MINI-REVIEW

The Models for Assessment of Chemopreventive Agents: Single Organ Models

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Abstract

Research in cancer chemoprevention involves a number of activities, the first and foremost of which is acquisition of detailed knowledge concerning the process of carcinogenesis and identification of points of intervention whereby the process can be reversed or stalled. Parallel to this is the search for ideal chemopreventive agents – natural or synthetic - and screening for their activity and efficacy in vitro and in vivo. For ethical reasons it is not possible to test new agents on humans, so preclinical studies are dependent on results first being obtained with suitable animal models. Since it is not possible for a single model to reflect the diversity and heterogeneity of human cancers, it is necessary to have as many different models as possible, depending on the requirement of the studies on different aspects of cancer biology.

Advances in research on carcinogenesis and chemoprevention therefore have to be accompanied by development of appropriate laboratory animal models using a variety of carcinogens that produce tumours at different sites. Animal models have contributed significantly to our understanding of carcinogenesis and ways to intervene in the underlying processes. Many animal carcinogenesis and tumour models have been found to mirror corresponding human cancers with respect to cell of origin, morphogenesis, phenotype markers and genetic alteration. In spite of the fact that interpolation of data from animal studies to humans is difficult for various reasons, animal models are widely used for assessment of new compounds with cancer chemopreventive potential and for preclinical trials. So despite the movements of animal rights activists, animal models will continue to be used for biomedical research for saving human lives. In doing so, care should be taken to treat and handle the animals with minimal discomfort to them and ensuring that alternatives are used whenever possible.

Key Words: Skin papilloma - lung carcinoma - black tea - epigallocatechin gallate - theaflavins - proliferation - apoptosis - caspase-3 - Cox-2

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Introduction

Investigations over the past decades on carcinogenesis and anti-carcinogenesis have laid the foundation for further approaches towards prevention of cancer including chemoprevention. Researches in cancer chemoprevention involves a number of activities the first and foremost of which is the acquisition of detailed knowledge concerning the process of carcinogenesis and identification of points of intervention whereby the process can be reversed or stalled. Parallel to this is the search for ideal chemopreventive agents – natural or synthetic, and screening for their activity and efficacy as well as determination of safe dosage. Only after obtaining adequate information as above through investigations *in vitro* and *in vivo*, clinical trials and human intervention studies for chemoprevention can be attempted.

Conventionally in the absence of convincing data on humans, information collected from animal studies are considered as evidence for carcinogenic risk to humans, although all agents or mixtures that cause cancer in experimental animals may not necessarily be carcinogenic to humans. It has been reported that 44 agents and mixtures for which there is sufficient or limited evidence of carcinogenicity to humans, about 37 were found to produce cancer in at least one animal species (Wilbourn et al., 1986). Similarly new compounds with cancer chemopreventive potential need to be assessed on animal carcinogenesis models (Steele et al., 1994) although interpolation of data from animal studies to humans require caution.

Since the beginning of studies on pathogenesis for cancer

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the need for suitable reproducible models for understanding the cellular, molecular and genetic events that are associated with initial transformation and gradual progression from precancer to invasive phenotypes was felt. Therefore advances in researches on carcinogenesis had to be accompanied by development of excellent models which formed the biological material for the experiments. It is not possible for a single model to reflect the diversity and heterogeneity of human cancers (Nicolson and Poste, 1983), so it is necessary to have as many as possible depending on the requirement of the studies on different aspects of cancer biology. Although a number of relatively non-animal procedures and tests have been developed which does not involve direct use of animals, they can at best supplement but cannot replace animal models. Therefore despite the movements of animal rights activists animal models will continue to be used for biomedical research for saving human lives.

Animal models have contributed significantly to our understanding of carcinogenesis and ways to interfere with the process. Many models were found to be identical to corresponding human cancers with respect to cell of origin, morphogenesis, phenotype markers and genetic alteration (Yuspa and Poirier, 1988).

Rodents viz. mouse and rats are the animals of choice for cancer research because they have a relatively short life span (2-3 years) that allow observation of the progressive pathophysiological changes in the host during carcinogenesis within a reasonable time frame. Moreover they physiologically resemble humans in many respects and it is possible to provoke a carcinogenic response by a diversity of experimental procedures. Assessment of carcinogenicity and the possible role of modifying factors across species and target organs under controlled conditions is also relatively easier in this group due to convenience of handling and caging.

It is now universally recognized that advances in the understanding of the pathogenesis of many human cancers would not have been possible without the well defined reproducible animal cancer models. Notwithstanding the problems related to interpretation and interpolation of data, animal tumour models have proved their worth, though the choice of a specific model, depending on the experimental design and information saught, is important.

Interest in the components of food and beverages, the micronutrients and phytochemicals in plant food in particular, for prevention of human cancer continues to increase. Controlled experiments on suitable animal carcinogenesis models are required to identify and determine the efficacy of these agents and for appropriate testing of hypothesis based on observational studies on humans. Given that the majority of human cancers are of epithelial origin, many chemically induced epithelial cancer models have been developed which are widely used for such studies.

Many vital information and basic concepts of multistage carcinogenesis have been derived from the classical two stage mouse skin model (Slaga, 1984) where Dimethylbenz(a) anthracene (DMBA) is used to initiate the process followed by application of a promoter, Croton oil or TPA its active chemical component, to induce skin papillomas and squamous cell carcinomas. Papillomas appear after 6-7 weeks of application of the promoter and it is possible to follow the development of these precancers to squamous cell tumours both morphologically and histopathologically. It therefore forms a very useful and extensively used single organ model for measurement of chemopreventive activity, which can be expressed as percent reduction in incidence of papillomas and carcinomas (Boone et al., 1990). This model has been included in our laboratory for screening of natural and synthetic compounds for their chemopreventive activity as well as to evaluate the effect of oral administration of a variety of fruits and spices during carcinogenesis (Ganguly et al., 2000; De et al., 2000a; Sengupta et al., 2001; De and Das, 2001; Saha and Das, 2002; Saha and Das, 2003).

Chronic exposure to methyl cholanthrene (MC) or benzo(a)pyrene (BP) can induce carcinoma in mouse uterine cervix. The progress of precancerous dysplasia through carcinoma *in situ* to invasive carcinoma can be easily followed by examination of vaginal exfoliated cells and histology of cervix uteri (Kehar and Wahi, 1967; De et al., 2000b). This is a very useful model for screening of new chemopreventive agents for cancer cervix as the model displays preneoplastic cellular characteristics similar to those noted in humans. We have successfully used our information on the chemopreventive role of alpha tocopherol based on this model (De et al., 2000c) to human intervention study (Ganguly et al., 2001).

The colon carcinogenesis model in rats which can be induced by azoxymethane (AOM) to produce colorectal adenocarcinomas within 40 weeks with approximately 70% incidence (Boone et al., 1990) has proved to be useful for preclinical assessment of chemopreventive agents for colorectal cancer. Distinct aberrant crypt foci (ACF) like those noted in humans and considered to be the earliest recognisable precancerous histopathological change (Bird, 1995; Pretlow et al., 1991), are formed during carcinogenesis that serve as useful intermediate histological markers. This model is being extensively used in our laboratory (Sengupta et al., 2002; Sengupta et al., 2003a; Sengupta et al., 2003b).

A benzo(a)pyrene induced lung carcinogenesis model in mice (Yun et al., 1995) allows analysis of the progressive changes in cellular and histilogical features in lung during transformation. We are using this model for validation of our hypothesis that black tea drinking may protect from lung cancer as postinitiation alterations in the lung leading to development of a mixed cell type tumour can be followed histopathologically.

Other single organ model for carcinogenesis, recommended by the NCI chemoprevention drug development programme, which have found wide use for chemopreventive studies include representatives of high-incidence human cancers like rat mammary gland, mouse urinary bladder and hamster respiratory tract.

Mammary tumours (adenocarcinoma, adenoma and fibroadenoma) are induced by N-methyl-N- nitrosourea (MNU) or DMBA (Moon and Mehta, 1989). Like human breast cancer, these chemically induced animal tumours are hormone dependent. DMBA needs activation in the liver so this model is useful for testing agents, which are associated with inhibition of carcinogen activation.

In the mouse bladder model N-butyl-N-(4-hydroxybutyl) nitrosamine (OH-BBN) produce invasive transitional cell carcinomas that morphologically resemble a human type (Becci et al., 1981).

The respiratoty tract models are MNU induced tracheal squamous cell carcinomas (Moon and Mehta, 1989) and (DEN) induced lung adenocarcinomas (Moon et al., 1992).

Assessment of the Anticarcinogenic Activity of Black Tea and Theaflavins:

Among the three major beverages of the world viz. tea, coffee and cocoa, tea is the most popular and widely consumed for its invigorating and stimulatory effects. Evidences from experimental and epidemiological studies mostly on green tea have indicated beneficial health effects and disease preventive properties of this beverage (Mitscher et al., 1997; Weisburger, 1999). Role of green tea and its compounds on cancer prevention has been extensively investigated (Chung et al., 1998; Stoner and Mukhtar, 1995; Yang et al., 1996; Katiyar and Mukhtar, 1996; Katiyar and Mukhtar, 1998; Steele et al., 2000) and the anticarcinogenic action of green tea is attributed to the presence of four major antioxidant flavanols (catechins) viz. epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC) and epigallocatechin gallate (EGCG) of which EGCG was found to be the most effective (Dreosti, 1996). Steele et al (2000) reported that most black and green tea extracts as well as EC, EGC and EGCG strongly inhibited neoplastic transformation in mouse mammary organ cultures, rat tracheal epithelial cells and human lung tumour epithelial cells. The interest in tea in relation to cancer therefore stems from the high antioxidant polyphenols present in green and black tea.

Much of the catechins present in tea leaves, to which health protective effects are attributed, undergo oxidative polymerization during processing of black tea (Bronner and Beecher, 1998). Theaflavins and thearubigins the major compounds of black tea also possess antioxidant properties (Halder and Bhaduri, 1998). Although, a large body of experimental evidences point towards varieties of mechanisms of action of green tea at several stages of carcinogenesis, including detoxification, inhibition of DNA damage, lipid peroxidation and cell proliferation (Yang et al., 1998; Hernaez et al., 1998; Hibasami et al., 1998), which support a putative role of tea and tea catechins in cancer chemoprevention (Siddiqi and Das, 1999), the action of theaflavins and other black tea components during carcinogenesis has not yet been fully investigated.

Therefore we attempted a focussed research on black tea during carcinogenesis using animal models.

For our study we selected the murine system (using outbred adult Swiss / A mice from our animal colony), because they have certain advantages over other systems. It was reported that human and murine proteins have in many cases amino acid identity (Breuinger et al., 1995) and so it is more likely that the results obtained in mouse would be closer to the humans (Lorico et al., 1997). Also, outbreed Swiss mice closely resemble genetically heterogeneous human population (Rice and O'Brien, 1980) and can withstand the experimental stress better and are easy to handle and maintain.

Evaluation of the Chemopreventive Effect of Black Tea, EGCG & TF:

I. Mouse Skin Model:

The chemical carcinogen, 9,10-dimethyl benz(a)anthracene (DMBA, Sigma Chemicals) was applied topically in a shaved portion of the skin on the dorsal surface of the mice, at a dose of 1mg / 100 µl acetone / mouse twice at an interval of three days. This was followed by application of croton oil (100ml of 1% croton oil in acetone) on the same place twice weekly for 8 weeks. Normally papilloma started to appear after 6 weeks which progressed to carcinoma in situ around 14th week (Fig. 1a-e). The experimental period was 12 weeks when severe dysplasia were noted.

24 common commercial brands of Black tea, available in Indian market were analyzed for quantitative measurement of theaflavins and catechins by HPLC method and the brand "Chuapara" which contained the maximum of these two compounds i.e. theaflavins and epigallocatechin gallate, (37.80mg & 4.41mg per gram tea respectively) and was in general high in total antioxidant content (46.43 mg /gm of tea leaves) was used for this study. Tea infusions (1, 2 & 5 %) were prepared by brewing tea leaves in boiling water for 5 minutes which were administered orally. Each mouse received 0.5 ml of this brew every day starting from the first day of application of DMBA and continued during the experimental period. Since 2% tea infusion exerted the most beneficial effect with respect to papilloma incidence and multiplicity, all other studies were made using this dose of tea infusion.

Black tea extract, containing 80% theaflavins and EGCG were purchased from Sigma chemicals. Theaflavin rich black tea extract (TF) was administered at a dose of 0.02 mg and EGCG at a dose of 0.01 mg per mouse per day by intraperitoneal injections. Selection of dose of TF and EGCG was based on the amount of these two components present in 4 cups of 2% tea (each cup of 100ml) because an average of 4 cups of tea per day is recommended for beneficial health effect on humans. Treatment was started from the same day as application of carcinogen and continued till termination of the experiment.

The effect of two active tea compounds viz. EGCG and TF were examined on incidence of papilloma, activities of

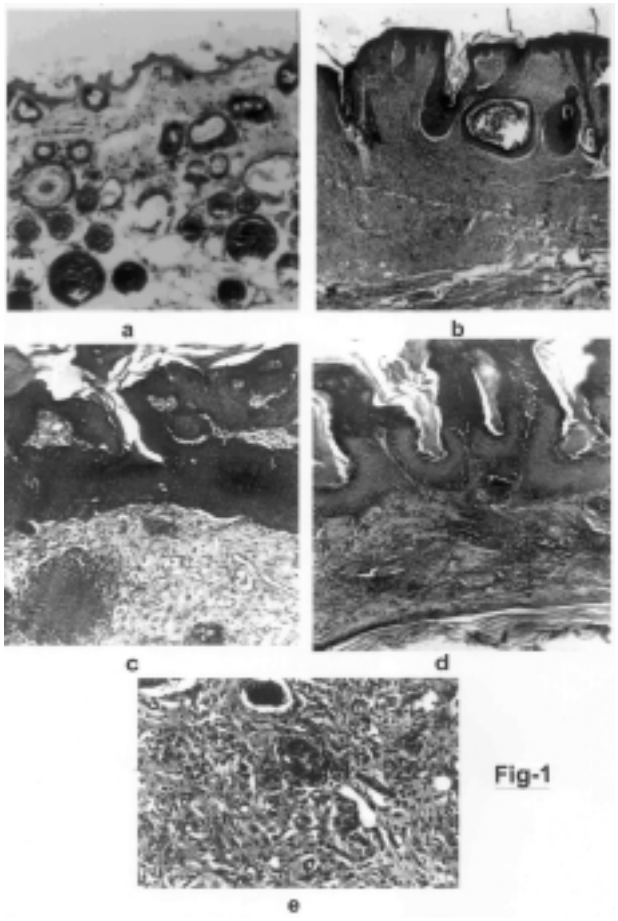

Figure 1. Microphotographs of HE preparations of mouse skin.

(a) Normal skin section showing uniformly arranged epidermal and dermal layers with cross section view of hair follicles. X 100. (b) Section through papilloma after 6 weeks of DMBA-croton oil induced carcinogenesis showing thickened epidermis with irregular proliferation and formation of reteridges and keratinized pearl X 40. (c) Severe dysplasia noted after 12 weeks X100. (d) Carcinoma in situ noted after 14 weeks X40. (e) Magnified portion of (d) to show polymorphic cells with altered nucleo-cytoplasmic ratio X250.

some Phase II detoxification enzymes, cell proliferation and apoptosis *in situ* as well as on the expressions of COX II and Caspase 3 as markers of proliferation and apoptosis respectively in skin lesion during DMBA induced murine skin carcinogenesis.

Detection of Papilloma:

The mice were kept under observation for 12 weeks and for the evaluation of chemopreventive activity skin papillomas (precancer growth) were used as intermediate markers. Morphological observation was confirmed by histopathology of the growth. The first incidence of papillomas and the number of papillomas were counted every week till the experiments ended after 12th week. Inhibition of multiplicity was calculated on the basis of observation made in the 12th. week as follows : (total number of papillomas in carcinogen control – total number of papillomas in treated group) X 100 / Total number of papillomas in carcinogen control.

Biochemical Parameters:

Activities of the hepatic Phase II detoxification enzymes, Glutathione-S-transferase (GST), Glutathione peroxidase (GPx), Superoxide dismutase (SOD) and Catalase (CAT) were determined at day 15 and after 12 weeks in the carcinogen control (untreated group) and following treatment (treatment groups). Effects on lipid peroxidation in liver during carcinogenenic exposure and following treatment was also assessed, as this physiological process is associated with damage of cellular macromolecules and tissue injury, due to generation of various reactive oxygen species, and suggested to play a key role in human cancer development.

Detection of in situ proliferation and apoptosis in skin lesion:

In situ proliferation and apoptosis were studied by immunohistochemical method, using commercially available detection kits (Boehringer Mannheim) as immunochemical techniques allow visualization of dividing or apoptotic cells in their natural environment and position in the tissues. The method for detecting cell proliferation is based on BrdU labeling of DNA in proliferating cells and its detection using monoclonal antibody directed against BrdU. In situ apoptosis assay, measure cell death by detecting DNA strand breaks in individual cells using terminal transferase (TdT) to label free 3'OH ends in genomic DNA. It is known that increased cell proliferation and removal of damaged cells (cells with DNA damage facilitate accumulation of sequential mutations resulting in tumour development) by apoptosis play an important role in the onset and progression of carcinogenesis.

Therefore these two parameters were studied to correlate the proportion of proliferation and apoptotis occurring in the precancer lesion of skin at the same period of time following exposure to the carcinogen and after intervention.

Expression of Cycloxygenase II (COX II) and Caspase 3 in skin lesion:

The inducible cycloxygenase-2 (COX-2) enzyme is upregulated in inflammatory diseases, as well as in epithelial cancers, and has an established role in cell proliferation. Therefore its expression is an important molecular marker for assessment of the effect of chemopreventive intervention during the process of carcinogenesis. Programmed cell death (apoptosis) is a part of normal physiology for most metazoan species by virtue of which unwanted cells are removed during development, making important contributions to morphogenesis and organogenesis. The molecular signaling responsible for apoptosis can be linked to the activation of a family of cystein aspertic acid proteases, known as caspases. One of the effector caspases - caspase 3, associated with induction of apoptosis was selected as a marker for the present study. The effect of chemopreventive treatment was assessed by Western Blot analysis of expression of markers in the target site.

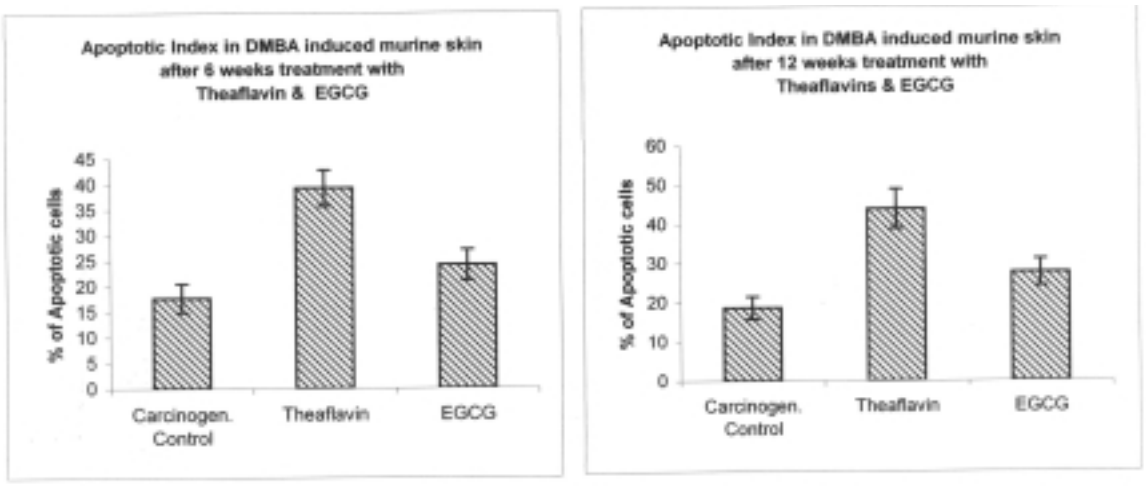

Figure 2. Effect of Black Tea Infusion, Theaflavin and Epigallocatechin Gallate on Apoptosis in Mouse Skin on 6th week and 12th week during DMBA-croton Oil Induced Carcinogenesis

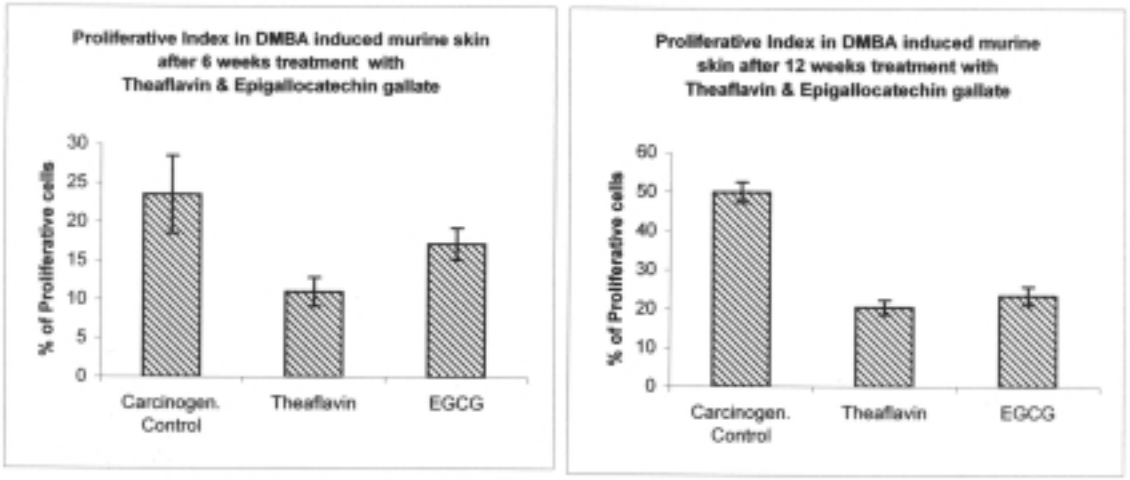

Fig. 3. Effect of Black Tea Infusion, Theaflavin and Epigallocatechin Gallate on Proliferation in Mouse Skin on 6th week and 12th week during DMBA-croton Oil Induced Carcinogenesis.

Observations:

The incidence of papillomas in carcinogen control group receiving DMBA and croton oil only was found to be 70 % with the number of papilloma per mouse in average was 3.20. These figures for papilloma incidence were reduced to 50%, 40% and 40% and multiplicity were 1.5, 2.0 and 1.50 per mouse following treatment with 2% black tea infusion, EGCG and TF respectively. We also noted elevation of the Phase II enzymes studied viz GST, GPx, SOD and CAT which was accompanied by reduction of lipid peroxidation following treatment with tea and the two of its components, suggesting their role in physiological detoxification and prevention of cellular damage by controlling generation of reactive oxygen species. These results have already been reported (Saha and Das, 2002).

It is interesting to note that treatment with EGCG and TF resulted in induction of apoptosis and inhibition of proliferation as detected by immunohistochemistry (Figs.2-5). This is further supported by Western blot analysis of COX-2 and caspase 3 proteins from skin lesion (Fig.6). It was revealed that after the application of DMBA there is over expression of both COX II and Caspase 3 in skin in comparison to normal mice. Expression of COX II could be down regulated by treatment of carcinogen treated mice with the tea compounds whereas there was further enhancement of caspase 3 expression by the same treatment.

II. Mouse Lung Model:

This is a very useful model for following the post-initiation changes during lung carcinogenesis and its modulation. New born mice (24-48 hours, Strain A) were injected subcutaneously in the sub scapular region with a suspension of benzo(a)pyrene in 1% gelatin solution at a dose of 0.2 mg per mouse. They were separated from the mother after 4 weeks and male and females separated. Treatment with black tea infusion, EGCG and TF were started from this period (post initiation) and continued throughout the experimental period at the same dose as in

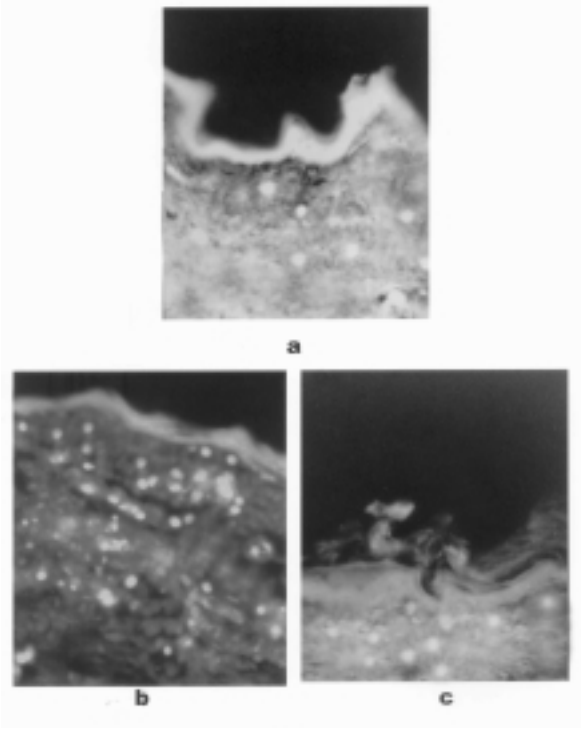

Figure 4. *In situ* Localization of Apoptotic Cells in Mouse Skin after 12 Weeks during DMBA-croton Oil Induced Carcinogenesis before Treatment, (a) and after Treatment with Theaflavin (b) and Epigallocatechin Gallate (c). X 45

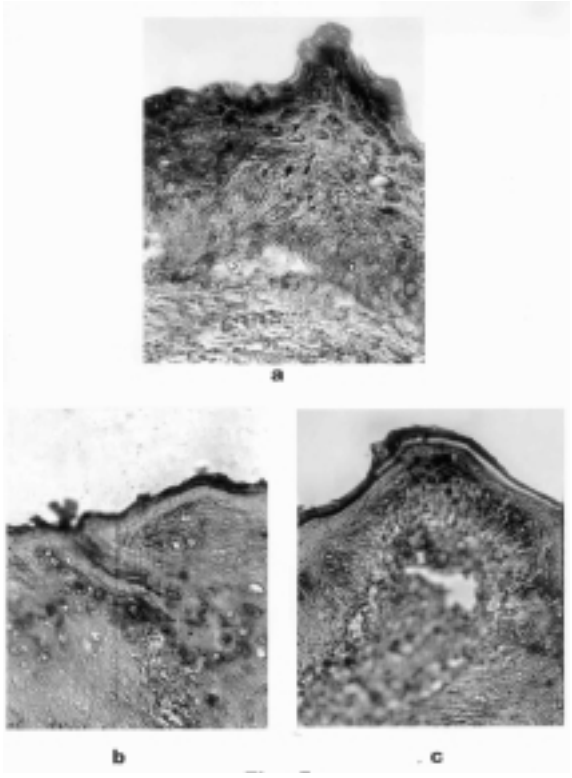

Fig.5. Proliferating Cells Localized *in situ* after 12 Weeks during DMBA-croton Oil Induced Carcinogenesis before Treatment, (a) and after Treatment with Theaflavin (b) and Epigallocatechin Gallate (c). X 45

case of the skin model. So far only histopathological study was made in this model.

Observation:

Histopathological changes can be detected from eighth week onwards. Early cellular changes noted in the 8th week gradually progress through mild to severe dysplasia leading to *carcinoma in situ* and development of mixed cell type tumour (Figs 7a-e).

Histopathological characterization of the effect of treatment with black tea infusion (2 %), EGCG (0.01 mg) and TF (0.01 mg) were undertaken and compared with the untreated group during carcinogenesis. Observation made on the seventeenth week following treatment, when distinct pre-neoplastic changes were evident in the untreated group, is presented here. The effect of black tea infusion, EGCG and theaflavin on the incidence of dysplastic changes at 17th week of treatment is shown in the Table 1. The histological appearance of lung tissue from the 17th week of the treated groups were found to be similar to that noted in the carcinogen control group at 6th week. The results indicate reduced incidence and delayed progress of carcinogenesis following treatment (Fig. 8a-e).

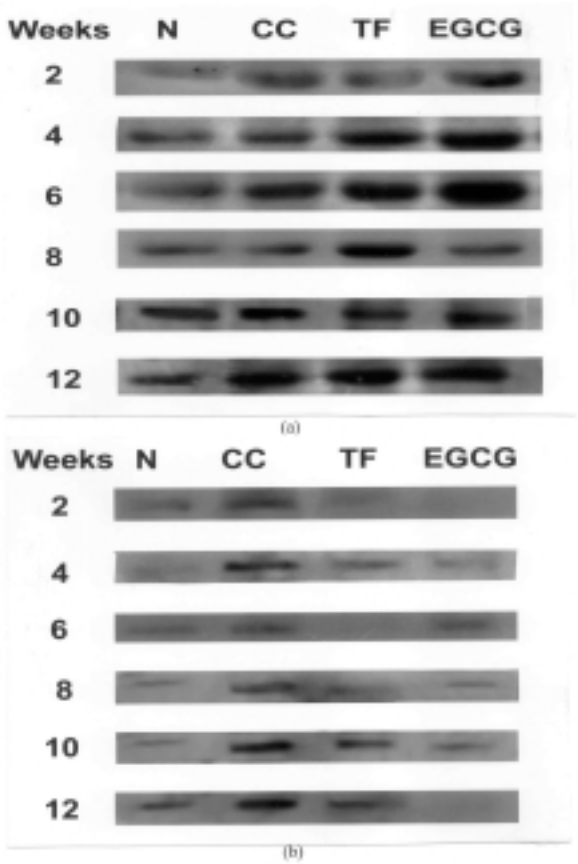

Figure 6. Expression of Caspase 3 and COX II Proteins by Western Blot Analysis.
(a) shows upregulation of caspase 3 following treatment with theaflavin and epigallocatechin gallate and (b) shows reduced expression of COX II following treatment with theaflavin and epigallocatechin gallate.

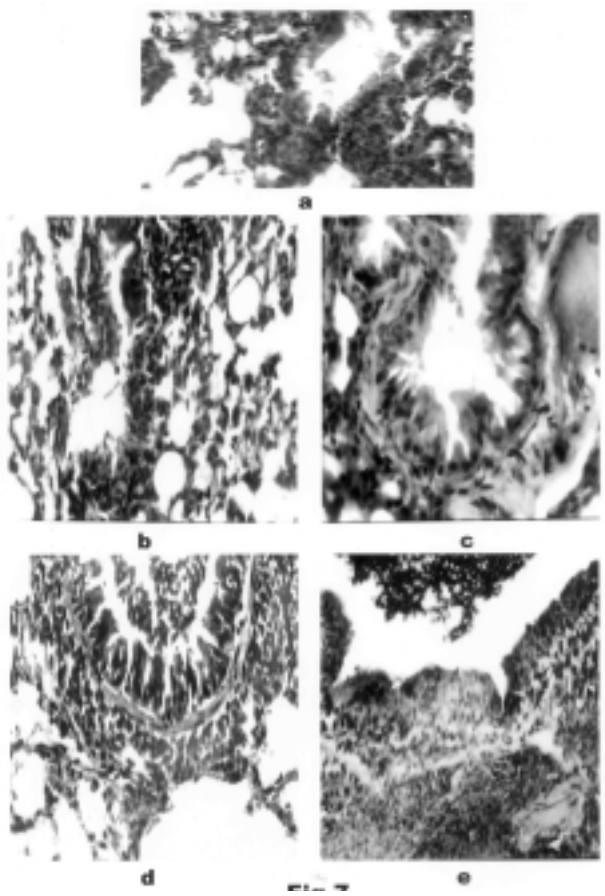

Figure 7. Microphotographs of HE Preparations of Mouse Lung showing Progressive Histopathological Changes at Different Time Intervals during Benzo(a)pyrene induced Carcinogenesis X 400.
(a) Hyperplastic epithelium noted with cellular and nuclear polymorphism after 8 weeks, dysplastic changes evident in alveolar region (b) and bronchiole (c) after 17 weeks, (d) carcinoma in situ noted after 26 weeks showing thickened area of stratified squamous cells with hyperchromatic nuclei and intact basement membrane without any invasion, (e) after 36 weeks extensive proliferation of epithelial cells with formation of intercellular bridges noted, proliferative cells can be seen invading through the basement membrane, presence of massive inflammatory cells also noted.

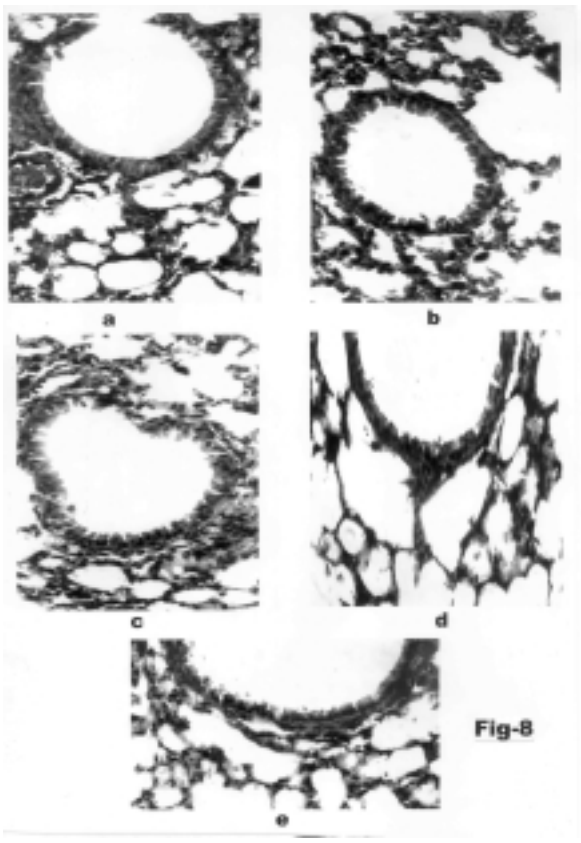

Figure 8. Microphotographs of lung sections from untreated mouse and from mice after treatment with tea infusion, theaflavin and epigallocatechin gallate. HE X 400 (a) Normal appearance of bronchiole and alveoli from untreated mouse. (b) Same as (a) noted after 6 weeks of Benzo(a)pyrene treatment. No apparent changes noted at this stage. Appearance of of bronchiole and alveoli after 17 weeks following treatment with carcinogen along with tea infusion (c), theaflavin (d) or epigallocatechin gallate (e)

Conclusion

Carcinogenesis is a long drawn process involving gene-environment interaction resulting in generation of mutated cell population that may progress to malignancy. During the process, molecular changes always precede histological alterations, whereby the regulatory control between cell proliferation and apoptosis becomes deranged. The molecular mechanism of control of such regulatory processes is the focus of attention of current researches in carcinogenesis and cancer chemoprevention. A better understanding of the obligate interrelations of important biological molecules, their functional interactions at different stages of carcinognesis and its modulation by specific natural compounds may well provide the basis for more rational cancer preventive and therapeutic approaches in the future.

A large body of experimental evidences has accumulated to suggest the anti-carcinogenic action of several phytochemicals. It has also been possible to have an insight into the varieties of mechanisms of action of these agents at different stages of carcinogenesis, including detoxification, inhibition of DNA damage, lipid peroxidation and cell proliferation, but the chemopreventive potential of many of them still remain largely unresolved. This is so because studies undertaken on a variety of *in vitro* and *in vivo* systems have generated contradictory reports that have created confusion. Moreover one agent with protective role in one particular organ may not elicit the same response at other sites. It could be due to a number of factors, which regulate genesis, and pathophysiology of a specific organ so the chemopreventive effect seen in one experimental study should not be generalized. The same agent has been shown to have both beneficial as well as harmful effects by different investigators again because the reports are based on experiments conducted on different systems. Therefore a

Table 1. Effect of Black Tea, Epigallocatechin and Theaflavin on the Incidence of Dysplastic Change in Mouse Lung after 17 Days of Exposure to Benzo(a)pyrene (n=10)

Groups	% Incidence	% Inhibition
Carcinogen Control (CC)	80 (8/10)	-
C + EGCG	30 (3/10)	62.5
C + TF	20 (2/10)	75
C + TI	40 (4/10)	50

more focussed research approach on specific experimental models is necessary to establish the role of any chemopreventive agent in prevention of carcinogenesis and protection from cancer. Ideally, following *in vitro* screening and understanding of the mechanism of action of a particular agent, pre-clinical studies should be undertaken to assess its activity on a number of selected single organ models *in vivo*. Apart from assessing the effect on the end point (in most cases incidence of tumours) the effect should be assessed at different stages of the process of carcinogenesis systematically. Efforts should also be made to understand the mechanism of action at the target site in the same model. The response of the host towards the test agent can also be followed and nature of toxicity if any identified. This would provide a more clear understanding on the nature of response of the host exposed to a particular carcinogen towards a specific agent. The single organ model is best suited for investigation in this line.

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