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ANTI-NEOPLASTIC AND ANTI-PEROXIDATIVE PROPERTIES OF NOBILETIN STUDIED IN BENZO(A) PYRENE INDUCED EXPERIMENTAL LUNG CANCER IN SWISS ALBINO MICE

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ABSTRACT

A subtype of adenocarcinoma, the bronchiol alveloar carcinoma is more common in female passive smokers. High CEA levels was found in 16-23% patients in early stage of lung cancer. Adenosine deaminase (ADA) is an important enzyme in purine metabolism and has been documented as a tumor marker and increased activities are found in rapidly growing malignancies. AHH levels were induced in pulmonary tissue and in serum of animals exposed to B(a)P. γ-GT is cell surface enzyme that cleaves extracellular glutathione there by providing the increased intracellular glutathione synthesis. Higher activities 5'-NT were reported in lung cancer patients. LDH is recognized as a potential tumor marker in assessing the proliferation of malignant cells and increased lung and serum LDH activity has been reported in experimental lung cancer. The free radical scavangers consequently to inhibit lipid peroxidation, chemical carcinogen compound strongly involved in the generation of reactive oxygen species.

KEYWORDS: Nobiletin, Benzo (a) Pyrene, Lipid Peroxidation, Tumor Marker.

INTRODUCTION

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death.[1] Lung cancer is the leading cause of cancer deaths in the United States and will claim more lives this year than cancer of the breast, prostate, and colon combined Lung cancer alliance 2015. World wide

lung cancer is the most common cancer in term of incidence and mortality with 1.35 million new cases and 1.18 million death in 2002.[2] By the year 2025, 85% of the world smoker will live in less developed countries.[3] It is expected that around 70% of all tobacco related death will occur in the world's poor and middle income nations compared with the current estimate of 50%.[4,5] International comparison of incidence of lung cancer with that seen in India showed a low figure (age adjusted rates of 66.5-100.4 in Europe and USA versus 2.0 to 14.6 per 10^5 in Indian males the same is 16.1 to 33.3 versus 0 to 3.7 in females) because of the overall population size.[6] A subtype of adenocarcinoma, the bronchiol alveloar carcinoma is more common in female passive smokers and may have different response to treatment.

MATERIALS AND METHODS

Animals

Healthy female Swiss albino mice (7-8 weeks old) weighing 20-25 g were used throughout the study. The animals were procured from Central Animal House Block, Dr. ALMPGIBMS, University of Madras, Taramani, Chennai-113 and maintained in a clean polypropylene, stainless steel grilled cages on 12 h light and 12h dark cycle, at a constant temperature (20–22°C) and humidity (76–78%). All animals were fed standard pelleted diet (Gold Mohor rat feed, Ms.Hindustan Lever Ltd., Mumbai) and water *ad libitum*. This research work on female Swiss albino mice was sanctioned and approved by our Institutional animal ethical committee (IAEC/No-01/18/14).

Chemicals and Reagents

Nobiletin, Benzo(a) Pyrene was obtained from (Sigma Aldrich, USA). All other chemicals used were of analytical grade.

Experimental Design

The experimental animals were divided into four groups, each group comprising of six animals.

Group I: Control animals treated with corn oil (as vehicle).

Group II: Animals treated with (Benzo(a)Pyrene) (50 mg/kg body weight dissolved in 1ml of corn oil orally) for 4 weeks of twice in a week, to induce lung cancer by 16^{th} week

Group III: Lung cancer bearing animals treated with Nobiletin (10mg/kg body weight dissolved in corn oil) orally and continued for 14 successive weeks

Group IV: Control animals treated with Nobiletin alone (as in group III).

At the end of the experimental period the animals were anaesthetized with diethyl ether and sacrificed. The blood samples were collected from experimental animals without any anticoagulant was centrifuged at 3000 g for 30 min to obtain serum. Lung tissue was removed and washed in ice-cold saline. A portion of the lung from groups 1 to 4 was homogenized in 0.1M Tris buffer pH 7.4 and used for various biochemical experiments. Another portion of lung tissue was stored in formal saline for histological analysis. Dilutions were decided according to protein concentration. The protein contents were measured by the method of Lowry*et al.*,(1951).[7]

MARKER ENZYMES

Assay of Adenosine Deaminase (ADA)

ADA was assayed by method of (Bergmeyer, 1983).[8] Monitor the A 265nm until constant, using a suitably thermo statted spectrophotometer. Immediately mix by inversion and record the decrease in the A 265nm for approximately 5 minutes. Obtain the A 265nm/minutes using the maximum linear rate for both the test and blank. The enzyme activity in tissue is expressed as µmoles of NH_3 liberated/mg protein/hr.

Assay of Arryl Hydrocarbon Hydroxylase (AHH)

AHH was assayed by method of (Mildred et al., 1981).[9] 1ml of the organic phase was taken added to 3ml of 1N NaOH and read at 540nm. The enzyme activity was expressed as nmoles of 3-hydroxy benzo(a)pyrene formed/min/mg protein.

Assay of γ-Glutamyl Transpeptidase (γ-GT)

γ -Glutamyl Transpeptidase in the tissues was assayed according to the method described by (Orlowski and Meister, 1965).[10] The extinction coefficient of 1 mol/ml of nitro aniline is 9.9. Read at 405nm in shimadzu UV spectrometer. The enzyme activity in tissue is expressed as moles of nitroaniline formed /min/mg protein and in serum as IU/L.

Assay of Lactate Dehydrogenase (LDH)

Lactate dehydrogenase was assayed according to the method of (King, 1965).[11] The color developed was measured at 420 nm in a Shimadzu UV spectrophotometer. The activity of the enzyme was expressed as µmole of pyruvate liberated/mg protein/hour.

LUNG TUMOR MARKERS

Estimation of Carcinoembryonic antigen (CEA)

The serum carcinoembryonic antigen was estimated by using the quantitative turbidimetric method. The absorbance was read at 450 nm with the Elisa microplate readers (Molecular Device). The values are expressed as ng/mL of serum.

LIPID PEROXIDATION (LPO)

The level of lipid peroxides in lung homogenate and serum was assayed by the method of (Ohkawa et al., 1979).[12] After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. The levels of lipid peroxides was expressed as nmoles of MDA formed/min/mg protein.

RESULTS

Body Weight, Lung Weight and Tumor Incidence

Fig. 1, 2 and Table 1 shows the body weight, lung weight and tumor incidence respectively of different groups of mice that were sacrificed at the end of the study. The final body weight of the B(a)P administered animals (group 2) was found to be significantly ($p<0.05$) lowered and the lung weight, tumor incidence was significantly increased ($p<0.05$) than that of untreated control animals (group 1). Nobiletin increased ($p<0.05$) the final body weight and significantly decreased lung weight and tumor incidence in group 3 animals when compared with group 2 lung cancer animals. There seems to be no significant difference between nobiletin alone treated animals (group 4) and control animals (group 1).

Serum Tumor Marker Enzymes

Fig. 3 depicts the levels of the tumor marker CEA in serum of control and experimental animals. CEA level were found to be significantly increased ($p<0.05$) in B(a)P-induced lung cancer bearing group 2 animals whereas their levels were significantly lowered on group 3 treatment with nobiletin when compared with group 2 lung cancer animals. There is no significant difference between nobiletin alone treated animals (group 4) and control animals (group 1).

Marker Enzymes

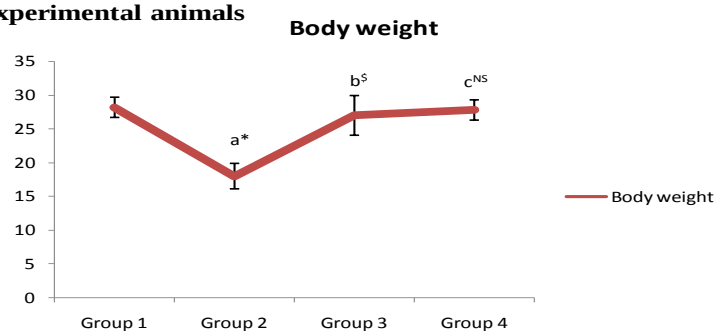
Fig. 4 represents the activities of marker enzymes in lung tissue of control and experimental groups. The activities of marker enzymes ADA, AHH, γ-GT, 5'NT and LDH were found to be significantly ($p<0.05$) increased in lung cancer bearing animals (group 2) when compared

to control animals of group1. Upon treatment the activities of these enzymes were reversed to near normalcy in nobiletin treated animals (group 3). when compared with group 2 lung cancer animals. However no significant difference was observed between the nobiletin alone treated (group 4) and control (group 1) animals.

Tissue and Serum Lipid Peroxidation

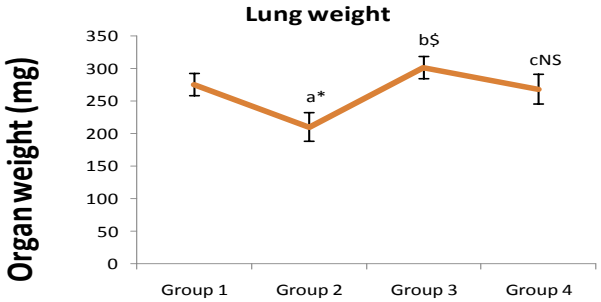
The levels of lipid peroxidation in lung and serum of control and experimental animals are depicted in fig.5. There found to be an increase in LPO in group 2 ($p<0.05$) cancer bearing animals group 2 when compared with control animals group 1. Nobiletin treatment resulted in significant decrease in the level of LPO in group 3 ($p<0.05$) animals when compare with group 2 animals. However the nobiletin alone treated group 4 animals when compared with group 1 control animals did not show any significant difference in the LPO levels.

Figure 1: Effect of Nobiletin on body weight changes in the control and experimental animals



The values are expressed as Mean ± SD for six animals. Group I (control)was compared with Group -II(B(a)P). The statistical significant levels were #p<0.001, *p<0.01,$p<0.005

Figure 2: Effect of Nobiletin on Lung weight changes in the control and experimental animals



The values are expressed as Mean ± SD for six animals. Group I (control)was compared with Group -II(B(a)P). The statistical significant levels were #p<0.001, *p<0.01,$p<0.005

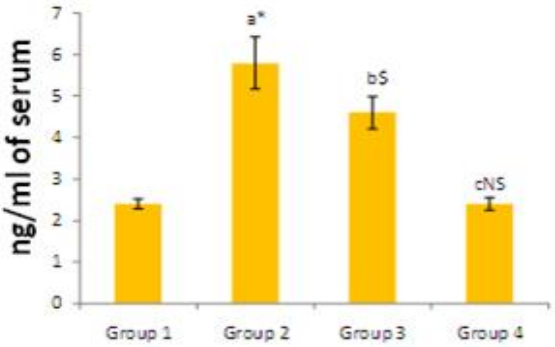
Table 1: Body weight, lung weight and tumor incidence in control and experimental animals.

Particulars	Group 1	Group 2	Group 3	Group 4
Body weight (g)	34.2± 3.4	18±2.21 [a*]	25.2±2.81 [b*]	32±3.20
Lung weight (mg)	279±27.9	346±37.6 [a*]	301.5±30.7 [b*]	266.5±26.5
Number of animals examined	6	6	6	6
Tumor incidence	0	6	3	0
No of tumor incidence / mice	0	3.98±0.38 [a*]	1.28±0.41 [b*]	0

Result are expressed as mean ± S.D (n=6)

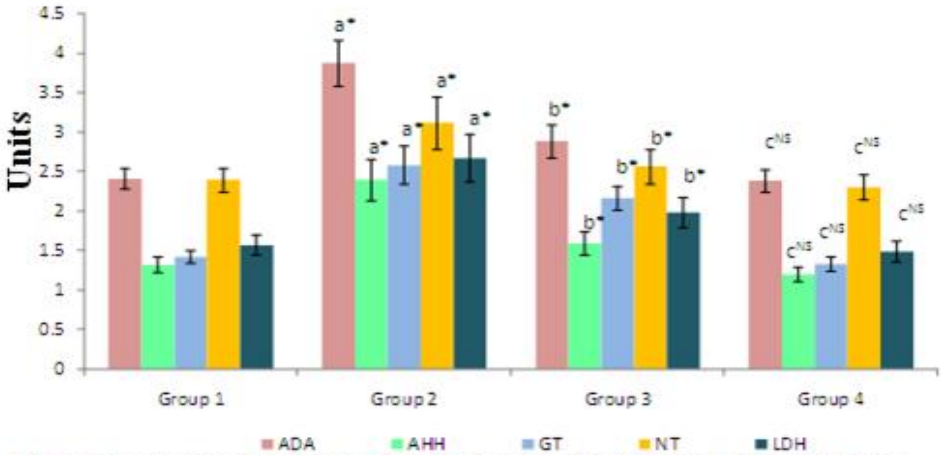
Statistical significance at *p<0.05, [a]group 2 compared with group 1 [b]group 2 compared with group 3.

Figure 3: Effect of Nobiletin on carcinoembryogenic antigen in the control and experimental animals



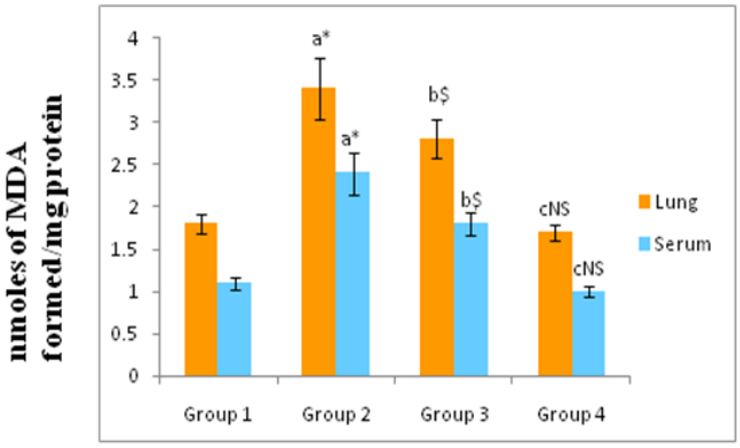
The values are expressed as Mean ± SD for six animals. Group I (control)was compared with Group -II(B(a)P). The statistical significant levels were #p<0.001, *p<0.01,$p<0.005

Figure 4: Effect of Nobiletin on Lung marker enzymes in the control and experimental animals



The values are expressed as Mean ± SD for six animals. Group I (control)was compared with Group -II(B(a)P). The statistical significant levels were #p<0.001, *p<0.01,$p<0.005

Figure 5: Effect of Nobiletin on Lipid peroxidation of Lung and Serum in the control and experimental animals



The values are expressed as Mean ± SD for six animals. Group I (control)was compared with Group -II(B(a)P). The statistical significant levels were #p<0.001, *p<0.01,$p<0.005

DISCUSSION

Nobiletin on body weight, lung weight and tumor incidences

In the present investigation the body weight loss observed in lung cancer bearing (Group 2) animals, could be because of cancer cachexia, annoexia which reported to contribute skeletal muscle and adipose tissue. Wasting are observed in cancer patients[13,14] malaborption.[15] The significant increase in body weight in Nobiletin administrated animals (Group 3) could be due to the inhibitory action of the drug on tumor growth, the gradual increase in body weight indicates the antineoplastic property of the drug. Drug control (Group 4) animals do not show any significant variations. These results indicate the positive nature of Nobiletin.

Nobiletin on Tumor Marker Enzymes

The abnormal variations in the marker enzymes reflect the overall change in metabolism that occurs during malignancy[16], serves as an indicator of cancer response to therapy. High CEA levels was found in 16-23% patients in early stage of lung cancer. The increased levels of serum CEA level in B(a)P administered animals could be associated with production rates of tumor, its location and stage, size, differentiation and vascularity. In the present investigation Nobiletin treatment lowered the levels of CEA in B(a) P induced lung carcinogenesis, which is a good prognosis for tumor regression, and inhibition of metastasis.

Nobiletin on Tissue Marker Enzymes

Adenosine deaminase (ADA) is an important enzyme in purine metabolism and has been documented as a tumor marker and increased activities are found in rapidly growing malignancies.[17] Increased ADA activity may give selective advantage to cancer cell by causing production of hypoxanthine guanine phosphoribosyl transferase (HGPRT) a key enzyme in salvage pathway which provide more mononucleotides to cancer cells patients with lung cancer shown to have elevated serum ADA levels.[18]

AHH converts polycyclic hydrocarbons to phenol, dihydrodiols, quinines and epioxides, this enzyme system that is highly inducible in mouse skin as well as in most mammalian tissue is positively correlated with susceptibility to B(a)P cytotoxicity leads to carcinogenesis.[19] Elevated AHH levels were induced in pulmonary tissue and in serum of animals exposed to B(a)P.

γ-GT is cell surface enzyme that cleaves extracellular glutathione there by providing the increased intracellular glutathione synthesis. Increased activities of γ-GT were observed in cancer cell. Chemical carcinogens that enter liver may initiate some systemic effects that induce γ-GT synthesis. Abnormal high levels of γ-GT are often observed in tumor of lung, liver and variety of tissues, including HCC, malignant squamous carcinoma of skin, mammary tumor and adenocarcinoma of the lung.[20]

5'-NT are enzymes that hydrolyses nucleotides with a phosphate group on carbon atom 5 of ribose it is found to be widely distributed in tumors tissues and increased activity of the enzymes in leukemia patients has been already reported.[21] Higher activities 5'-NT were reported in lung cancer patients. 5'-NT a fast moving 5'-nucleotide phosphodiesterase is found to be elevated in metastases to liver from tumor of the lungs.

Activity of LDH is found to be higher in malignant tissues the elevated activity of LDH may be due to the over production by tumour cells or it may be due to the release of isoenzymes from destroyed tissues. LDH is recognized as a potential tumor marker in assessing the proliferation of malignant cells and increased lung and serum LDH activity has been reported in experimental lung cancer[17] proliferating malignant cell inhibit very high rate of glycolysis which subsequently leads to elevate LDH activity.

In the present investigation study significant elevation in all the above serum marker enzymes were observed in B(a)P administrated lung cancer animals, decrease in the activities of the above mentioned marker enzymes on treatment with Nobiletin suggest the offers some protective against abnormal cell growth by changing the permeability or affecting cellular growth. This may be due to the antineoplastic property of Nobiletin. Thus the Nobiletin treatment brought down the levels of tissue and serum marker enzymes close to normal suggesting its membrane stabilizing and protective potential against lung cancer.

Nobiletin on the Status of Lipid Peroxidation in Lung and Serum

The B(a)P is a very effective carcinogen to induce enormous amounts of free radicals, which in turn reacts with lipids causing lipid peroxidation. Lung is exposed to higher levels of oxygen than most of other tissues, the level of reactive oxygen species (ROS) in the lung is further increased by cigarette smoke, inflammation, pollutants, chemicals and carcinogen. The effect of many antioxidants is to scavange radicals and consequently to inhibit lipid peroxidation, chemical carcinogen compound strongly involved in the generation of reactive oxygen species, such as peroxides, hydroxyl and superoxide anion radicals leading to cellular oxidative damage through DNA strand break and lipid peroxidation.[22]

The Nobiletin could protect the cell to the oxidative stress induced by exogenous addition of H_2O_2 could protect normal lymphocytes from diseases related to oxidative stress, including cancer. The nobiletin biotransformation extract have free radical scavenging activities and inhibit lipid peroxidation.

Nobiletin supplementation negatively controlled spontaneous MDA formation, plasma MDA concentration in supplemented animals were significantly lower than in not supplemented rats and also greatly inhibited the oxidation stress induced by B(a)P. Nobiletin generated a greater amount of ROS followed by more cell death, makes H_2O_2 scavenging system break down more rapidly with decrease of GSH.[23]

In the present investigation study, an increase in the levels of lipid peroxides were observed in B(a)P administrated lung cancer animals when compared to control animals and in Nobiletin treated animals the levels were significantly reduced after treatment when compared to control. There is extensive evidence that supplementation of Nobiletin can enhance antioxidant enzymes. The antioxidant enzymes may reduce the carcinogen-DNA

interaction by providing a large nucleophilic pool for electrophilic carcinogens thus quenching effect signifying its potent anti-peroxidative effect.

CONCLUSION

The significant increase in body weight in Nobiletin administrated animals could be due to the inhibitory action of the drug on tumor growth, the gradual increase in body weight indicates the antineoplastic property of the drug. Drug control animals do not show any significant variations. These results indicate the positive nature of Nobiletin. The potential role as a marker of early cancer and as a prognostic indicator. In the present investigation Nobiletin treatment lowered the levels of B(a)P induced lung carcinogenesis, which is a good prognosis for tumor regression, and inhibition of metastasis. In the present investigation the marker enzymes such as ADA, AHH, GGT, 5'-NT and LDH are specific indicators of lung damage. The antioxidant enzymes may reduce the carcinogen-DNA interaction by providing a large nucleophilic pool for electrophilic carcinogens thus quenching effect signifying its potent anti-peroxidative effect.

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