Clove (*Syzygium aromaticum* L.), a potential chemopreventive agent for lung cancer

Sarmistha Banerjee[1], Chinmay Kr.Panda[2] and Sukta Das[1,*]

[1]Department of Cancer Chemoprevention and [2]Department of Oncogene Regulation, Chittarajan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata 700026, India

*To whom correspondence should be addressed
Email: suk_tadas@yahoo.com

Spices and flavoring plants part rich in supposedly health-promoting phytochemicals are currently receiving much attention as a possible source of cancer chemopreventive compounds. Clove, the sun-dried unopened flower bud from the plant *Syzygium aromaticum* L. is a commonly used spice and food flavor. In the present work we assess the chemopreventive potential of aqueous infusion of clove during benzo[*a*]pyrene (BP)-induced lung carcinogenesis in strain A mice. Incidence of hyperplasia, dysplasia and carcinoma *in situ* evident in the carcinogen control group on the 8th, 17th and 26th weeks, respectively, were effectively reduced after treatment with clove infusion. Significant reduction in the number of proliferating cells and an increased number of apoptotic cells was also noted in these BP-induced lung lesions following clove treatment. Western blotting analysis revealed that clove infusion upregulates the expression of pro-apoptotic proteins p53 and Bax, and downregulates the expression of anti-apoptotic protein Bcl-2 in the precancerous stages. Expression of caspase 3 and its activation by clove infusion were evident from a very early stage of carcinogenesis (eighth week). Clove infusion was also found to downregulate the expression of some growth-promoting proteins, viz, COX-2, cMyc, Hras. The observations signify the chemopreventive potential of clove in view of its apoptogenic and anti-proliferative properties.

Introduction

Healthy eating and drinking habits are considered to protect from a variety of diseases, including cancer. The possibility of dietary modification and/or supplementation with specific agents for preventing cancer is currently being explored. A number of natural and synthetic agents have been identified, which can prevent cellular damages, associated with carcinogenesis, leading to the development of tumors. Chemopreventive strategies for cancer embrace intervention by the administration of natural or synthetic agents that can inhibit, delay, block or reverse the process of carcinogenesis, which can be used for the general population to protect from carcinogenic exposure and reduce cancer risk. Epidemiological and animal model studies do indicate that cancer risk may be influenced by dietary factors (1,2). It is now believed that a wide variety of naturally occurring substances from plant food offer protection from carcinogenic exposure (3). There has been a growing realization that spices possess anti-carcinogenic properties, which is supported by experimental evidences (4–7).

Among spices, clove (*Syzygium aromaticum* L.) is used as a spice to add flavor to exotic food preparations. It was used by the traditional Ayurvedic healers of India since ancient times to treat respiratory and digestive ailments. Aqueous clove infusion was found to inhibit the growth of germinated spores of *Bacillus subtilis*, and inhibit the pathogens *Campylobacter jejuni, Salmonella enterides* and *Escherichia coli*. Because of its antiseptic and antibiotic properties, clove is used to treat toothache and as an ingredient in a popular toothpaste and mouthwash in India. An anti-herpes virus compound eugenin was purified from clove, which could inhibit viral DNA synthesis. In addition water extracts of clove have shown the inhibitory effect on hepatitis C virus protease. Eugenol, the principle component of clove has been shown to prevent lipid peroxidation and also known as strong scavengers of active oxygen radicals (5).

Many different essential oils have been identified from clove, in which the most abundant one is eugenol (81.1%) (8,9). However, the activity of either the clove extracts or eugenol in cancer prevention is not known.

It is now well recognized that disturbances in the homeostatic mechanisms, which regulate cell proliferation and cell death (apoptosis), can contribute to the development and growth rate of a tumor. In fact, apoptosis has a pivotal role in limiting the population expansion of tumor cells early in the process of carcinogenesis, and inhibition of apoptosis has been shown to play an important role in the genesis of tumor (10). Apoptotic death may be initiated or modulated by the presence of some exogenous factors, such as drugs, radiations and also diet (11). If DNA damaged cells no longer respond to apoptosis, mutations may be acquired and fixed through further proliferation, which may lead to malignant neoplasia. In this context, it is noteworthy that apoptosis-inducing and proliferation-inhibiting ability may be considered as a primary factor in determining the efficacy of chemopreventive agents.

Among the positive and negative regulators of apoptosis, p53, the tumor suppressor gene, is an important defense against cancer as it suppresses tumor growth through cell-cycle regulation and also via apoptosis (12–14), each of which operates in a distinct context. Bax, the pro-apoptotic member of Bcl-2 family, is a p53 target and is transactivated in a number of systems during p53-mediated apoptosis (15). The upregulation of Bax expression and downregulation of Bcl-2, which lead to caspase 3 activation, the key executioner of apoptosis that results in the induction of apoptosis (16,17), have been well documented in a p53-dependent pathway of apoptosis (18).

Abbreviations: AI, Apoptotic index; BP, Benzo[*a*]pyrene; BrdU, 5-bromo-2-deoxyuridine; CIS, carcinoma *in situ*; COX-2, cycloxygenase; PI, Proliferative index.

Cyclooxygenase (COX) is a rate-limiting enzyme in the cellular production of prostaglandins (PGs) from arachidonic acid. Overexpression of cyclooxygenase-2 (COX-2) is associated with cell proliferation and the inhibition of programmed cell death, has been recognized as a potential target of chemoprevention during the pathogenesis of cancer (19,20).

Among different oncogenes, the *myc* oncogene family appears to play an important role in the pathogenesis of cancer. The cMyc protein, encoded by the c-*myc* proto-oncogene, is both a potent inducer of cell proliferation and of apoptosis (21,22). Another oncogene the *ras* genes play an important role in signal transduction and cellular proliferation [through the mitogen-activated protein kinase (MAPK) pathways]. The collaboration mechanism of the *ras* with *myc* activates the cyclin E-Cdk activity, which leads to the loss of p27 inhibition and induces S-phase and cell proliferation (23).

All these reports indicate that the proliferation and apoptosis regulating proteins are important factors for deciding the fate of a cell. However, the role of the spice, clove, in regulating the balance between cell proliferation and apoptosis via modulating the expression pattern of proliferation and apoptosis regulating genes in tumor cells, is not yet revealed.

Global incidence of lung cancer is rising continually per year (24) and as a consequence lung cancer is the leading cause of cancer mortality in most countries (25). Tobacco smoking is the major risk factor for lung cancer (26). Benzo[*a*]pyrene (BP), one of the important tobacco related carcinogen is significant for the formation of DNA adducts and ultimately cancer (27).

Surgical resection and/or radiation ablation or systemic chemotherapy is the main lines of treatment for most cancers, but in case of lung cancer post-treatment recurrence is quite frequent. Therefore another approach that focuses on the prevention of lung cancer is gaining ground. Although the cessation of tobacco smoking is important for lung cancer prevention, ex-smokers are still at a risk for developing lung cancer. An alternative and novel approach for the management of lung cancer is chemoprevention through the recommended intake of health protective food especially those present in vegetables, fruits, beverages and spices in daily diet.

Here we report for the first time the proliferation-inhibiting and apoptosis-inducing effects of aqueous infusion of clove on BP-induced lung carcinogenesis in Strain A mice.

In this study, we addressed the role of clove infusion on p53 and its downstream effectors Bax, Bcl-2 and caspase 3 in the induction of apoptosis and also the effect of aqueous infusion of clove on the expression status of COX-2, Hras and cMyc oncogene during BP-induced lung carcinogenesis. We suggest that such analysis could be useful for the individualization of chemopreventive strategies in the future.

Materials and methods

Materials and animals

BP was purchased from Sigma Chemical; USA. 5-Bromo-2-deoxyuridine (BrdU) labeling and detection kit II and *In situ* Cell Death Detection Kit II, AP was procured from Roche Molecular Biochemicals (Manheim, Germany). β-Actin, COX-2, caspase 3, p53, Bax Rabbit Polyclonal Antibody, and Hras, cMyc, Bcl-2 mouse monoclonal antibody (Primary antibody), anti-rabbit and anti-mouse IgG-horseradish peroxidase (HRP) (secondary antibody), were purchased from Santa Cruz Biotechnology, USA. Other chemicals were purchased from Sigma Chemical ; USA., Merck India and Roche Molecular Biochemicals.

Clove was purchased from a reputed governmental store, Kolkata, India. We wanted to evaluate the anticarcinogenic role of clove (*S.aromaticum* L.) that is commonly available and used by the Indian people during their food preparation. Cloves (available from governmental source) grow normally in South India in the open sandy loams and the laterite soils of south Kerala region, as clove is a tropical plant requiring warm and humid condition with a rainfall of 150–250 cm.

Newborn Strain A mice 24–48 h old was obtained from the animal colony of Chittaranjan National Cancer Institute (CNCI). They were maintained in plastic cages ($\sim$5 mice/cage) at an ambient temperature of $25 \pm 2°C$, on 12 h light/dark cycle with their mother. All animals were cared for, maintained, handled and killed following the guidelines of the animal ethical committee of the institute (Registration No.: IAEC-1.2/SD-1/2001–2003).

Preparation of aqueous infusion of clove

Cloves were powdered and soaked overnight in double distilled autoclaved water (2 g/100 ml) for the preparation of an infusion. The infusion was administered at a dose of 100 µl/mouse/day.

Development of lung carcinogenesis in mice and treatment with clove

Each newborn mouse (24–48 h) received a subcutaneous injection at the sub-scapular region with 0.02 ml of a suspension containing 0.2 mg concentration of BP in 1% aqueous gelatin solution (single dose). The carcinogen was used within 1 h after emulsification. Then the newborns were allowed to grow with their mother, provided with water and food pellets (Lipton India; India) *ad libitum*. After weaning (after fourth week) males and females were separated and the animals were divided into two groups.

Group I (GrI) was the carcinogen control group receiving 100 µl of distilled water orally every day from the fifth week of BP administration and continued up to the 26th week.

Group II (GrII) was the treatment group that received aqueous infusion of clove orally at a dose of 100 µl/mouse/day from the fifth week of BP administration and continued up to the 26th week.

Animals from both the groups were killed on the 8th, 17th and 26th weeks for the study of different parameters. The number of animals was 45 for each group ($N = 45$; $n = 15$ for each time point). Randomly selected 10 animals were used for histopathological and immunohistochemical analysis and 5 for immunoblotting analysis for each time point.

Tissue section preparation and histopathological evaluation

After having killed the mice from both groups at different time intervals all five lobes of the lungs from each mouse were collected, washed in phosphate-buffered saline (PBS) and soaked in blotting paper to remove the blood. Tissues were then fixed in 10% neutral buffered formalin for 24 h. The tissue samples were dehydrated in ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin to prepare the block. All five lobes of the lungs were sectioned, mounted on slides and stained. The 4 µm serial sections were used in such a way that the corresponding sections were used for staining with hematoxylin–eosin (HE) for light microscopy and histopathological evaluation as well as the determination of proliferating index (PI) and apoptotic index (AI). This process continued for the serial sections of the total lung.

Detection of in situ cell proliferation

The percentage of proliferative cells in the lungs was determined using BrdU labeling and detection kit II purchased from Roche Molecular Biochemicals. The mice were killed and the lungs were dissected out and placed into BrdU added pre-warmed (37°C) cell culture medium for labeling of DNA in proliferating S-phase cells. This was followed by fixation of tissues and preparation of paraffin sections. After de-paraffinization and re-hydration sections were rinsed in PBS and incubated with anti-BrdU monoclonal antibody at 37°C, 5% CO_2 for 30 min, in a humid chamber, which was detected *in situ* by an alkaline phosphatase-conjugate-antimouse-immunoglobulin antibody (antimouse Ig-AP). The bound antimouse Ig-AP was visualized using nitro-blue tetrazolium (NBT), an AP-substrate solution by light microscopy (28). The PI was determined by dividing the number of labeled cells by the total cells counted and multiplying by 100.

Detection of in situ cell death

Apoptotic cells in the lung section were detected using the terminal deoxy-nucleotidyl transferase (TdT)-mediated dUTP—fluorescein nick end labeling (TUNEL) method using *in situ* cell detection kit, AP (Roche Molecular Biochemicals, Germany). Deparaffinized tissue sections, after re-hydration were permeabilized using Triton-X-100 (Sigma Chemical, USA), washed in washing buffer and incubated with TUNEL reaction mixture (containing the TdT and fluorescein–dUTP) at 37°C, 5% CO_2 for 30 min. Slides were again washed with PBS for 10 min and NBT was added and kept for 10–15 min. Finally, the lides were washed and analyzed under light microscope (29). AI was

determined as the percentage of labeled cells with respect to total number of cells counted.

Protein extraction and western blot analysis

Protein was isolated for the analysis by western blotting. For protein isolation, lungs were collected from both groups, homogenized in 5 V/W 20 mM HEPES, 10% glycerol (pH 7.5), buffer containing a mixture of protease inhibitor (2 mM EDTA, 2 mM EGTA, 10 mM PMSF, 10 mg/ml leupeptin and 5 μg/ml aprotinin). Particulate fraction was prepared by centrifugation (13 000 r.p.m. for 1 h) and the amount of protein was quantified with the help of Lowry method (30). The gel was loaded with 50 μg of protein/lane and subjected to electrophoresis on 10% polyacrylamide gel. The separated proteins were then transferred to Millipore Immobilion-P-membrane (PVDF), blocked with blocking buffer (100 mM Tris–Cl and 150 mM NaCl and 0.1% Tween-20) and incubated with a 1:250 dilution of a specific primary antibody followed by 1:500 dilution of secondary antibody. The hybridized protein band was then detected using luminol reagent (Santa Cruz Biotechnology) and immunoreactive proteins were quantified with densitometer.

Statistical analysis

Tumor incidence results were analyzed by the χ^2-test. Data obtained from other experiments were analyzed by Student's *t*-test using MS-Excel and expressed as mean ± SD. The *P*-value of <0.05 was considered significant.

Results

Inhibition of BP-induced early lung lesion by aqueous infusion of clove

BP-induced early lung lesions in Strain A mice had been studied previously in our laboratory and we had histopathologically identified hyperplasia, dysplasia and carcinoma *in situ* (CIS) in the 8th, 17th and 26th week, respectively (31). Presently the effect of oral administration of aqueous infusion of clove from the fifth week onward of BP administration on the incidence of hyperplasia, dysplasia, CIS as well as the number of changed zone per mouse is reported.

Figure 1A shows the normal appearance of a lung section showing a single layer of bronchiolar epithelium. On the eighth week hyperplastic changes were noted in bronchiolar epithelial cells but not in alveolar epithelial cells of the carcinogen control group (Figure 1B). This change was restricted to almost normal condition in the treated group (Figure 1C). Effect of clove infusion was also noted on the 17th week where dysplasia observed in the carcinogen control group did not show such progression after clove treatment (Figure 1D and E). The histological appearance of CIS at the 26th week and the influence of clove infusion can be seen in Figure 1F and G. Only hyperplastic changes were noted in the treated group.

Incidence of BP-induced hyperplasia (Figure 2) was reduced following treatment with clove infusion. It may be noted that while 80% of BP-exposed untreated mice had hyperplasia in lung tissue, this was reduced to 70% by clove infusion showing 12.50% inhibition after such treatment. When the number of hyperplastic zones in the lung was considered, a significant difference (*P* < 0.01) was noted between the control and treated groups. While the average number of hyperplastic zones observed in the carcinogen control group was found to be 2.75 ± 0.53, treatment with clove infusion resulted in significant reduction to 1.6 ± 0.55 (Figure 3A).

Incidence of dysplasia (Figure 2), which was 70% in the BP-exposed group, came down to 50% following clove infusion treatment showing 28.57% inhibition after such treatment. Significant reduction in dysplastic zones both in the bronchiolar (*P* < 0.01) and alveolar (*P* < 0.05) regions of the lungs was also noted in the treatment group with respect to the carcinogen control group. It may be noted that in the BP administered group the number of dysplastic zones in bronchioles and

alveoli were 4.14 ± 0.69 and 3.14 ± 0.69 while the number of dysplastic zones in bronchiolar and alveoli region in the treatment group were 2 ± 1.41 and 1.67 ± 0.71 (Figure 3A and B).

The effect of clove infusion was very pronounced (*P* < 0.01) on the incidence of CIS (Figure 2). While 70% of BP-exposed animals had CIS, after treatment with clove infusion only 10% animals showed CIS indicating 85.71% inhibition after such treatment. Significant reduction (*P* < 0.001) in the number of CIS lesions per mouse was noted in the treatment group both in bronchiolar and alveolar regions as compared with control (Figure 3A and B). The number of bronchiolar and alveolar CIS lesions per mouse was 7.71 ± 0.76 and 6.29 ± 0.76, respectively in the carcinogen control group, and 4.50 ± 0.71 and 3.5 ± 0.71, respectively in the treatment group.

Inhibition of cell proliferation in BP-induced lung lesions by clove infusion

Effect of clove on *in situ* cell proliferation was assessed by BrdU incorporation and immunohistochemical analysis, which confirms our histopathological observation. It may be noted in Figure 4 that immunostain positive BrdU labeled cells in the bronchiolar region were lower in the treated group (B, D and F) than in the carcinogen control (A, C and E). PI on the eighth week of BP exposure was found to be 41.49 ± 0.98, which went up to 52.12 ± 0.81 on the 17th week and 70.66 ± 0.84 on the 26th week in the carcinogen control group. Treatment with clove infusion reduced the PI to 35.86 ± 0.57, 32.59 ± 0.196 and 25.23 ± 0.58 on the 8th, 17th and 26th weeks, respectively indicating 13.56, 37.47 and 64.29% inhibition (Figure 5). This result demonstrates a significant (*P* < 0.001) inhibitory effect of cove infusion on cell proliferation during carcinogenesis.

Induction of apoptosis by clove infusion in lung lesions during carcinogenesis

The effect of treatment with the test material on apoptosis was also assessed by immunohistochemical analysis. It is interesting to note that simultaneously with the inhibition of proliferation at the targeted site clove infusion also induced cell death (Figure 6). While AI on the eighth week was 1.56 ± 0.25 in the BP-exposed group, in the treatment group it was found to be 1.72 ± 0.21. During the progression of carcinogenesis AI in the BP-exposed group was 1.27 ± 0.11 on the 17th week that was further reduced to 0.82 ± 0.23 on the 26th week. Induction of apoptosis after the treatment with clove infusion was 1.91 ± 0.41 on the 17th week and 5 ± 0.18 on the 26th week (Figure 7).

Effect of clove infusion on the expression of pro- and anti-apoptotic proteins

After confirming that clove infusion induces apoptosis during lung carcinogenesis, our next attempt was to determine the impact of clove on the expression of genes associated with apoptosis. As it is well established that various pro- and anti-apoptotic proteins play a crucial role in programmed cell death, we examined whether or not clove infusion can affect the expression of pro-apoptotic proteins, p53 and Bax as well as anti-apoptotic protein, Bcl-2 in our mice model. Interestingly, it was observed that clove infusion could elevate the expression of p53 and Bax in the lesions identified as dysplasia and CIS (14.12 and 46.53% for p53; 16.11 and 53.88% for Bax, on the 17th and 26th weeks, respectively) as shown in Figure 8, A1 and A2 for p53 and Figure 8, B1 and B2 for Bax. Bcl-2
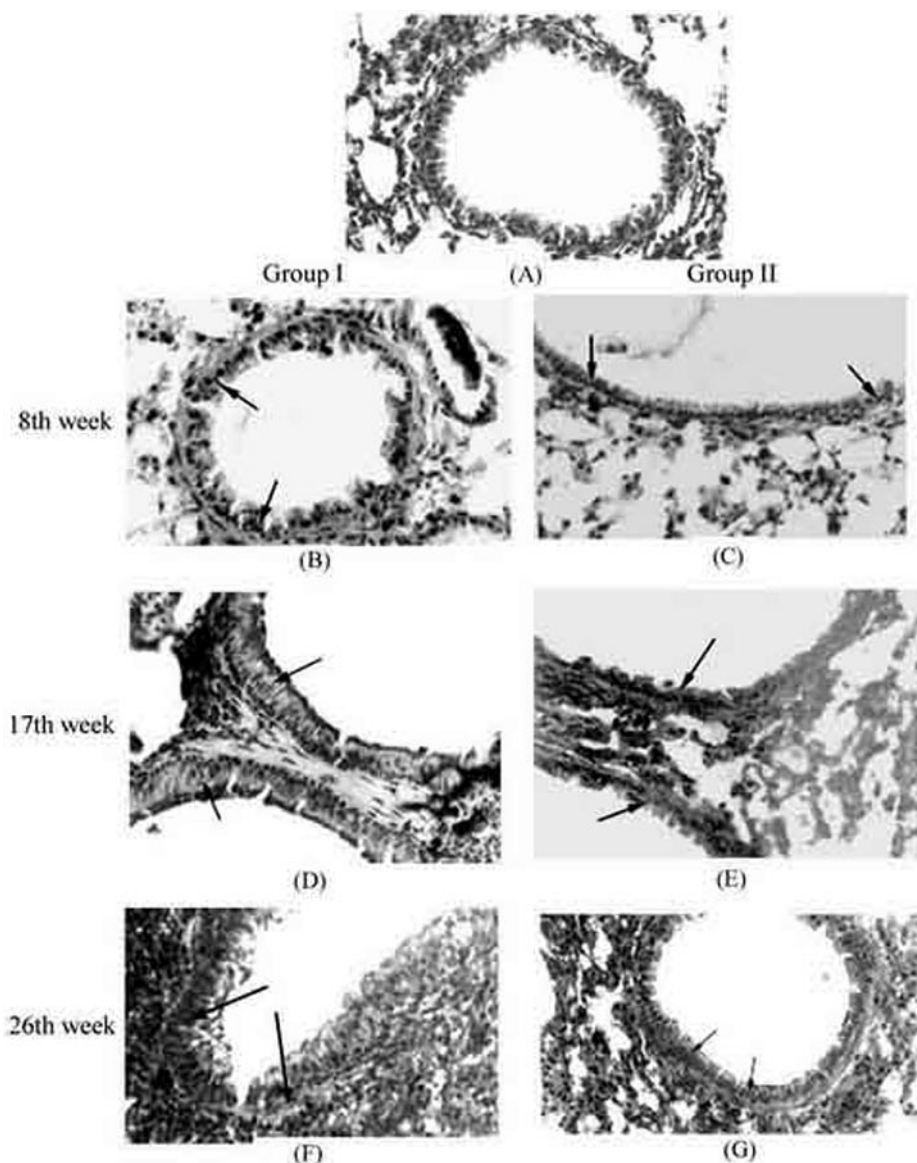
Fig. 1. Histopathology of lung lesion of carcinogen-exposed and clove-treated mice X400. (**A**) Normal lung bronchiolar epithelium. (**B**) Hyperplastic lesion in lung at the eighth week of carcinogen exposure. (**C**) Bronchiolar epithelium appears normal at the eighth week of carcinogen exposure after clove treatment. (**D**) At the 17th week dysplastic changes noted in carcinogen control. (**E**) Restriction of histopathological changes following treatment with clove may be noted and the appearance at the 17th week is almost normal showing a single layer of epithelial cells. (**F**) CIS noted at the 26th week of carcinogen exposure. (**G**) Clove treatment restricted the changes seen at the 26th week of carcinogen exposure (CIS) at hyperplasia.

expression was found to be downregulated by clove infusion at the same stage of carcinogenesis (47.29 and 56.15% reduction, respectively on the 17th and 26th weeks), thereby resulting in a decrease in Bcl-2:Bax ratio (Figure 8, C1 and C2).

Effect of clove infusion on caspase 3 expression and its activation

Accumulating evidence indicate that high level of p53 increases the ratio of Bax:Bcl-2 that leads to upregulation of caspase 3 expression, its activation and ultimately the induction of apoptosis (32). After assessing that clove infusion increased the Bax:Bcl-2 ratio, our next interest was to obtain evidence whether clove infusion increased the level of caspase 3 expression and its activation via upregulation of Bax:Bcl-2 expression or not. Interestingly it has been found that clove infusion not only increased caspase 3 expression and its

activation on the 17th and 26th weeks but also on the 8th week though clove infusion had no effect on Bax:Bcl-2 ratio during hyperplasia. Treatment was most effective in this respect during CIS (Figure 8, D1 and D2 for procaspase 3; Figure 8, E1 and E2 for 19 kDa caspase 3; and Figure 8, F1 and F2 for 17 kDa caspase 3).

Effect of clove infusion on COX-2 expression during lung carcinogenesis

Since COX-2 is a promising target for chemopreventive agents due to its presence during the early stage of lung carcinogenesis (33), our attempt was to find out the role of clove infusion on COX-2 expression in BP-induced early lung lesions. Clove infusion reduced the COX-2 level on the 17th and 26th weeks (13.49 and 55.93%) but not on the 8th week (Figure 8, G1 and G2).
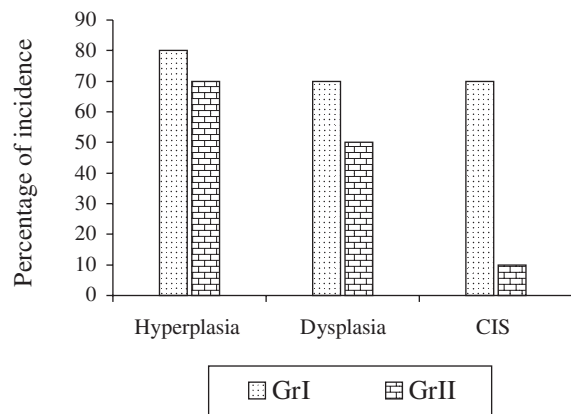
Fig. 2. Inhibitory effect of clove on incidence of hyperplasia, dysplasia, CIS at the 8th, 17th and 26th weeks, respectively, during BP-induced mouse lung carcinogenesis. Significant difference (χ^2-test) between carcinogen control group (GrI) and treatment group (GrII) were obtained only at CIS stage, $P < 0.01$.
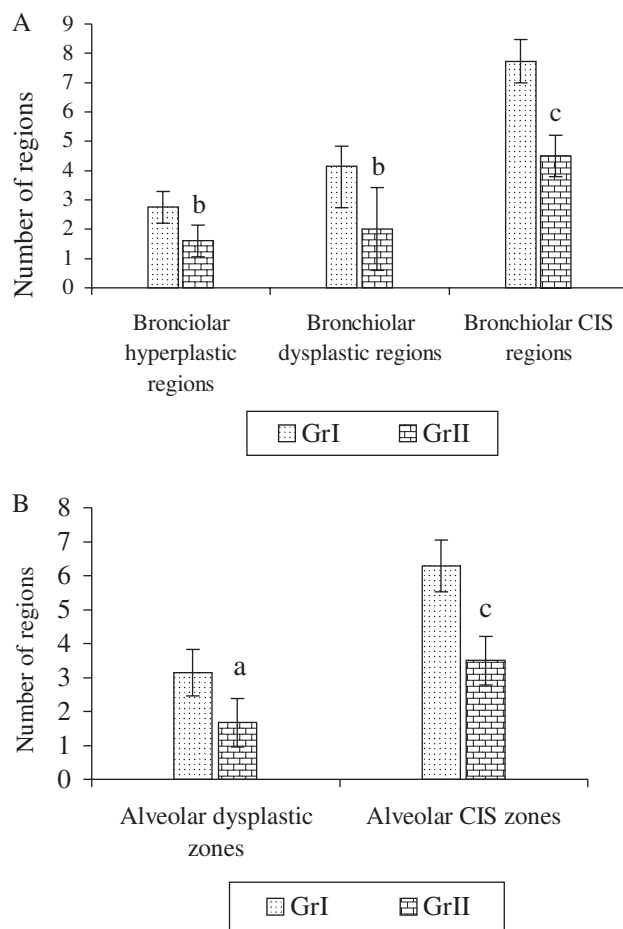

Fig. 3. The protective effect of clove on the number of hyperplastic, dysplastic and CIS regions during mouse lung carcinogenesis induced by BP. (**A**) Altered bronchiolar changed zone. Values are represented as mean ± SD. b, $P < 0.01$; c, $P < 0.001$. (**B**) Changed alveolar region. Values are represented as mean ± SD. a, $P < 0.05$; c, $P < 0.001$.

Effect of clove infusion on the expression of oncogenic protein during lung carcinogenesis

The protein products of many dominant oncogenes are capable of inducing both cell proliferation and apoptosis (34).

A large body of physiological evidence shows that either upregulation or downregulation of intracellular cMyc activity has profound consequences on cell-cycle progression (35). Similarly various cancers have been found to have mutations and overexpression of *ras* genes as an early or late event in the carcinogenesis process (36). In view of this the influence of clove infusion, if any on the expression of cMyc and Hras during the preneoplastic condition of lung carcinogenesis was investigated. While clove infusion did not have any effect on the expression of cMyc and Hras at a very early stage (eighth week), there was minimum reduction in the expression of these oncogenic proteins at 17th week (2.63 and 4.24%, respectively). The most effective result was noted on the 26th week (15.16 and 45.52%, respectively for cMyc and Hras, respectively) (Figure 8, H1 and H2 for cMyc; Figure 8, I1 and I2 for Hras).

Discussion

Chemoprevention has become an emerging area of cancer research that in addition to providing a practical approach to identifying potentially useful inhibitors of malignant transformation affords opportunities to study the mechanism of anticarcinogenesis. In recent years considerable efforts have been made to develop chemopreventive agents that would inhibit, retard or reverse the multistage carcinogenesis (37). Chemopreventive agent can act in any stage of carcinogenesis i.e. initiation, promotion or progression. The intervention of cancer at the post-initiation stage, however, seems to be the most appropriate and practical since this stage is reversible (38). According to the report of the chemoprevention-working group of the American Association of Cancer Research, one of the major strategies adopted in chemoprevention is to suppress the carcinogenesis process after initiation (39).

In the present study we report that aqueous infusion of clove can elicit strong pro-apoptotic effect during early lesion of lung carcinogenesis and also affect the *in situ* cell proliferation. The dose of aqueous infusion of clove (100 μl/mouse/day) adopted in this study was derived from our previous experiments on 7,12-dimethylbenz[*a*]anthracene (DMBA)-croton oil induced two-stage skin carcinogenesis model where inhibition of papilloma formation and reduction in tumor burden by clove was noted (40).

During BP-induced lung carcinogenesis in mice distinct histopathological changes could be identified as hyperplasia on the 8th week, dysplasia on the 17th week and CIS on the 26th week as early lesions of lung cancer. Clove infusion was found to inhibit or delay this progression. The incidence of hyperplasia was reduced after treatment with clove infusion. Moreover, the numbers of hyperplastic regions were also reduced and their appearance delayed in the treatment groups. While hyperplastic changes were noted in the carcinogen control group around the eighth week, such changes were observed in the clove infusion treatment group only at the 26th week. Likewise an inhibition was also observed on the incidence as well as the number of dysplastic regions (the precancer lesion) in the treatment group. Impact of such restrictive effect of clove was reflected as significance reduction of incidence of CIS in the treatment groups. The number of CIS lesions both in the alveoli and bronchiole also significantly reduced. Histopathological analysis of the different stages of lung carcinogenesis thus suggest a protective role of clove infusion
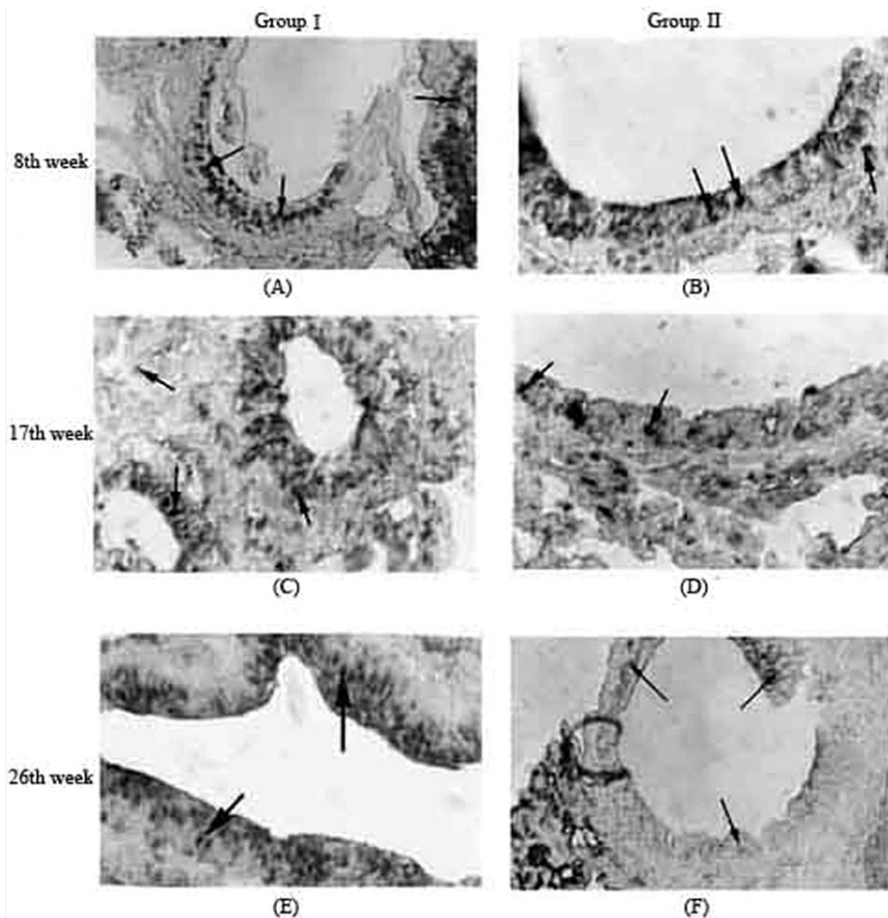
Fig. 4. Immunohistochemical localization of proliferating cells in carcinogen-exposed lung lesion (**A**, **C** and **E**). Clove treatment resulted in the reduction of proliferating cells at all preneoplastic stages during lung carcinogenesis (**B**, **D** and **F**). ×400.
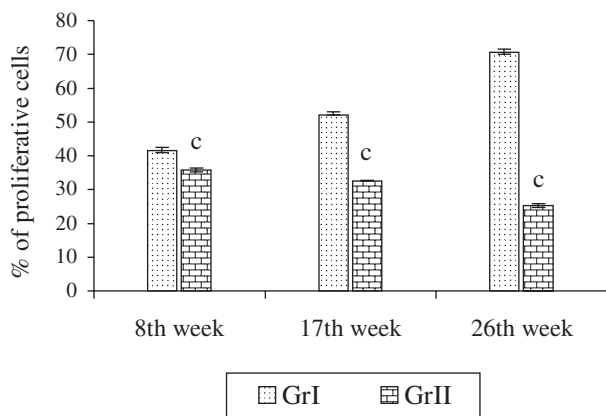

Fig. 5. Inhibitory effect of clove on the percentage of BrdU labeled proliferating cells on the 8th, 17th and 26th weeks of BP exposure, showing a significant difference between carcinogen control group (GrI) and treated groups (GrII). Values are represented as mean ± SD. c, $P < 0.001$.

which restricted the process of carcinogenesis and halted or delayed the appearance of early lesions by preventive intervention at the post-initiation phase.

It is clearly conceivable that increased or uncontrolled proliferation and an impaired apoptosis play a decisive role in the accumulation of malignant cells and eventually in the genesis of multistage carcinogenesis (41). The result of the present study demonstrated that clove infusion could lower the PI at different stages of experimental lung carcinogenesis in mice model. Number of BrdU labeled cells at the hyperplastic stage was significantly reduced after clove infusion treatment of BP-exposed mice in comparison with the carcinogen control group. The inhibitory effect of clove infusion on cell proliferation continued through the 17th week up to the 26th week. The delayed progression of histopathological changes from hyperplasia to CIS in treated groups may be attributed to their anti-proliferative action noted. Consequently, an enhanced but balanced rate of apoptosis would provide an important protective mechanism by any chemopreventive agents. Such an apoptotis inducing property of clove infusion was noted at different stages of lung carcinogenesis (8th, 17th and 26th weeks) in the present study.

Since clove infusion was found to be effective in the inhibition of cell proliferation and the induction of apoptosis in BP-induced lung carcinogenesis, attempts were made to explore the possible mechanisms of aqueous infusion of clove in the induction of apoptosis and the inhibition of proliferation. The expression of some apoptosis-inducing and growth-promoting proteins during BP-induced early lung lesions was therefore analyzed.

It is well established that when a cell would be committed to apoptosis partly depends upon the balance between proteins that mediate cell death, e.g. p53, Bax and proteins that promote
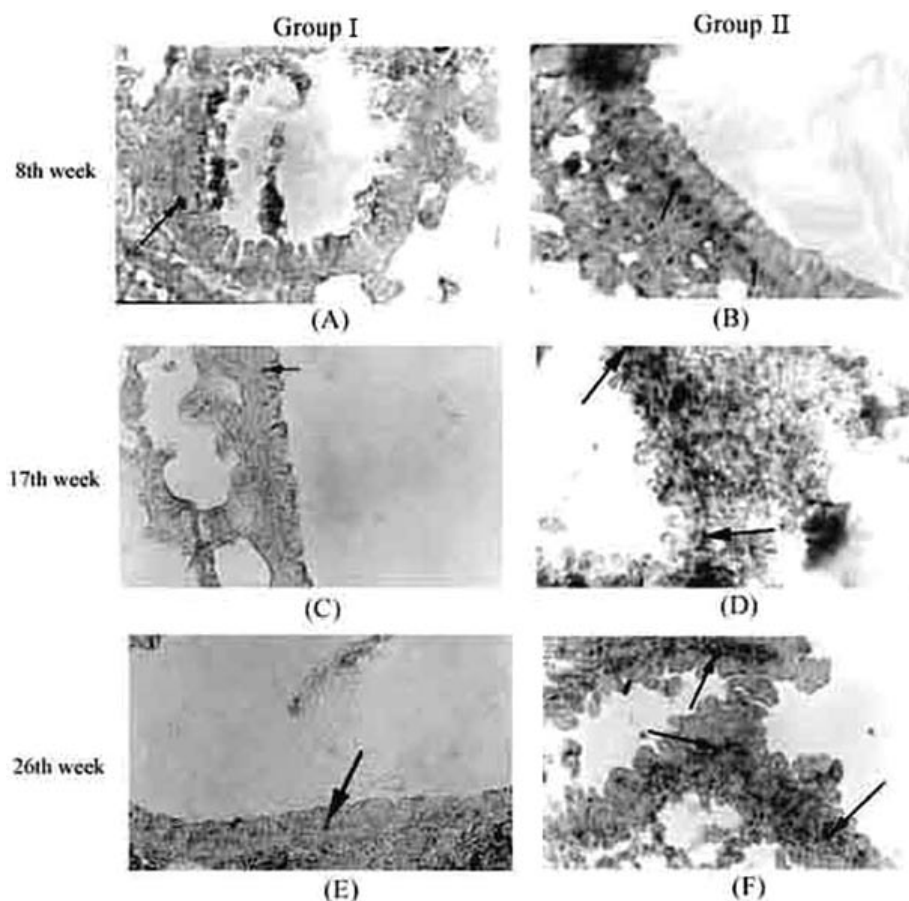
Fig. 6. *In situ* localization of apoptotic cells in lung tissue during BP induced carcinogenesis in carcinogen control group (**A, C** and **E**) and treated group (**B, D** and **F**). Treatment with clove resulted in an increase in apoptotic cells in the bronchiolar epithelium. ×400.
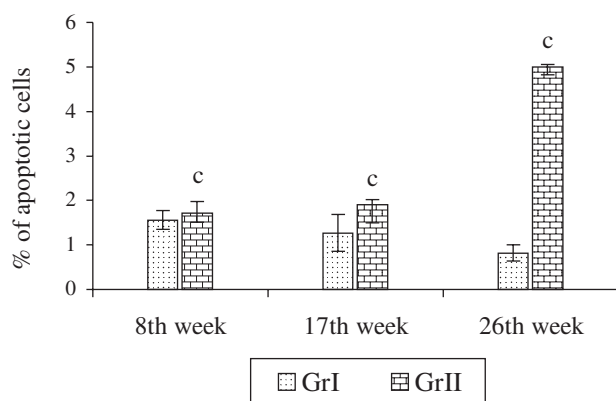

Fig. 7. Effect of clove on the percentage of apoptotic cells in mouse lung on the 8th, 17th and 26th weeks of exposure to BP showing a gradual increase with maximum effect on the twenty-sixth week. Values are expressed as mean ± SD. c, $P < 0.001$.

cell viability, e.g. Bcl-2 (42,43). Among these proteins, p53 has been found to facilitate apoptosis in various cancers. In this study we show that clove infusion can increase the p53 expression. It is known that p53 may induce genes in response to stress signals thereby contributing to apoptosis. The Bax is an apoptosis-promoting member of the Bcl-2 protein family. The Bcl-2 protein is known to form heterodimers with the Bax protein *in vivo* and the molar ratio of Bcl-2 to Bax determines whether apoptosis is induced or inhibited in several tissues (44). The Bax protein controls cell death through its participation in disruption of mitochondria and subsequent cytochrome *c* release and is also considered to be one of the primary p53 targets (45). In our experiment, treatment with clove infusion decreased the expression level of Bcl-2 and increased Bax concentration thereby decreasing the Bcl-2:Bax ratio. The modulatory effect of clove infusion was noted only after the lesion progressed beyond hyperplasia.

Caspase 3 is the ultimate executioner of apoptotic pathway. Activation of procaspase 3 (32 kDa) requires proteolytic processing of its inactive zymogen into activated p17 and p19 subunits (46). Bax drives the release of cytochrome *c* from the mitochondria and the released cytochrome *c* activates caspase 3 (47). Treatment with clove infusion has been shown to not only upregulate the expression of procaspase 3 but also its activation during the preneoplastic condition of BP-induced lung carcinogenesis. Therefore it implied the pro-apoptotic efficacy of aqueous infusion of clove. However, many questions remain to be answered, including how clove induces apoptosis or activates caspase 3 during hyperplasia, though p53 and or Bcl-2:Bax ratio remained unaffected at this stage. There are other p53 independent pathways involved
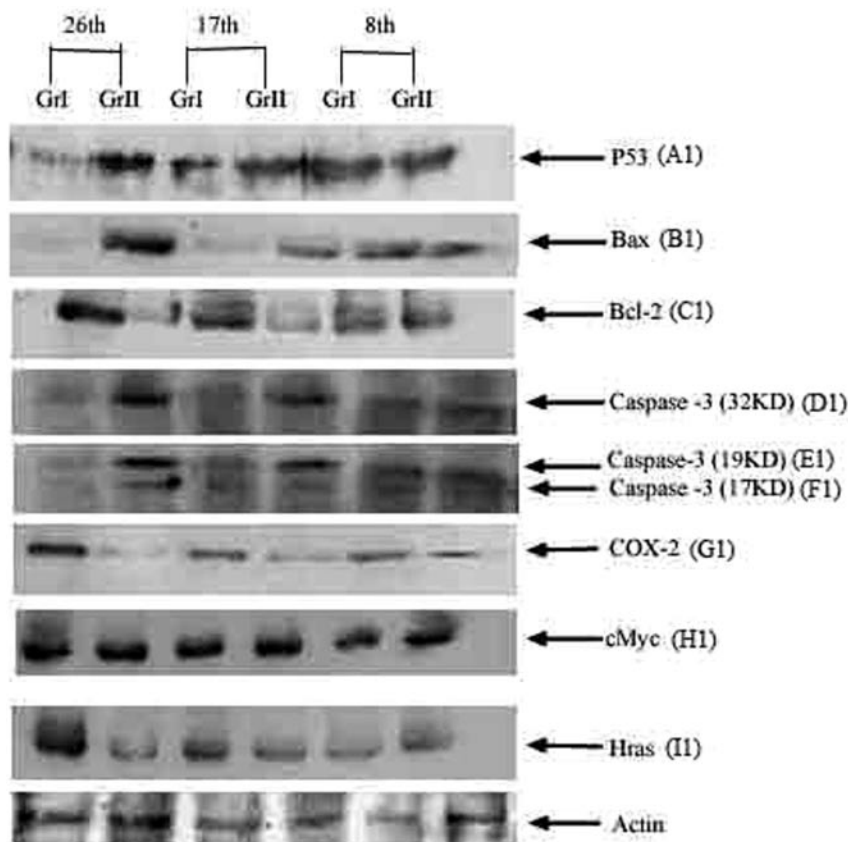
Fig. 8. Western blotting analysis shows the effect of clove infusion on the expression of pro-apoptotic, anti-apoptotic and growth-related genes. Treatment with clove resulted in the induction of apoptosis-associated genes such as p53 (**A1**), Bax (**B1**), caspase 3 (**D1, E1** and **F1**) and the inhibition of anti-apoptotic gene Bcl-2 (**C1**) and growth-promoting genes such as COX-2 (**G1**), Hras (**H1**), cMyc (**I1**).

in the regulation of apoptosis (48), which need to be explored in our system to account for this observation.

There is a growing body of compelling evidence that targeted inhibition of COX-2 expression or activity is valuable for not only alleviating inflammation, but also preventing cancer. Since chronic inflammation predisposes to malignancy (49), the inhibition of COX-2 by clove seems likely to contribute to both anti-inflammatory and anticarcinogenic action for chemoprevention of lung carcinogenesis induced by BP.

One of the key biological functions of oncogene cMyc is its ability to promote cell-cycle progression (50–53). After mitogenic stimulation cMyc proteins rapidly induce the cells to enter the G_1 phase of cell cycle. Thereafter the proteins are detectable in proliferating cells. Western blotting analysis of cMyc indicated that the expression of this oncogenic protein was not affected by clove infusion during hyperplasia and dysplasia of lung carcinogenesis, but the effect of treatment was noted at CIS stage in the form of downregulation of cMyc oncogenic expressions. Consequently, the oncoprotein ras, a 21 kDa guanine nucleotide-binding protein, is encoded by a member (Harvey-, Kirsten-, and Neural-ras) of the ras proto-oncogene family. Mutational activation transforms ras into an oncogenic form, results in the loss of intrinsic GTPase function and therefore the protein is constitutively in the active, GTP-bound state and is continuously sending signals

for cell growth (54, 55). Statistics reveal that 10–25% of all human malignancies in clinics were found to harbor a variety of ras mutations (56, 57), making ras one of the most important targets to suppress tumor cell growth (58, 59). Therefore the development of inhibitors of the ras as potential anticancer agents is a very promising strategy to prevent cancer. This investigation is the first report to demonstrate that aqueous infusion of clove represses ras oncoprotein expression most prominently during lung carcinogenesis at the CIS suggesting clove infusion to be a potent inhibitor of oncogenic induction.

All these observations indicate the relationship between p53 status, Bcl-2/Bax ratio, and caspase 3 activation and enhanced apoptosis induced by clove infusion result in the restriction of BP-induced lung carcinogenesis. In addition, the expression status of COX-2, and some oncogene, viz, cMyc, Hras and the inhibition of cell proliferation added to the anticarcinogenic action of clove infusion during lung carcinogenesis. To conclude, our results imply an apoptosis-enhancing and proliferation-inhibiting capability of clove infusion during the post-initiation phase of lung carcinogenesis by modulating the balance between pro- and anti-apoptotic factors and also by regulating some growth-promoting genes. The most notable implication of our work is that the oral infusion of clove could result in a significant inhibition in the progression of cancer in the animal model that emulates human disease.
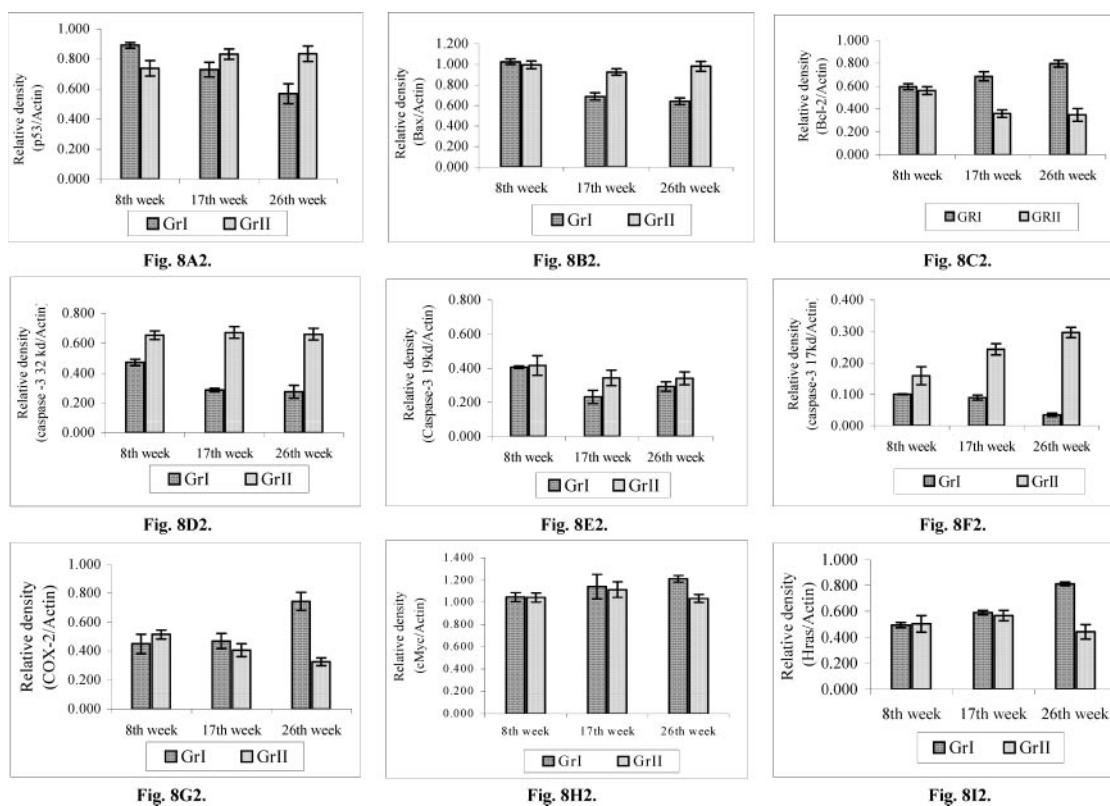
Fig. 8A2.

Fig. 8B2.

Fig. 8C2.

Fig. 8D2.

Fig. 8E2.

Fig. 8F2.

Fig. 8G2.

Fig. 8H2.

Fig. 8I2.

Fig. 8 (Continued). Western blotting analysis shows the effect of clove infusion on the expression of pro-apoptotic, anti-apoptotic and growth-related genes. Treatment with clove resulted in the induction of apoptosis-associated genes such as p53 (**A1**), Bax (**B1**), caspase 3 (**D1, E1** and **F1**) and the inhibition of anti-apoptotic gene Bcl-2 (**C1**) and growth-promoting genes such as COX-2 (**G1**), Hras (**H1**), cMyc (**I1**). Actin was used as an internal control. The quantification of expression of these genes with respect to internal control (actin) is represented from A2 to I2. (**A2**), Effect of clove on p53 expression in mouse lung during BP-induced carcinogenesis. Quantification of p53 expression was normalized to actin using densitometer. Increased p53 expression was observed in the clove-treated group which was the maximum at the 26th week; (**B2**), Effect of clove on the expression of Bax during BP-induced mouse lung carcinogenesis. Quantification of Bax expression was normalized to actin using densitometer. Maximum Bax expression was observed in the clove-treated group at the 26th week; (**C2**), Inhibitory effect of clove on the expression of Bcl-2 during mouse lung carcinogenesis induced by BP. Quantification of Bcl-2 expression was normalized to actin using densitometer. Maximum inhibition of Bcl-2 expression was observed in the clove-treated group at the 26th week; (**D2**),Effect of clove on the expression of procaspase 3 (32 kDa) during mouse lung carcinogenesis induced by BP. Quantification of expression was normalized to actin using densitometer. (**E2**) Effect of clove on the expression of active caspase 3 (19 kDa) during mouse lung carcinogenesis induced by BP. Quantification of expression of caspase 3 (19 kDa) was normalized to actin using densitometer; (**F2**), Effect of clove on the expression of active caspase 3 (17 kDa) during mouse lung carcinogenesis induced by BP. Quantification of expression of caspase 3 (17 kDa) was normalized to actin using densitometer; (**G2**) Inhibitory effect of clove on BP-induced COX-2 expression. Quantification of COX-2 expression was normalized to actin using densitometer. Maximum inhibition of COX-2 expression was observed in the clove-treated group at the 26th week; (**H2**), Inhibitory effect of clove on cMyc during BP-induced lung carcinogenesis. Quantification of cMyc expression was normalized to actin using densitometer. Inhibition of cMyc expression was the maximum in the clove-treated group at the 26th week; (**I2**), Inhibitory effect of clove on Hras during BP-induced lung carcinogenesis. Quantification of Hras expression was normalized to actin using densitometer. Maximum inhibition of Hras expression was observed in the clove-treated group at the 26th week.

Acknowledgements

References

1. Doll,R. (1992) The lessons of life: keynote address to the nutrition and cancer conference. *Cancer Res.*, **52**, 2024s–2029s.
2. Wattenberg,L.W. (1992) Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.*, **52**, 2085s–2091s.
3. Das,S. (2004a) Food phytochemicals and cancer chemoprevention. *Sci. Cult.*, **70**, 131–135.
4. Unnikrishnan,M.C. and Kuttan,R. (1990) Tumour reducing and anticarcinogenic activity of selected spices. *Cancer Lett.*, **51**, 85–89.
5. Das,S. (2004b) Anticancer potential of flavouring agents and their active principles- garlic, saffron, clove. *Int. J. Cancer Prev.*, **1**, 89–97.
6. Das,I., Chakrabarty,R.N. and Das,S. (2004) Saffron can prevent chemically induced skin carcinogenesis in Swiss albino mice. *Asian Pacific J. Cancer Prev.*, **5**, 70–76.
7. Sengupta,A., Ghosh,S. and Bhattacharjee,S. (2005) Dietary cardamom inhibits the formation of azoxymethane induced aberrant crypt foci in mice and reduces cox-2 and iNos expression in colon. *Asian Pacific J. Cancer Prev.*, **6**, 118–122.
8. Zheng,G.Q., Kenney,P.M. and Lam,L.K. (1992) Sesquiterpenes from clove (*Eugenia caryophyllata*) as potential anticarcinogenic agents. *J. Nat. Prod.*, **55**, 999–1003.
9. Srivastava,K.C. and Malhotra,N. (1991) Acetyl eugenol, a component of oil of cloves (*Sygium aromaticum L*) inhibits aggregation and alters arachidonic acid metabolism in human blood platelets. *Prostaglandins Leukot. Essent. Fatty Acids*, **42**, 73–81.
10. Naik,P., Karrim,J. and Hanahan,D. (1996) The rise and fall of apoptosis during multistage tumorigenesis: down-modulation contributes to tumor progression from angiogenic progenitors. *Genes Dev.*, **10**, 2105–2116.

11. Hong,M.Y., Chang,W.C., Chapkin,R.S. and Lupton,J.R. (1997) Relationship among colonocyte proliferation, differentiation and apoptosis as a function of diet and carcinogen. *Nutr. Cancer*, **28**, 20–29.

12. Das,T., Sa,G., Sinha,P. and Ray,P.K. (1999) Induction of cell proliferation and apoptosis: dependence on the dose of the inducer. *Biochem. Biophys. Res. Commun.*, **260**, 105–110.

13. Basu,A. and Haldar,S. (1998) The relationship between BcI2, Bax and p53:consequences for cell cycle progression and cell death. *Mol. Hum. Reprod.*, **4**, 1099–1109.

14. Levin,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.

15. Miyashita,T. and Reed,J.C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*, **80**, 293–299.

16. Bickers,D.R. and Athur,M. (2000) Novel approaches to chemoprevention of skin cancer. *J. Dermatol.*, **27**, 691–695.

17. Shibata,M.A., Maroulakour,I.G., Jorcyk,C.L., Gold,L.G., Ward,J.M. and Green,J.E. (1996) p53-independent apoptosis during mammary tumor progression in C3(1)/SV40 large T antigen transgenic mice: suppression of apoptosis during the transition from preneoplasia to carcinoma. *Cancer Res.*, **56**, 2998–3003.

18. Miyashita,T., Krajewski,S., Krajewska,M., Wang,H.G., Lin,H.K., Liebermann,D.A., Hoffman,B. and Reed,J.C. (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. *Oncogene*, **9**, 1799–1805.

19. Tsujii,M. and DuBois,R.N. (1995) Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell*, **83**, 493–501.

20. DuBois,R.N., Shao,J., Tsujii,M., Sheng,H. and Beauchamp,R.D. (1996) G1 delay in cells overexpressing prostaglandin endoperoxide synthase-2. *Cancer Res.*, **56**, 733–737.

21. Askew,D.S., Ashmun,R.A., Simmons,B.C. and Cleveland,J.L. (1991) Constitutive c-*myc* expression in IL-3-dependent myeloid cell line suppresses cycle arrest and accelerates apoptosis. *Oncogene*, **6**, 1915–1922.

22. Evan,G.I., Wyllie,A.H., Gilbert,C.S., Littlewood,T.D., Land,H., Brooks,M., Waters,C.M., Penn,L.Z. and Hancock,D.C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell*, **69**, 119–128.

23. Leone,G., DeGregori,J., Sears,R., Jakoi,L. and Nevins,J.R. (1997) Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. *Nature*, **387**, 422–426.

24. Jemal,A., Murray,T., Ward,E., Samuels,A., Tiwari,R.C., Ghafoor,A., Feuer,E.J. and Thun,M.J. (2005) Cancer Statistics, 2005. *CA Cancer J. Clin.*, **55**, 10–30.

25. Sethi,T. (2002) Lung cancer: introduction. *Thorax*, **57**, 992–993.

26. Hecht,S.S., Carmella,S.G., Murphy,S.E., Foiles,P.G. and Chung,F.L. (1993) Carcinogen biomarkers related to smoking and upper aerodigestive tract cancer. *J. Cell Biochem.*, **17F**, 27–35.

27. King,H.W., Osborne,M.R. and Brookes,P. (1979) The *in vitro* and *in vivo* reaction at the *N*7-position of guanine of the ultimate carcinogen derived from benzo(*a*)pyrene. *Chem. Biol. Interact.*, **24**, 345–353.

28. Davidson,L.A., Brown,R.E., Chang,W.C., Morris,J.S., Wang,N., Carroll,R.J., Turner,N.D., Lupton,J.R. and Chapkin,R.S. (2000) Morphodensitometric analysis of protein kinase expression C beta(II) expression in rat colon: modulation by diet and relation to *in situ* cell proliferation and apoptosis. *Carcinogenesis*, **21**, 1513–1519.

29. Caderni,G., De Filippo,C., Luceri,C., Salvadori,M., Giannini,A., Biggeri,A., Remy,S., Cheynier,V. and Dolara,P. (2000) Effects of black tea, green tea and wine extracts on intestinal carcinogenesis induced by azoxymethane in F344 rats. *Carcinogenesis*, **21**, 1965–1969.

30. Lowry,O.H., Rosenbrough,N.J., Farr,A.L. and Randall,R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–276.

31. Banerjee,S. and Das,S. (2004) Chemopreventive efficacy of black tea on benzo(*a*)pyrene induced lung carcinogenesis. *Int. J. Cancer Prev.*, **1**, 129–136.

32. He,Z., Ma,W.Y., Hashimoto,T., Bode,A.M., Yang,C.S. and Dong,Z. (2003) Induction of apoptosis by caffeine is mediated by the p53, Bax, and caspase 3 pathways. *Cancer Res.*, **63**, 4396–4401.

33. Wardlaw,S.A., March,T.H. and Belinsky,S.A. (2000) Cyclooxygenase–2 expression is abundant in alveolar type II cells in lung cancer-sensitive mouse strains and in premalignant lesions. *Carcinogenesis*, **21**, 1371–1377.

34. Pelengaris,S., Rudolph,B. and Littlewood,T. (2000) Action of Myc *in vivo*—proliferation and apoptosis. *Curr. Opin. Genet. Dev.*, **10**, 100–105.

35. Obaya,A.J., Mateyak,M.K. and Sedivy,J.M. (1999) Mysterious liaisons: the relationship between c-Myc and the cell cycle. *Oncogene*, **18**, 2934–2941.

36. Zachos,G. and Spandidos,D.A. (1997) Expression of ras proto-oncogenes: regulation and implications in the development of human tumors. *Crit. Rev. Oncol. Hematol.*, **26**, 65–75.

37. Weinstein,I.B. (1991) Cancer prevention: recent progress and future opportunities. *Cancer Res.*, **51**, 5080s–5085s.

38. DiGiovanni,J. (1992) Multistage carcinogenesis in mouse skin. *Pharmacol. Ther.*, **54**, 63–128.

39. AACR Chemoprevention Working Group (1999), Prevention of cancer in the next millennium: Report of the chemoprevention working group to the American Association for Cancer Research. *Cancer Res.*, **59**, 4743–4758.

40. Banerjee,S. and Das,S. (2005) Anticarcinogenic effect of aqueous infusion of clove on skin carcinogenesis. *Asian Pacific J. Cancer Prev.*, **6**, 304–308.

41. Bedi,A., Pasricha,P.J., Akhtar,A.J., Barber,J.P., Bedi,G.C., Giardiello,F.M., Zehnbauer,B.A., Hamilton,S.R. and Jones,R.J. (1995) Inhibition of apoptosis during development of colorectal cancer. *Cancer Res.*, **55**, 1811–1816.

42. Chinni,S.R., Li,Y., Upadhyay,S., Koppolu,P.K. and Sarkar,F.H. (2001) Indole-3-carbinol (I3C) induced cell growth inhibition, G_1 cell cycle arrest and apoptosis in prostate cancer cells. *Oncogene*, **20**, 2927–2936.

43. Giannkakou,P., Robey,R., Fojo,T. and Blagosklonny,M.V. (2001) Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G_1/G_2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. *Oncogene*, **20**, 3806–3813.

44. Oltvai,Z.N., Milliman,C.L. and Korsmeyer,S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609–619.

45. Marzo,I., Brenner,C., Zamzami,N. *et al.* (1998) Bax and adenine nucleotide translocator cooperate in the mitochondrial control of apoptosis. *Science*, **281**, 2027–2031.

46. Hoshi,T., Sasano,H., Kato,K., Yabuki,N., Ohara,S., Konno,R., Asaki,S., Toyota,T., Tateno,H. and Nagura,H. (1998) Immunohistochemistry of caspase-3/CPP32 in human stomach and its correlation with cell proliferation and apoptosis. *Anticancer Res.*, **18**, 4347–4353.

47. Wang,X. (2001) The expanding role of mitochondria in apoptosis. *Genes Dev.*, **15**, 2922–2933.

48. Gupta,S. (2003) Molecular signaling in death receptor and mitochondrial pathways of apoptosis (review). *Int. J. Oncol.*, **22**, 15–20.

49. Bennett,A. (1986) The production of prostonoids in human cancers and their implications for tumor progression. *Prog. Lipid Res.*, **25**, 539–542.

50. Amati,B., Alevizopoulos,K. and Vlach,J. (1998) Myc and the cell cycle. *Front Biosci.*, **3**, D250–D268.

51. Dang,C.V. (1999) C-MYC target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell. Biol.*, **19**, 1–11.

52. Eilers,M. (1999) Control of cell proliferation by Myc family genes. *Mol. Cells.*, **9**, 1–6.

53. Amati,B. (2001) Integrating Myc and TGF-beta signaling in cell cycle control. *Nat. Cell Biol.*, **3**, E112–E113.

54. Rayter,S.I., Iwata,K.K., Michitsch,R.W., Sorvillo,J.M., Valenzuela,D.M. and Foulkes,J.G. (1989) Biochemical functions of oncogenes. In: Glover,D.M., Hames,B.D. (eds.), *Oncogene*. IRL Press, Oxford, pp. 113–189.

55. Maculuso,M., Russo,G., Cinti,C., Bazan,V., Gebbia,N. and Russo,A. (2002) Ras family genes: an interesting link between cell cycle and cancer. *J. Cell Physiol.*, **192**, 125–130.

56. Barbacid,M. (1987) *Ras* gene. *Annu. Rev. Biochem.*, **56**, 779–827.

57. Bos,J.L. (1989) *Ras* oncogene in human cancer: a review. *Cancer Res.*, **49**, 4689–4692.

58. Rinker-Schaeffer,C.W., Graff,J.R., De Benedetti,A., Zimmer,S.G. and Rhoads,R.E. (1993) Decreasing the level of translation initiation factor 4E with antisense RNA causes reversal of ras-mediated transformation and tumorigenesis of cloned rat embryo fibroblasts. *Int. J. Cancer*, **55**, 841–847.

59. Prendergast,G.C., Davide,J.P., Lebowitz,P.F., Wechsler-Reya,R. and Kohl,N.E. (1996) Resistance of a variant ras-transformed cell line to phenotypic reversion by farnesyl transferase inhibitors. *Cancer Res.*, **56**, 2626–2632.