RESEARCH COMMUNICATION

Chemopreventive Role of Sulforaphane by Upholding the GSH Redox Cycle in Pre- and Post-initiation Phases of Experimental Lung Carcinogenesis

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Abstract

Sulforaphane (SFN) is a natural, biologically active compound extracted from cruciferous vegetables such as broccoli and cabbage with anti-inflammatory and anti-cancer properties. The present study was carried to assess cytoprotective potential in alleviating oxidative stress, to influence the initiation and subsequent carcinogenesis caused by benzo(a)pyrene [B(a)P] administration in the pre- and post-initiation phases of carcinogenesis in Swiss albino mice. Sulforaphane, supplemented orally at a dose of 9μmoles /mouse/day was found to greatly lessen the damaging effects of B(a)P in mice by increasing the availability of reducing equivalents to fulfil the futile GSH redox cycle and replenish GSH biosynthesis, stabilizing the thiol status. Activity of superoxide dismutase and catalase in native gel prove their differential activities in cancer induced and treated animals. SFN was also found to prevent formation of leaky membranes by boosting the antioxidant status leading to maintenance of ATPase activity in B(a)P treated animals. Histopathological analysis confirmed reduction of carcinogen-associated morphological changes in the lung tissue. The results suggest that SFN has potential as a chemopreventive phytochemical against B(a)P induced lung damage in the processes of carcinogenesis.

Keywords: Chemoprevention - glutathione metabolism - lung carcinogenesis

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Introduction

Lung cancer is the most common fatal malignancy among men and women in most countries of the world and the gender difference is narrowing (Bal, 2001). In India, the incidence of lung cancer is increasing rapidly, mainly due to progressive change in life style (Majumdar et al., 2001). Cigarette smoke is a multipotent carcinogenic mixture that can cause cancer in numerous different organs, and a strong independent risk factor for lung cancer and cancers of oral cavity, upper respiratory tract, and upper gastrointestinal tract (Salaspuro et al., 2006). The lung is more susceptible to oxidative injury than any other organ in the body because of constant exposure to air that might contain toxic particles or oxidant gases such as nitrogen oxide or ozone (Nehls et al., 1997). Lungs have a high rate of blood perfusion, which makes them more likely to be exposed to xenobiotics (Vallyathan and Shi, 1997). Reactive oxygen species are generated in the normal metabolism of all aerobic cells. Cell cytoplasm is an important site of ROS production. These reactive molecules interact with DNA in proliferating epithelium to produce permanent genomic alteration (De Marzo et al., 1999).

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed environmental genotoxins. Benzo(a)pyrene [B(a)P] is a member of PAH family, and is often used as a model compound for PAH toxicity studies and has been shown to be a potent lung carcinogen in animal models of lung cancer (Harrigan et al., 2003). The current concept of oxidative stress emphasizes the balance between oxidants and antioxidants. The imbalance of these biological end points is an important step in the development of cancer. Previous findings suggest that malignant cells of different cancer types exhibit heterogeneity in levels of oxidative stress, associated with various expression levels of SOD and other antioxidant, detoxifying enzymes (Kanbagli et al., 2000).

Dietary manipulations by cruciferous vegetables are known to act at molecular level and prevent the development of cancer (Bonnensen et al., 2001). Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane], a naturally occurring member of the isothiocyanates family, is the most potent known inducer of phase II enzyme involved in the detoxification of xenobiotics (Gerhauser et al., 1997). It attributes to modulate the carcinogenic metabolism by induction of phase II detoxification enzymes and inhibition of cytochrome P- 450-dependent monooxygenase and histone deacetylase (HDAC) (Langouët et al., 2000; Myzak et al., 2004). This causes effective clearance of potential carcinogens as well as endogenous reactive oxygen species and can consequently lead to cellular

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protection against DNA or other cellular damage and thereby block the initiation of carcinogenesis. Cancer chemoprevention by Sulforaphane has been observed against benzo(a)pyrene induced fore stomach cancer in mice (Fahey et al., 2002). This study was initiated to investigate the effect of B(a)P on lung and liver antioxidant status in control mice and those treated with sulforaphane.

Materials and Methods

Animals

Healthy female Swiss albino mice (4-6 weeks old) weighing 15-20g were used throughout the study. Mice were acclimated to laboratory condition with regular temperature control ranging from 23±2˚C and were given ad libitum access to balanced diet (Gold Mohor rat feed, Ms. Hindustan Lever Ltd., Mumbai) and water. All the experiments were performed in compliance with the regulation of our institutional Animal Care and Use Committee. They were maintained in a controlled environment condition of alternative12h light/dark cycles. This research work on female Swiss albino mice was sanctioned and approved by our Institutional animal ethical committee (IAEC/No-02/077/07).

Drugs and chemicals

Benzo(a)pyrene was purchased from Sigma Chemical Company, USA. Sulforaphane (>99% purity) was purchased from LKT Laboratories (St Paul, MN). All other chemicals used were of analytical grade obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Glaxo Laboratories, CDH division, Mumbai, India.

Experimental set-up

The animals were divided into five groups with six mice in each group. Group I served as control animals treated with corn oil (vehicle) intraperitoneally. Group II animals were treated with Benzo(a)pyrene [B(a)P] (100mg/kg body weight dissolved in corn oil, intraperitoneally) thrice a week and kept for 20 weeks to induce lung cancer. The method of cancer induction was adopted from Lee W. Wattenberg et al., (1997) with slight modifications and treatment conditions were accordingly decided in the current study. Group III comprised of control animals treated orally with sulforaphane (9μmoles/mouse/day) on alternate days for two weeks prior (pre-initiation) to first dose of the carcinogen and treated continuously until the 12th week and sacrificed (Pre-treatment group). Group IV were B(a)P treated animals as in group II, treated with sulforaphane (post- initiation) from the 12th week to the 20th week (Post- treatment group) as in group IIIanimals. Control animals treated with sulforaphane alone as in group III served as group V.

After the experimental period the animals were killed by cervical dislocation and blood, lung and liver tissues were used for the further analyses. Both the organs were excised immediately and was washed in ice cold saline to remove any extraneous matter, cleaned, blotted to dryness in filter paper. A 10% homogenate of lung and liver tissues were prepared in was prepared in Tris-HCl buffer 0.1M (pH-7.4) using a Potter Elvejhem glass homogenizer as necessitated by the protocol. Dilutions were decided based on the protein concentration. The method of Lowry et al., (1951) was adopted for the estimation of protein content in the serum and tissue homogenates.

Histopathological Analysis of Lung Tissue

A portion of the lung tissue immediately after sacrifice was kept in 10% formalin to fix the tissue. The tissues were washed in running tap water, dehydrated in the descending grades of isopropanol and finally cleaned in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5μm thickness, stained with hematoxylin and eosin (H&E) and were then viewed under light microscope (Nikon microscope ECLIPSE E 400, model 115, Japan) for histopathological changes in the lung of control and experimental animals.

Biochemical Protocols

The activity of γ-Glutamyl cysteine synthase (γ-GCS) was measured by Mooz and Meister (1971). After centrifugation inorganic phosphorous liberated was determined in the supernatant by the method of Fiske and Subbarow (1925) and expressed as μmoles of Pi liberated/ min/mg protein. The activity of glutathione reductase (GR) was assayed by the method of Stahl et al., (1969) and expressed as nmoles of NADPH oxidized/min/mg protein. Glucose-6-phosphate dehydrogenase (G6PDH) was assayed by the method of Beutler (1983) and expressed in terms of nmoles of NADPH formed /min/mg protein.

Reduced Glutathione (GSH) was determined by the method of Moron et al., (1979) which is based on the reaction of GSH with 2,2'-dithio-bis- nitrobenzoic acid (DTNB) to give a coloured compound that absorbs at 412nm. The level of ascorbic acid (AsA) was estimated by the method of Omaye et al., (1979) and expressed as μg/ mg protein. The level of vitamin E was estimated by the method of Desai (1984) and expressed as μg/ mg tissue. The levels of TSH and NPSH were estimated by the method of Sedlack and Lindsay (1968) and their concentration expressed as nmoles/mg protein using the calibration curve obtained with GSH as standard.

Blood collected with 5% EDTA was used for erythrocyte isolation according to the method of Dodge et al., (1963) and modified by Quist (1980). Na^+ K^+ ATPase activity was assayed by the method of Bonting (1970). Ca^{++} ATPase was estimated by the method of Hjerten and Pan (1983). Mg^{++} ATPase was assayed according to the method of Ohinishi et al., (1982). Liberated inorganic phosphorus was estimated by the method of Fiske and Subbarow (1925). All enzyme activities were expressed as μmoles of inorganic phosphorus liberated/min/mg protein.

Superoxide dismutase (SOD) was estimated by the method of Marklund and Marklund (1974). The enzyme activity is defined as Units /mg protein. The activity of catalase (CAT) was estimated by the method of Sinha (1972) and expressed as nmoles of H_2O_2 consumed/min/ mg protein. Activity of glutathione peroxidase (GPX) was assayed by the method of Rotruck et al. (1973), expressed as μmoles of GSH oxidised/min/mg protein.

Activity staining of native gels

a) Superoxide dismutase. The superoxide dismutase

(SOD) activity staining was carried out in native gel as described by Beauchamp and Fridovich (1971). Samples were separated on a native gel under non-reducing conditions. SOD activity was visualized by soaking the gel in 2.45×10^{-3}M nitroblue tetrazolium (NBT) for 20 min, followed by an immersion for 15 min in phosphate buffer (0.036M potassium phosphate, pH 7.8, containing 0.028M N,N,N'-tetramethylethylenediamine (TEMED) and 2.8×10^{-5} M riboflavin). The gels were then subjected to illumination until optimum contrast between the achromatic zones of SOD and uniformly stained blue background had been achieved. The gels were scanned and images quantified with the help of a densitometer and dried in the gel dryer.

b) Catalase. The activity staining of catalase in native gel was carried out as described by Sun et al., (1988). Samples were electrophoresed on a native gel under non-reducing conditions. Catalase was visualized by incubating the gel in 0.01% H_2O_2 for 10 min, followed by a brief rinsing in distilled water. Then, the gel was immersed for 10 min in a freshly prepared solution containing equal volumes of 2% ferric chloride and potassium ferricyanide. The gels were rinsed with distilled water and the catalase activity was visualized as faint yellow bands against a dark green background. The gels were scanned and images quantified using a densitometer and dried using gel dryer.

Statistical Analysis

Data are presented as the mean ± standard deviation (SD).One-way analysis of variance (ANOVA) was used to detect the significant changes between the groups. The student least significant difference (LSD) method was used to compare the means of different groups and the significance was denoted by 'P' value. A commercial software SPSS version 10.0 was employed to find out the statistical significance between the groups.

Results

Table 1 represents the body and organ weights of the control and experimental mice recorded periodically once in a week from the day of tumor induction, till the completion of the experimental period. Initially, there wasn't any significant change in the body and organ weight of the control and experimental animals, but for the later period an explicit decrease in the body weight and increase in Lung weight was observed with the induction group indicating the severity in the assault of the carcinogen. On treatment with sulforaphane, a gradual improvement was noticed in both the treated groups (group III and IV), being more significant with the pre-treatment (group III). The control and drug control animals in group I and V did not show any change in the body and organ weight throughout the experimental period.

Table 2 represents the effect of sulforaphane on the GSH metabolising enzymes in Lung and Liver respectively. The activities were profoundly decreased signifying with $p<0.001$ and $p<0.05$ for GCS in lung and liver of the induction group animals and increased in the pre- and post treatment with a significance of $p<0.001$ and $p<0.01$ for GCS in Lung and $p<0.05$ in liver. Similarly there was an intense decrease in the activity of G6PDH in both the tissues with a significance of $p<0.001$ in the induced animals and increased with a significance of $p<0.01$ in the pre-treated group in both the tissues and with $p<0.01$ and $p<0.05$ in the post- treated group of lung and liver respectively. A considerable reduction in the activity of GPx in the lung ($p<0.001$) and liver ($p<0.01$) in the induction group, and a sufficient increment ($p<0.05$) in the pre- and post- treated groups in both tissues was observed. Similarly a noteworthy reduction of glutathione reductase ($p<0.001$) was noticed in cancer induced animals in both tissues. There was a slight difference in the increment of this enzyme in the pre-treated groups having a significance of $p<0.01$ and $p<0.001$ in lung and liver respectively. The post -treated group shared the same significance of $p<0.05$ in both tissues. The sulforaphane alone treated animals (group V) did not show any significant change when compared with control animals (group I).

Table 3 illustrates the antioxidant status in Lung and liver tissues of control and experimental animals.

Table 1. Effects of Sulforaphane on Body and Organ Weights of Control and Experimental Animals

	Group I	Group II	Group III	Group IV	Group V
Body wt (g)	20.3±0.88	15.8±1.45$^{a\$}$	22.1±0.49$^{b\$}$	18.5±0.54$^{c\$}$	21.5±1.22
Lung wt (mg)	250.3±3.26	261.7±7.22$^{a\$}$	251.0±1.67$^{b\$}$	256.3±6.34^{c*}	250.5±3.01

Mean ± SD (n=6 mice); ^{a}compared with Group I; ^{b,c}compared with Group II; * $p<0.05$; $^{\#}p<0.01$; $^{\$}p<0.001$; NS= Not significant

Table 2. Effects of Sulforaphane on the Antioxidant Status

		Group I	Group II	Group III	Group IV	Group V
Lung	SOD	5.9±0.77	9.1±0.84$^{a\$}$	6.9±0.45$^{b\$}$	7.9±0.74^{c*}	6.1±0.56
	CAT	51.1±9.04	35.6±5.88$^{a\$}$	53.7±14.6$^{b\$}$	54.4±8.03$^{c\#}$	51.1±12.5
	Vit C	1.0±0.10	0.7±0.09$^{a\$}$	0.94±0.11$^{b\#}$	0.9±0.19^{c*}	0.99±0.11
	Vit E	0.96±0.14	0.58±0.08$^{a\$}$	0.82±0.10$^{b\#}$	0.83±0.10$^{c\$}$	0.95±0.12
Liver	SOD	27.8±3.00	14.9±3.71$^{a\$}$	27.5±5.03$^{b\$}$	28.1±4.59$^{c\$}$	26.4±3.89
	CAT	137.4±9.25	99.6±9.96$^{a\$}$	127.3±10.7$^{b\$}$	111.3±9.21^{c*}	143.4±9.74
	Vit C	1.4±0.26	0.75±0.13$^{a\$}$	1.22±0.21$^{b\#}$	0.97±0.15^{c*}	1.28±0.26
	Vit E	1.5±0.25	0.93±0.17$^{a\$}$	1.34±0.23$^{b\#}$	1.39±0.24$^{c\#}$	1.46±0.25

Values are mean ± SD (n=6 mice); ^{a}as compared with Group I; ^{b,c}as compared with Group II; * $p<0.05$; $^{\#}p<0.01$; $^{\$}p<0.001$; NS= Not significant; SOD-1, amount of enzyme required to inhibit 50% auto-oxidation of pyrogallol; CAT, µmoles and nmoles of H_2O_2 consumed/min/mg protein; Vit C and E, µg/mg tissue

Table 3. Effects of Sulforaphane on the Glutathione Metabolizing Enzymes

		Group I	Group II	Group III	Group IV	Group V
Lung	γGCS	3.52±0.58	2.23±0.28$^{a\$}$	3.11±0.48$^{b\$}$	2.93±0.70$^{c\#}$	3.81±0.55
	G6PDH	3.98±0.51	2.78±0.42$^{a\$}$	3.72±0.75$^{b\#}$	3.55±0.70$^{c\#}$	4.09±0.42
	GPx	8.48±0.86	5.39±0.66$^{a\$}$	6.47±0.95^{b*}	6.15±0.61^{c*}	8.28±0.62
	GR	0.07±0.01	0.05±0.01$^{a\$}$	0.06±0.01$^{b\#}$	0.06±0.01^{c*}	0.07±0.01
Liver	γGCS	4.05±0.28	3.25±0.58^{a*}	3.93±0.62^{b*}	3.94±0.63^{c*}	4.03±0.55
	G6PDH	4.26±0.35	3.17±0.60$^{a\$}$	4.02±0.52$^{b\#}$	4.00±0.52$^{c\#}$	4.16±0.51
	GPx	15.9±1.45	13.2±1.45$^{a\#}$	14.9±1.59^{b*}	15.3±1.33^{c*}	15.1±1.28
	GR	0.62±0.06	0.41±0.03$^{a\$}$	0.55±0.07$^{b\$}$	0.52±0.05$^{c\#}$	0.59±0.06

Values are mean ± SD (n=6 mice); [a]as compared with Group I; [b,c]as compared with Group II; * p<0.05; [#]p<0.01; [$]p<0.001; NS= Not significant; γGCS, μmoles of Pi liberated/min/mg protein; G6PDH, μmoles of NADPH formed /min/mg protein; GR, μmoles of NADPH oxidised/min/mg protein; GPx, μmoles of GSH oxidised/min/mg protein

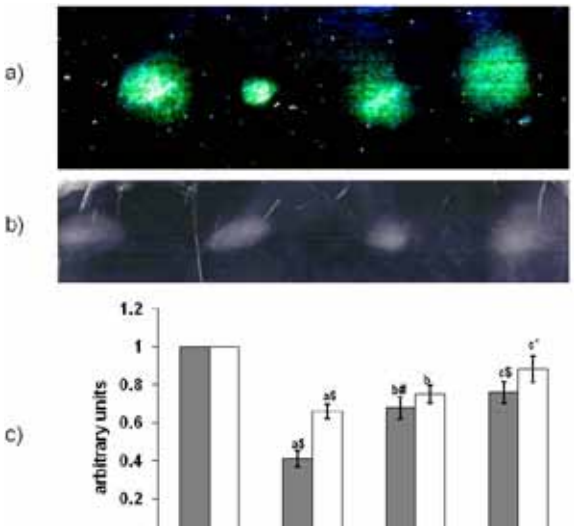

Figure 1. Native Gels Showing Differential Staining for (a) Catalase and (b) SOD Activity in Mice Pulmonary Tissue. Lanes1-4 represent Group I-IV. The graph (c) Results corresponding to the bands

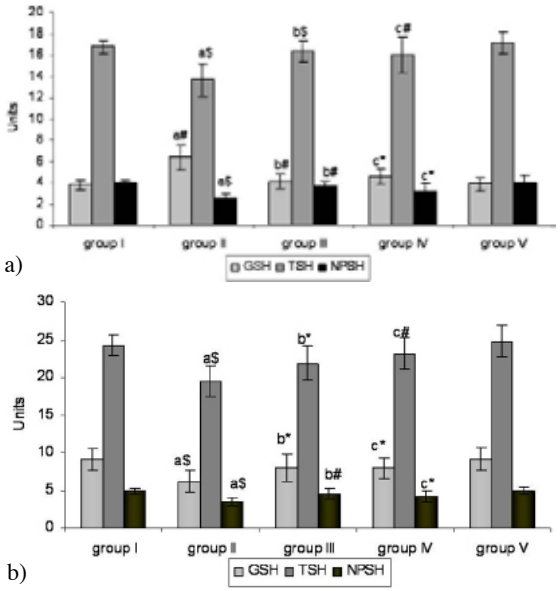

Figure 2 (a), (b). Effect of SFN on the Thiol Status in (a) Lung and (b) Liver. Mean ± SD (n=6); [a]compared with group I; [b and c]: compared with group II; *p<0.05; #p<0.01; $ p<0.001; NS, Not significant. Units: TSH & NPSH- nmoles / mg protein; GSH- microgram / mg tissue

Carcinogen treated mice (group II) showed a significant (p<0.001) reduction in the activity of both enzymic and nonenzymic antioxidants when compared with the control animals (group I) in both lung and liver tissues. These changes were reversed to near normal values in Sulforaphane treatment group III and group IV. There was a significant (p<0.001) increase in the pre-treatment group in the activities of SOD in Lung and Liver. In the post-treatment group a significance of (p<0.05; p<0.01) for SOD and CAT in the lung tissue and (p<0.001; p<0.05) in the liver tissue were observed when compared with the carcinogen induced animals (group II). Sulforaphane treated group III and group IV caused a significant increase the levels of vitamin C in lung (p<0.01) and liver (p<0.05). Similarly vitamin E showed a significance of (p<0.01) in the pre treatment group and (p<0.001; p<0.01) in the post treated groups in lung and liver respectively. However, drug control animals (group V) did not show any remarkable change when compared with control animals (group I). The gels stained for SOD and catalase activities are shown in Figure 1(a) and (b). The graph gives a clear notion of the differential expression of both the enzymes in group II B(a)P treated animals and their modulation by sulforaphane in the pre- and post-initiation phases.

Figures 2(a) and (b) represents the levels of GSH, protein and non-protein thiols (T-SH and NP-SH) in lung and liver of control and treated mice. Cancer bearing animals (group II) showed a significant (p<0.01) reduction in the GSH levels when compared with the control animals (group I). The adverse changes were reversed to near normal values by sulforaphane during pre-treatment (group III) period (p<0.01) in lung and (p<0.05) in liver and post-treatment (p<0.05) in both the tissues of group IV animals, when compared with the cancer induced animals (group II). The T-SH and NP-SH was significantly decreased (p<0.001) in both lung and liver tissue. This unpleasant effect was reversed on pre- and post- treatment with sulforaphane in group III signifying (p<0.001in lung and (p<0.05) in liver for T-SH and (p<0.01) for NP-SH in both lung and liver. In the post- treatment group IV animals the increase was alike in both tissues signifying (p<0.01) and (p<0.05) in lung and liver respectively.

Figures 3 (a) and (b) shows the effect of sulforaphane on the activities of tissue ATPases in Lung and liver of control and experimental animals. In lung a decrease (p<0.001) in the activities of Na^+/K^+, Ca2+ and Mg^{2+}ATPases (p<0.01) were seen in cancer induced (group II) animals when compared with control animals. Likewise in liver a decrease signifying (p<0.01) for Ca^{2+} and Mg^{2+} ATPases

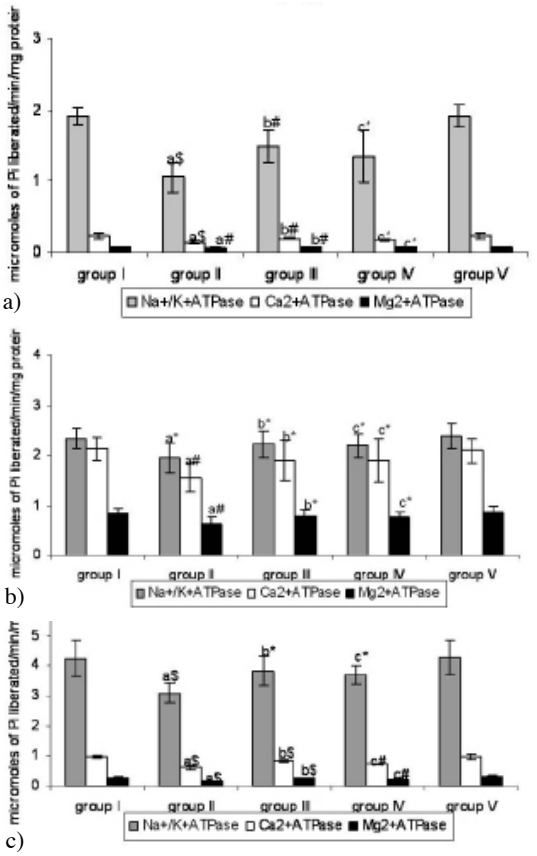

Figure 3. Effects of SFN on Membrane Bound Enzymes of (a) Lung (b) Liver and (c) Erythrocytes in Control and Experimental Mice Mean ± SD (n=6); [a]compared with group I; [b and c]compared with group II; * p<0.05; # p<0.01; $ p<0.001; NS= Not significant

activities and p<0.05 for Na^+/K^+ ATPase were noticed. On pre- and post-treatment with sulforaphane an enhancement in these activities signifying with p<0.01 and p<0.05 were observed respectively for all three ATPases in lung and with p<0.05 in liver. There was no significance between group V and group I control animals.

Figure 3(c) represents an extensive decrease (P<0.001) in Na^+/K^+, Mg^{2+} and Ca^{2+} ATPases in the erythrocytes of cancer induced group II animals when compared with controls. This change in the ATPases activities were significantly reversed in group III pre- treated animals with a significance of p<0.001 and p<0.05 for Ca^{2+}, Mg^{2+} ATPases and p< 0.05 for Na^+/K^+ ATPase. Likewise a similar significance was noticed in the group IV post-treated animals signifying p<0.01 for Ca^{2+} and Mg^{2+} ATPases and p<0.05 for Na^+/K^+ ATPase. Drug control group V animals showed no significance when compared with group I animals.

Histological analysis of lung tissue revealed a normal alveolar architecture in control animals (Group I and V). In lung adenocarcinoma induced animals (Group II), the sections showed hyperproliferating alveolar epithelial cells with severe dysplasia and pleomorphic nuclei (see Figure 4). Group III animals showed almost near normal glandular architecture with diffuse inflammation. Group IV animals had a greatly lessened hyperplasia due to the SFN treatment.

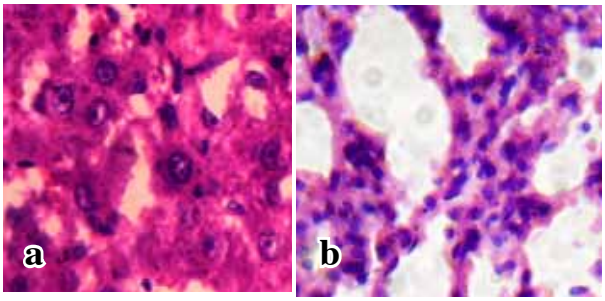

Figure 4. Histopathological Appearance a) Group II, b) Group IV

Discussion

In lung cancer induced (group II) animals, there was a sharp drop in their body weight. This may be due to the cancer cachexia which results in progressive loss of body weight, and is mainly accounted by wasting of host body compartments such as skeletal muscle and adipose tissue as observed earlier in cancer patients (Tessitore et al., 1994). Pain et al., (1984) reported that the drop in body weight results due to the fewer amounts of food intake and/or absorption, which contribute to muscle wasting in tumor cachexia. The weight gain in the SFN pre- and post-treatment groups involve a recovery period indicating lessened damage to the tissues and muscles.

To examine whether enzymatic defence strategies participate in the protection against intracellular ROS accumulation, we analyzed the alterations in the antioxidant enzyme activities. We observed a significant elevation in the SOD activity concomitant with the down regulation of catalase activity along with the other antioxidant enzymes in the cancer induced animals (group II). Nevertheless, the pulmonary system has a series of defence mechanisms including SOD, CAT, GPx, GR, and GSH to protect the cell against these toxic oxygen metabolites, such as $O_2^{-}\cdot$, H_2O_2 and hydroxyl radicals (OH·). SFN also induces glutathione peroxidase (GPx), an enzyme that catalyzes the reduction of organic hydroperoxide and H_2O_2 in mouse Hepa1c1c7 cells (Keck and Finley, 2006). In general, it is proposed that GPx is more efficient at low intracellular H_2O_2 concentrations whereas high amounts are preferentially removed by catalase (De Bleser et al., 1999). Furthermore, the CAT enzyme works on cooperating with GPx to scavenge excess H_2O_2 (unpublished result) as well as lipid peroxides in response to oxidative stress. This interaction suggests SOD, CAT and GPx constitute a mutually supportive team of enzymes that provide a defence against the oxidative insult caused by B(a)P which also confirms Kono et al's and Blum et al's hypothesis (Kono and Fridovich, 1982; Blum and Fridovich, 1985).

Since thiol status is an indicator of the oxidative stress response, we evaluated the thiol status following B(a)P treatment. Thiols are potentially powerful antioxidants and can conjugate xenobiotics to aid their elimination (Lannan et al., 1994). Reduced glutathione contributes around 90% of the intracellular NPSH. The remaining 10% is made up of other small thiol compounds such as cysteine and methionine (Jacobson et al., 1990). GSH is mainly involved in the maintenance of the intracellular redox

homeostasis including removal of hydrogen peroxide which will otherwise undergo oxidation to form GSSG and a thiyl group, which are toxic to the cells (Gilbert, 1990; Reed, 1990). Cells depleted of cysteine were more oxidized and had lower levels of GSH, but addition of cysteine provided a rapid recovery of GSH/GSSG redox and an overshoot in GSH concentrations (Miller et al., 2002). The above explanation justifies the thiol depletion in B(a)P treated animals. Its restoration is evidenced in SFN treated animals which improves the redox regulation and increases the GSH synthesis by enhancing the cysteine uptake thus preventing its oxidation.

Glutathione is the predominant non-protein thiol, redox buffer, and substrate for keeping the ascorbate in reduced form in the ascorbate-glutathione pathway (Noctor and Foyer, 1998) and a major antioxidant used to eliminate peroxides (Buettner, 1993). Thus any fluctuation in the GSH levels will be reflected on the reduction of ascorbate availability thereby increasing α-tocopheryl radical as seen in B(a)P-treated animals. α-tocopherol (vitamin E) plays a major role in blocking the chain reaction of lipid peroxidation (Sies et al., 1992). α-tocopherol and ascorbic acid (AsA) cooperate in cellular defence against ROS. Deficiency of ascorbic acid is expected to result in the depletion of tissue tocopherol by inhibiting the recycling of oxidized α-tocopheryl radical to tocopherol (Freeman and Crapo, 1982) resulting in the elevation of LPO reactions. Interestingly, the AsA and vitamin E levels were restored in SFN pre-treated animals, suggesting its antioxidant potential against B(a)P-induced free radical generation in both the tissues.

As a result of various oxidative stresses, B(a)P has been reported to alter the glutathione-metabolizing enzymes in animal studies (Daniel L. Stout and Frederick F. Becker, 1986) which is in accordance with our results. Hence a significant depletion of glutathione-metabolizing enzymes were seen in group II B(a)P administered mice due to the inhibition of cytosolic [NAD(P)]-dependent dehydrogenases that could limit the availability of NADPH in cells to maintain the reduced state of cellular glutathione. An upregulation of catalase and GSH was encountered on treatment with SFN in group III and IV animals thus favouring the conversion of superoxide anion to H_2O_2 which is effectively removed by GPx in the presence of GSH to fulfil the futile GSH redox cycle along with GR, replenishing GSH synthesis. Additionally SFN has also been reported to stabilize the glutathione-metabolizing system under oxidative stress (Fahey and Talalay, 1999). This is suggestive of that, the pulmonary antioxidant defence system of treated animals reacted positively to combat ROS toxicity enabling an adaptive response to oxidative stress.

Lipid peroxidation is primarily an outcome of the formation of free radicals and observations from earlier studies revealed that significant reduction of both enzymic and non-enzymic antioxidants is associated with a concomitant increase of lipid peroxidation in tumor bearing rats (Chidambaram and Baradarajan, 1995) and similar results were noticed in B(a)P challenged animals (group II). Destructive lipid peroxidation products lead to breakdown of membrane structure and function (Girotti et al., 1998). In our study, SFN brought about a reduction in LPO level and increased the GSH levels in pulmonary and hepatic tissues, suggesting the antioxidant and free-radical scavenging activity of SFN may reduce the toxic effects of B(a)P. Thus the preservation of cellular membrane integrity depends on the protection or repair mechanisms capable of neutralizing the oxidative reactions.

One of the most serious damaging effects on cells is the peroxidation of membrane lipids that severely affect the functional and structural integrity of biological membranes, leading to leakage of potassium ion and other solutes (Shah et al., 2001). The loss of total -SH content might be responsible for the low level of these membrane bound enzyme activities in B(a)P treated mice. Na^+/K^+-ATPase maintains the electrochemical potential gradient of Na^+ and K^+ ions in the membranes of cells, and plays an important role in the transport of substances, energy conversion and information transfer (Glitsch et al., 2001; Zhao et al., 2003) and is in accord with our results. The decrease of its activity indicates substances and energy dysmetabolism (Guo et al., 2004). A shutting down of Na^+/K^+ATPase activity by B(a)P impairs ion pumping function of the Na^+/K^+-ATPase, which could increase [Ca^{2+}] accumulation and open mitochondrial ATP-sensitive K channel (mitoKATP) and further stimulate the production of ROS (Guo et al., 2004). Increase in Na^+/K^+- ATPase activity is associated with an increased recruitment of active Na^+/K^+- ATPase molecules to the plasma membrane (Ridge et al., 2002). This data further support the importance of both, motion and incorporation into plasma membrane of Na^+/K^+-ATPase-containing vesicles, as part of the mechanism by which SFN may increase Na^+/K^+-ATPase activity.

Inhibition of the plasma membrane or the endoplasmic reticulum Ca^{2+}ATPase (Rohn et al., 1993), associated with the loss of critical protein sulphydryl groups (Nicotera et al., 1985) is common during exposure to oxidants. We suspected that thiol oxidation due to excess ROS in B(a) P treated mice resulted in restorable damage to the plasma membrane Ca^{2+}pump depending on the levels of ROS as reported earlier (Hofer et al., 1996). Mg^{2+} has been observed to inhibit Ca^{2+} channels (Agus et al., 1989) and Na^+/Ca^{2+} exchange across cell membranes (Kimura, 1996). Release of intracellular enzymes is a consequence of cell damage and cell membrane alterations. Modulation of ROS metabolism and/or reduction of Ca^{2+} accumulation may be the underlying mechanism(s) for the protective action of Mg^{2+}ATPase. Hence a decrease in the LDH (unpublished result) activity was observed in the SFN treated group indicating the restoration of cell membrane damage and hence the Ca^{2+}ATPase and Mg^{2+}ATPase activities in both erythrocytes and tissues.

Lung plays a central role in toxicological response and pollutant-induced pathological changes which can be observed at the level of lung histology. The analysis of the microscopical findings of the tumoral sections characterize the neoplastic alterations found (pleomorphism suggesting adenocarcinoma) in Group II cancer induced animals which is mainly used for the classification of the tumor (Franklin, 2000). Miranda et al., (2003) considered the hyperplasic injury as one of the alterations associated

to the development of the lung cancer. This finding was obvious in our mode of cancer induction. In the pre-treated the damage was not initiated due the protection afforded by SFN well in advance to the carcinogen administration and in the post treated group the damage was significantly attenuated which suggests a preventive and a therapeutic role of SFN in the pre- and post- initiation phases of carcinogenesis.

In conclusion, the oxidative stress during the initiation of tumorigenesis is manipulated by sulforaphane by fine-tuning the activities of enzymic and non-enzymic antioxidants, glutathione metabolising enzymes and membrane bound enzymes in B(a)P treated mice. But still, the resulting excess of non-detoxified free radicals may mediate some molecular events linked to tumor promotion that causes cell injury and death. The plausible biochemical mechanisms showed significance for the stimulating effects of sulforaphane on the production of the synthase and the reductase of cellular GSH. With this, we conclude that the increase in GSH levels by the modulation of some metabolic/ antioxidative enzymes by orally administered sulforaphane may well be the mechanism by which it protects against cellular toxicity and formation of leaky membranes by free radical intermediates generated during the biotransformation of B(a)P induced carcinogenesis.

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References

Agus ZS, Kelepouris E, Dukes I, et al (1989). Cytosolic magnesium modulates calcium channel activity in mammalian ventricular cells. *Am J Physiol Cell Physiol*, **256**, C452-5.

Bal S (2001). Screening for lung cancer. *Hospital Today*, **6**, 609-11.

Beauchamp C and Fridovich I (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem*, **44**, 276-87.

Beutler E (1983). Active transport of glutathione disulphide from erythrocytes, In 'Functions of glutathione: Biochemical, physiological, toxicological and clinical aspects', Eds Larsson A, Orrenius S, Holmgren A and Mannervick B. Raven press, New York pp 65-74.

Blum J and Fridovich I (1985). Inactivation of glutathione peroxidase by superoxide radical. *Arch Biochem Biophys*, **240**, 500-8.

Bonnesen C, Eggleston IM, Hayes JD (2001). Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Res*, **61**, 6120-30.

Bonting SL (1970). In 'Membranes and ion transport', Eds Bittar EE. *Wiley-InterScience, London*, pp257-63.

Buettner GR (1993). The pecking order of free radicals and antioxidants: lipid peroxidation, alpha-tocopherol, and ascorbate. *Arch Biochem Biophys*, **300**, 535-43.

Chidambaram N and Baradarajan A (1995). The effects of selenium on the antioxidative enzymes in rats with mammary tumor induced by 7,12- dimethylbenz(a)anthracene. *J Clin Biochem Nutr*, **18**, 89-95.

Stout DL and Becker FF (1986). Xenobiotic metabolizing enzymes in genetically and chemically initiated mouse liver tumors. *Cancer Res*, **46**, 2693-6.

De Bleser PJ, Xu G, Rombouts K, et al (1999). Glutathione levels discriminate between oxidative stress and transforming growth factor-beta signaling in activated rat hepatic stellate cells. *J Biol Chem*, **274**, 33881-7.

DeMarzo AM, Marchi VL, Epstein JI, et al (1999). Proliferative inflammatory atrophy of the prostate: Implications for prostatic carcinogenesis. *Am J Pathol*, **155**, 1985-92.

Desai ID (1984). Vitamin E - analysis methods for animal tissues. *Methods Enzymol*, **105**, 138-44.

Dodge JD, Mitchell G, Honatian DJ (1963). The preparation and chemical characteristics of hemoglobin free ghosts of human red blood cells. *Arch Biochem Biophys*, **180**, 119-30.

Fahey JW and Talalay P (1999). Antioxidant functions of sulforaphone: a potent inducer of phase II detoxication enzymes. *Food Chem Toxicol*, **37**, 973-9.

Fahey JW, Haristoy X, Dolan PM, et al (2002). Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a] pyrene-induced stomach tumors. *Proc Natl Acad Sci USA*, **99**, 7610-5.

Fiske CH and Subbarow Y (1925). The colorimetric determination of phosphorus. *J Biol Chem*, **66**, 375-400.

Franklin WA (2000). Diagnosis of lung cancer - pathology of invasive and preinvasive neoplasia. *Chest*, **117**, S80-9.

Freeman BA and Crapo JD (1982). Biology of disease: free radicals and tissue injury. *Lab Invest*, **47**, 412-26.

Gerhauser C, You M, Liu J, et al (1997). Cancer chemopreventive potential of sulforamate, a novel analogue of sulforaphane that induces phase 2 drug-metabolizing enzymes. *Cancer Res*, **57**, 272-8.

Gilbert HF (1990). Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol Relat Areas Mol Biol*, **63**, 69-172.

Girotti AW (1998). Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J Lipid Res*, **39**, 1529-42.

Glitsch HG (2001). Electrophysiology of the sodium-potassium-ATPase in cardiac cells. *Physiol Rev*, **81**, 1791-826.

Guo JR, Cui JJ, Ding JQ, et al (2004). Effects of propofol on energy metabolism, Na, K-ATPase activity and lipid peroxidation of hippocampus mitochondrial following global ischemia-reperfusion in rats. *Chin J Clin Pharmacol Ther*, **9**, 893-6.

Harrigan JA, Vezina CM, Mc Garrigle BP, et al (2003). DNA adduct formation in precision-cut rat liver and lung slices exposed to benzo[a]pyrene. *Toxicol Sci*, **77**, 307-14.

Hjerten S and Pan H (1983). Purification and characterisation of two forms of a low affinity calcium ion ATPase from erythrocyte membranes. *Biochim Biophys Acta*, **728**, 281-8.

Hofer AM, Curci S, Machen TE, et al (1996). ATP regulates calcium leak from agonist-sensitive internal calcium stores. *FASEB J*, **10**, 302-8.

Hursting SD, Slaga TJ, Fischer SM, et al (1999). Mechanism-based cancer prevention approaches: targets, examples, and the use of transgenic mice. *J Natl Cancer Inst*, **91**, 215-25.

Jacobson JM, Michael JR, Jafri MH, et al (1990). Antioxidants and antioxidant enzymes protect against pulmonary oxygen toxicity in the rabbit. *J Appl Physiol*, **68**, 1252-9.

Kanbagli O, Ozdemirler G, Bulut T, et al (2000). Mitochondrial

lipid peroxides and antioxidant enzymes in colorectal adenocarcinoma tissues. *Jpn J Cancer Res*, **91**, 1258-63.

Keck AS and Finley JW (2006). Aqueous extracts of selenium-fertilized broccoli increase selenoprotein activity and inhibit DNA single-strand breaks, but decrease the activity of quinone reductase in Hepa 1c1c7 cells. *Food Chem Toxicol*, **44**, 695-703.

Kimura J (1996). Effects of external Mg^{2+} on the Na^{+}/Ca^{++} exchange current in guinea pig cardiac myocytes. *Ann NY Acad Sci*, **779**, 515-20.

Kono Y and Fridovich I (1982). Superoxide radical inhibits catalase. *J Biol Chem*, **257**, 5751-4.

Langouët S, Furge LL, Kerriguy N, et al (2000). Inhibition of human cytochrome P450 enzymes by 1,2-dithiole-3-thione, oltipraz and its derivatives, and sulforaphane. *Chem Res Toxicol*, **13**, 245-52.

Lannan S, Donaldson K, Brown D, et al (1994). Effects of cigarette smoke and its condensates on alveolar cell injury in vitro. *Am J Physiol 266 (Lung Cell Mol Physiol 10)*, L92-100.

Wattenberg LW, Wiedmann TS, Estensen RD, et al (1997). Chemoprevention of pulmonary carcinogenesis by aerosolized budesonide in female A/J mice. *Cancer Res*, **57**, 5489-92.

Lowry OH, Rosenbrough NJ, Farr AL, et al (1951). Protein measurement with the Folin's phenol reagent. *J Biol Chem*, **193**, 265-76.

Majumdar SK, Das, Nanda S, Goirola M (2001). Brachytherapy in lung cancer. *Hospital Today*, **6**, 612-5.

Marklund S and Marklund G (1974). Involvement of superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem*, **47**, 469-74.

Miller LT, Watson WH, Kirlin WG, et al (2002). Oxidation of the glutathione/glutathione disulfide redox state is induced by cysteine deficiency in human colon carcinoma HT29 cells. *J Nutr*, **132**, 2303-6.

Miranda DGN, Jamnik S, Santoro IL, et al (2003). Avaliaçãodo escarro induzido no diagnóstico do carcinoma brônquico. *Revista Brasileira de Cancerologia*, **49**, 91-8.

Mooz ED and Meister A (1971). Glutathione biosynthesis: In γ-glutamyl-cysteine synthetase (Hog liver). *Methods Enzymol*, **17**, 483-8.

Moron MS, DePierre JW, Manerwik KB (1979). Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. *Biochim Biophys Acta*, **582**, 67-8.

Myzak MC, Karplus PA, Chung FL, et al (2004). A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. *Cancer Res*, **64**, 5767-74.

Nehls P, Seiler F, Rehn B, et al (1997). Formation and persistence of 8-oxoguanine in rat lung cells as an important determinant for tumor formation following particle exposure. *Environ Hlth Perspect*, **105**, 1291-6.

Nicotera P, Moore M, Bellomo G, et al (1985). Demonstration and partial characterization of glutathione disulfide-stimulated ATPase activity in the plasma membrane fraction from rat hepatocytes. *J Biol Chem*, **260**, 1999-2002.

Noctor G and Foyer CH (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol*, **49**, 249-79.

Ohinishi T, Suzuki T, Suzuki Y, et al (1982). A comparative study of plasma membrane magnesium ion ATPase activities in normal, regenerating and malignant cells. *Biochim Biophys Acta*, **684**, 67-74.

Omaye ST, Turabull JD, Sauberlich HE (1979). Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. *Methods Enzymol*, **62**, 1-11.

Pain VM, Randall DP, Garlick PJ (1984). Protein synthesis in liver and skeletal muscle of mice bearing ascites tumour. *Cancer Res*, **44**, 1054-7.

Pick E and Kesari Y(1981). Superoxide anion and H2O2 production by chemically elicited peritoneal macrophages -induction by multiple non-phagocytic stimuli. *Cell Immunol*, **59**, 301-18.

Quist EE (1980). Regulation of erythrocyte membrane shape by calcium ion. *Biochem Biophys Res Commun*, **92**, 631-7.

Reed DJ (1990). Glutathione: toxicological implications. *Annu Rev Pharmacol Toxicol*, **30**, 603-31.

Ridge KM, Dada L, Lecuona E, et al (2002). Dopamine-induced exocytosis of Na,K-ATPase is dependent on activation of protein kinase C-epsilon, and -delta. *Mol Biol Cell*, **13**, 1381-9.

Rohn TT, Hinds TR, Vincenzi FF (1993). Ion transport ATPases as targets for free radical damage: Protection by an aminosteroid of the Ca^{2+} pump atpase and Na^{+}/K^{+} pump ATPase of human red blood cell membranes. *Biochem. Pharmacol*, **46**, 525-34.

Rotruck JT, Pope AL, Ganther HE, et al (1973). Selenium: Biochemical role as a component of glutathione peroxidase purification and assay. *Science*, **179**, 588-90.

Salaspuro VJ, Hietala JM, Marvola ML, et al (2006). Eliminating Carcinogenic Acetaldehyde By Cysteine From Saliva During Smoking. *Cancer Epidemiol Biomarkers Prev*, **15**, 146-9.

Sedlack J and Lindsay RH (1968). Estimation of total, protein-bound and non-protein sulfhydryl groups in the tissue with Ellman's reagent. *Anal Biochem*, **25**, 192-205.

Shah K, Kumar RG, Verma S, et al (2001). Effects of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedings. *Plant Science*, **161**, 1135-44.

Sies H, Stahl W, Sundquist AR (1992). Antioxidant functions of vitamins. Vitamins E and C, beta-carotene, and other carotenoids. *Ann N Y Acad Sci*, **669**, 7-20.

Sinha AK (1972). Colorimetric assay of catalase. *Anal Biochem*, **47**, 389-94.

Stahl GEJ, Visser J, Veeger C (1969). Purification and properties of glutathione reducatse of human erythrocytes. *Biochim Biophys Acta*, **185**, 39-48.

Sun Y, Elwell JH, Oberley LW (1988). A simultaneous visualization of the antioxidant enzymes glutathione peroxidase and catalase on polyacrylamide gels. *Free Radic Res Commun*, **5**, 67-75.

Tessitore L, Costelli P, Baccino MF (1994). Pharmacological interference with tissue hypercatabolism in tumour-bearing rats. *Biochem J*, **299**, 71-8.

Vallyathan V and Shi X (1997). The role of oxygen free radicals in occupational and environmental lung diseases. *Environ Hlth Perspect*, **105**, 165-77.

Zhao SHM, He XJ, Huang LJ, et al (2003). Effect of acute hypoxia on SOD, MDA, NO and Na, K-ATPase in rabbits. *J Clin Exp Med*, **2**, 155-7.