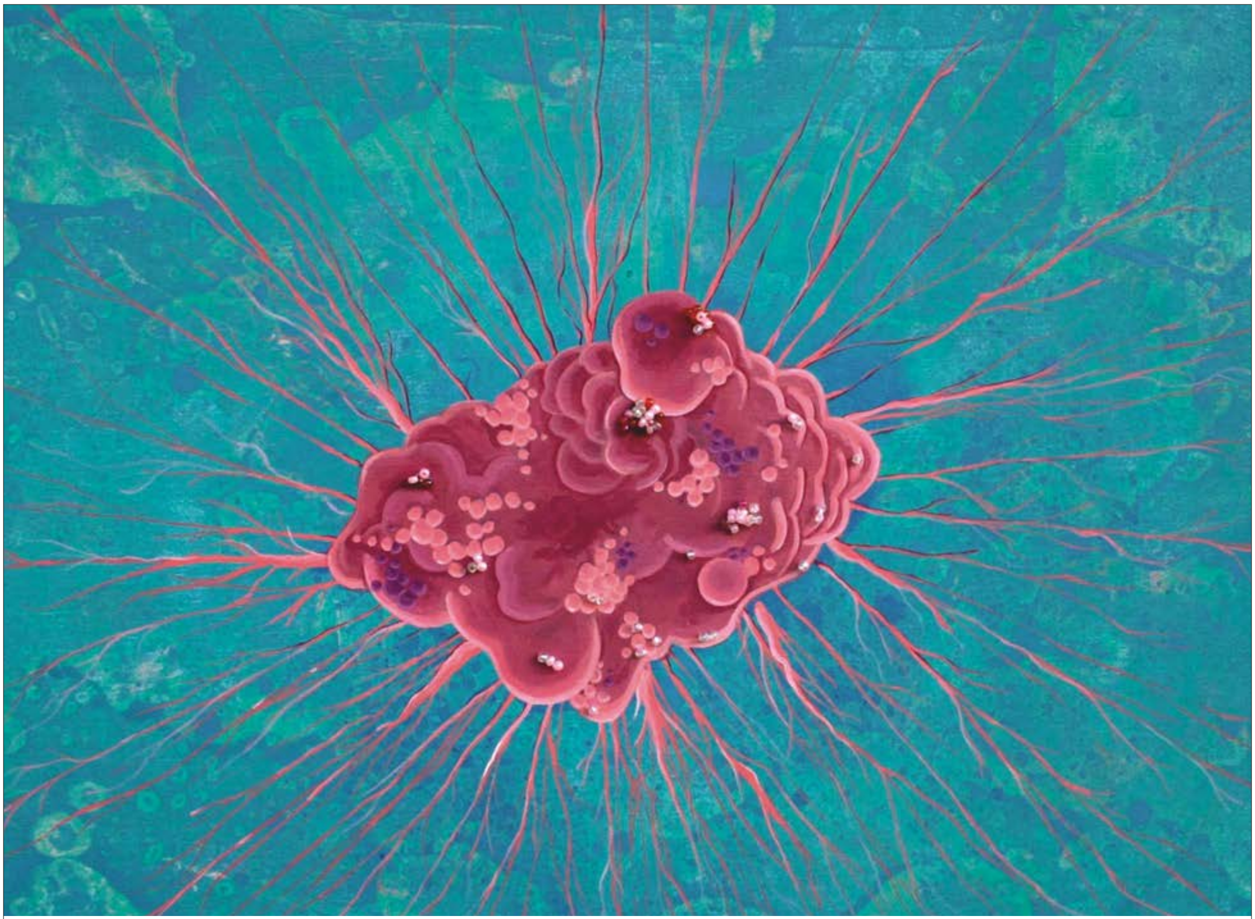

International Journal of

Cancer Research

ISSN 1811-9727

Academic
Journals Inc.

www.academicjournals.com

International Journal of Cancer Research 2 (3): 224-233, 2006
ISSN 1811-9727
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Chemopreventive Role of Lycopene and D-arginine in Benzo (a) Pyrene Induced Lung Cancer with Reference to Lipid Peroxidation, Antioxidant System and Tumor Marker Enzymes

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Abstract: The chemo preventive efficacy of Lycopene and D-arginine with regard to lung carcinogenesis was investigated using benzo (a) pyrene induced swiss albino mice a lung cancer model. A number of natural and synthetic antioxidants are known to retard chemical carcinogenesis in experimental animal model. Here lycopene is a pure antioxidant constituent of tomato and D-arginine complex amino acid having anticancer and antiproliferative effect which inhibits tumour growth was found to suppress benzo(a) pyrene induced lung cancer in Swiss albino mice as revealed by the increase in activity of enzymic antioxidant (Superoxide dismutase, Catalase and Gluthathione peroxidase) and nonenzymic antioxidant (reduced glutathione, vitamine E and vitamine C) levels when compared to lung cancer bearing animals. Values in animals treated with lycopene 10 mg kg^{-1} body weight concomitant with D-arginine 500 mg kg^{-1} body weight shows the reversal to near normal. Further confirmed by increase in the tumor marker enzymes (Aryl hydrocarbon hydroxylase, γ-glutamyl transpeptidase, 5'-Nucleotidase, lactate dehydrogenase and Adenosine deaminase). The above studies also confirm the induction of lung cancer in Benzo (a) pyrene administered mice. Post oral treated with Lycopene and D-arginine for 10 weeks prevents the alterations and restores the enzymes activated to near normal. These findings through overall data demonstrate that the animals post treated with Lycopene and D-arginine may prevent lung cancer and hence will aid in establishing the chemopreventive effect on combinations when administered orally on lung cancer bearing animals.

Key words: Benzo(a)pyrene, lipid peroxidation, enzymic, non-enzymic antioxidants and tumour markers

Introduction

Lung cancer is a major cause of mortality and morbidity worldwide. An estimate 1-2 million people are diagnosed with lung cancer annually and 1-1 million people die from the disease. (Parkin *et al*., 2000) Cancer chemoprevention is defined as the use of natural or synthetic agents to reverse, prevent or delay carcinogenic progression to invasive cancer (Hong *et al*., 1997). Epidemiological and experimental studies however suggest that an increase dietary intake of lycopene play a beneficial role in lowering lung cancer rates (Lenore *et al*., 2002). Lycopene

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suggested having antioxidant anti-cell proliferative and anticarcinogenic activities (Rao and Agarwal, 1999; Stahl and Sies, 1996). Lycopene a naturally occurring carotenoid has focused on its antioxidant profile and plays an important protection role in several human cancers (Lenore *et al*., 2002). Lycopene supplementation at both low and high doses for weeks significantly increased the concentration of lycopene in both plasma and lung tissues (Chun *et al*., 2003). D-arginine is one of the most promising anticancer agents for lung cancer which retard the growth of tumour by enhancing immune functions by increasing T-cell function response to tumour, retard and prolong median survival time (Reynolds *et al*., 1990, Critselis, 1977; Barbul, 1981). A large number of chemopreventive agents have been elucidated in epidemiological and experimental studies, preclinical and clinical observations (Tang *et al*., 1995). However the toxic side effect produced by some of these agents have limited their extensive use. Therefore there is a need to identify synthetic or natural compounds that have significant chemopreventive potential without undesirable toxic effects. In combination each drug has a different mechanism of action but can be given to the individual according to their maximum tolerated dose (Kalant and Roschlau, 1998). Since arginine supplementation in mice provide significant enhancement of cytotoxic T-lymphocytes, natural killer cell active, interleukin-2 receptor and give immune improvement (Reynolds *et al*., 1990). Here an attempt has been made to improve the therapeutic efficacy of lycopene by combining with immunomodulatory D-arginine in experimentally lung cancer bearing animals. The purpose of our present research is to demonstrate the protective efficacy of lycopene and D-arginine by modulating Enzymic and Non Enzymic antioxidants defense system and in elevated marker enzymes in lung carcinogenesis induce by benzo (a) pyrene.

Materials and Methods

Chemicals

Benzo (a) pyrene was provided by National cancer institute MRI Missouri USA. Lycopene was kindly provided by Jagsonpal Pharma, New Delhi, India represented by LycoRed. Natural Product Industries Ltd, Beer Sheva, Israel. All other chemicals were of analytical grade.

Animals

Healthy male Swiss albino mice (7-8 weeks old) were used throughout the study. They were maintained in a controlled environment condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed standard pellet diet (Gold Mohor mice feed, Ms. Hindustan Lever Ltd., Mumbai and water *ad libitum*.

Experimental Protocol

The animals were divided into five groups and each groups consisted of six animals. Group1.Served as control animals and was given corn oil (vehicle) (25 mL kg^{-1} body weight) orally for 16 weeks. Group 2 animals were treated with benzo (a) pyrene (50 mg kg^{-1} body weight) dissolved in corn oil orally twice weekly for 4 successive weeks to induce lung cancer. Group 3 animals post treated with Lycopene alone (10 mg kg^{-1} body weight) p.o. for ten weeks. Group 4 animals were post treated with lycopene as above along with D-arginine (500 mg kg^{-1} body weight) orally for 10 weeks. Group 5 Control animals treated with lycopene and D-arginine alone as above. The post initiations were used to study the chemopreventive and/or chemotherapeutic efficacies of lycopene and D-arginine combination in the experimental animals.

Table 1: Effect of Lycopene and D-arginine on lipid peroxidation, enzymic and non-enzymic antioxidants induced benzo(a)pyrene in lungs of mice

Particulars	Group 1	Group 2	Group 3	Group 4	Group 5
LPO	0.55±0.04	0.91±0.09^{a*}	0.64±0.05^{b*}	0.59±0.07$^{b*c\ NS}$	0.53±0.05
SOD	4.47±0.40	2.62±0.18^{a*}	4.37±0.36^{b*}	4.44±0.40$^{b*c\ NS}$	4.51±0.34
CAT	248±19.00	119±13.08^{a*}	159±14.00$^{b@}$	236±21.00^{b*c}	251±25.00
GPx	43.50±3.04	21.60±2.18^{a*}	39.50±4.53^{b*}	39.47±3.08$^{b*c\#}$	44.20±3.76
GSH	1.49±0.08	0.82±0.07$^{a@}$	1.30±0.15$^{b@}$	1.03±0.09$^{b\#c\ NS}$	1.52±0.141
Vitamin E	0.54±0.06	0.03±0.02^{a*}	0.36±0.05^{b*}	0.49±0.04$^{b*c\ NS}$	0.52±0.05
Vitamin C	0.42±0.04	0.25±0.02^{a*}	0.38±0.04$^{b@}$	0.30±0.03$^{b*c\ \#}$	0.45±0.05

Each value expressed as mean±SD for six mice in each group. LPO: nmoles of MDA released /mg protein; SOD: units/min/mg protein; CAT: μmoles of H_2O_2 consumed/min/mg protein; GPx μmoles of GSH oxidized/min/mg protein; GSH: µg/mg protein; α-tocopherol: µg/mg protein. Ascorbic acid: µg/mg protein

a: Group 2 compared with Group 1. b: Group 2 compared with Group 3 and Group 4. c: Group 3 compared with Group 4. Statististical significance: * $p<0.001$, $^{@}$ $p<0.01$, $^{\#}p<0.05$, NS-Not significan

Biochemical Analysis

At the end of the experiment period, the animals were killed by cervical decapitation. Lung tissues were immediately excised, weighed and then homogenized in Tris-HCl buffer 0.1 M (pH 7.4). These homogenates were taken for the analyses described below. Total protein was estimated by the method of Lowery *et al*. (1951). Lipid peroxidation was estimated by the method of Ohkawa *et al*. (1979). Superoxide dismutase (SOD) was estimated by method of Marklund and Marklund (1974). Catalase (CAT) by the Sinha, (1972) and gluthathione (GPx) by that of Rotruck *et al*. (1973) Reduced glutathione (GSH) was determined by the method of Moron *et al*. (1979) vitamin E (Vit E) was estimated by the method of Desai, (1984) and vitamin C (Vit C) was measured by the method of Omaye *et al*. (1979). The marker enzymes Aryl hydrocarbon hydroxylase (AHH) was estimated by Mildred *et al*. (1981) A γ-glutamyl transpeptidase was estimated according to the method of Orlowski and Meister, (1965) 5'-Nucleotidase was assayed by the method of Luly *et al*. (1972). The activity of lactate dehydrogenase was assayed by the method of King (1965) Adenosine deaminase activity was assayed by the method of (Baggott *et al*., 1986).

Statistical Analysis

All data were analyzed with SPSS 12 student software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test. The values are expressed as mean S.D. p-values of less than 0.05 were considered to indicate statistically significance.

Results

A highly significant increase in the extent of lipid peroxidation in the tumour bearing mice (Group 2) was observed. These adverse changes were reversed to near normal values in (Group 4) lycopene 10 mg kg^{-1} +D-arginine 500 mg kg^{-1} and to some extent in (Group 3) lycopene alone 10 mg kg^{-1} (Table 1). However the Lycopene and D-arginine (Group 5) did not show any significant changes when compared with control animals (Group 1). The activities of SOD, CAT and GPx were found to be significantly ($p<0.001$) decreased in cancer induced (Group 2). On administration of lycopene Group 3, there found to be a significant ($p<0.001$; $p<0.01$) increase in

Table 2: Effect of lycopene along with D-arginine on the activity of marker enzymes in the serum of control and experimental animals

Particulars	Group 1	Group 2	Group 3	Group 4	Group 5
AHH	0.52± 0.04	0.83±0.07^{a*}	0.69±0.07^{b*}	0.57±0.05$^{b*c\,@}$	0.50±0.04
γ-GT	1.13±0.08	1.88±0.03^{a*}	1.43±0.16^{b*}	1.27±0.16$^{b*c\,\#}$	1.11±0.09
ADA	250±28.00	384±33.00^{a*}	258±26.00^{b*}	255±29.00$^{b\#c\,NS}$	253±23.00
5'Nucleotidase	1.76±0.13	2.63±0.21^{a*}	2.52±0.31$^{b\#}$	1.92±0.18^{b*c}	1.77±0.12
LDH	1.06±0.09	1.85±0.18^{a*}	1.46±0.17^{b*}	1.24±0.14$^{b*c\#}$	1.07±0.07

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

Units: AHH-μmoles of fluorescent phenolic metabolites formed/min/mg protein. γ-GT-nmoles of p-nitroaniline formed /min/mg protein. ADA-μmoles of NH_3 liberated /mg protein/hour. 5'-Nucleotidase-nmoles of Pi liberated/min/mg protein. LDH-μmoles of pyruvate liberated/min/mg protein

a: Group 2 compared with Group 1. b: Group 2 compared with Group 3 and Group 4. c: Group 3 compared with Group 4. Statististical significance: * $p<0.001$, $^{@}$ $p<0.01$, $^{\#}p<0.05$, NS – Not significant

these enzyme activities. Cancer bearing group treated with both lycopene and D-arginine (Group 4) showed much more significant ($p<0.001$) increase in the activity of the antioxidant enzymes. Also there found to a significant ($p<0.001$; $p<0.01$) changes between lycopene alone treated Group 3 and in combination with D-arginine (Group 4). No significant difference in the enzymic activities between the control animals treated with lycopene and D-arginine (Group 5) was seen.

The effect of lycopene along with D-arginine on the levels of non-enzymic antioxidants in the lung of control and experimental animals. The level of GSH, Vitamin E and Vitamin C were found to be significantly ($p<0.01$; $p<0.001$) decreased in cancer bearing group (Group 2) when compared with the control group (Group 1). Administration of lycopene (Group 3), lead to significant ($p<0.001$; $p<0.01$) increase in the levels of these antioxidants when compared with cancer bearing group. Combination treatment with lycopene and D-arginine (Group 4) caused a considerable significant ($p<0.05$; $p<0.001$) increase in their levels when compared with the cancer bearing animals. There found to be no significant difference in the antioxidant levels between the control animals and the control animals treated with the combination of lycopene and D-arginine (Group 5). When lycopene treated (Group 3) and combination treated animals (Group 4) were compared there found to be a significant ($p<0.05$) difference in the levels of Vitamic C.

Table 2 shows the influence of lycopene and D-arginine combination on the activities of some marker enzymes such as AHH, γ-GT, 5'-Nucleotidase LDH and ADA in the lung of control and experimental animals. The activities of these marker enzymes were found to be significantly ($p<0.001$) increased in carcinogen treated animals (Group 2) when compared with the control animals (Group 1). On treatment with lycopene Group 3 there found to be significant ($p<0.001$; $p<0.05$) decreased in the activity of these marker enzymes when compared with cancer bearing group. Combination treatment with lycopene and D-arginine Group 4 caused a much significant ($p<0.001$) decreased in their activities when compared with cancer bearing (Group 2) animals. Lycopene treated (Group 3) and combination (Group 4) also showed a significant ($p<0.01$; $p<0.05$; $p<0.001$) increase in the activities of marker enzymes when compared with each other. However there found to be no significant difference in the activities of these marker enzymes between the control animals and the control animals treated with lycopene and D-arginine Group 5.

Discussion

LPO is regarded as one of the basic mechanisms of cellular damage caused by free radicals. The B(a)P is a very effective carcinogen in interacting with membrane lipids and consequently inducing free radical formation (Sikkim *et al*., 2000). Free radicals react with lipids causing peroxidation, resulting in the release of products such as malondialdehyde, hydrogen peroxide and hydroxyl radicals. An increase in lipid peroxides indicates serious damage to cell membranes, inhibition of several enzymes, cellular function and cell death (Pompella *et al*., 1991; Mikhail *et al*., 1996). Lycopene and D-arginine significantly reduced the membrane and plasma lipid peroxides in cancer bearing animals. There is a evidence that supplementation of lycopene and D-arginine can enhance antioxidant enzymes from Table 1. The antioxidant enzymes may reduce the carcinogen-DNA interaction by providing a large nucleophilic pool for the electrophilic carcinogens.

In malignancy it is well known that SOD, CAT, GPx plays an important role as protective enzymes against LPO in tissues. The primary antioxidant enzymes catalase possess a slow catalytic activity at low intra cellular level of its substrate H_2O_2 and under this conditions, GPx plays a predominant role in the detoxification of peroxides from the cell or tissues. The source of H_2O_2 in cells is mainly through superoxide dismutasee mediated by O_2, the later generated in the tissue by several endogeneous enzyme systems as well as the non-enzymic pathway (Sun *et al*., 1999). The activity of GPx is dependant on the availability of GSH, which in turn is maintained by de novo synthesis via. Glutathione reductase and by the level of NADPH via GR.

GPx coupled with glutathione reductase, catalyses the conversion of oxidized glutathione to reduce glutathione and simultaneously NADPH is oxidised to $NADP^+$. B(a)P induces the oxidation of mitochondrial NADPH (Zuohan *et al*., 1994). This causes an increase in the NADP+/NADPH ratio. The low availability of the substrate, NADPH, may be responsible for the decrease in the activity of GR. The lowered GR activity reduces the conversion of oxidized glutathione into reduced glutathione, which inturn decrease the activity of GPx.

Antioxidant enzymes the main scavengers of free radicals are altered during carcinogenesis or after tumor formation (Sun, 1990). Superoxide dismutase protects against oxygen free radicals by catalyzing the removal of superoxide radical (Freiglben and Packer, 1993), which damages the membrane and biological structures, Glutathione peroxidase metabolize peroxides such as H_2O_2 and protects cell membrane from lipid peroxidation. Glutathione reductase is an important enzyme for maintaining the intracellular level of reduced glutathione.

In the present study, the lung cancer bearing Group 2 animals showed a reduction in the activities of SOD, CAT, GPx, and GR. The animals treated with lycopene along with D-arginine showed increased activities of these enzymes in Group 3 and 4 animals. Hence, it is suggested that the lycopene and D-arginine during the treatment could have protected the cells and tissues against the cytotoxic effect of B(a)P. This may be due to the direct reaction of lycopene and the D-arginine with superoxide, hydroxyl radical and alkoxyl radicals with an increase in the levels of GSH by recycling mechanism, which in turn increase the formation of reducing equivalents and also elevates the activity of related enzymes.

Apart from the enzymic antioxidants, non-enzymic antioxidants like reduced glutathione, ascorbic acid and alpha-tocopherol play an excellent role in preventing the cells from oxidative threats. Reduced glutathione play an important role in a variety of detoxification process, including nullification of peroxidative damage (Yu, 1994). Kosower (1983) have shown a direct link between the thiol status of the membrane and cellular glutathione. The function of glutathione is to serve as an agent for reducing membrane protein disulphides and to arrest membrane oxidation.

Glutathione act as a most important antioxidant in living systems because it is a remover of H_2O_2, lipid peroxides and their product like 4-hydroxynonental (Duffy *et al*., 1998). In the present study a decrease in the level of glutathione has been observed and this may be due to enhanced oxidative damage and enhanced utilization of glutathione by the enzyme glutathione peroxidase and a reduction in the activities of the glutathione synthesizing enzymes like glucose-6-phosphate dehydrogenase and glutathione peroxidase, neutralizes hydroxyl radicals and singlet oxygen. Since, it is present in high concentration in the cells, it protects cells from free radical attack (Gopalakrishnan *et al*., 1996).

Antioxidant vitamins C and E are particularly vulnerable to attack by peroxidation products. A reduction in the concentration of these antioxidant vitamins observed in the present study could be due to an increase in oxidative stress as well as due to impairments in the absorption of these vitamins from the intestine (Goodwin *et al*., 1983; Cocharg *et al*., 1990).

Ascorbic acid is the most widely cited form of water, soluble antioxidants, which prevents oxidative damage to cell membrane, induced by aqueous radicals. In addition, recycling of tocopheroxyl radicals to tocopherol is achieved by reaction with ascorbic acid (Freiglben and Packer, 1993).

Alpha-Tocopherol (Vitamin E), a known biological antioxidant, protects the biological membranes and plasma lipoproteins from oxidative stress because it is hydrophobic and can quench free radicals (Traber and Sies, 1996). Vitamin E is the major lipid soluble peroxyl radical scavenger, which can limit lipid peroxidation by terminating chain reactions initiatied in the membrane lipids (Wiseman and Halliwell, 1993) Ascorbic acid causes regeneration of tocopherol from its oxidized from as a result of which tocopherol continues to scavenge the free radicals within the membrane (Hansen *et al*., 1991).

In our present study a decreased level of GSH found in carcinogen treated animals might due to excess utilization of this antioxidant by tumor cells. The vitamics C and E also exist in inter convertible forms and participate in neutralizing free radicals. When there is a reduction in GSH level, the levels of vitamin C and vitamin E were also lowered (Woodside, 2001). But these conditions were found to be reverted in lycopene and D-arginine treated animals. The combination chemotherapy has enhanced various cellular antioxidants and thiol content in tissues, which in turn reduces free radical, mediated cellular damage.

Analysis of cancer marker enzymes serves as an indicator of cancer response to therapy. Distribution of many biochemical, immunological and molecular properties of the host has been observed in B(a)P mediated cancer conditions (Mikhail *et al*., 1996). The marker enzymes such as AHH, ADA, GGT, 5'-ND, and LDH are specific indicators of lung damage (Durak *et al*., 1993; Ferrigno and Buccheri, 1994; Yildrim *et al*., 1999). The increase in the activities of these enzymes may be due to the increased tumour incidence. The AHH and ADA activities were significantly increased in lung cancer bearing animals. There was found to be a reduction in the activities of AHH and ADA on piperine supplementation during initiation and post initiation periods. Recently, reported (Chen and Liu, 2000), new biomarker AHH helps in early diagnosis of lung cancer and has been proved to strongly inhibit the AHH activity in pulmonary and hepatic tissues.

γ-Glutamyl transpeptidase is not only useful in diagnosis but also has prognostic value in malignancies such as lung cancer and malignant melanoma. γ-GT activity serves as a specific marker for the progress of carcinogenic events. The enzyme is membrane bound and its active site is oriented on the outer surface of cell membranes. γ-GT is a cell surface enzyme that cleaves extracellular glutathione thereby providing the component for increased intracellular glutathione synthesis (Durham *et al*., 1997).

Elevated activities of γ-GT were observed in cancer conditions. Chemical carcinogens that enter the liver may initiate some systematic effects that induce γ-GT synthesis (Vanisree and Shymaladeve, 1998). Increase levels of γ-GT was observed in cancerous cells (Ngo and Nutler, 1994). This elevation may indicate the basic tumour burden. Reports show that γ-GT activity in lung and liver was significantly higher in lymphoma mice than in normal mice (Komlosh *et al*., 2002). Administration of lycopene and D-arginine caused recoupment of their activity to near normal values.

5'-nucleotidase enzyme hydrolyzes nucleotides with a phosphate group on carbon atom 5 of the ribose. It is found to be widely distributed in tumor tissues. A fast moving 5'-nucleotide phosphodiesterase is found to be elevated in metastases to liver from tumour of the lung breast (Vanisree and Shyamaladevi *et al*., 1998).

5'-Nucleotidase activity was found to be elevated in cancerous animals (Dao *et al*., 1980) have reported that the increased activity of 5'-nucleotidase seems to have originated from the proliferating tumour cells. The elevation of marker enzymes may be correlated with the progression of malignancy (Durak *et al*. 1993) have reported higher activities of 5'-nucleotidase in lung cancer patients. Lycopene and D-arginine to lung caner bearing mice brought back the activity to near normal values indicating their antitumour property. The Combination of an antioxidant with a complex amino acid D-arginine has been proved to have antiproliferative effect on lung cancers.

LDH is a tetrameric enzyme and is recognized as a potential tumour marker in assessing the proliferation of malignant cells. LDH is fairly sensitive marker for solid neoplasms. Elevation of serum LDH activity is common in myocardial dysfunction and in neoplasms. The elevated levels of LDH may be due to the over production by tumour cells (Helmes *et al*., 1998), or it may be due to the release of isoenzyme from destroyed tissues. Numerous reports revealed increased LDH activity in various types of tumors (Engan and Hannisda, 1990; Nano *et al*., 1989). This may be due to higher glycolysis in cancerous conditions, which is the only energy producing pathway for the uncontrolled proliferating malignant cells. Recoupment of the above mentioned marker enzymes on treatment with lycopene and D-arginine give some protection against abnormal cell growth by changing the permeability of membrane or affecting cellular growth. The chemopreventive and chemotherapeutic effect of lycopene and D-arginine may be attributed to its antioxidant property/or through the enhancement of detoxification enzymes/or throught its metabolites formed during the metabolism.

In conclusion, our present data suggest that oral treatment with 500 mg kg^{-1} of D-arginine along with 10 mg kg^{-1} of lycopene supplementation significantly decreases the toxicity of oxygen species by increasing the levels of free radical scavenging enzymes. Based on the significance observed between lycopene alone and lycopene+D-arginine treated animals, it may be suggested that D-arginine exerts more beneficial effect than Lycopene alone and it can be used as a potential chemotherapeutic agent along with lycopene in the treatment of experimental lung cancer.

Acknowledgement

We acknowledge with gratitude Dr. Lesile Karlin, Research associate National Cancer Institute, MRI, Missoruri, USA for her kind gift of benzo(a)pyrene and Jagsonpal Pharma, New Delhi, India represented by LycoRed Natural Product Industries Ltd, Beer Sheva, Israel, for providing us Lycopene to carrying out the experiments successfully.

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