Inhibitory effect of a mixture containing vitamin C, lysine, proline, epigallocatechin gallate, zinc and alpha-1-antitrypsin on lung carcinogenesis induced by benzo(a) pyrene in mice

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INTRODUCTION

Lung cancer is one of the most lethal cancers of the 20th century and still the most common cancer in the world causing up to 3 million deaths annually, and it is increasing at a rapid rate.[1,2] In Egypt, official statistics showed that lung cancer is the second most common cancer in men and second leading cause of cancer death, after bladder cancer.[3] Lung cancer often takes many years to develop. It usually starts with premalignant changes in the epithelium and develops from squamous metaplasia through carcinoma in situ to frank invasive cancer, which can break away and spread to other parts of the body.[4]

Lung cancer is largely attributable to environmental carcinogens. By far, tobacco smoke is the most important environmental carcinogen leading to lung cancer. Today, the epidemiology of lung cancer is the epidemiology of smoking. Experts estimate that 85-90% of lung cancers are caused by tobacco smoke.[5,6] Tobacco smoke contains over 4,000 chemical compounds. More than 50 chemical compounds have been recognized as human carcinogens. However, the major tobacco-related carcinogens are polycyclic aromatic hydrocarbons typified by benzo(a) pyrene and nitrosamine, which are likely to play major roles in lung cancer induction in smokers.[7]

Treatment for lung cancer revolves around surgery, radiotherapy and chemotherapy used either alone or in combination. All these forms have their downsides as they focuses exclusively on destroying the cancerous cells, but fail to deal with the principal anabolic imbalance that set the stage for the cancer development.[8] Moreover, the high mortality and the marginal improvement in the survival, taken together with the toxic side effects of these therapeutic approaches necessitate the sighting of novel agents with the potential to reduce the risk of lung cancer.[9]

The search for new chemopreventive and antitumor agents that are more effective and less toxic has kindled great interest in the naturally occurring constituents...
mainly in the nutritional substances. Nutrients can directly affect tumor growth and metastases and a good nutritional plan can complement conventional cancer treatments by helping to strengthen the immune system, avoiding malnutrition, selectively starve the cancer cells and reduce the toxicity of medical therapies with enhancement of the anticancer activity of chemotherapeutic drugs. Also, nutrients act as biological response modifiers and can protect living cells against damage from free radicals. In addition, nutrients can revert cancer cells back to healthy cells in the early stages of cancer. Researchers have shown that nutritional supplements, such as vitamins, essential fatty acids, essential amino acids, bioflavonoids and minerals may play a vital role in filling the nutrient gap and can help the body to fight cancer. Previously an international report demonstrated that a unique formulation composed of lysine, proline, vitamin C and epigallocatechin gallate exerts a chemopreventive effect on several types of cancer. Also, earlier studies illustrate that zinc, an essential trace mineral; can play an important role in prevention and treatment of various types of cancer. Moreover, the deficiency of alpha-1-antitrypsin protein can greatly increase the risk for the development of lung cancer. Based on the foregoing, a mixture composed of vitamin C, lysine, proline, zinc and epigallocatechin gallate along with alpha-1-antitrypsin protein was formulated in the present work to study its protective effects on different physiologic aspects involved in experimental lung tumorigenesis induced by B(a)P carcinogen in Swiss albino mice.

MATERIALS AND METHODS

Animals

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland and according to approval from the ethical committee for animals care at the National Research Centre, Egypt. Healthy male Swiss albino mice (6-7 weeks old) weighing 17-20 g were used in the present study. The animals were purchased from the animal house laboratory of the National Research Center, Cairo-Egypt. They were maintained under standard laboratory conditions of temperature and humidity on alternatively 12-h light-dark regimen and monitored for the duration of the study.

Experimental design

After housing the animals for a week, the mice were divided into two experiments. Eighty mice were used in experiment (1), which lasted for 28 weeks. Those animals were subdivided into five groups, Group I (15 mice) served as control and were given vehicle only. Group II (15 mice) were injected intraperitoneal (i.p.) twice weekly with the protector mixture till the end of the experiment. Group III (20 mice) were gavaged orally once with (100 mg B(a)P/kg body weight dissolved in corn oil). Group IV (15 mice) were treated with the protector mixture as group II, simultaneously with B(a)P dose as group III. Group V (15 mice) were treated with B(a)P dose as group III, then after 7 weeks received the protector mixture as group II.

In experiment, (2) 50 mice were used and the experiment lasted for 16 weeks. Animals were subdivided into five groups each contain 10 mice. Group I served as control and was given vehicle only. Group II animals were injected i.p. twice weekly with the protector mixture till the end of the experiment. Group III animals were gavaged orally with 8 doses of B(a)P (each dose has 50 mg/kg body weight dissolved in corn oil). Group IV animals were gavaged with B(a)P as group III, and treated i.p. with the protector mixture as group II, after the first carcinogen dose. Group V animals were gavaged with B(a)P as group III, then treated i.p. with the protector mixture after the last carcinogen dose till the end of the experiment.

Protector composition

The constituents of the protector mixture for each mouse are 1 mg lysine, 0.75 mg proline, 0.7 mg vitamin C, 0.1 mg epigallocatechin gallate, 0.2 mg ZnCl₂ and 0.1 mg alpha-1-antitrypsin. The selected dose based on preliminary study performed in our department.

Biochemical analysis in serum

Total sialic acid (TSA) level was estimated by periodate-resorcinol microassay according to Surangkul et al. [21] vascular epithelial growth factor concentration (VEGF) was determined using ELISA kit (Koma Biotech Inc., Korea) according to Kim, [22] alanine and aspartate transaminases (ALT and AST) were estimated as liver function tests, also urea and creatinine were estimated as kidney function tests were determined calorimetrically according to standard procedures using commercially available diagnostic kit (Biodiagnostic, Egypt).

Biochemical analysis in lung tissues

Lipid peroxidation was estimated by thioribarbituric acid reactive substances (TBARS) assay according to Le’evre et al. [23] the gelatinases was estimated by zymography method according to Frederiks and Mook, [24] the elastase activity was assayed according to the method of Zay et al. [25] collagen breakdown was determined by hydroxyproline assay by the method of Edwards and O’Brien. [26]

Histopathological examination

For histopathological examination, portions of lung tissues were fixed in 10% formalin in saline, then embedded in paraffin wax, serially sectioned and stained according to Conn et al. [27] using a standard method of hematoxylin and eosin (H and E).
Statistical analysis

The statistical analysis of data were carried out by the aid of excel data sheets and statistical package for the social sciences software program (Texas, USA) (SPSS) version 9.0 the data then analyzed statistically using one-way analysis of variance (ANOVA); the levels of significance were evaluated with \( P \) values less than 0.05.

RESULTS

The mean serum total sialic acid levels of study groups are shown in Figure 1. TSA levels were significantly (\( P < 0.001 \)) elevated in groups received B(a)P when compared with control animals of experiment (1) and (2). On administration of the protector mixture in Group IV and Group V of both experiments there was a significant (\( P < 0.001 \)) decrease in TSA levels when compared with Group III. Where TSA level was improved in Group IV\(_1\) and V\(_1\) by approximately 31% from Group III\(_1\) and improved in Group IV\(_2\) by 41% and 35% in Group V\(_2\) from Group III\(_2\).

The mean TBARS levels in lung tissues of various groups are shown in Figure 2. TBARS levels were significantly elevated in groups III\(_1\) (\( P < 0.001 \)), IV\(_1\) (\( P < 0.01 \)) and V\(_1\) (\( P < 0.001 \)) when compared with Group I\(_1\) of experiment (1). Similarly in experiment (2) TBARS levels significantly increased (\( P < 0.001 \)) in groups III\(_2\), IV\(_2\) and V\(_2\) when compared with Group I\(_2\). On the other hand, administration of the protector mixture in Group IV and Group V of both experiments was resulted in a significant (\( P < 0.001 \)) reduction in TBARS levels when compared with Group III. Where TBARS level was improved in Group IV\(_1\) by 55% and 43% in Group V\(_1\) from Group III\(_1\) and improved in Group IV\(_2\) and V\(_2\) by approximately 40% from Group III\(_2\).

The mean VEGF levels of various groups are shown in Figure 3. VEGF level was found to be significantly (\( P < 0.001 \)) increased in Group III, which received B(a)P only of experiment (1) and (2) when compared with control group of experiment (1). This adverse change in VEGF level was reversed to near normal on administration of the protector mixture in either Group IV\(_1\) or Group V\(_1\) with significant decrease (\( P < 0.001 \)) when compared with Group III\(_1\), corresponding to approximately 47% reduction. However, VEGF level decreased significantly, in Group IV\(_2\) (\( P < 0.001 \)) and V\(_2\) (\( P < 0.05 \)) when compared with Group III\(_2\) with 57% reduction in Group IV\(_2\) and 19% in Group V\(_2\).

The mean hydroxyproline levels of various groups are shown in Figure 4. In experiment (1) Group III, the hydroxyproline level was significantly increased (\( P < 0.001 \)) when compared with Group I\(_1\). However, administration of the protector in groups IV\(_1\) and V\(_1\) showed a significant reduction (\( P < 0.001 \)) when compared with Group III, corresponding to 40% and 28%, respectively. In experiment, (2) hydroxyproline level also significantly increased (\( P < 0.001 \)) in the group received carcinogen when compared with control animals. However, protector administration in both Group IV\(_2\) and Group V\(_2\) showed a significant decrease (\( P < 0.001 \)) with approximately 37% reduction when compared with Group III\(_2\).

The mean elastase activity of various groups is shown in Figure 5. Elastase activity in both experiments was significantly increased (\( P < 0.001 \)) in groups received carcinogen when compared with control groups. On the other hand, administration of the protector in Group IV\(_1\) showed a significant decrease (\( P < 0.001 \)) with 25% reduction in elastase activity when compared with Group III\(_1\) in experiment (1). Also, protector treated groups (IV\(_1\) and V\(_1\)) in experiment (2) showed a significant decrease (\( P < 0.001 \)) in the activity of elastase enzyme when compared with Group III\(_2\) corresponding to 38% and 35% reduction.

A representative gelatin zymogram comparing gelatinolytic activity between the different groups is shown in Figure 6.
Gelatin zymography identified distinct gelatin lysis bands, corresponding to expression of MMP-9 and MMP-2 enzymes either latent or active forms. Interestingly, the gelatinase profile of both MMP-9 and MMP-2 was altered by treatment of the mice with the protector mixture.

The histological investigation of lung tissues from different studied groups revealed that, lung section taken from the control group demonstrating normal alveolar patterns shown in Figure 7a, also, sections taken from groups received the protector only indicated no pathological abnormalities [Figure 7b]. Pulmonary lesions induced after 28 weeks in Group III, showed squamous metaplasia where glandular epithelium transformed to squamous flat scale-like cells [Figure 7c], comparing to control group. Treatment with the protector in Group IV, showed similar histology to control group in 20% of samples while 80% of samples showed only moderate inflammation [Figure 7d]. But, Group V, showed moderate inflammation and mucosal thickening [Figure 7e]. In experiment (2) lung lesions induced after 16 weeks in Group III, were variable cellular pleiomorphism, coarse uneven chromatin, little cell maturation, and well-differentiated columnar cells with pleomorphic nuclei expanding within the lung parenchyma as finger-like projections various grades of dysplasia and papillary hyperplasia [Figure 7f]. Treatment with the protector in Group IV, improved the pulmonary lesions where only inflammation ranging from bronchitis to interstitial pneumonitis appeared [Figure 7g]. Whereas in Group V, moderate grade dysplasia was seen in Figure 7h.

**DISCUSSION**

The result of this study demonstrated that the combination of lysine, proline, vitamin C, EGCG and zinc nutrients in addition to alpha-1-antitrypsin protein shows great potential to inhibit B(a)P induced lung injuries via their synergy, and has the ability to modulate lung tumorigenesis in male Swiss albino mice using a safe and multi-targeted approach.

It is found that sialic acid, a sensitive marker for the progression of tumor growth[28] was significantly elevated in experiment (1) and (2) in the groups exposed to B(a)P as compared to control animals. It is noticeably to mention that serum TSA reached its highest level in groups received B(a)P only. According to Anandakumar et al.[29] B(a)P can induce
deleterious alterations in protein-bound carbohydrate components including sialic acid in Swiss albino mice. Suresh et al.\(^{30}\) also mentioned that the cause of sialic acid elevation during tumorigenesis is through the shedding of sialic acid from the tumor cell surface or possibly as a product of the tumor itself. However, administration of the protector mixture in Group IV and Group V causes significant decrease in TSA levels when compared with Group III of the two experiments. This reduction in sialic acid level indicates that the present protector mixture has the ability to suppress neoplastic alteration by maintaining TSA status.

In the present study, we have observed significant increased levels of lipid peroxidation, assayed as TBARs, in the groups exposed to B(a)P, when compared with control groups. Such elevation in TBARs in these groups is due to the genotoxic property of B(a)P, which is a very effective carcinogen enhancing oxidative stress and consequently inducing free radical formation, which in turn react with lipids in the cell membrane causing lipid peroxidation.\(^{31}\) On the other hand, the administration of the protector mixture in groups (IV) and (V) significantly reduces lipid peroxidation comparing with Group III in both experiments. This demonstrates that such protector mixture can modulate lipid peroxidation due to the strong antioxidant effects of vitamin C, EGCG and zinc in this protector mixture. According to Kato et al.\(^{32}\), Weyers et al.\(^{33}\) vitamin C is a potential scavenger of superoxide radical and singlet oxygen and can intercept free radical induced chain reaction and prevent further oxidation. Moreover, vitamin C is in a unique position to scavenge destructive peroxyl radicals before they have a chance to damage lipids. Also, EGCG has been shown to have strong antioxidant activity as it is capable of protecting cells against oxidative stress by direct scavenging of reactive oxygen species and inhibiting lipid peroxidation.\(^{34}\) EGCG may also function indirectly by inhibiting both redox-sensitive transcription factors and pro-oxidant enzymes.\(^{35}\) In addition, it is reported that zinc is required for the activity of some antioxidant enzymes and results in induction of some other ultimate antioxidants proteins. Also, its presence in zinc-containing enzymes was found to protect specific regions of these enzymes from free radical attack.\(^{36}\)

VEGF as reliable marker of angiogenic activity sustaining tumor growth was evaluated in the present work.\(^{37}\) In both experiments (1) and (2) VEGF level was found to be significantly increased in Group III when compared with control groups. According to Bergers and Benjamin\(^{38}\) inhibiting tumor angiogenesis, can block one of the fundamental requirements for tumor growth. In this study, it was found that protector administration improves VEGF level, in both experiments. VEGF level decreased significantly in Group IV and Group V when compared with Group III. This reduction in VEGF value after the protector administration could be related to the antiangiogenic actions of the protector mixture.
Several studies also showed that vitamin C has antiangiogenic properties. Ashino et al.\textsuperscript{[39]} mentioned that vitamin C affects angiogenesis through its antioxidant activity and through stimulating collagen synthesis where angiogenesis involves degradation of the extracellular matrix proteins surrounding the pre-existing vessels. Also, Mikirova et al.\textsuperscript{[40]} hypothesized that vitamin C may exert antiangiogenic effects through its inhibition of nitric oxide, the major stimulus of tumor angiogenesis. Shankar et al.\textsuperscript{[41]} reported that EGCG also has anti-angiogenic property and reduces tumor growth through suppression of a variety of VEGF-induced angiogenic processes such as the inhibition of protein kinases B and vascular endothelial-cadherin phosphorylation. Also, EGCG could inhibit angiogenesis by suppressing cellular production of VEGF itself and inhibits formation of VEGF-receptor complex.\textsuperscript{[42]} According to Uzzo et al.\textsuperscript{[43]} zinc supplementation may have important implications for inhibiting the angiogenic potentials of malignant cells, predominantly through suppression of NF-B signaling, the transcription modulator of VEGF.

Tumor growth and invasion essentially requires the degradation of basement membrane components and extracellular matrix macromolecules, which considered the physical protective barriers in restraining the expanding growth of tumor cells.\textsuperscript{[44]} Proteinases are vital proteolytic enzymes that play a central role in facilitating neovascularization and tumor growth by degrading or remodeling such extracellular matrix components that constitute the pericellular connective tissue, therefore the status of the pulmonary connective tissue was monitored. The data of our study revealed that all animals received B(a)P in the two experiments had significantly altered proteolytic enzymes comparing to the control animals, which is represented by highly expressed gelatinase-A and gelatinase-B, with elevation of both elastase activity and hydroxyproline content the marker of collagen breakdown. However, these parameters in all groups received the mixture together with B(a)P showed a significant reduction when compared with groups received the carcinogen only.

From these findings we postulated that this mixture could be a powerful natural protector against matrix degradation and could strengthen the connective tissue. This can be explained by the fact that nutrients such as lysine, proline and vitamin C acid could act as natural inhibitors of matrix proteolysis through increased connective tissue strength and stability surrounding tumor cells and, as such, they have the potential to modulate tumor growth, contributing to encapsulation of the tumor.\textsuperscript{[19]} Furthermore, the presence of alpha-1-antitrypsin (the major circulating inhibitor of the proteases) in the mixture, could reverse the biochemical abnormalities caused by excessive collagenolytic activity and could interact with elastase enzyme to shield the lungs from its effect by neutralizing its action on increased lysis of extracellular matrix macromolecules.\textsuperscript{[45,46]} Also, vitamin C has a critical role in stability of extracellular matrix structure and prevents degradation action of the ground substance surrounding the tumor.\textsuperscript{[19]} As well, it is an effective biocatalyst that modifies lysine and proline needed for collagen support. Moreover, vitamin C itself stimulates the production of new collagen and strengthens connective tissue.\textsuperscript{[47]} Furthermore, Maeda-Yamamoto et al.\textsuperscript{[48]} Hazgui et al.\textsuperscript{[49]} demonstrated that EGCG is a direct and potent inhibitor of gelatinases due to its ability to form a reversible complex with them. At the same time, it can cause suppression of extracellular signal-regulated kinase phosphorylation, which results in the inhibition of gelatinases expression, leading to the reduction of their enzyme activities in cancer cells. Moreover, EGCG is a potent natural inhibitor of elastase enzyme thus reducing elastase-mediated progression to tumor invasion.\textsuperscript{[50,51]}

Histopathological findings confirmed that groups received B(a)P only in the two experiments have lung lesions that were distributed in the lung alveoli and bronchi. Such abnormalities were basal cell hyperplasia and squamous metaplasia and various grades of dysplasia, which was considered to be important precancerous lesions. On the other hand, treatment of groups with the protector mixture was shown to markedly reduce the lesions resulted from B(a)P and restored the abnormalities in lung tissues towards normal with minimal histological changes.

In conclusion, the results of the present study demonstrate that the dosage of B(a)P carcinogen highly affects on many parameters estimated in the present study such as TSA, elastase enzyme, and hydroxyproline. Where such parameters were highly altered in experiment (2), which received higher B(a)P dose than experiment (1) which received lower dose of the same carcinogen. Furthermore, the actions of such mixture on almost all parameters are more effective in groups received the mixture simultaneously with B(a)P than in groups received the mixture consecutively after the carcinogen dose indicating that, the present mixture has more prophylactic action rather than therapeutic action and it can be used as a safe protective agent because it has no adverse effects on liver and kidney since there is no change in both liver and kidney function tests (data not shown).

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