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Chemopreventive Efficacy of Fisetin against Benzo(a)pyrene Induced Lung Carcinogenesis in Experimental Animals Swiss Albino Mice

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ABSTRACT

The present study was designed to evaluate the effects of oral administration of the fisetin on lung tumour initiation by orally applied Benzo(a)pyrene (B(a) P). Its effects on biochemical markers enzymes as well as pathological markers were evaluated. The pathological enzyme activities were increased (P<0.05) in lung cancer bearing animals when compared with control group animals. Fisetin-treated animals from groups-3 and- 4 showed a significant (P<0.05) decreases in the levels of these enzymes when compared to B(a)P induced group-2 animals. Levels of glycoprotein and activities of membrane ATPases play an important role in the carcinogenesis. Hence, objective this study was aimed to evaluate the effect of fisetin on the changes in glycoprotein components (hexose, hexosamine and sialic acid) and ATPases(Na^+/K^+ATPase, Ca^{2+}ATPase and Mg^{2+}ATPase) in control and lung carcinoma bearing animals. A significant increase in the levels of glycoproteins and activities of membrane ATPases were observed in animals with lung cancer. The administration of fisetin, these changes were reverted back to near normal levels of glycoproteins and membrane ATPases. Furthermore, anti-proliferative efficacy of fisetin was assessed by immunofluorescence analysis of proliferating cell nuclear antigen (PCNA) in B(a)P induced mice showed increased PCNA expression which was restored upon fisetin administration. Together, our results were depicts that fisetin can be used as a chemopreventive agent against lung cancer.

Keywords: Benzo(a)pyrene; Fisetin; Glycoproteins; Membrane ATPases; PCNA

INTRODUCTION

Lung cancer is leading cause of deaths in the developing countries and it was estimated that 1.4 million new cases would be diagnosed and approximately 1.2 million deaths every year. There are two major types of lung cancer such as small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Carcinogens from cigarette smoke link between nicotine addictionsleads to lung cancer, contributing to a tenfold increase in risk in long-term smokers compared with non-smokers [1]. Tobacco contents of smoke, the polycyclic aromatic hydrocarbons (PAHs) such as B(a)P, that play a major role in induction of lung carcinogenesis [2]. B(a)P is metabolized to (±)-B(a)P-r-7,t-8-dihydrodiol-t-9,10-epoxide (BPDE), the ultimate carcinogen. BPDE isomers then bind to the hexocyclic nitrogen of deoxyguanosine in DNA via trans-addition of the C-10 position in the epoxide molecule and this adduct might also cause activation of proto-oncogenes [3]. Glycoproteins are important groups of compounds involved in the cellular function and its play a significant role in contributing to the surface properties of the cells and also an

important role in tumor genesis and as mediators of immunological specificity. Carbohydrates moieties of glycoproteins have also been implicated in the transport of metabolites across cell membranes and also observed a direct relationship between glycoproteins and tumor genesis). Many chemical changes in the host's lung are detectable before the onset of secondary physiological and nutritional changes that may be associated with the condition of tumor bearing host [4]. The significant alteration of lung reduced glutathione (GSH) level, extent of lipid peroxidation and GPx activity during the lung carcinogenesis, their inter-relationship and role in characterizing the progression of tumor, have been successfully established in recent reports [5]. On the basis of our work and the available literature on the role of flavonoids fisetin may also interact with chemotherapeutic drugs used in cancer treatment through the induction or inhibition of their metabolism in modulating the carcinogenic process [6]. There is an increased interest in the scientific community on the use of plant based polyphenols based on their varied biological properties including antioxidative, antimicrobial, anticarcinogenic as well as cardio protective activity [7]. An important advantage with plant based polyphenols, especially those from dietary sources, is that they are perceived as non-toxic and have wide human acceptance. Several non-nutritive, macronutrient phytochemicals are being evaluated for the management of cancer and other diseases [8]. Fisetin (3,7,3',4'-tetrahydroxyflavone (Figure 1) belongs to the flavonol subgroup of flavonoids along with quercetin, myricetin and kaempferol and its present in many fruits and vegetables most notably strawberries, apples, persimmons, cucumbers and onions [9]. The bioavailability of fisetin has been studied following intravenous and oral administration [10], anti-proliferative [11], neuroprotective [12] and antioxidant activities [5] were reported.

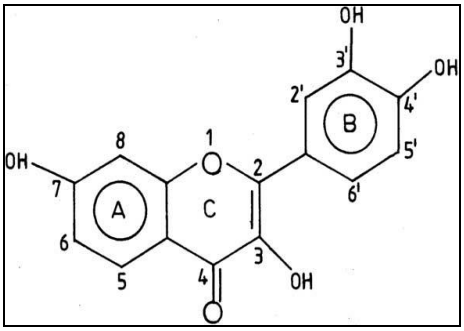


Figure 1: Structure of fisetin (3,7,3',4'-tetrahydroxyflavone)

This study attempts to characterize and document the exact role of fisetin in prolonging survival of themice with B(a)P induced lung carcinogenesis by investigating the important biochemical parameters for chemopreventive effects.

EXPERIMENTAL SECTION

Chemicals

Fisetin and Benzo(a)pyrene were purchased from Sigma chemicals Co (St. Louis, MO, USA). Proteins with primary antibody rabbit polyclonal IgG to mouse PCNA Santa Cruz biotech (USA). All other chemicals and reagents used were of the highest analytical grade commercially available.

Animals

Male Swiss albino mice (6-8 weeks old) were used throughout the study. Animals were purchased from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai, India and maintained in a controlled condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed standard pellet diet (Hindustan Lever Ltd., Mumbai) and water *ad libitum*. This study was conducted according to the ethical norms approved by Ministry of Social Justices and Empowerment, Government of India and by Animal Ethics Committee Guidelines of our Institution (IAEC No.04/011/09).

Experimental Protocol

The experimental animals were divided into five groups, each group comprising of six animals (Figure 2). Group-1 served as normal control. Group-2 animals were administered with B(a)P (50 mg/kg body weight dissolved in corn oil, orally) twice a week for 4 successive weeks to induce lung cancer by 16th week. Group-3 animals were pre-treated with fisetin (25 mg/kg body weight, dissolved in 0.1% DMSO, twice aweek, orally) (according to the

optimum dosage fixation study) one week before the first dose of B(a)P induction and continued for 16 weeks. Group-4 animals were post-treated with fisetin (Group-3) from 8th week of B(a)P induction till the end of the experiment (16th week). Group-5 animals were treated with fisetin alone (as in Group-3) for 16 weeks to study the cytotoxicity (if any) induced by fisetin. The pre-and post- treatment groups were used to study the chemopreventive and chemotherapeutic efficacies of fisetin in the experimental animals. The dose of fisetin was chosen based on our previous study [5]. At the end of the experimental period, the animals were killed by cervical decapitation. Blood and lung tissues were collected; tissues were immediately excised, weighed and then homogenized in Tris-HCl buffer 0.1 M (pH 7.4). Whole blood was collected in serum tube and serum was recovered by centrifugation (2000 rpm, 5 min; 4ºC).

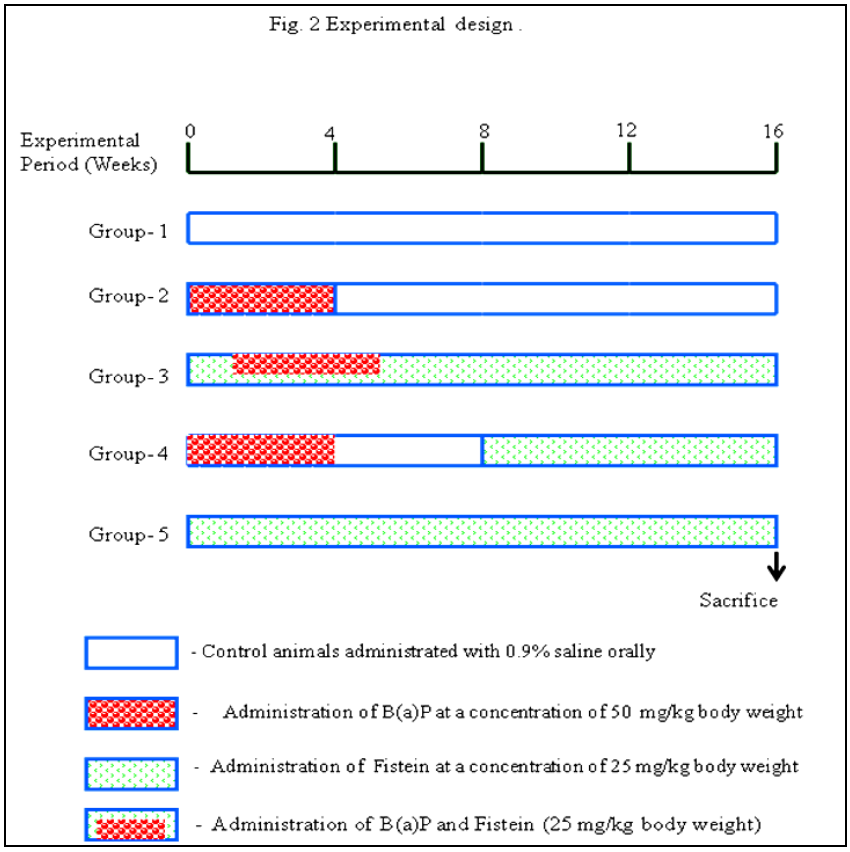


Figure 2: Experimental section

Pathological Markers that Enzyme Analysis

Estimation of aminotransaminase determined by the method of [13], estimation of acid phosphatase [14] and estimation of alkaline phosphatase [15].

Glycoprotein Analysis

For the glycoprotein analysis, a known amount of lung tissue were defatted by dissolving in hexane and taken in test tube, to which 1 ml of 2 N HCl was added and the tubes were sealed. Hydrolysis was complete by keeping the sealed tubes in 100ºC for 16-18 h. After hydrolysis, the contents were neutralized with NaOH and made up to known volume and aliquots were used for glycoprotein determination. Hexose was estimated by the method of [16]. The neutralized sample was mixed with orcinol-H_2SO_4 reagent, heated at 80ºC, cooled and left in the dark for 25 min for colour development. The absorbance was read at 540 nm. Hexosamine was estimated by the method of [17]. The acetyl acetone reagent consisting of trisodium phosphate and potassium tetra borate with acetyl acetone was added to the neutralized sample and boiled. After cooling, Ehrlich'sreagent was added and the pink colour developed was measured at 540 nm. Sialic acid was determined by themethod of [18], with modifications by [16]. The neutralized sample was mixed with 0.25 M periodate (0.1 N H_2SO_4) and the reaction was inhibited after 30 min

by arsenite solution, thiobarbituric acid was added and the contents were heated. The pink colour that developed on cooling was measured at 540 nm.

Analysis of Membrane ATPases

Na^+, K^+ ATPase was estimated by the method of [19]. The activity of Ca^{2+}ATPase was assayed according to the method of [20] and Mg^{2+}ATPase by the method of [21].

Immunofluorescence Analysis

Briefly, paraffin-embedded tissue sections of 5 µm thickness were rehydrated first in xylene and then in graded ethanol solutions. For antigen retrieval, the slides were incubated in citrate buffer (pH 6.0) in a microwave oven. The sections were then allowed to cool to room temperature and then rinsed with Tris buffered saline (TBS) and treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. Non-specific binding was blocked with 5% bovine serum albumin (BSA) in TBS for 2 h. The sections were then incubated with primary rabbit polyclonal IgG to mouse PCNA (Santacruz Biotech, USA), diluted 1:1000 with 3% BSA in TBS and incubated overnight at 4°C. The slides were washed thrice with TBS and then incubated with goat anti-mouse fluorescence isothiocyanate (FITC) conjugated secondary antibody (Bangalore Genei, India), diluted 1:40 with TBS and incubated in dark for 2 h at room temperature. The sections were then washed with TBS and counter stained using nucleus specific counter stain propidium iodide (PI), respectively, to highlight cell nuclei. Slides were visualized under a fluorescent microscope (Nikon TE 300) using Excitation wave length/Emission wavelength of PI-529 nm/620 nm and 494 nm/525 nm for FITC.

Statistical Analysis

All the grouped data were evaluated with SPSS/10 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference [LSD] test. P values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as mean ±S.D. for six animals in each group.

RESULTS AND DISCUSSION

Pathological Markers Enzymes

The activities of pathological marker enzymes such as AST, ALT, ACP and ALP in the serum of control and experimental groups of animals are shown in Table 1. Animals from group- 2 exhibited a significant (P<0.05) increase in the activity of AST, ALT, ACP and ALP in blood serum as compared to group-1. Fisetin-treated animals from groups-3 and-4 showed a significant (P<0.05) decrease in the levels of these enzymes when compared to group-2 B(a)P-induced lung cancer animals. There was no significant difference in the level of marker enzyme between fisetin alone treated group-5 and group-1.

Table 1: Effect of fisetin on the levels of AST, ALT, ACP and ALP in the serum of control and experimental groups of animals

Parameters	Group-1	Group-2	Group-3	Group-4	Group-5
AST	3.64 ± 0.15	6.01 ± 0.37[a]	4.35 ± 0.23[bc]	5.34 ± 0.30[b]	3.57 ± 0.16
ALT	22.35 ± 1.79	40.21 ± 4.50[a]	29.64 ± 2.79[bc]	35.21 ± 3.34[b]	21.34 ± 1.75
ACP	25.47 ± 1.91	43.67 ± 5.23[a]	31.24 ± 2.69[bc]	38.43 ± 3.65[b]	25.15 ± 2.01
ALP	132.50 ± 11.26	263.14 ± 32.37[a]	173.20 ± 16.97[bc]	184.20 ± 25.05[b]	131.20 ± 16.14

Each value is expressed as mean ± S.D for six determinations in each experimental group. Units: µmoles of pyruvate liberated mg protein per min for AST and ALT; µmoles of phenol liberated mg protein per min ACP and ALP. Statistical significance: p<0.05. a) Group 2 compared with Group 1. b) Group 3 and Group 4 compared with Group 2. c) Group 3 compared with Group 4. *Group 3 and Group 4 when compared with Group 1 (Control) showed near normal values. Non significant - [#]Group 5 compared with Group 1.

Protein Bound Carbohydrates

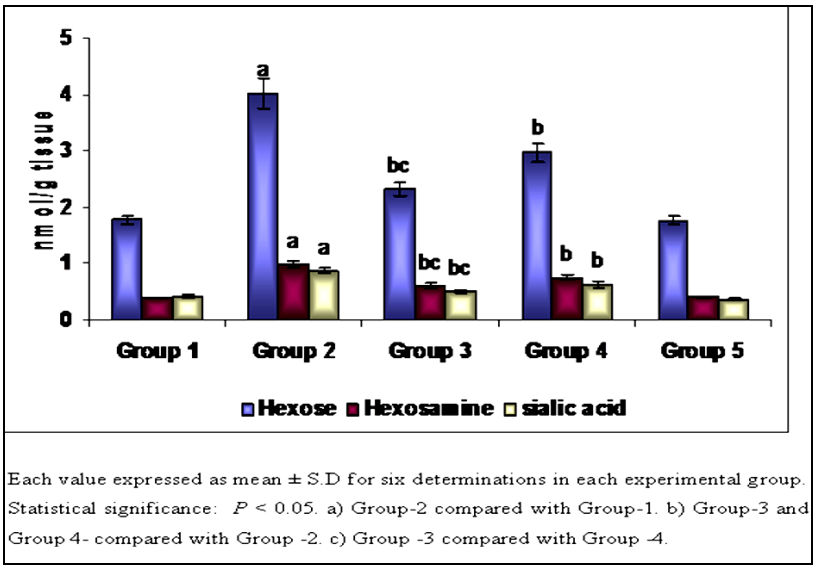
As shown in Figure 3, level of glycoproteins in the lung tissues of control and experimental animals. There was a significant (P<0.05) increase in the levels of hexose, hexosamine and sialic acid in group-2 cancer bearing animals in comparison to the control. By contrast, treatment with fisetin, these levels were found to be decreased significantly (P<0.05) in groups-3 and group-4 than when compared to group-2 animals. There was no significant difference in the level of glycoproteins between fisetin alone treated groups-5 animals and the control.

Membrane Bound ATPases

Figure 4 shows the effect of fisetin on the activities of ATPases in lung tissues of control and experimental animals. ATPase enzymes were evaluated Na^+, K^+ATPase and Mg^{2+}ATPase enzyme activities were decreased and Ca^{2+}ATPase increased (P<0.05) in of B(a)P induced lung cancer bearing animals (group-2) compared with control (group-1). The above alterations were significantly (P<0.05) normalized in pre (group-3) and post-treatment (group-4) of fisetin when compared with group-2 animals. There was no significant difference in the activity of ATPases between group-5 animals and the control.

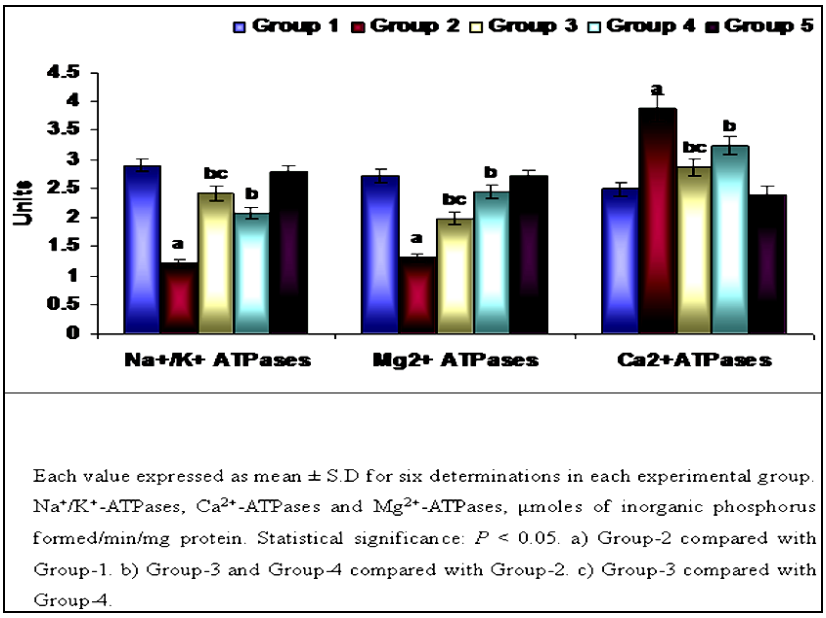
Expression of PCNA

Figure 5 shows the immunofluorescence analysis to confirm the expression levels of PCNA in the lung of mice in control and experimental groups. The expression of PCNA was significantly high in B(a)P-induced (group-2) lung cancer mice as compared to the control mice (group-1). Fisetin treatment caused a significant reduction in the levels of these expression in pre-treated (group-3) and post-treated animals (group-4) when compared with B(a)P-induced animals (group-2), whereas fisetin alone-treated mice (group-5) showed expression similar to control animals (group-1).



Each value expressed as mean ± S.D for six determinations in each experimental group. Statistical significance: P < 0.05. a) Group-2 compared with Group-1. b) Group-3 and Group 4- compared with Group -2. c) Group -3 compared with Group -4.

Figure 3: Level of glycoprotein components in lung of control and experimental groups of animals



Each value expressed as mean ± S.D for six determinations in each experimental group. Na^+/K^+-ATPases, Ca^{2+}-ATPases and Mg^{2+}-ATPases, µmoles of inorganic phosphorus formed/min/mg protein. Statistical significance: $P < 0.05$. a) Group-2 compared with Group-1. b) Group-3 and Group-4 compared with Group-2. c) Group-3 compared with Group-4.

Figure 4: Effect of fisetin on the activities of ATPases in the lung tissues of control and experimental group's animals

Chemoprevention has become an important area of cancer research and numerous studies have indicated that lung cancer is not the result of a sudden transformation in the bronchial epithelium, but rather a multi-step accumulation of genetic and epigenetic alterations. These are mostly caused by chronic exposure to carcinogens such as B(a)P, which are predominantly present in tobacco smoke and automobile exhaust fumes, either actively or passively [22]. Therefore, a strategy to arrest or reverse preneoplastic changes in the bronchial epithelium by natural orsynthetic agents before invasive cancer develops is a rational approach for reducing the burden of lung cancer [23]. During carcinogenesis, some enzymes can be used as biochemical indicators of tumor response to therapy. Biochemical tumour markers are used to screen tumorous conditions for differential diagnosis, prognosisand for monitoring progress and assessing the response to therapy [24] changes in their activities reflect the effect of proliferation of cells growth potential and where metabolic turnover is dramatically different from that of normal cells. The rise in their activities is shown to be in good correlation with the number of transformed cells in cancer conditions [25]. changes in their activities reflect the effect of proliferation of cells growth potential and where metabolic turnover is dramatically different from that of normal cells.

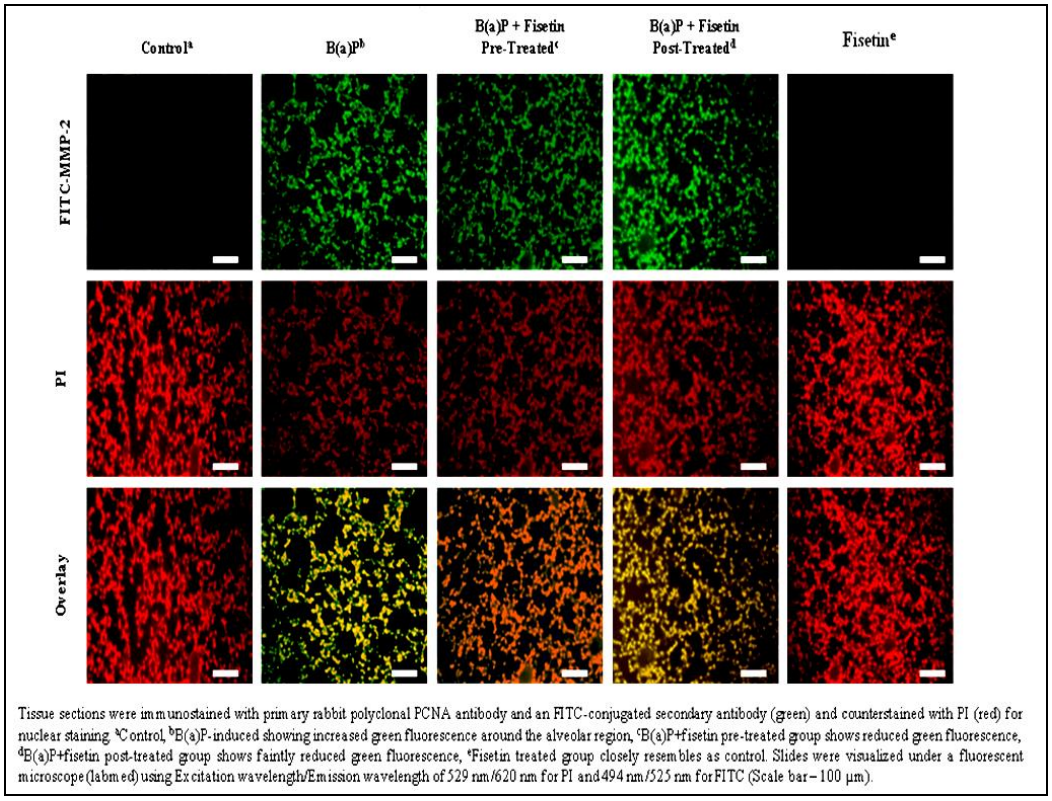


Figure 5: Immunofluorescence expression of PCNA in control and experimental groups of animals

The rise in their activities is shown to be in good correlation with the number of transformed cells in cancer conditions [25]. AST and ALT activities in blood serum are generally accepted as an index of lung damage and this tendency is also known to be distinct in rodents [26]. There was a good correlation between the activities of ALT and AST with tumor volume during therapy. The stable pattern of these enzymes was noticed inpatients with lung carcinoma malignancy after chemotherapy, while patients fail to respond for drug treatment showed progressive increase in the level of these enzymes. Kuznietsova et al. [27] observed similar results in dimethyl hydrazine-induced colon cancer in rats. The altered activities of ALT and AST in serum were normalized in a time dependent manner with fisetin treatment. Serum ACP activities in patients with lung cancer became a factor of great importance to us because of an erroneous diagnosis of metastatic cancer of the prostate in a man with raised serum ACP who was shown at necropsy to have disseminated bronchial oat-cell carcinoma [28]. ALP is the main alkaline phosphatase component constituting more than 90% of the total alkaline phosphatase catalytic activity in the serum. During bone growth and hepatobiliary diseases, the activity of ALP in serum was elevated and ALP was a valuable serum marker for the conditions [29]. Concurrent with the above findings an elevated serum aminotransferase activity was observed in animals bearing lung carcinoma. Fisetin treatment significantly attenuated this alteration, thereby, showing its anticarcinogenic activity. Carbohydrate moieties of glycoproteins have been implicated in thetransport of metabolites across cell membranes and a direct relationship between glycoproteins and tumorigenesis has been observed. Many chemical changes in glycoproteins are detectable before the onset of secondary physiological and nutritional changes that may be associated with the condition of tumor-bearing animals [4]. Increased levels of tissue glycoproteins during malignancy reflect either a local or systemic tissue response to tumour, which arises from the tumour itself. It was reported that natural components and major flavonoids capsaicin decreased glycoprotein levels in B(a)P induced cancer animals [30]. These findings agreed with our present observations where we noticed an increase in the tissue levels of hexose, hexosamine and Sialic acid in lung cancer bearing animals. Fisetin treatment significantly reduced the levels of these glycoproteins to normal. This reduction in the levels of glycoprotein components indicates that fisetin has the ability to suppress malignancy by modulating cellular transformation, signifying its chemoprotective function. The activities of the ATPases in lung tissues have been found to be lowered in B(a)P treated animals. The membrane bound enzymes such as Na^+/K^+ATPase, Mg^{2+}ATPase and Ca^{2+}ATPase are responsible for the transport of sodium/potassium, magnesium and calcium ions

across the cell membranes at the expense of ATP by hydrolysis [31]. The activities of all the three ATPases in lung tissues have been found to be inhibited in carcinoma bearing animals. These findings are similar to those reported by [32]. The decreased activities of Na^+/K^+ and Mg^{2+}ATPase in lung cancer bearing animals may be due to increased LPO which occur in cancer conditions. Previously we have observed LPO levels increased in cancer animals [5]. Peroxidation of membrane lipids initiates the loss of membrane integrity and membrane bound enzyme activities, which in turn leads to a disruption in cellular homeostasis [33]. Abnormal lipidperoxides affect membrane bound ATPases activities and their levels were decreased due to the excessive production of thiobarbituric acid reactive substances [34]. Ca^{2+}ATPase, the enzyme responsible for active calcium transport, is extremely sensitive to hydroperoxides and this may lead to its inhibition. The impairment in this enzymemay is due to the peroxidative stress, which may act on the sulphydryl groups present in the active sites of the Ca^{2+}ATPase. In addition, there are several reports stating that flavonoids such as piperine and capsaicin controls membrane bound ATPases in various experimental cancer studies [30]. In the present study, decrease in the activities of Na^+/K^+ATPase, Mg^{2+}ATPase and increase activities Ca^{2+}ATPase were found in B(a)P induced animals. The restoration of activities of all the three ATPases to near normal values was observed in fisetin treated animals. Cell proliferation plays an important role in multi-stage carcinogenesis with multiple genetic changes. Increased cell proliferation has been proposed to be a biomarker of increased susceptibility to lung cancer [35]. Thus, over expression of PCNA observed in the current study reflects increased cell proliferation, which was confirmed by immunofluracence analysis in lung tumors. B(a)P induced animals treated with fisetin showed a decline in the number of PCNA positive cells that in turn reflects a decrease in S phase cells and thus reduced proliferative activity. Most potential chemo-preventive agents against chemical-induced lung carcinogenesis suppress cell proliferation activity through PCNA index [36]. It was reported that natural components and major flavonoids such as morin and capsaicin suppress various cancers by altering cell proliferation. In addition, there are several reports stating that fisetin controls cell proliferation in various experimental cancer studies [37]. This is in accordance with the present study which shows that the fisetin has the ability to suppress the cell proliferation, as confirmed by PCNA index. In conclusion, the results of the present study indicate that fisetin supplementation alleviated the disruptions in markers enzymes, glycoprotein levels and membrane-bound enzyme activity levels during B(a)P-induced lung cancer, Further, the study clearly shows that ability of fisetin in inhibiting cell proliferation and tumor development. The preliminary studies in the present investigation depicts that fisetin effect is more pronounced when used as a chemo preventive agent against B(a)P induced lung carcinogenesis.

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