-\Delta\Delta Ct}$, which was the amount of gene products, normalised to the endogenous control and relative to the control sample.

Statistical analysis

Values are expressed as mean ± standard deviation (SD). Statistical comparisons were performed by one-way analysis of variance followed by Duncan's Multiple Range Test. The results were considered to statistically significant if the *p* values were less than 0.05.

RESULTS

The tumor incidence, tumor volume and tumor burden of control and experimental hamsters are shown in Table 2. We observed 100% tumor formation with mean tumor volume (321.72 mm^3) and tumor burden (932.98 mm^3) in hamsters treated with DMBA alone. The tumor was histopathologically confirmed as well differentiated squamous cell carcinoma. Oral administration of coumarin at a dose of 100 mg/kg bw significantly prevented the tumor incidence, tumor volume and burden and reduced the severities of hyperplasia, dysplasia and hyperkerotosis in hamsters treated with DMBA. Tumors were not formed in control hamsters painted with liquid paraffin alone as well as hamsters administered with coumarin alone.

The immunoexpression pattern and score of positively stained cells of apoptotic (p53, Bcl-2 and Bax), cell proliferative (PCNA), and angiogenic (VEGF) markers in control and experimental hamsters in each group are depicted in figure 1 and table 3 respectively. Over expression of all these markers except Bax was noticed in hamsters treated with DMBA alone. Oral administration of coumarin at a dose of 100 mg/kg bw to hamsters treated with DMBA significantly restored the expression of above markers. Hamsters treated with coumarin alone revealed expression similar to that of control hamsters.

The status of COX-2 and c-fos in the buccal mucosa of control and experimental hamsters in each group is shown in figure 2. The activities of COX-2 and c-fos were increased in hamsters treated with DMBA alone. Oral administration of coumarin to hamsters treated with DMBA brought back the status of above markers to near normal range. No significant difference was noticed in the status of above markers in control hamsters and hamsters administered with coumarin alone.

The NF-κB and cyclin D1 mRNA expression pattern of control and experimental hamsters in each group is depicted in figure 3. The expression of NF-κB and cyclin D1 were significantly higher in hamsters treated with DMBA alone as compared to control hamsters. Oral administration of coumarin to hamsters treated with DMBA suppressed the expression of cyclin D1 and NF-κB. Similar expression pattern of cyclin D1 and NF-κB was observed in control hamsters and hamsters administered with coumarin alone.

DISCUSSION

Cancer arises due to abnormalities in the genes that regulate normal cellular growth processes. Malignant neoplasm arises not only due to increased cell proliferation but also due to decreased cell death or increased cell survival of genetically altered cells. Deregulation of the cell cycle proteins and apoptosis are the hallmark of malignant transformation (Sousa et al., 2009). Oral squamous cell carcinoma is constantly associated with heterogenetic changes within the key cell cycle molecules of squamous cells. Investigation of natural or synthetic entities with anti-angiogenic, anti-inflammatory, anti-cell proliferative and apoptotic po-

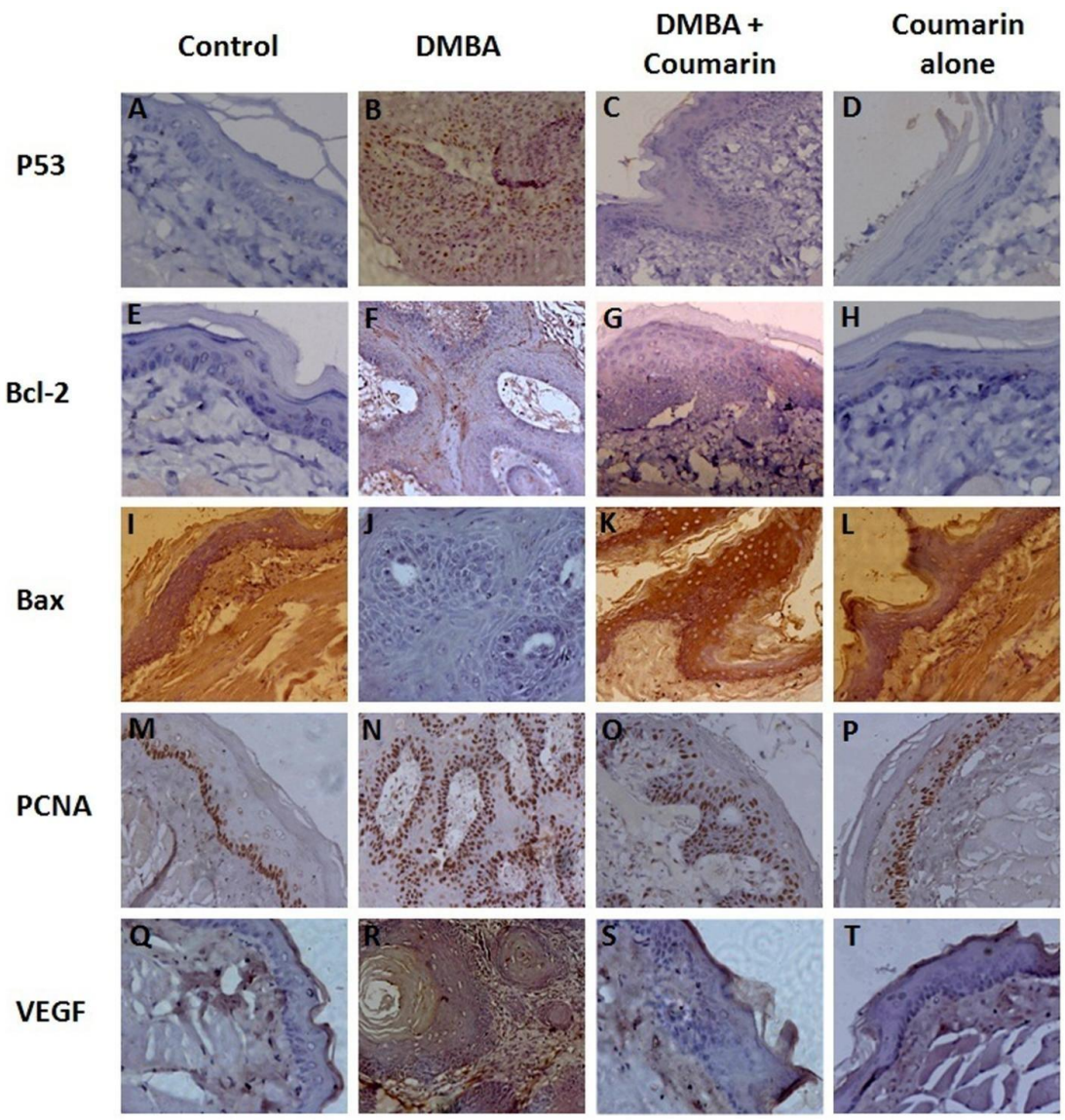

Figure 1: Immunoexpression pattern of p53, Bcl-2, Bax, PCNA and VEGF proteins observed in the buccal mucosa of control and experimental hamsters in each group (40X).

P53: A and D - Control and coumarin alone (expression not detectable), B - DMBA alone (over expressed), C - DMBA + coumarin (down regulated). Bcl-2: E and H - Control and coumarin alone (expression not detectable); F - DMBA alone (over expression); G - DMBA + coumarin (down regulated). Bax: I and L - Control and coumarin alone (nuclear expression positive); J - DMBA alone (nuclear expression negative); K - DMBA + coumarin (nuclear and cytoplasmic expression positive). PCNA: M and P - Control and coumarin alone (expression not detectable), N - DMBA alone (over expressed), O - DMBA + coumarin (down regulated). VEGF: Q and T - Control and coumarin alone (expression not detectable), R - DMBA alone (over expressed), S - DMBA + coumarin (down regulated).

tential is a promising strategy in the treatment of solid tumors (Manoharan et al., 2011).

Immunohistochemical expression pattern of p53, Bcl-2, Bax, PCNA and VEGF could serve as an indicator of malignant transformation. In the present study, over expression of p53, Bcl-2, PCNA and VEGF accompanied with decreased expression of Bax was noticed in the buccal mucosa of hamsters treated with DMBA alone (tumor bearing hamsters). In the present study, p53 positivity significantly increased in the buccal mucosa of tumor bearing hamsters as compared to control hamsters. p53 mutation is the most commonly observed phenomenon in several types of malignancies, including oral cancer (Balakrishnan et al., 2010). Around half of oral cancer cases were reported to have defected in p53 function (Sousa et al., 2009). Soares et al. (2006) demonstrated that p53 over expression assessed by quantitative and tissue distribution measurements could help to verify the severity of malignant transformation of oral lesions. Our results corroborate these findings. Teni et al. (2002) have demonstrated over expression of p53, Bcl-2 and Bax in the tobacco chewing associated Indian oral cancer patients. Bcl-2 plays an important role in a survival rate of genetically

Table 3: The score of positively stained cells of PCNA, p53, Bcl-2, Bax and VEGF in control and experimental hamsters in each group

Groups / Markers	P53				Bcl-2				Bax				PCNA				VEGF			
	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+	0	1+	2+	3+
Control	10	0	0	0	10	0	0	0	2	4	2	2	10	0	0	0	10	0	0	0
DMBA	0	2	3	5	0	1	3	6	8	1	1	0	0	1	1	8	0	1	3	6
DMBA + coumarin	4	3	2	1	5	3	2	0	5	4	1	0	0	7	2	1	7	2	1	0
Coumarin alone	10	0	0	0	10	0	0	0	3	3	2	2	10	0	0	0	10	0	0	0

Values are given as number of hamsters (n = 10). The percentage positive cells were scored as: 3+ = strong staining, more than 50% of cells were stained, 2+ = moderate staining, between 20 and 50% of cells were stained 1+ = week staining, between 1 and 20% of cells were stained, 0 = negative, less than 1% of cell staining.

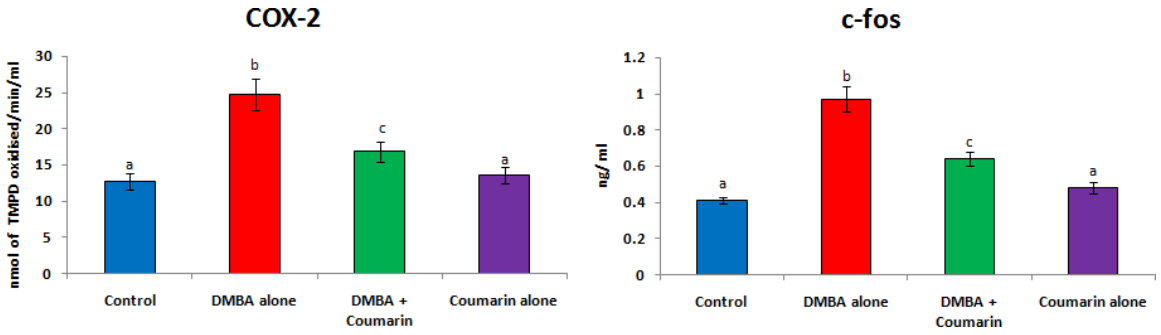

Figure 2: COX-2 and c-fos activities in control and experimental hamsters in each group

Values are expressed as mean ± SD for 10 hamsters in each group. Values that do not share a common superscript letter between groups differ significantly at $p < 0.05$. (Analysis of variance followed by DMRT).

altered cell. Bcl-2 expression was reported from dysplastic stage to metastatic stage of oral carcinogenesis (Sousa et al., 2009). A large number of studies pointed out that Bcl-2 over expression occurs during early phases of carcinogenesis, favoring apoptosis impairment (Manjunatha et al., 2011; Panjamurthy et al., 2009). Over expression of Bcl-2 in the buccal mucosa of tumor bearing animals indicates that Bcl-2 increased the survival rate of tumor cells rather than stimulating proliferation. Increased p53 and Bcl-2 expression accompanied by decreased Bax expression indicates a deregulation of the apoptotic mechanisms during DMBA-induced oral carcinogenesis.

Expression of PCNA is associated closely with the invasion and metastasis of malignant neoplasms and their prognosis. Extensive literatures showed a correlation between PCNA expression and degree of malignancy, vessel invasion, distant metastasis and prognosis (Jiang et al., 2002; Yue et al., 2003). Investigation of natural products that target the inhibition of Cyclin D1 expression will emerge as a novel antitumor agent. Cyclin D1, a key sensor and integrator of extracellular signals of cells in early to mid-G1 phase, was over expressed in various tumor cells including oral cancer (Priyadarsini et al., 2011). Over expression of cyclin, D1 was observed in 30% of patients with oral squamous cell carcinoma (Chen et al., 2009). c-Fos plays an important role in cell differentiation in several organs. Over expression of c-Fos has been implicated in the pathogenesis of human oral carcinogenesis (Mishra et al., 2010). c-Fos was over expressed in dysplastic lesions as well as in oral squamous cell carcinomas (Sachdev et al., 2008). Decreased expression of cyclin D1, PCNA and c-Fos in hamsters treated with DMBA + coumarin suggests that coumarin might have inhibited cell proliferation by inhibiting their expression during DMBA-induced hamster buccal pouch carcinogenesis.

Recent studies on cancer chemoprevention suggested that agents that inhibit the angiogenic process during carcinogenesis could be a promising candidature for cancer prevention (Manoharan et al., 2011). Angiogenesis is stimulated during early phases of carcinogenesis to expand the tumor growth as well as to facilitate metastasis. VEGF, a powerful promoter of angiogenesis, increases vessel permeability and enhances endothelial cell growth, proliferation, migration and differentiation (Ferrara et al., 2003). The results of the present study showed over expression of VEGF during in the buccal mucosa of tumor bearing hamsters, which indicates VEGF might have played a crucial role in the vascular development during oral carcinogenesis. Oral administration of coumarin down regulated the expression of VEGF in the buccal mucosa of hamsters treated with DMBA. The present results indicate that coumarin

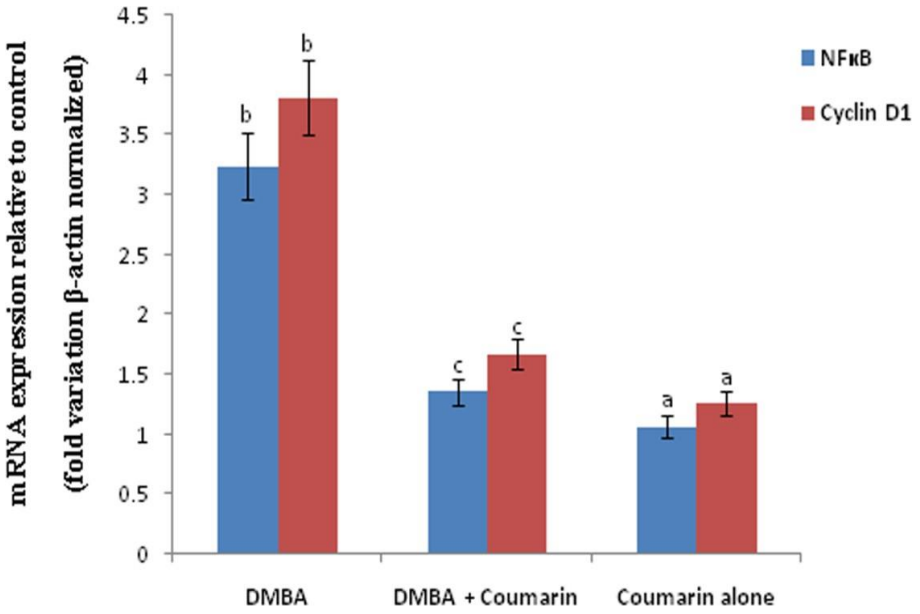

Figure 3: Fold increase in the mRNA expression pattern for NF-κB and Cyclin D1 in hamsters treated with DMBA alone, DMBA + coumarin and coumarin alone

Values are expressed as mean ± SD for 10 hamsters in each group. Values that do not share a common superscript letter in the same column differ significantly at $p < 0.05$. (Analysis of variance followed by DMRT).

might have inhibited the process of angiogenesis during DMBA-induced hamster buccal pouch carcinogenesis.

Over expression of COX-2 is not only associated with abnormal cell proliferation but also in the inhibition of apoptosis and immune surveillance, promotion of angiogenesis and increase in cancer invasiveness and metastasis. Extensive studies pointed out that COX-2 was over expressed in well differentiated carcinoma than the poorly differentiated carcinomas (Goto et al., 2008; Manoharan et al. 2011). Over expression of NF-κB, a ubiquitous nuclear transcription factor, has been implicated in the pathogenesis of inflammation and carcinogenesis (Karabela et al., 2012). Inhibition of NF-kB activation may lead to down regulation of Cyclin D1expression in oral tumor tissues. Sawhney et al. (2007) reported that NF-κB activation and COX-2 over expression occurs in parallel during oral precancer and cancer.

In recent years, coumarins have attracted the intense attention from the researchers working on chemoprevention due to their diverse pharmacological and biochemical properties. Elinos-Baez et al. (2005) reported that coumarin and its synthetic derivative 7-hydroxycoumarin decreased Bcl-2 expression and increased Bax expression in A427 human lung cancer cells. Chuang et al. (2007) reported that coumarin induced cell cycle arrest and apoptosis in human cervical cancer HeLa cells through a mitochondria and caspase-3 dependent mechanism and NF-κB down-regulation. Coumarin induced cell cycle arrest by decreasing the expression of anti-apoptotic protein Bcl-2 and increasing the expression of pro-apoptotic protein Bax in human cervical cancer HeLa cells (Chuang et al., 2007). In the present study, oral administration of coumarin at a dose of 100 mg/kg bw restored the expression pattern of markers of apoptosis, cell proliferation, inflammation and angiogenesis in the buccal mucosa of hamsters treated with DMBA. The results of the present studysuggests that coumarin modulated the expression of above mentioned markers in favor of inhibiting the abnormal cell proliferation occurring in DMBA induced hamster buccal pouch carcinogenesis. The present study thus concludes that the apoptotic, anti-cell proliferative, anti-inflammatory and anti-angiogenic potential of coumarin might have played a possible role in the prevention of oral tumors during DMBA-induced hamster buccal pouchcarcinogenesis. .

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