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Original Article

ORAL ADMINISTRATION OF THYMOQUINONE ATTENUATES BENZO (A) PYRENE INDUCED LUNG CARCINOGENESIS IN MALE SWISS ALBINO MICE

GAJENDRAN NITHYA[1*], REVATHI MANI[1], DHANAPAL SAKTHISEKARAN[1]

Department of Medical Biochemistry, Dr.ALM PG Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Chennai 600113, India
Email: nithyaunom@gmail.com

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ABSTRACT

Objective: The present study has been designed to unravel the anticancer potential of thymoquinone against Benzo(a)pyrene induced lung cancer in male swiss albino mice. Thymoquinone ($C_{10}H_{12}O_{20}$) is a bioactive compound derived from the medicinal plant Nigella sativa. Thymoquinone (TQ) has been shown to exert anticancer effect on various cancer cell lines and there is no study on the efficacy of TQ on Benzo(a)pyrene [B(a)P] induced lung carcinogenesis in male swiss albino mice.

Methods: The changes in heme indices (RBC, Hb, WBC, monocytes, lymphocytes and neutrophils), membrane bound ATPases (Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase) in control and experimental animals were analysed in serum and lung tissue homogenate.

Results: Lung cancer induced animals showed a considerably altered levels of heme indices with concomitant decreased levels of membrane bound ATPases in the lung tissue and erythrocyte membrane. Oral administration of TQ at a dose of 20mg/kg b.w brought back the levels of the biochemical parameters to near normal.

Conclusion: TQ supplementation restored the detrimental effects induced by B (a) P, indicating its anticancer potential in the treatment of experimental lung carcinogenesis.

Keywords: Benzo(a)pyrene,Lung cancer, Thymoquinone, Heme indices, Membrane bound ATPases.

INTRODUCTION

Cancer is the leading cause of morbidity and mortality throughout the world and Lung cancer is the second leading cause of cancer deaths. Approximately 90% of patients with lung cancer ultimately die from metastatic disease. Polycyclic aromatic hydrocarbons (PAHs) are environmental and tobacco carcinogens and are suspect agents in the causation of human lung cancer [1].Benzo(a)pyrene [B(a)P)], a potent chemical carcinogen present in tobacco smoke and environmental pollution causes lung cancer in humans and in experimental systems. The radical cationic forms of B (a)P may be involved in both the mechanism and metabolic activation leading to the formation of DNA adducts, which are key components for tumor initiation process[2].

Although there are no magic bullets that can cure cancer completely and conquer it, the risk can be reduced by eliminating identified carcinogens or least minimizing the exposure to them. During the past few decades, mouse has proved to be a useful model animal in mechanistic studies of chemical carcinogenesis. While there are differences in the process of carcinogenesis between mouse and human, mice do develop tumors in the same tissues and with similar histopathology as humans. Most of the earlier models of spontaneous and chemically induced mouse lung tumors more closely resemble human lung adenocarcinoma than other subtypes in morphology and molecular characteristics [3].

Recently, there is a growing body of gene expression array studies that add our understanding of the molecular mechanisms of human and mouse lung carcinogenesis. In the current study, male swiss albino mice has been used to study the biochemical alternations on B(a)P administration and TQ treatment.

Phytochemicals are well established to exert anticancer activities, partially based on their ability to quench reactive oxygen species and thereby protecting critical cellular targets (DNA, Proteins, lipids) from oxidative insult [4].Phytochemicals may also interfere with intracellular signalling pathways, such as those which regulate proliferation, induction of apoptosis and response to oxidative stress. The World Health Organization (WHO) estimates that 80% of the populations in some Asian and African countries are mostly dependent on traditional medicine for their health care. Thymoquinone (TQ) is the predominant bioactive constituent present in black seed oil (Nigella sativa) and has been tested for its anticancer property in various cell lines. In previous studies it has been demonstrated that thymoquinone inhibits cell proliferation, decreases cellular viability, induces apoptosis, arrests cell cycle, modulates multiple molecular targets including p53, p73, PTEN, STAT3, PPAR-γ, activation of caspases and generation of ROS in in vivo and in vitro conditions of different cancer types.The anti-tumor effects of thymoquinone have also been investigated in tumor xenograft mice models for colon, prostate, and pancreatic cancer [5].

However, to our knowledge, studies on the effect of TQ on lung cancer remain unexplored. Hence, the present study was designed to explicate the protective role of TQ on B(a)P induced lung carcinogenesis by evaluating its potential in maintaining membrane integrity and levels of heme indices in control and experimental animals.

MATERIALS AND METHODS

Chemicals

Benzo(a)pyrene [B(a)P] and Thymoquinone were purchased from M/s.Sigma chemicals, St. Louis, USA. All other chemicals were of analytical grade, procured from M/s. SRL Chemicals Pvt.Ltd., Mumbai.

Animals

Healthy male Swiss albino mice (6-8 weeks old) were used throughout the study. Mice were acclimated to laboratory condition with regular temperature control ranging from 23±2 °C and with balanced diet (Gold Mohor rat feed, M/s. Hindustan Lever Ltd., Mumbai) and water *ad libitum.* 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 alternative 12h light / dark cycles. This research work on male Swiss albino mice was sanctioned and approved by the Institutional animal ethical committee (IAEC. No. 01/027/2010).

Experimental Design

The animals were divided into **FIVE** groups and each group consists of six animals.

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

Group II:B(a)P treated animals (50 mg/kg body weight dissolved in corn oil, orally) twice weekly for 4 successive and left until 20 weeks to induce lung cancer.

Group III:Cancer bearing animals treated with thymoquinone on alternate days (20mg/kg body weight dissolved in corn oil, orally) for two weeks prior to the first dose of carcinogen and continued till 12^{th} week.

Group IV:Cancer bearing animals treated with thymoquinone as in group group III, (20mg/kg body weight dissolved in corn oil, orally) after 12^{th}, till 20^{th} week.

Group V:Control animals treated with thymoquinone alone as in group III.

The group III and group IV animals were used to study the chemopreventive and chemotherapeutic efficacies of thymoquinone, respectively.

Collection of blood and lung tissue

At the end of the experimental period, the animals were fasted overnight and killed by cervical decapitation. The blood and lung tissues were used for further analyses. Both the samples 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 tissue was prepared by homogenizing the tissue with motor driven teflon coated homogeniser in ice-cold 0.1M Tris-HCl buffer pH 7.4. Dilutions were decided based on the protein concentrations.

Packed cells remaining after the removal of plasma were washed with isotonic saline to remove the buffy coat. Four ml of packed cells were then washed thrice with isotonic Tris-HCl buffer, 0.31 M, pH 7.4. Haemolysis was performed by pipetting out the washed red blood cell suspension into propylene centrifuge tubes which contained hypotonic buffer (Tris-HCl buffer, 0.015M, pH 7.2). Erythrocyte ghosts were sedimented in a high speed refrigerated centrifuge at 20,000xg for 40 minutes. The supernatant haemolysate was decanted carefully and used for further analysis.

Estimation of hematological parameters

Hemoglobin (Hb) content in the blood samples was assessed by cyanmethemoglobin method using Drablin's solution [6]. Red blood cell (RBC) count and white blood cell (WBC) count were determined [7, 8]. Differential count of WBC was carried out with Leishman stained blood smears [6].

Biochemical analysis of Membrane Integrity Markers

Na+, K+-ATPase was estimated by the method of Bonting [9], the activity of Ca2+-ATPase was assayed according to the method of Hjerten and Pan [10], the activity of Mg2+ATPase was assayed by the method of Ohinishi *et al.* [11]. The enzyme activity was expressed as μmoles of phosphorous liberated/min/mg protein under incubation conditions. Total protein of membrane was estimated in an aliquot of diluted membrane extract in Tris-HCl buffer by the method of Lowry *et al.* [12] the inorganic phosphorous was estimated by the method of Fiske and Subbarow [13].

Statistical Analysis

Statistical analysis was performed using SPSS 20 package. Values represent Mean ± SD for six mice in each group and the significance of difference between mean values were determined by one-way analysis of variance (ANOVA) followed by Turkey's multiple comparison test.

RESULTS

Haematological Changes

Table 1 shows the effect of thymoquinone on haematological parameters in serum of control and experimental animals. B(a)P induced lung cancer bearing (Group II) animals showed a significantly ($p<0.001$) decreased levels of haemoglobin, RBC, lymphocytes and monocytes count with increased levels of WBC and neutrophil count when compared to (Group I) animals. These changes were significantly altered in thymoquinone treated group III and group IV animals ($p<0.001$, $p<0.01$ and $p<0.05$) when compared with cancer bearing animals. However, thymoquinone alone treated animals (Group V) did not show any significant changes in their levels when compared with control animals (Group I).

Membrane Integrity Markers

Fig. 1 depicts the effect of thymoquinone on the activities of ATPases in erythrocyte of control and experimental animals. The activities of Na^+/K^+ and Mg^{2+} ATPases were found to be significantly ($p<0.001$) decreased and the activity of Ca^{2+} ATPase was also significantly ($p<0.001$) decreased in cancer bearing group II animals when compared with the group I control animals. This change in the activities of ATPases were significantly ($p<0.001$) reverted in Group III animals and Group IV ($p<0.01$) thymoquinone treated animals to near normal values with no significant difference between the levels of Group V animals and controls.

Table 2 elicit the activities of membrane bound ATPases in the lung of control and experimental animals. The decrease ($p<0.001$) in the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase were seen in cancer bearing group II animals when compared with control animals. While the activities of Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase were significantly increased ($p<0.001$; $p<0.01$) and decreased ($p<0.001$; $p<0.05$) respectively in Group III and Group IV animals treated with thymoquinone when compared with Group II animals. There was no significant difference in the activities of ATPases between Group V animals and drug control animals.

DISCUSSION AND CONCLUSION

Defensive role of thymoquinone on heme indices

Blood is the principal tissue in human body wherever abnormal modification in its parameters indicates the toxic effects of chemicals leading to diseases. In fact, changes in RBCs have been detected in a number of human pathologic conditions or after exposure to xenobiotics displaying oxidative stress. Erythrocytes are permanently in contact with potentially damaging levels of oxygen, but their metabolic activity is capable of reversing this injury under normal conditions, which are equipped by many defense systems representing their antioxidant capacity [14].Further, oxidative stress in red blood cells is an indicator of overall oxidative stress besides RBC-related disorders. Thus, the present study investigates the potential protective and curative effect of TQ in erythrocyte oxidative damage in B(a)P induced lung carcinogenesis.

In our present study, lung cancer bearing animals showed reduction in haemoglobin percentage and RBC count, which is an indication of anemia. It may be assumed that the free radicals resulting from B(a)P metabolism caused liver injury and a proportion of these free radicals were liberated from the liver into the blood and may affect the membranes of circulating red cells. The depression in RBC count and Hb content recorded in the present work could be attributed to disturbed hematopoiesis, destruction of erythrocytes, and reduction in the rate of their formation and/or their enhanced removal from circulation.

The reduction in the values of blood parameters (RBC and Hb) may be attributed to the hyperactivity of bone marrow, which leads to production of red blood cells with impaired integrity that are easily destroyed in the circulation.[15].The complications of hypoxia of all organs and tumor hypoxia are considered as potential therapeutic

problem. Continued hypoxia condition may result in cellular changes leading to a more aggressive tumor phenotype as reflected by accelerated malignant progression, increased potential for local invasiveness and tumor spreading [16].Restoration of Hb contents and RBC count in mice that received TQ after B(a)P induction indicates that TQ might have protected the tissue from hypoxia and reduced the extent of tumorigenesis. White blood cell populations, lymphocytes and neutrophils play a crucial role in the systemic inflammatory response, often observed in cancer patients. Increase in WBC count and alterations in differential count (lymohocytes, monocytes and neutrophils) have been suggested as one of the hallmarks of carcinogenesis. In the current study the lung cancer bearing animals showed elevated WBC count and neutrophil count with reduced lymphocyte and monocyte count, which was in line with the previous findings [17]. Our results agree with the results of Saeed *et al*., who attributed the increase in the WBC count to antioxidant activity of vitamin E and TQ [18]. Similarly, TQ could increase the WBC count, due to its role in free radical scavenging [19]. Lymphocytes play the key role in all immune reactions and are always directed against the specific foreign antigens (toxins).Lymphocytes were significantly decreased in number in response to stressful condition. Additionally, lymphocytes migrate to the site of inflammations which may be resulted due to toxic effect of B (a)P. We observed a significant difference in circulating WBC after TQ treatment and no significant difference was observed between control and TQ alone treated animals.

Membrane stabilising effect of thymoquinone

The membrane bound enzymes play an important role in the maintenance of the ionic gradients between the intracellular and extracellular compartment of the cell. Any disturbance or inactivation of these enzymes can inturn alters the concentration of ions. Changes in ionic concentrations can bring about diverse types of cell injury and ultimate cell death [20].Membrane bound ATPases are the biochemical expressors of specific active transport systems. ATPases characterize the membrane state of constituents and their environment dynamical interactions. Any perturbations in the activity of these enzymes bring about changes in energetics and normal homeostasis. ATPases are lipid dependent membrane bound enzymes and alterations in membrane lipid environment changes the ATPases activity and inturn normal cellular functions.

ATPases are responsible for the transport of Na^+, K^+, Mg^{2+} and Ca^{2+} ions across the cell membranes at the expense of ATP by hydrolysis [21]. Injury to cell membrane by free radicals has been a recent focus since the vital activities of the cell are challenged. The three important ATPases of the plasma membrane are the Mg^{2+} ATPase, Na^+K^+ATPase, and Ca^{2+} ATPase. The regulatory role of Mg^{2+} in normal cells via membrane activity contrasts with loss of its regulatory function in neoplastic cells. The latter statement is based on the observation that lowering the Mg^{2+} concentration in transformed cells either by external Mg^{2+}-deprivation, or by maximizing contact between the cells at very high density normalizes the appearance, the Ca^{2+} concentration and the growth behaviour of transformed cells [22]. Ample evidence had long existed for abnormal membrane properties of transformed cells including their decreased mutual adhesiveness and structural differences visualized on surfaces of normal and carcinomatous cells [23]. Recent molecular studies confirm that membrane alterations play a significant role in the neoplastic phenotype. For example, carcinoembryonic antigen is a tumor marker in 50% of human cancer cases; it functions as an intercellular adhesion molecule and is overexpressed in many human cancers [24, 25].such overexpression changes the adhesive properties of the cells and could reduce their capacity to bind divalent cations [26]. This would account at least partly for the low Ca^{2+} content of neoplastic cells and a higher cytosolic free Mg^{2+} due to its release from the internal surface of the plasma membrane [27, 28]. The normalization of transformed cells by maximized contact at very high population density suggests that their plasma membranes are stabilized by mutual adhesions between the cells which restored the normal cation-binding capacity on both sides of the phospholipid bilayer. Na^+K^+ATPase uses energy derived from the hydrolysis of ATP to keep a high K^+ and a low Na^+ concentration in the cytoplasm which in turn provides the driving force for the net movement of other substances such as Ca^{2+}, aminoacids, and H^+[29].

Decrease in the activity of Na^+K^+ ATPase and Mg^{2+} ATPase occurs during tumor growth, particularly in malignancy. This is well correlated with the current study wherein a similar decrease in the activities were found in cancer bearing animals (group II), which suggests the condition of malignancy and progression of cancer. The decreased activity might also be due to lipid peroxides induced by benzo (a) pyrene which could have altered membrane structure.

Table1: Effect of thymoquinone on hematological parameters in control and experimental animals.

Particulars	Group I (Control)	Group II B(a)P induced	Group III TQ+ B(a)P	Group IV B(a)P+TQ	Group V TQ alone
RBC(10^6 Cells/ml)	6.43±0.31	$3.80±0.29^{a*}$	$5.62±0.25^{b*}$	$4.91±0.28^{b*c@}$	6.61±0.25
Haemoglobin (gm %)	12.22±0.42	$8.27±0.32^{a*}$	$11.15±0.31^{b*}$	$10.87±0.39^{b*cNS}$	12.41±0.38
WBC(10^3 Cells/ml)	5.25±0.36	$11.89±0.72^{a*}$	$7.02±0.30^{b*}$	$8.27±0.42^{b*c@}$	5.27±0.34
Monocytes (%)	0.94 ± 0.06	$0.43 ±0.03^{a*}$	$0.82±0.04^{b*}$	$0.71±0.03^{b\#cNS}$	0.91±0.05
Lymphocytes (%)	68.77±2.94	$27.02±1.88^{a*}$	$60.75±2.14^{b*}$	$54.57±2.01^{b*c@}$	68.81±2.97
Neutrophils (%)	21.70±1.06	$50.42±2.03^{a*}$	$28.65±1.25^{b*}$	$33.62±1.83^{b*c@}$	21.74±1.05

Each value is expressed as mean ± SD for six mice in each group, a - as compared with Group I,b - as compared with Group II, c - as compared with Group III, Statistical significance - $^*p<0.001$, $^\#p<0.01$, $^@p<0.05$, NS - Not significant

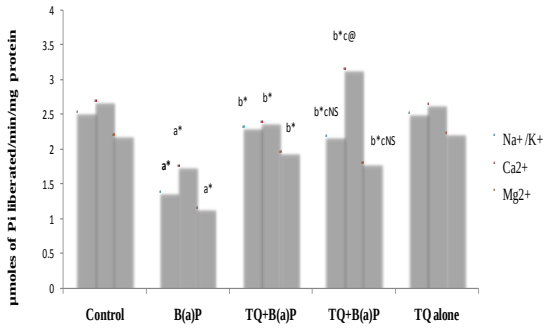
Table 2: Effect of thymoquinone on the activities of ATPases in lung of control and experimental animals

Particulars	Group I (Control)	Group II B(a)P induced	Group III TQ+ B(a)P	Group IV B(a)P+TQ	Group V TQ alone
Na^+K^+ATPase	2.93±0.24	$1.66±0.16^{a*}$	$2.48±0.22^{b*}$	$2.26±0.22^{b\#c@}$	2.89±0.23
Ca^{2+}ATPase	2.62±0.20	$1.61±0.26^{a*}$	$2.11±0.21^{b*}$	$1.99±0.17^{b@cNS}$	2.64±0.22
Mg^{2+}ATPase	2.55±0.24	$1.53±0.14^{a*}$	$2.22±0.18^{b*}$	$1.92±0.14^{b@c@}$	2.49±0.23

Each value is expressed as mean ± SD for six mice in each group. Units: ATPase activities - µmoles of Pi liberated/min/mg protein, a - as compared with Group I; b - as compared with Group II; c - as compared with Group III, Statistical significance - $^*p<0.001$, $^\#p<0.01$, $^@p<0.05$, NS - Not significant

Ca^{2+} ATPase activity was found to be decreased in cancer bearing animals. Free intracellular calcium, acting as a second messenger, is crucial for a diverse range of biological functions. Intracellular calcium signalling is also a key regulator of proliferation, cell cycle progression and apoptosis [30].The plasma membrane Ca^{2+}ATPase (PMCA) or pump belongs to the family of P-Type ATPases and is a critical regulator of free intracellular Ca^{2+}. There are two isoforms of PMCA (PMCA1-4). PMCA alterations are also found to be associated with tumorigenesis [31]. The decrease in the activity of cancer bearing animals suggests that there is a high concentration of Ca^{2+}

inside the cells due to toxicity created by B(a)P, which the calcium pump tries to eliminate, to keep its level low. Subsequently increase in the activity was recorded after treatment with thymoquinone suggesting its protective role. Further Ca^{2+} ATPase activity is mainly impaired due to oxidative modification of thiol groups present in this enzyme which in turn is due to the generation of free radicals [32].More literature evidences showed that TQ protects the cell against ROS under various disease conditions.



Each value is expressed as mean ± SD for six mice in each group, a: as compared with group - I; b: as compared with group - II; c: as compared with group – III, Statistical significance: *p<0.001, #p<0.01, @p<0.05 and NS - Not significant

Fig. 1: Effect of thymoquinone on the activities of membrane bound ATPases in the erythrocyte membrane of control and experimental animals

CONCLUSION

In the current study, significant increase in the activities of these membrane integrity enzymes in TQ treated animals indicate the protective role of thymoquinone in maintaining membrane bound ATPases. From the above results, it can be inferred that TQ possess significant anticancer effect through its role in prevention of erythrocyte membrane damage and restoration of membrane integrity.

CONFLICT OF INTERESTS

Declared None

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