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Protective Role of Nobiletin on Antioxidants Activity and Xenobiotic Metabolizing Enzymes against Benzo(a)Pyrene Induced Lung Cancer and in Swiss Albino Mice

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ABSTRACT

About 1,685,210 new cancer cases are expected to be diagnosed in 2016. Superoxide dismutase protect against oxygen free radicals by catalyzing the removal of superoxide radicals hydrogen peroxide mediated LPO. B(a)P induces the oxidation of mitochondrial NADPH. An increase in the $NADP^+$/NADPH ratio, the low availability of substrate NADPH may be responsible for the decrease in the activity of GR. Reduced glutathione, vitamin C and α-tocopherol comprise the non-enzymatic antioxidant components which protects the cells against the deleterious effects of the free radicals. Phase I & II metabolizing enzymes plays an important role in the detoxification of electrophilic toxicants and their induction protects against carcinogenesis and mutagenesis.

Keywords : Benzo(a)Pyrene, Nobiletin, Detoxification, Antioxidants

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INTRODUCTION

Lung cancer is a serious health problem in most developed countries and its incidence rate is profusely increasing. Lung cancer is a major cause of morbidity and mortality worldwide in both men and women, accounting for 29 % of all cancers. Siegel et al.,2016 reviewed recent cancer data and estimated a total of 239,320 new cases of lung cancer and 161,250 deaths from lung cancer in the United States in 2010. The statistics reflect data from 2007 and, therefore, likely underestimate the current lung cancer burden. Lung cancer will continue to be a major cause of death throughout the world with in the foreseeable future, it is estimated that by 2030 lung cancer will be the sixth most common cause of death compared with its current ranking of ninth[1]. The age-adjusted incidence rate of lung cancer is 62 per 100,000 men and women per year in the United States, with the incidence rate higher in men than in women (75.2 vs 52.3 per 100,000). Lung cancer in both genders tops the list on the number of estimated deaths yearly (85,600) or 28% of all cancer deaths for men, and 71,340, or 26% of all cancer deaths for women. About 1,685,210 new cancer cases are expected to be diagnosed in 2016.

MATERIALS AND METHOD

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 12 h 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 16th 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)[2]

Assay of tumor marker enzyme

Assay of SOD

Superoxide dismutase was assayed by the method of (Misra and Fridovich,1972)[3]. The increase in absorbance at 480 nm was measured. The enzyme activity is expressed as 50% inhibition of epinephrine auto oxidation. The enzyme activity was expressed as units/mg protein.

Assay of CAT

The activity of catalase was assayed by the method of (Bergmeyer et al.,1974)[4].The color developed was measured at 240 nm. The activity was expressed as μmole of H_2O_2 consumed/min/mg protein.

Assay of Glutathione Peroxidase (GPx)

The activity of Glutathione peroxidase was assayed by the method of (Rotruck et al.,1973)[5]. The colour developed was read at 420 nm. The activity was expressed as micromoles of glutathione utilized /mg protein.

Estimation of Glucose-6-phoshate dehydrogenase (G6PD)

The levels of glucose-6-phosphate dehydrogenase was measured by the method of (Glock and Mclean,1953) [6]. The optical density was read at 640 nm against water blank. The enzyme activity was expressed as units/mg of protein.

Estimation of Vitamin E

The level of vitamin E estimation was performed as according to the method of (Desai, 1984)[7]. The colour developed was read at 530 nm. The level of vitamin E was expressed as μg/mg protein.

Estimation of Ascorbic Acid

The level of ascorbic acid was estimated as advocated by (Omaye et al.,1979)[8]. The colour developed was read at 520 nm. The level of ascorbic acid was expressed as μg/mg protein.

Microsomal enzymes

Estimation of Cytochrome P_{450}

Cytochrome P_{450} was estimated by the method of (Omura and Sato, 1964)[9]. The absorbances of the samples were scanned at 400-500 nm.The level of cytochrome P_{450} was expressed as nmole/mg protein based on molar extinction coefficient 91.

Estimation of Cytochrome b_5

The amount of cytochrome b_5 was measured by the method of (Omura and Sato,1964)[9]. The level of cytochrome b_5 was calculated using the molar extinction coefficient of 185 mM/cm between 409-424 nm and was expressed as nmoles/mg protein

Assay of NADPH-Cytochrome P_{450} Reductase

The activity of NADPH-cytochrome P_{450} reductase was assayed by the method of (Phillips and Langdon,1962)[10]. The optical density was recorded at 30 seconds intervals for 3 min at 550 nm. The activity of NADPH-cytochrome P_{450} reductase was expressed as nmoles of cytochrome C oxidized/min/mg protein.

Assay of Epoxide Hydrolase (EH)

The activity of Epoxide Hydrolase was assayed by the method of (Hasegawa and Hammock,1992)[11]. The reaction mixture incubated at 37°C for 20 min, the change in optical density was recorded 229 nm.The activity of Epoxoide Hydeolase was expressed as nmoles of TSO/min/mg protein.

Assay of UDP Glucuronyl Transferase (UDP-GT)

The UDP-GT was assayed by the method of (Isselbacher et al., 1962)[12] modified by (Hollman and Touster, 1962)[13]. The precipitate was centrifuged and the supernatant of 1ml was taken 0.25 ml of NaOH was added and read at 450 nm.The activity of UDP-glucuronyl transferase was expressed as nmoles of p-nitrophenol/min/mg protein.

Assay of DT-diaphorase (DTD)

The activity of DT-diaphorase was assayed according to the method of (Ernster,1967)[14]. Reading were taken at 30 seconds interval for 2 minutes at 600 nm. Activity of DT-diaphorase was expressed as Cytochrome C reduced/min/mg of protein.

Assay of Glutathione-S-Transferas (GST)

The activity of glutathione-S-transferase was assayed by the method of (Habig et al.,1974)[15]. The optical density was read at 340nm. The glutathione-S-transferase activity was expressed as μmoles of CDNB conjugated/min/mg of protein.

RESULTS AND DISCUSSION

Enzymic Antioxidants

Table 1 antioxidants such represent the cellular enzymic as SOD, CAT, GPx and G6PD in serum and lung of the various experimental groups. A highly significant ($p<0.05$) reduction in the enzymic antioxidants activity of lung cancer bearing animals was observed, when compared with group 1 animals (control). In case of group 3 which is treated with nobiletin the changes were bring back to near normal when compared to group 2 animals. However, drug control (Nobiletin) alone group 4 animals did not show any changes when compared with group 1 animals. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism. ROS/RNS are known to act as secondary messengers controlling various normal physiological functions of the organism and therefore the production of NO• by NOS and superoxide by NAD(P)H is tightly regulated by hormones, cytokines, and other mechanisms. In addition, ROS and RNS participate in various redox-regulatory mechanisms of cells in order to protect cells against oxidative stress and maintenance of cellular "redox homeostasis". The most prominent examples of such mechanisms involve the inhibition of NOS by NO• and the oxidative induction of protective enzymes by the redox-sensitive bacterial OxyR protein. Excess amount of ROS is due to over stimulation of NAD(P)H by cytokines and by the xanthine oxidase in mitochondrial electron transport chain which leads to the oxidative stress. Oxidative stress vital process that acts as a mediator of damage to cell structures.

Table:1 Effect of Nobiletin on enzymic antioxidants enzymes in the lung of control and experimental animals

Particulars	Group 1	Group 2	Group 3	Group 4
SOD	6.20±0.40	$4.12\pm0.50^{a*}$	$5.67\pm0.56^{b\$}$	6.18 ± 0.46^{cNS}
CAT	284±16.63	$134\pm14.58^{a*}$	$177\pm12.6^{b\$}$	289 ± 15.54^{cNS}
GPx	51.70±3.49	$32.31\pm4.02^{a*}$	$42.86\pm4.12^{b\$}$	51.75 ± 3.7^{cNS}
G6PD	204±13.44	$157\pm20.04^{a*}$	$225\pm19.8^{b\$}$	203 ± 15.12^{cNS}

Each value is expressed as mean ± SD for six mice in each group.

Units: SOD - units/min/mg protein; CAT - μmoles of H_2O_2 liberated/min/mg protein; GPx - μmoles of GSH oxidised/min/mg protein;

a compared with Group I; b compared with Group II; c compared with Group III Statistical significance - $^{*}p<0.001$, $^{\$}p<0.01$, $p<0.05$, NS - Not significant

Superoxide dismutase protect against oxygen free radicals by catalyzing the removal of superoxide radicals hydrogen peroxide mediated LPO[16]. Which in metabolized by CAT and GPx that damage the membrane and biological structure. Catalase has not usually been connected to malignancies, but its expression in mesothelioma is high[17]. Since this catalase may also be connected to highly resistant invasive tumors. B(a)P induces the oxidation of mitochondrial NADPH[18].This causes an increase in the $NADP^+$/NADPH ratio. The decrease in the activity of GR is because of the low availability of substrate NADPH. The lowered activity of GR decreases the conversion of oxidized GSH level, which in turn decreases the conversion of oxidized GSH in to reduced GSH level, which in turn decreases the activity of GPx. Catalyzing the detoxification of electrophilic compound to protective against peroxidative damage in which GST is a multifunctional protein [19]

Non Enzymic Antioxidants

Figure 1 represent the non-enzymatic antioxidants (GSH, vitamin E and vitamin C) in serum and lung of the various experimental groups. A highly significant ($p<0.05$) reduction in the activity of non-enzymatic antioxidants in the tumor bearing animals (group 2) was observed. When compared with control animals group1. In group 3 (B(a)P+Nobiletin) animals the changes were reverse back to normal when compared to group 2 animals. In group 4 drug control alone (nobieltin) treated animals did not show any changes when compared with group 1 animals. Reduced glutathione, vitamin C and α-tocopherol comprise the non-enzymatic antioxidant components which protects the cells against the deleterious effects of the free radicals. In our study, we observed a decline in GSH levels in lung cancer bearing animals, which may be due to excess amout of antioxidant for cancer cell proliferation, but after the administration of nobiletin GSH levels in group 3 animals is elevated. The excessive utilization of non enzymic antioxidants for quenching enormous free radicals produced in this conditions leads to decreased the vitamin E content in group 2 animals. This is because of Vitamin E acts a chain breaking antioxidant by alkoxyl radicals intermediates of LPO and donating its labile hydrogen atom from phenolic OH group to propogating lipid peroxyl , thus terminating the chain reactions[20].

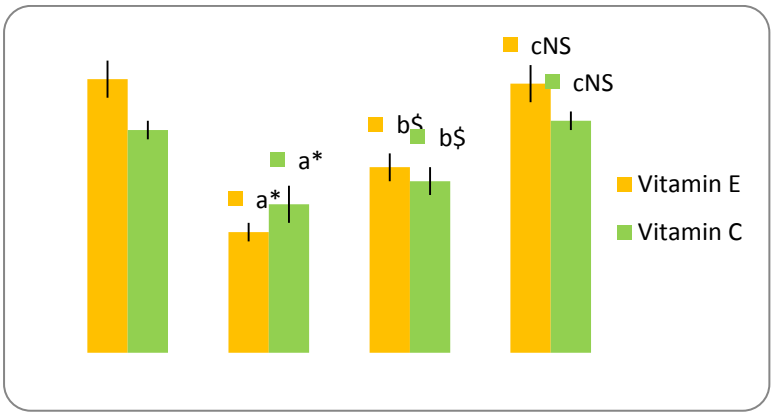

Figure 1: Effect of Nobiletin on Non enzymic antioxidants enzymes in the control and experimental animals

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<0.05, NS-not significant

Xenobiotic Metabolising Enzymes

Phase I Enzymes

Figure 2 & Figure 3 depicts the phase I enzyme activities in lung both control and experimental animals. The levels of Cyt.P_{450}, Cyt.b_5 , NADPH Cyt. P_{450} and Epoxide hydrolase were significantly increased in group 2 ($p<0.05$) cancer bearing animals when compared to group 1 animals. Nobiletin treatment resulted in significant decrease in the activities of these enzymes in group 3 ($p<0.05$) animals when compared to group 2 animals. There seems to be no significant difference between nobiletin alone treated animals (group 4) and control animals (group 1). Cytochrome P_{450} enzymes are responsible for the metabolic conversion of many drugs to the polar metabolites via Phase I and Phase II reactions. It is widely accepted that metabolic activation of xenobiotics by phase I enzymes is required for their cytotoxic, mutagenic and carcinogenic activities[21]. Most chemical carcinogens require transformation by phase I metabolizing enzymes into a more reactive form able to bind to DNA. The blocking chemopreventive effect of nobiletin and other monoterpenes during the initiation phase of mammary carcinogenesis are likely to the induction of phase I. Several lines of evidence indicate that phase II xenobiotic metabolizing enzymes, such as glutathione-S–transferase (GST) and NAD(P)H (quinine-acceptor) oxidoreductase (NQO), play a major role in the cellular detoxification of oxidative damaging, genotoxic and carcinogenic chemicals (Yoshimasa,1998). Phase II detoxification enzymes such as GST,UDP- GT and DTD are consider to be a major mechanism of protection against chemical

stress and initiation of carcinogenesis. GSH for their catalytic activity with the multidrug resistance proteins (MRPs) in the transport of various drugs from the cells.

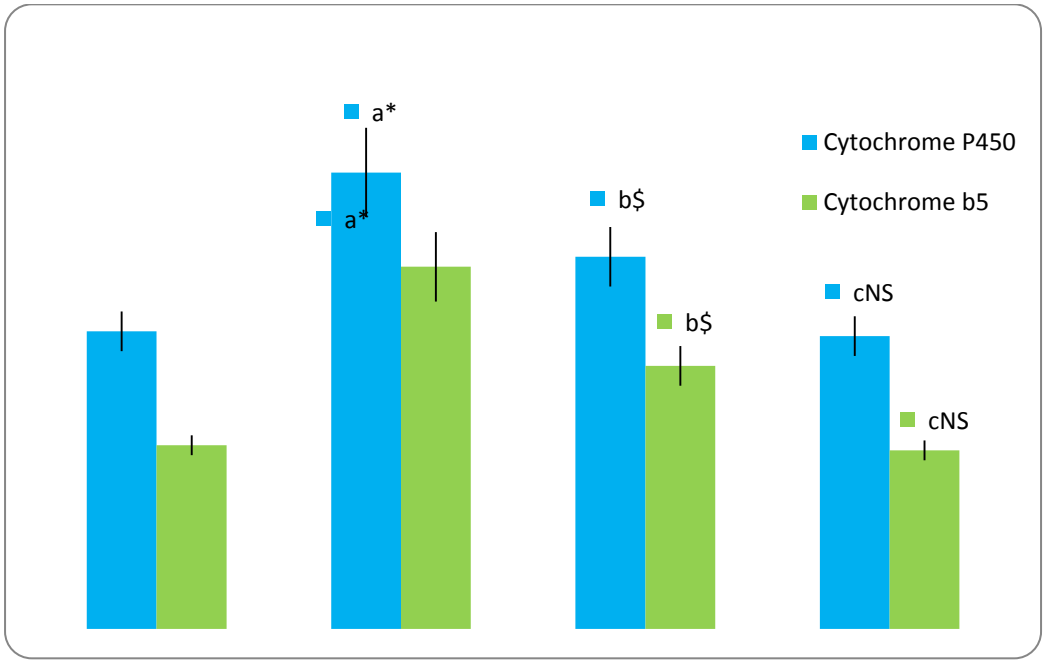

Figure 2: Effect of Nobiletin on Lung microsomal phase I metabolizing enzymes in the control and experimental animals

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<0.05, NS-not significant

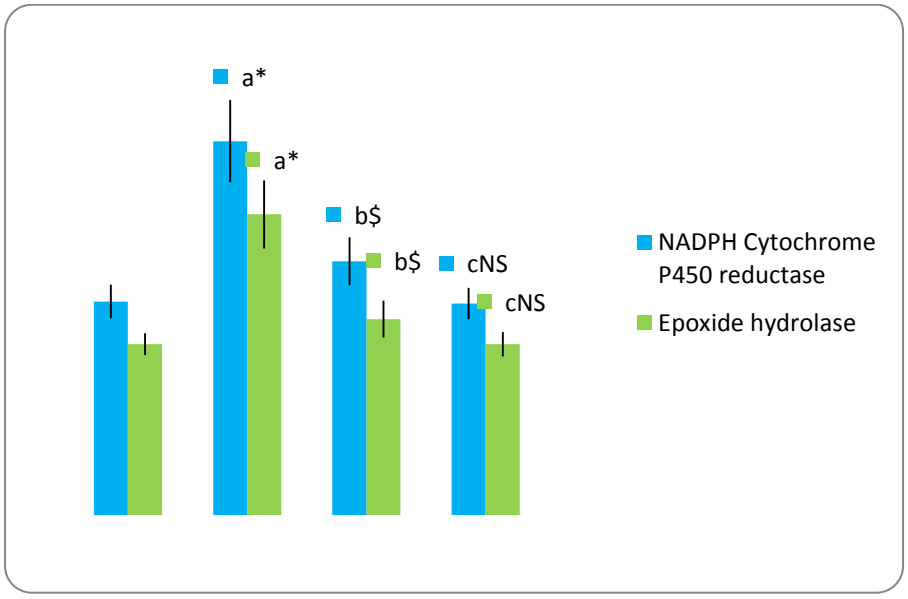

Figure 3: Effect of Nobiletin on Lung microsomal phase II metabolizing enzymes in the control and experimental animals

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<0.05, NS-not significant

Phase II Enzymes

Figure 4 represent the phase II detoxification enzyme activities in lung of control and experimental animals. The levels of GST, UDP-GT, DTD were decreased in group 2 (p<0.05) cancer bearing animals when compared to group 1 animals. Nobiletin treatment resulted in significant increase in the activities of these enzymes in group 3 (p<0.05) animals when compared to group 2 animals. There seems to be no significant difference between nobiletin alone treated animals (group 4) and control animals (group 1). It has been reported that antioxidant properties of Nobiletin would related to induction of total cytochrome P_{450}[22] and of phase II enzymes glutathione-s-transferase and UDP-glucronyl transferase . Several phytochemicals are known to cause elevation in the activites in the GST through the induction of the microsomal detoxification enzymes UDP-GT gene complexes. DTD another phase II enzymes is a flavoprotein that catalyze two-electron reduction of quinines, quinione imines and nitrogen oxide. DTD act as an early cellular defense against tumorigenesis[23]. There is substantial evidence that phase II drug metabolizing enzymes, e.g., GST, NQO1, epoxidehydrolase, hemoxygenase and UDP-glucuronosyl-transferase, plays an important role in the detoxification of electrophilic toxicants and their induction protects against carcinogenesis and mutagenesis.

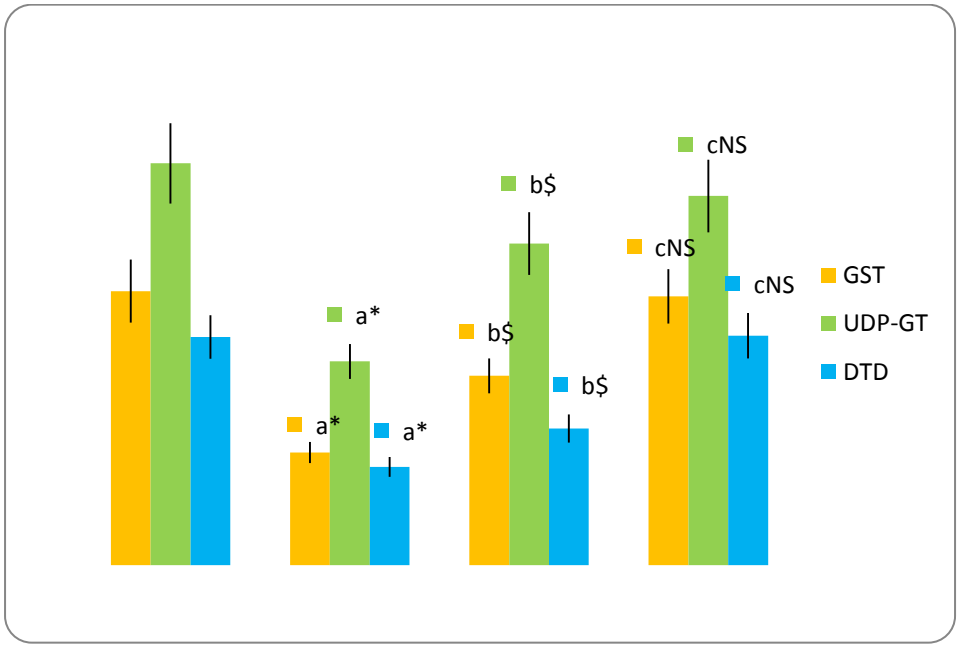

Figure 4: Effect of Nobiletin on Lung microsomal phase II metabolizing enzymes in the control and experimental animals

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<0.05, NS-not significant

CONCLUSION

In the present investigation the increased levels of Cyt P_{450}, Cyt b5 and increased activities of phase I xenobiotic metabolizing enzymes namely NADPH Cyt p450 reductase and epoxide hydrolase in B(a)P administered lung cancer bearing animals. Phase II carcinogen metabolizing enzymes, resulting in carcinogen detoxification. This study also suggest that the enzmic and non enzymic antioxidants modifying capability of nobiletin might plays an important role in its anticarcinogenesis potency against B(a)P induced experimental lung cancer.

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