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## Protective Effect of Berberine on Expression Pattern of Apoptotic, Cell Proliferative, Inflammatory and Angiogenic Markers During 7,12-dimethylbenz(a)anthracene Induced Hamster Buccal Pouch Carcinogenesis

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**Abstract:** Investigation of expression pattern of molecular markers in oral epithelial tissues would help to assess the cell differentiation and proliferation as well as early diagnosis of precancerous and cancerous lesions of the oral cavity. Aim of the present study was to investigate the protective effect of berberine on expression pattern of apoptotic, cell proliferative, inflammatory and angiogenic markers during 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Immunohistochemical staining [p53, Bcl-2, Bax, Proliferating Cell Nuclear Antigen (PCNA) and Vascular Endothelial Growth Factor (VEGF)], Enzyme Linked Immuno Sorbent Assay (ELISA) [c-fos, COX-2, caspase-3 and -9] and Real-Time PCR [Cyclin D1 and NFκB] were utilized to assess the expression pattern of molecular markers in DMBA induced hamster buccal pouch carcinogenesis. Over expression of mutant p53, PCNA, Bcl-2 and VEGF were noticed in hamsters treated with DMBA alone. Decreased expression of Bax protein was noticed in hamsters treated with DMBA alone. Increased expression of C-fos, COX-2, NFκB and Cyclin D1 and decreased activities of caspase-3 and -9 were also noticed in hamsters treated with DMBA alone. Oral administration of berberine at a dose of 75 mg kg<sup>-1</sup> b.w. brought back the expression of above mentioned molecular markers to near normal pattern in hamsters treated with DMBA. The present results thus suggest that berberine has potent anti-inflammatory, anti-angiogenic, anti-cell proliferative and apoptosis inducing properties in DMBA induced oral carcinogenesis.

**Key words:** Oral cancer, berberine, caspases, apoptosis, inflammation, cell proliferation

### INTRODUCTION

Oral cancer, the most life threatening of all dental diseases, accounts for 3-4% of all cancers in Western European countries and 40-50% of all cancers in developing countries (Senthil *et al.*, 2007). Around 90% of all malignant neoplasms in the mouth are Squamous Cell Carcinomas (SCC) (Attar *et al.*, 2010; Moore *et al.*, 2000). Tobacco chewing and smoking are identified as principal risk factors of oral carcinogenesis. Alcohol consumption, viruses and immunodeficiency were reported as co-carcinogens in the development of oral cancer (Pelucchi *et al.*, 2008). The five year survival rate

of oral cancer patients is still at 50% which is probably due to delay in the diagnosis of oral cancerous lesions (Curado and Hashibe, 2009). 7,12-dimethylbenz(a)anthracene (DMBA), a Polycyclic Aromatic Hydrocarbon (PAH), is one of the most commonly used chemical carcinogens to induce oral carcinogenesis in golden Syrian hamsters. Hamster buccal pouch carcinogenesis mimics biochemical, histological and molecular aspects of human oral carcinogenesis (Kavitha *et al.*, 2006; Suresh *et al.*, 2006). Accumulating evidences suggested that over-production of Reactive Oxygen Species (ROS), induction of chronic inflammation, oxidative DNA damage and DNA adduct formations are the possible mechanisms

of DMBA mediated carcinogenesis (El-Mofty *et al.*, 2001; Renju *et al.*, 2007; Pugalendhi and Manoharan, 2010). DMBA induced hamster buccal pouch carcinogenesis is an ideal experimental model for evaluating biochemical and molecular alterations in oral carcinogenesis due to the fact that the premalignant and malignant lesions induced by DMBA in the hamster buccal pouch closely resembles those that take place during analogous development in human oral carcinogenesis (Miyata *et al.*, 2001; Muller *et al.*, 2007; Kitakawa *et al.*, 2006). Investigation of expression pattern of molecular markers of cell proliferation, inflammation, apoptosis and angiogenesis in oral epithelial tissues could help to assess cell differentiation and proliferation status as well as serve as definitive indicators of premalignancy and in the early diagnosis of oral cancer (Garg *et al.*, 2008; Chin *et al.*, 2004).

Cyclooxygenase -2 (COX-2), a key enzyme involved in the modulation of inflammatory process, catalyses the rate-limiting step in the formation of prostaglandins (PGs) from arachidonic acid. COX -2 was up regulated in many pathological conditions and a constitutive expression of COX-2 was triggered since the earliest steps of carcinogenesis (Levita *et al.*, 2010; Al-Turki *et al.*, 2010). Over expression of COX-2 has been reported in human and experimental oral carcinogenesis (Pandey *et al.*, 2008). COX-2 was also over expressed in a variety of premalignant conditions including oral leukoplakia (Sakagami, 2010). Over expression of COX-2 was associated with the progression of different cancers (Cerella *et al.*, 2010). A poor prognosis is common and is frequently associated with COX-2 over-expression (Sun *et al.*, 2008). It has been reported that prevention of over expression of COX-2 could help to prevent the neoplastic transformation of cells (Kapoor *et al.*, 2010). Targeting COX-2 expression in cancer cells could therefore, represent a promising strategy to investigate the anticancer efficacy of natural products or synthetic entities.

The Nuclear Factor-kappa B (NFκB) signaling pathway plays a crucial role in the inflammatory as well as immune responses. Uncontrolled chronic inflammatory responses are the driving force in the premalignant and malignant transformation of cells (Cichocki *et al.*, 2010). NFκB activation was stimulated by UV radiation, bacterial or viral infection and ROS. Improper up-regulation of NFκB initiated by proinflammatory stimuli has been implicated in the pathogenesis of several cancers (Wiebe *et al.*, 2010).

C-fos, identified as a proto-oncogene in virology research, is present in many tissues at very low levels

(Lu *et al.*, 2005). The Activator Protein-1 (AP-1) transcription factor family, with chief constituents, c-jun and c-fos, plays important role in cell proliferation, apoptosis, cell survival and cell differentiation and, thus, is an important target for therapeutic applications (Hagemann *et al.*, 2008). c-fos expression is stimulated by a variety of extracellular stimuli, including growth factors, cytokines, neurotransmitters, polypeptide hormones and stress. Increased expression of c-fos has been reported in several cancerous conditions (Mukhopadhyay *et al.*, 2009; Reddy and Mossman, 2002).

Proliferating Cell Nuclear Antigen (PCNA) has been used as a marker for cells undergoing proliferation (Motiwale *et al.*, 2005; Amin, 2009). The antigen can be detected in proliferating tumor cells using immunohistochemical staining. PCNA, an auxiliary protein of DNA polymerase δ is directly involved in DNA synthesis and replication, repair, cell cycle and play an important role in the regulation of cell proliferation (Mishra *et al.*, 2010). PCNA is necessary for cells traverse from the G1 into the S phase and was found to be over expressed in many cancers including oral carcinoma (Lawall and Crivelini, 2006).

Cyclin D1, a pivotal cell cycle-regulatory molecule, is a key regulator of cell-cycle progression (Fouladdel *et al.*, 2005; Guo *et al.*, 2009). It is responsible for the progression of cell cycle from the G1 phase to S phase. Cyclin D1 is also involved in the cellular response to DNA damage. Profound studies demonstrated early deregulation and amplification of Cyclin D1 expression in oral carcinogenesis (Abou *et al.*, 2006; Gautschi *et al.*, 2007).

Apoptosis, programmed cell death, plays an important role not only in the embryogenesis and normal homeostasis but also in carcinogenesis (Okada and Mak, 2004). Apoptosis is central to the pathogenesis of many pathological processes including cancer. Apoptosis was characterized by chromatin condensation, cell shrinkage, DNA fragmentation, mitochondrial swelling and apoptotic body generation (Rodriguez-Berriguete *et al.*, 2011). Measurement of relative rates of cell replication and programmed cell death or both would have information about net rate of cell proliferation. Evasion of apoptosis is a critical phenomenon in tumor cells which is due to down regulation of death receptors, over expression of the antiapoptotic proteins and/or reduced expression of the proapoptotic proteins and increased cell proliferation and inhibition of apoptosis thus plays an important role during the process of multistage carcinogenesis (Das *et al.*, 2005). p53, a tumor suppressor gene located on chromosome 17 p13.1 is involved in cell cycle

regulation, DNA repair and apoptosis. p53 restricts cellular growth by inducing senescence, cell cycle arrest at G1 and/or G2 phase or apoptosis (Hsu *et al.*, 2011). p53 has an important role in several biological processes including induction of apoptosis (Rai *et al.*, 2011). p53 mutation is associated with poor clinical outcome and over expression of mutant p53 was reported in several cancers including oral cancer (Chin *et al.*, 2004).

Bcl-2 plays an important role in promoting cell survival and inhibition of apoptosis, whereas Bax is involved in the induction of apoptosis. Bcl-2 and Bax, together, regulate the mitochondrial transmembrane passage of cytochrome c which in turn activates caspase proteins. Bax/Bcl-2 ratio determines cell survival or death (Zhang *et al.*, 2009). Over expression of Bcl-2 and down regulation of Bax were reported in several cancers including oral carcinoma (Kang *et al.*, 2010).

Caspases, the initiators and executioners of apoptosis, may contribute to the pathogenesis of proliferative disorders such as cancer, if the activity is drastically altered (Gupta *et al.*, 2004; Ansah *et al.*, 2008). Caspases are activated by cleavage after an aspartate residue, leading to the liberation of the large and small subunits which together constitute the catalytic form of the enzyme. The caspase cascade is initiated via initiator caspases, caspase-2, caspase-8, caspase-9 and caspase-10 which in turn, cleave and activate the executioner caspases, caspases -3, -6 and -7 (Peng *et al.*, 2010). Caspase-9, the essential initiator of mitochondrial mediated apoptotic pathway determines whether the cell is susceptible or resistant to apoptosis (Arduino *et al.*, 2009). Down regulation of caspase 9 was reported in several cancers including oral cancer (Shultz and Chalfant, 2011). The active form of caspase-3 plays a crucial role in cleaving cellular components associated with DNA repair (Sriram *et al.*, 2009). The activation of caspase-3 was repressed in many tumor cells including oral tumors (Cagnol *et al.*, 2011).

As tumors grow, invade and metastasize, the process of neovascularization is essential. Angiogenesis in tumors results due to the degradation of the extracellular matrix, endothelial cell proliferation, migration and assembly of endothelial cells into higher order structures (Sappayatosok *et al.*, 2009). VEGF over expression was reported in several types of cancers including oral cancer (Faratzis *et al.*, 2009). Deregulation of VEGF resulted in the progression of Squamous Cell Carcinomas (SCC) in the oral cavity (Martins *et al.*, 2011).

Natural products that modulate cell proliferation, apoptosis, inflammation and angiogenesis are considered to have potent anticancer properties. Berberine is a

quaternary protoberberine alkaloid and is a major constituent of many medicinal plants of the families *Papaveraceae*, *Berberidaceae*, *Fumariaceae*, *Menispermaceae*, *Ranunculaceae*, *Rutaceae*, *Annonaceae* etc. (Grycova *et al.*, 2007; Khan *et al.*, 2010). Berberine possesses diverse biological activities which include antibacterial (Samosorn *et al.*, 2009), antifungal (Iwazaki *et al.*, 2010), antimalarial (Stermitz *et al.*, 2000), antileishmanial (Kalla and Singh, 1996) and antidiarrheal (Rabbani *et al.*, 1987) activities. Profound studies reported its cardioprotective (Huang *et al.*, 1992; Wu *et al.*, 2010), anti-diabetic (Lee *et al.*, 2006), antihyperlipidemic (Zhou *et al.*, 2008) and anti-cancer (Ho *et al.*, 2009) potential. Previous studies from our laboratory have shown the anticlastogenic potential of berberine in DMBA induced clastogenesis (Sindhu and Manoharan, 2010). Also its protective effect on cellular integrity was shown during DMBA induced oral carcinogenesis. Recently, we demonstrated the chemopreventive potential of berberine in DMBA induced hamster buccal pouch carcinogenesis (Manoharan *et al.*, 2011). To scientifically validate the chemopreventive potential of berberine, the present study investigated the modulating effect of berberine on the expression pattern of inflammatory (NF $\kappa$ B, COX-2 and c-fos), angiogenic (VEGF), cell proliferative (Cyclin D1 and PCNA) and apoptotic (p53, Bcl-2, Bax, caspase-3 and -9) markers during DMBA induced oral carcinogenesis.

## MATERIALS AND METHODS

**Chemicals:** Berberine ( $\geq 95\%$  purity, CAS NO: 633-65-8) and DMBA (95% purity, CAS NO: 57-97-6, soluble in liquid paraffin) were purchased from Sigma Aldrich Chemical Pvt. Ltd., Bangalore, India. PCNA, Bcl-2, Bax, VEGF and p53 primary antibodies were purchased from Dako, Carpinteria, CA, USA. Power Block™ 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. The caspase-3 and -9 colorimetric assay kits were purchased from Biovision, Mountain View, CA, USA. 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, India. All other chemicals used were of analytical grade, purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India.

**Animals:** Male golden Syrian hamsters, 8-10 weeks old 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, Annamalainagar, India. The animals were housed in polypropylene cages and were provided with standard pellet diet (Amrut laboratory Animal Feed Mysore Feeds Limited, Bangalore, India) and water *ad libitum*. The standard pellet diet is composed of 21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorous, 3.4% glucose, 2% vitamin and 55% nitrogen-free extract (carbohydrates). The animals were maintained under controlled conditions of temperature ( $27\pm 2^{\circ}\text{C}$ ) and humidity ( $55\pm 5\%$ ) with a 12 h light/dark cycle.

**Experimental design:** The local institutional animal ethics committee (Registration number 160/1999/CPCSEA) of Annamalai University approved the experimental design [proposal No. 548 dated 20.03.2008]. The animals were maintained following the principles and the guidelines of 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 categorized into 4 groups of 10 hamsters each. Group 1 animals served as control and were painted with liquid paraffin (Vehicle) three times a week for 14 weeks on their left buccal pouches. Animals in groups 2 and 3 were painted with a 0.5% solution of DMBA in liquid paraffin three times per weeks for 14 weeks. Animals in group 2 received no other treatment. Group 3 animals were orally administered with berberine at a concentration of  $75\text{ mg kg}^{-1}$  bw three times per week on days alternate to DMBA application, starting 1 week before the exposure to the carcinogen and continued until one week after the final exposure of the carcinogen. Group 4 animals were received oral administration of berberine  $75\text{ mg kg}^{-1}$  b.w alone throughout the experimental period. All animals were sacrificed by cervical dislocation at the end of experimental period.

**Immunoexpression of p53, Bcl-2, Bax, PCNA and VEGF:** Buccal mucosa from control and experimental animals were fixed in 10% formalin and were routinely processed and paraffin embedded, 2-3  $\mu\text{m}$  sections were cut in a rotary microtome and mounted on polylysine coated clean glass slides, dried at  $37^{\circ}\text{C}$  and used for immunohistochemical staining.

Paraffin embedded tissue sections were dewaxed and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3%  $\text{H}_2\text{O}_2$  in methanol for 10 min. The antigen retrieval was achieved by microwave in citrate buffer solution (2.1 g citric acid  $\text{L}^{-1}$   $\text{D.H}_2\text{O}$ ; 0.37 g EDTA  $\text{L}^{-1}$   $\text{D.H}_2\text{O}$ ; 0.2 g Trypsin) (pH 6.0) for 10 min, followed by washing step with Tris-buffered saline (8 g NaCl; 0.605 g Tris) (pH 7.6). The tissue section was then incubated with power Block™ reagent for 15 min 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) overnight at  $4^{\circ}\text{C}$ . The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase for 30 min at room temperature. After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3,3'-diaminobenzidine, 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. The percentage of positive cells was scored according to the method of Lyzogubow *et al.* (2008).

**Estimation of c-fos, COX-2, caspase-3 and -9 activities by enzyme linked immunosorbent assay [ELISA]:** The activities of c-fos, COX-2, caspase-3 and -9 were assayed in the buccal mucosa using ELISA kit for c-fos, Cayman's COX activity assay kit for COX-2, colorimetric assay kits for caspase-3 and caspase-9 according to the manufacturer's instructions respectively. 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. The caspase-3 and -9 assays are based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA and LEHD-pNA, respectively at 405 nm in a microtiter plate reader.

**Expression of NF $\kappa$ b and cyclin D1 using real time PCR:** Total RNA from the buccal mucosa was extracted with Trizol reagent. The RNA integrity and concentration was

determined by electrophoresis on agarose gel and nanodrop analysis at 260 nm. Isolated total RNA (1 µg) 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 NFκB, Cyclin D1 and β-actin with SYBR green fluorophore following the manufacturer's 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 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 product, normalised to the endogenous control and relative to the control sample.

**Statistical analysis:** The data are expressed as Mean±SD. Statistical comparisons were performed by one way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) using SPSS version 12.0 for windows (SPSS Inc.chicago; <http://www.spss.com>). The results were considered statistically significant if the p-values were 0.05 or less.

**RESULTS**

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 are depicted in Fig. 1-3 and Table 2, respectively. Over expression of all these markers except Bax was noticed in hamsters treated with DMBA alone. Oral administration of berberine at a dose of 75 mg kg<sup>-1</sup> bw to hamsters treated with DMBA

**Table 1: List of primers used for real-time PCR analysis**

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

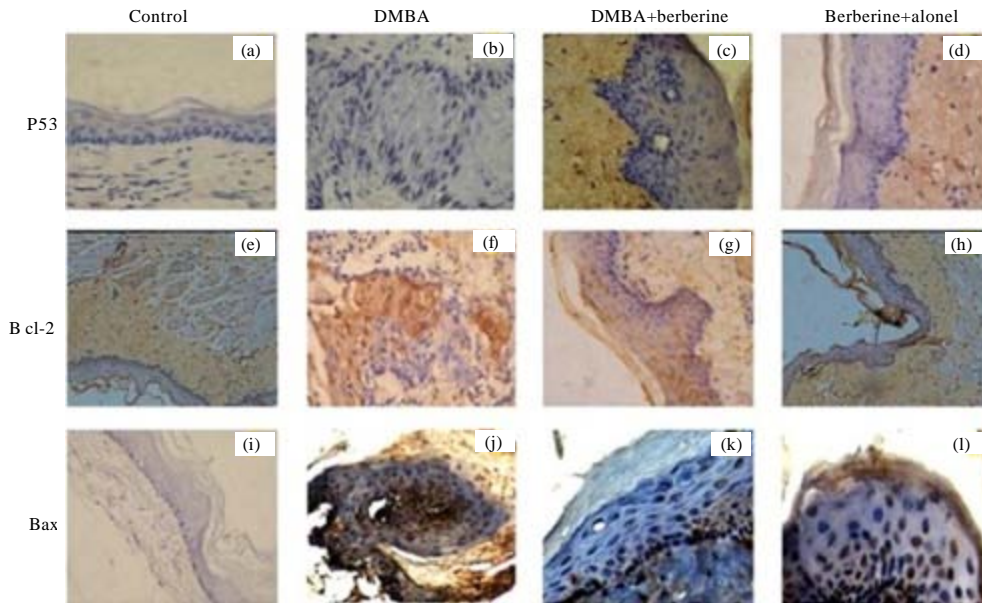


Fig. 1 (a-l):Immunoexpression pattern of p53, Bcl-2 and Bax proteins observed buccal mucosa of control and experimental hamsters (x 400). P53: a and d: Control and berberine alone. (expression not detectable), b: DMBA alone. (over expression), c: DMBA+berberine (down regulated). Bcl-2: e and h: Control and berberine alone (expression not detectable), f: DMBA alone (over expression), g: DMBA+berberine (down regulated). Bax: i and l: Control and berberine alone (mild expression), j: DMBA alone (down regulated); k: DMBA+berberine (up regulated)

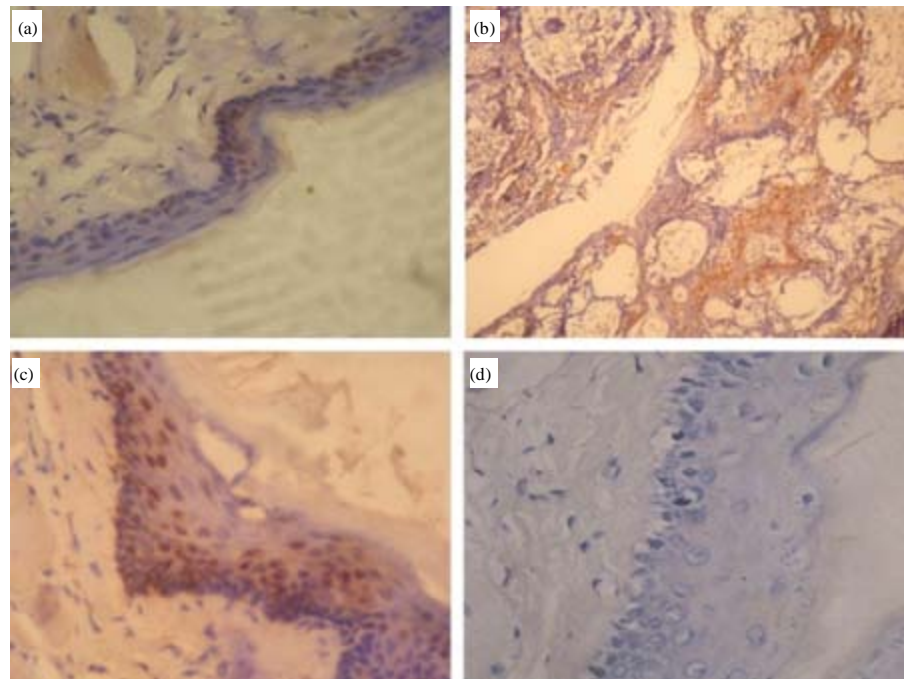


Fig. 2 (a-d): Immunoeexpression pattern of PCNA protein in control and experimental hamsters (x 400). A and D: Control and berberine alone (expression not detectable), B: DMBA alone (over expressed) and C: DMBA+berberine (down regulated)

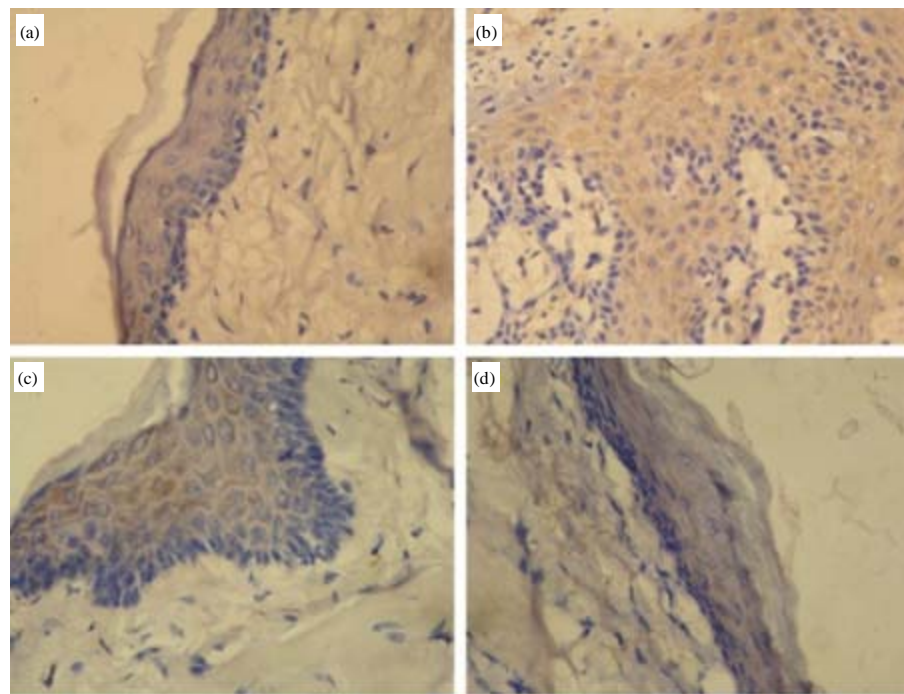


Fig. 3 (a-d): Immunoeexpression pattern of VEGF protein in control and experimental hamsters (x 400). A and D: Control and berberine alone (expression not detectable), B: DMBA alone (over expressed) and C : DMBA+berberine (down regulated)

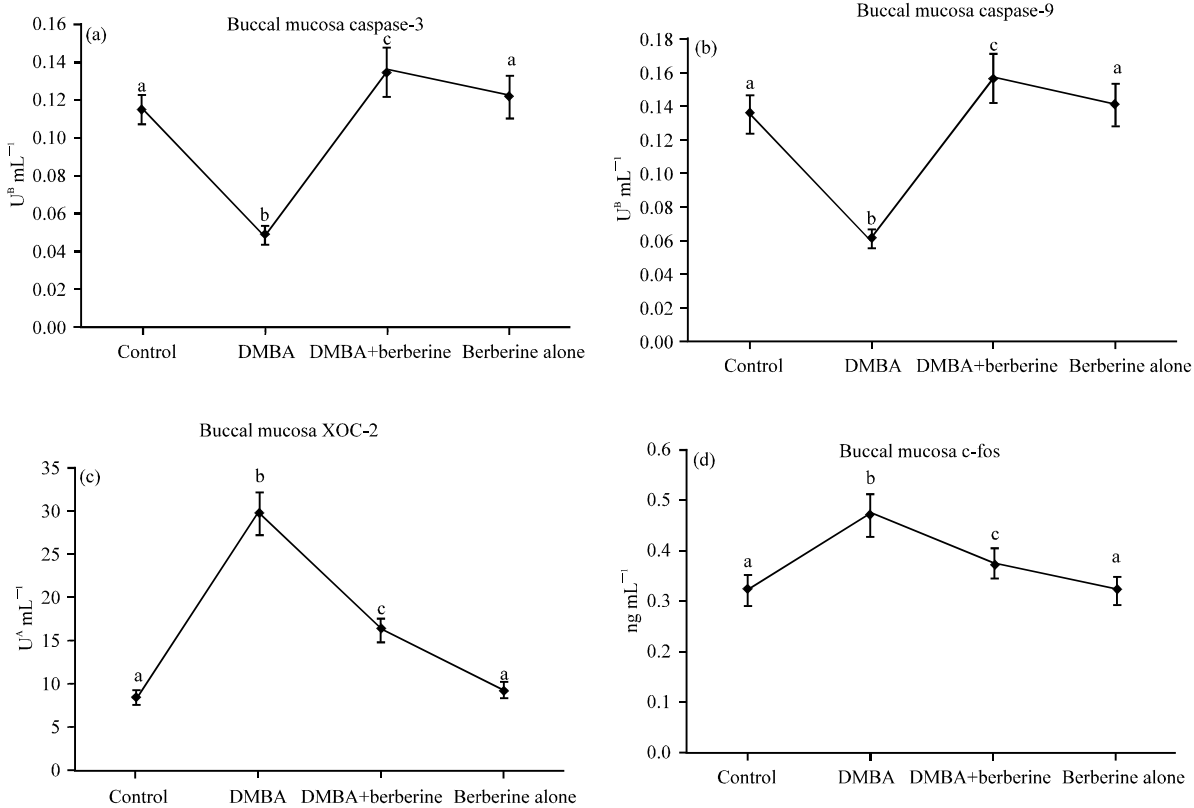


Fig. 4 (a-d): (a-b) ELISA assay showing effect of berberine on caspase-3 and -9, c-fos and COX-2 activity in control and experimental hamsters. Values are expressed as Mean±SD for 10 animals in each group. Values that do not share a common superscript letter in the same trend line differ significantly at  $p < 0.05$ . (Analysis of variance followed by DMRT). (A) nmol of TMPD oxidized  $\text{min}^{-1}$  and (B)  $\mu\text{mol}$  of pNA formed  $\text{min}^{-1}$

Table 2: Score of positively stained cells of p53, PCNA, Bcl-2, Bax and VEGF in control and experimental hamsters

Groups/Markers	P53				PCNA				Bcl2				Bax				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	1	9	0	0	9	1	0	0	6	4	0	0	10	0	0	0
DMBA	0	2	4	4	0	1	2	7	0	2	5	3	9	1	0	0	0	2	7	1
DMBA+berberine	7	2	1	0	0	8	2	0	3	6	1	0	0	3	5	2	8	2	0	0
Berberine alone	10	0	0	0	0	10	0	0	9	1	0	0	7	3	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+ = weak staining, between 1 and 20% of cells were stained, 0 = negative, less than 1% of cell staining

significantly restored the expression of above markers. Hamsters treated with berberine alone revealed expression similar to that of control hamsters.

Buccal mucosa caspase 3 and 9, COX-2 and c-fos status of control and experimental hamsters is shown in Fig. 4a-d. The activities of caspase -3 and -9 were significantly decreased whereas COX-2 and c-fos were increased in hamsters treated with DMBA alone. Oral administration of berberine 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 treated with berberine alone.

The Cyclin D1 and NFκB mRNA expression pattern of control and experimental hamsters is depicted in Fig. 5a-c. The expression of NFκB and Cyclin D1 was significantly higher in hamsters treated with DMBA alone as compared to control hamsters. Oral administration of berberine to hamsters treated with DMBA suppressed the expression of Cyclin D1 and NFκB. Similar pattern of Cyclin D1 and NFκB was observed in control hamsters and hamsters treated with berberine alone.



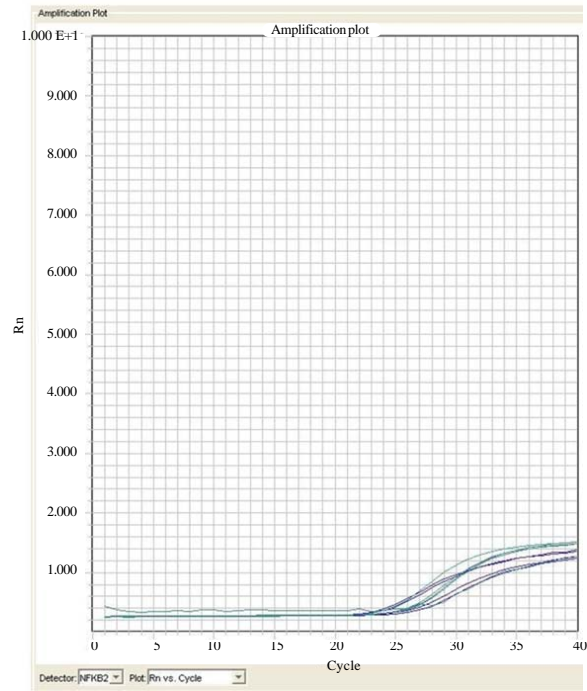


Fig. 5 (a): Real-time PCR analysis of mRNA expression in buccal mucosa of control and experimental hamsters. a: Relative quantification of  $\beta$ -actin and NF $\kappa$ B mRNA expression in buccal mucosa of control and experimental hamsters

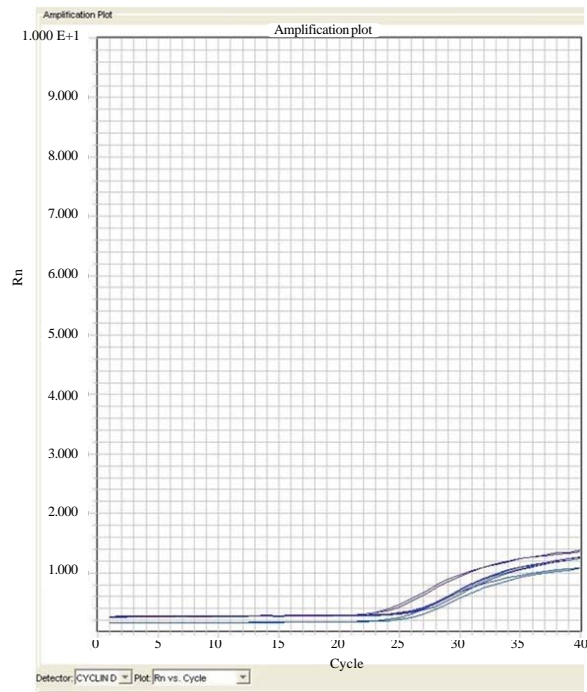


Fig. 5b: Real-time PCR analysis of mRNA expression in buccal mucosa of control and experimental hamsters. Relative quantification of  $\beta$ -actin and Cyclin D1 mRNA expression in buccal mucosa of control and experimental hamsters

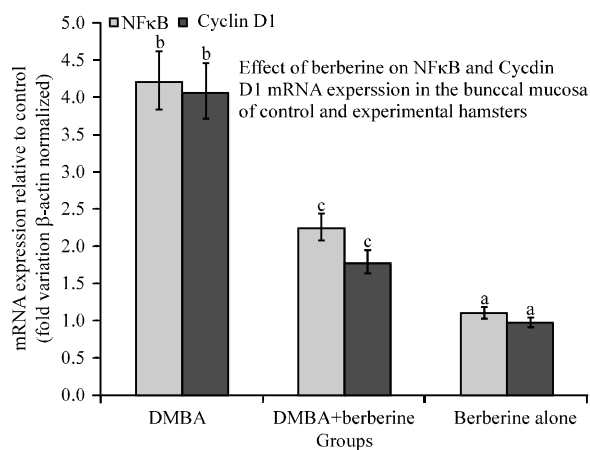


Fig. 5c: Effect of berberine and DMBA on NFκB and Cyclin D1 mRNA expression in buccal mucosa of control and experimental hamsters. Values are expressed as Mean±SD for 10 animals in each group. Values that do not share a common superscript letter in the same bar differ significantly at  $p < 0.05$ . (Analysis of variance followed by DMRT)

## DISCUSSION

In the present study, we noticed deregulation of apoptotic, cell proliferative and inflammatory markers in hamsters treated with DMBA alone. COX-2 expression could be used as predictor of overall survival of cancer patients. Over expression of COX-2 were reported in oral cancers both *in vivo* and *in vitro* (Abrahao *et al.*, 2010). Studies also reported that COX-2 expression was increased as the tumor progresses. Deregulation of COX-2 expression in the cell can lead to DNA damage, increased cell proliferation, oxidative stress and resistance to apoptosis. COX-2 expression analysis would help to assess the lymph node metastasis in patients with oral squamous cell carcinoma (Sappayatosok *et al.*, 2009). Over expression of COX-2 was noticed in oral cancer patients with lymph node metastasis than in those without lymph node metastasis (Ohtsu *et al.*, 2010). Extensive studies suggested that inflammation is one of the early pathological alterations occurring in DMBA induced hamster buccal pouch carcinogenesis (Hao *et al.*, 2009). Present results support these findings. Over expression of COX-2 in buccal mucosa of tumor bearing hamsters is probably due to inflammatory processes within the tumor microenvironment. Agent which acts as inhibitor of COX-2 is considered to have a potent chemopreventive activity against oral carcinogenesis (Garg *et al.*, 2008).

NfκB, a well known transcription factor, plays a crucial role in the pathogenesis of neoplastic transformations. It has been reported that constitutive activation of NFκB exerted abnormal cell proliferative and antiapoptotic functions in several cancer cells including oral cancer (Cichocki *et al.*, 2010). Over expression of NFκB was noticed in the buccal mucosa tissues of tumor bearing hamsters (Philip *et al.*, 2004). Our results are in line with these findings. Targeting NFκB signaling pathways with natural products could help to identify promising chemopreventive and chemotherapeutic agents against oral carcinogenesis (Yun *et al.*, 2009).

Deregulated expression of c-fos could result in neoplastic transformation. Over expression of c-fos was noticed in several malignancies including oral carcinoma (Garg *et al.*, 2008). Our results corroborate these observations. Abnormal expression of inflammatory markers, NFκB, COX-2 and c-fos, in hamsters treated with DMBA alone suggested that DMBA might have induced oral carcinogenesis through chronic inflammation in the buccal pouch of the hamsters.

PCNA expression pattern can be used to assess cell proliferation and in the comprehension of the neoplastic process (Motiwale *et al.*, 2005). A gradual increase in the expression of PCNA has been reported from normal epithelium to malignant epithelium (Lawall and Crivelini, 2006). Over expression of PCNA has been demonstrated in DMBA induced hamster buccal pouch carcinogenesis (Chen and Lin, 2000). Our results lend credibility to these observations. In the present study, immunohistochemical analysis revealed over expression of PCNA in hamsters treated with DMBA alone. Over expression of PCNA in the buccal mucosa of hamsters treated with DMBA alone indicated abnormal proliferation of cells occurring in DMBA induced oral carcinogenesis.

Cyclin D1, a positive regulator of G1 to S phase cell cycle transition, plays crucial role in modulating the cell response to DNA damage (Abou *et al.*, 2006). Several studies pointed out that Cyclin D1 deregulation is an early event in oral carcinogenesis (Cheng *et al.*, 2007). Over expression of Cyclin D1 has been reported in precancerous oral epithelial dysplastic lesions (Todd *et al.*, 2002). Cyclin D1 is also regarded as a marker of cell proliferation and its over-expression has been demonstrated in several malignancies including oral cancer (Alam *et al.*, 2011). The results of the present study are in line with these findings. Over expression of the cell proliferation markers, Cyclin D1 and PCNA, in hamsters treated with DMBA alone suggested abnormal cell proliferation occurring in DMBA induced oral carcinogenesis.

In the present study, immunohistochemical analysis revealed over expression of VEGF in tumor tissues of hamsters treated with DMBA. Deregulation of VEGF, an important angiogenic growth factor, is involved in the tumor progression and aggressiveness. A positive correlation between VEGF expression and micro vessel density, disease severity and poor prognosis has been reported (Sauter *et al.*, 1999). Extensive studies suggested that oral tumor tissues showed higher expression of VEGF as compared to normal oral tissues (Smith *et al.*, 2000; O-charoenrat *et al.*, 2001; Chin *et al.*, 2004). Our results support these findings.

P53, a best characterized tumor suppressor gene, performs an important role in the induction of cell cycle arrest and apoptosis, when there is a DNA damage or oxidative stress in the human cells (Sousa *et al.*, 2009). Over expression of p53 mutant protein has been demonstrated in precancerous and cancerous lesions of the oral cavity (Chin *et al.*, 2004; Lawall and Crivelini, 2006). Mutant p53 proteins were noticed in 50% of all oral cancer cases (Panjamurthy *et al.*, 2008). P53 over expression were also reported in DMBA induced hamster buccal pouch carcinogenesis (Hsue *et al.*, 2008). Present results are in line with these findings.

Bcl-2 family proteins serve as an important regulator of mitochondria-mediated apoptosis. Under normal conditions, Bcl-2/Bax ratio regulates the process of apoptosis through maintaining the mitochondrial integrity and inhibiting the activation of caspase cascade (Shu *et al.*, 2011). Siow *et al.* (2011) reported that over expression of Bcl-2 favors the cell towards abnormal proliferation rather than inhibiting apoptosis. A negative correlation between the expression of antiapoptotic Bcl-2 and proapoptotic Bax has been shown in DMBA induced hamster buccal pouch carcinogenesis (Balakrishnan *et al.*, 2010). The results of the present study support these findings.

Imbalance in apoptotic machinery is a characteristic feature of neoplastic cells. Caspases play pivotal role in the initiation and execution of apoptosis (Rodríguez-Berriguete *et al.*, 2011). It has been suggested that the apoptotic proteins such as Bcl-2 and Bax are involved in the activation of several caspases especially caspase-3 (Okada and Mak, 2004). In the present study we noticed decreased activities of caspase-3 and -9 in hamsters treated with DMBA alone. Present results suggest that there was an evasion of apoptotic mechanism in hamsters treated with DMBA alone.

Any agents that inhibit cell proliferation activities through cell cycle arrest and induce caspase mediated apoptosis are considered as good anticancer agents.

Chemopreventive agents mediate their antitumor initiative potential probably by inhibiting cell proliferation, inflammatory process, angiogenesis and inducing apoptosis. The present study assessed the chemopreventive potential of berberine by investigating its modulating effect on the expression pattern of cell proliferative, inflammatory, angiogenic and apoptotic markers during DMBA induced oral cancer. Studies have demonstrated that berberine induced apoptosis through cell cycle arrest at G0/G1, G1 and/or G2/M, phase (Tsang *et al.*, 2009). Berberine inhibited cell proliferation through p53 dependent G1 arrest and p53 independent G2 arrest (Khan *et al.*, 2010; Piyamuch *et al.*, 2007). Berberine triggered cell death by increasing Bax/Bcl-2 ratio in human glioblastoma T98G cells (Eom *et al.*, 2008). It has been demonstrated that berberine induced cell cycle arrest in G1-phase through down regulation of the proto oncogene Cyclin D1 (Liu *et al.*, 2011). Berberine significantly down regulated the expression of PCNA in tumor cells (Khan *et al.*, 2010). Berberine suppressed the activation of Cyclin D1 and activated the levels of caspase -3 and caspase-9 (Inoue *et al.*, 2005). It has been reported that berberine modulated inflammatory responses through the inhibition of transcription factors such as NFκB (Letasiova *et al.*, 2005). It has been reported that berberine prevented invasion and metastasis of human lung cancer cells through the reduction of phosphorylation of c-fos and NFκB (Kim *et al.*, 2008; Tsang *et al.*, 2009). Berberine also inhibited the expression of c-fos in oral cancer cells as well as COX-2 expression in colon cancer cells (Kuo *et al.*, 2004). Berberine suppressed the expression of cyclooxygenase-2 in human colon cancer cells (Fukuda *et al.*, 1999). Berberine attenuated the growth and metastasis of tumor cells probably by inhibiting of vascular endothelial cell proliferation, as evidenced by down regulation of VEGF (Jie *et al.*, 2011). In the present study oral administration of berberine at a dose of 75 mg kg<sup>-1</sup> body weight down regulated the expression of inflammatory markers (COX-2 and NFκB) and cell proliferation markers (CyclinD1, PCNA, VEGF and c-fos). Also berberine enhanced the expression of Bax, caspase -3 and -9 and down regulated the expression of p53 and Bcl-2. Oral administration of berberine thus restored the expression of cell proliferation, inflammatory and apoptotic markers in hamsters treated with DMBA alone. The present study concludes that the chemopreventive potential of berberine might be due to its anti-inflammatory, anti-cell proliferative, anti-angiogenic and apoptotic potential during DMBA induced oral carcinogenesis.

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