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ORIGINAL ARTICLE

Elucidating the Immunomodulatory Effect of Daidzein in Benzo(a)pyrene -Induced Lung Cancer Mice Model through Modulation of Proliferating Cell Nuclear Antigen, NF-κB, CYP1A1, and NRF

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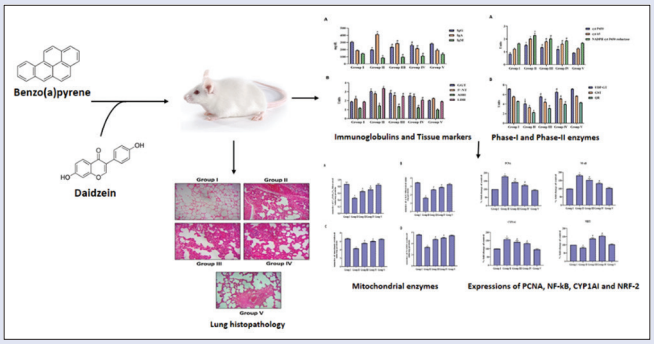
ABSTRACT

Background: Lung cancer is the second most predominant reason for cancer deaths globally. It is estimated to be approximately 30% among the cancer deaths. Daidzein (DAZ) is a polyphenolic compound present commonly in soy-based plants and is proven to have various therapeutic properties. **Objectives:** In this study, we aimed to understand the immunomodulatory activity of DAZ in the mice model with benzo(a)pyrene (B(a)P)-induced lung carcinoma. **Materials and Methods:** The mice were divided into five groups: Group I served was the control group; Group II animals were challenged with B(a)P; Group III animals were treated with DAZ before challenge with B(a)P; Group IV animals were treated with DAZ after challenging the animals with B(a)P; and Group V animals were treated with DAZ alone till the end of the experimental period. Tumor incidence was calculated, and the following parameters were analyzed: Body weight, lung weight, total number of tumors, percentage of inhibition, immunoglobulin (Ig) levels (immunoglobulin G, immunoglobulin A, and immunoglobulin M), key marker enzymes, and proinflammatory cytokines in both treated and normal mice. The lung tissues were analyzed through the histopathological analysis. **Results:** According to our results, all the markers that favor the growth of cancer were increased in the lung cancer group. After the administration of DAZ, all the markers returned to their original levels. **Conclusion:** In conclusion, DAZ protected the cells against the B(a)P-induced inflammatory responses in lung cancer.
Key words: Benzo(a)pyrene, carcinoembryonic antigen, CYP1A1, daidzein, lung cancer, tumor necrosis factor-alpha

SUMMARY

- Lung cancer is known to be one of the most prevalent cancers which cause higher mortality globally
- DAZ initiation in B(a)P-induced lung cancer modulates the antioxidant activities and immune responses of the animals
- DAZ might possess the ability to alter the responses of immune cells, either scavenge or inhibit the formation of reactive oxygen species.



Abbreviations used: DAZ: Daidzein; PAHs: Polycyclic aromatic hydrocarbons; B(a)P: Benzo(a) pyrene; ICDH -Isocitrate dehydrogenase; KDH: α-Ketoglutarate dehydrogenase; SDH: Succinate dehydrogenase; MDH: Malate dehydrogenase.

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INTRODUCTION

Worldwide, lung cancer is known to be one of the leading causes of cancer deaths. Statistical reports describe that approximately 2 lakh new lung cancer cases were found including both male and female, among which approximately more than 1 lakh deaths have been reported in 2020.[1,2] Previous studies have shown that diet plays a major role in initiating the process of cancer.[3,4] It has been reported that soy food helps in minimizing the risk of lung cancer, especially aggressive cancers.[5-7]

The most frequently used therapeutic strategies are surgery, radiotherapy, and chemotherapy. Although modern chemotherapy is effective during the initial days of treatment, the long duration of chemotherapy can lead to chemoresistance, which is the major reason for treatment failure. Therefore, there is an increasing demand to identify novel drugs with minimal risk for the treatment of cancer. Tobacco smoking is the leading

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cause of lung cancer,[8] which is caused mainly due to the presence of polycyclic aromatic hydrocarbons (PAHs).[9] The major component having the carcinogenic property of PAHs is benzo(a)pyrene (B(a)P). It is present in the great quantities in cigarettes.[10] One of the key roles played by B(a)P is the formation of DNA adduct, which in turn leads to the initiation of tumor formation. However, an imbalance in the processes of detoxification and metabolic activation processes increases the risk of cancer in an individual.[11,12] Plant-based compounds initiate various signaling pathways and thus help in inhibiting the damages caused by the carcinogens.[13]

Daidzein (DAZ), also called as 7-hydroxy-3-4-hydroxyphenyl chromen-4-one, is a polyphenolic compound derived from various soy-based plants. Previous studies suggest that DAZ is associated with the isoflavones group shows antioxidative, antidysrhythmic, and anti-inflammatory properties.[14,15] Studies also show that isoflavone minimize the risk of cancer.[16,17] In addition, previous reports state that DAZ induces cell death in different types of cancer cells.[18,19] Many experiments have proven that DAZ inhibits the growth of tumor cells by initiating apoptosis.[20,21] However, to the best of our knowledge, there are no studies conducted to test the beneficial effects of DAZ against lung cancer. Therefore, in this study, we aimed to discover the therapeutic activity of DAZ against the B(a)P-induced lung cancer in mice.

MATERIALS AND METHODS

Animals

In this study, healthy male Swiss albino mice (aged from 6 to 10 weeks) weighing 20–30 g were employed. Animals were purchased from the Institutional Animal Facility and were maintained in a 12 h/12 h light–dark cycle. The temperature and humidity were constantly regulated.

Chemicals

All the fine chemicals including B(a)P (purity: ≥96%), DAZ (purity: ≥98%), and solvents were obtained from Sigma-Aldrich (USA).

Study design

All animals were randomly divided into five groups with six animals in each group. Group I animals were fed with 0.2 mL of corn oil orally for 16 weeks. Group II animals were orally fed with B(a)P (50 mg/kg b. w. dissolved in maize oil) twice a week for 4 weeks, and then were continued with or without the administration of vehicle for another 12 weeks.[22] Group III mice were administered with DAZ (20 mg/kg b. w. dissolved in corn oil orally) daily for 6 weeks and then B(a)P (as in group II) twice a week for 10 weeks. For the postinitiating experiments, animals in Group IV were administered with B(a)P (as in Group II) for 6 weeks and then with DAZ (as in Group III) for 10 weeks. Histopathological analysis validated the lung carcinoma inductions. DAZ (as in Group III) was administered to Group V animals alone for 16 weeks to verify if DAZ caused any cytotoxicity. The chemopreventive potentials of DAZ in experimental animals were studied using the initiation and postinitiation treatment of DAZ.

Body weight, lung weight, and tumor incidence

The body weight (BW) of the animals was recorded throughout the experimental period, once at the start of the trial and then once a week until the experiment was completed. Following the completion of the experiment, the mice were anesthetized using ketamine/xylazine (90/10 mg/kg). Subsequently, the animals were sacrificed, and their lungs removed, cleaned in saline, and weighed. Tumor incidence (TI) was calculated through manual counting.

Estimation of serum immunoglobins G, A, and M (immunoglobulin G, immunoglobulin A, and immunoglobulin M, respectively)

The serum levels of immunoglobulin G (IgG), immunoglobulin M (IgM), and immunoglobulin A (IgA) in the blood samples of control and treated mice were examined by using the previously described procedures.[23,24]

Evaluation of biochemical parameters

The lung homogenate was subjected to the following biochemical analyses: Adenosine deaminase (ADA),[25] aryl hydrocarbon hydroxylase (AHH),[26] gamma glutamyl transferase (GGT),[27] 5′-nucleotidase,[28] and lactate dehydrogenase (LDH).[29]

Phase-I enzymes

NADPH-cytochrome c reductase activity was measured using Wharton and Tzagoloff's technique.[30] Omura and Sato's approach[31] was used to calculate the activity of cytochromes P450 and b5.

Phase II enzymes: Assay for detoxification enzymes

The activity of QR was measured using 2,6-dichlorophenol-indophenol as an electron acceptor.[32] The activity of cytosolic fraction of UDP glucuronyltransferase Uridine 5'-diphospho-glucuronosyltransferase (UDP-GT) was evaluated using *p*-nitrophenol.[33] The glutathione S-transferase (GST) activity was measured using the protocol described by Habig *et al*.[34]

Evaluation of carcinoembryonic antigen and CK-19 fragment (CYFRA 21-1)

CYFRA 21-1 and carcinoembryonic antigen (CEA) were quantified in the serum samples of normal and treated animals using a chemiluminescent immunoassay on a SIEMENS ADVIA Centaur (Bayer, USA).[35]

Analysis of proinflammatory cytokines

In this study, we measured the level of interleukin (IL) 1 β, IL-6, and tumor necrosis factor alpha (TNF-α) in the cancer tissues. Briefly, tissue homogenate (10%) of the tumor tissue was prepared in phosphate-buffered saline (0.01 M, pH 7.4), and the supernatant was centrifuged for 20 min at 10,000 g. The supernatant was used to quantify the levels of IL1, IL6, and TNF-α (USA).

Biochemical analysis

Lung tissues were removed, weighed, and homogenized in 0.1 M Tris–HCl buffer (pH 7.4). The method described by Johnson and Lardy[36] was used to isolate the mitochondria from the lungs and liver. The total protein content in serum and tissue samples was quantified by the method of Lowry *et al*.[37]

Evaluation of mitochondrial enzymes

The level of mitochondrial enzymes, namely, isocitrate dehydrogenase (ICDH),[29] α-ketoglutarate dehydrogenase (KDH),[38] succinate dehydrogenase (SDH),[39] and malate dehydrogenase (MDH) (L-malate: NAD oxidoreductase)[40] was measured.

Electron transport chain complex assay

The activities of electron transport chain complexes I II,[41] III,[42] and IV,[43] were measured as described earlier. The method of Lowry *et al*.[37] was used to estimate the protein content.

Adenosine-5-triphosphate measurements in mice

Adenosine-5-triphosphate (ATP) levels in mice were analyzed using the ATP lite assay kit (Perkin Elmer) immediately after the collection of serum. The assay was conducted as per the manufacturer's instructions. As previously mentioned, (Cicko *et al.*, 2010),[44] the cell lysis phase was avoided to evade the contamination of intracellular ATP.

RNA isolation and reverse transcriptase quantitative polymerase chain reaction assay

For the expression analysis of proliferating cell nuclear antigen (PCNA), NF-κB, CYP1A1, and NRF2, and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) were used. The RNA was extracted from the tumor tissues with the help of RNA kit, and the assay was conducted based on the manufacturer's protocol. Spectrophotometer was used to determine the total RNA content (Jasco V-700, Japan). We followed the manufacturer's instructions to synthesize cDNA from 2 µg total RNA using the QuantiNova Reverse Transcription Kit (Qiagen, USA). The SYBR green PCR Master Mix (Qiagen, USA) was used to perform qPCR in a Rotor Gene Q 5Plex HRM equipment (Qiagen, USA). The control gene was the actin gene. GenBank was used to create gene-specific primers (NCBI, Bethesda, MD, USA). PCR results were quantified using the ΔCq method.[45]

Histopathological analysis

The lungs of the experimental animals were fixed in 10% formalin and processed for paraffin block production. Hematoxylin and eosin staining was performed on paraffin slices with a thickness of 5 µm. In a microscope (Olympus, Tokyo, Japan), the slides were seen and photographed at ×40 magnification.[46]

Statistical analysis

Data were analyzed using the SPSS software version 19 (SPSS Inc., IBM Corp, NY, USA). We performed the one-way analysis of variance by Tukey's *pos hoc* assay to find out the level of significance. The results were displayed as mean ± standard deviation of 6 mice. For significance, $P < 0.05$ and $P < 0.01$ were considered statistically significant.

RESULTS

Impact of daidzein on body weight and lung weight with tumor incidence

Table 1 shows the data on the general parameters including BW, lung weight, and TI. In comparison with all the groups, Group II mice demonstrated low BW. The mice which were supplemented as preinitiation with DAZ and B(a)P (Group III) had a higher impact on BW related to mice which were supplemented as postinitiation with DAZ and B(a)P (Group IV). Group II mice demonstrated a significantly increased lung weight followed by the Group III and Group IV mice. When compared with the control group, mice in all the treated groups showed an elevation in the lung weight. The TI rate was negligible in both Groups I and V. The TI was significantly high in Group II. There was a significant decrease in the TI in both the DAZ initiation with B(a)P groups.

Impact of daidzein on Ig levels

Figure 1a shows the expression levels of IgG, IgA, and IgM in both control and treated groups. There was a significant decrease in the levels of IgG and IgM in the treated animals when compared to the control animals. The level of IgA was remarkably ($P < 0.05$) increased in B(a)P-challenged mice. DAZ remarkably increased the levels of IgG and IgM and decreased the levels of IgA in the treated mice. Group V mice did not exhibit any changes in the Ig levels compared with control animals.

Impact of daidzein on tissue marker enzymes and xenobiotic agents

Figure 1b shows the results obtained for tissue marker enzymes and xenobiotics (GGT, 5′NT, AHH, LDH) analyzed in the samples obtained from both treated and control animals. All the enzymes were markedly ($P < 0.05$) augmented in the B(a)P-induced animals in comparison to control group animals. This significant increase might be because of the damage caused to the lungs which increased TI. DAZ supplemented mice showed a reduction in the enzyme activity. Moreover, we did not find variations in the mice that were treated with DAZ alone and control animals.

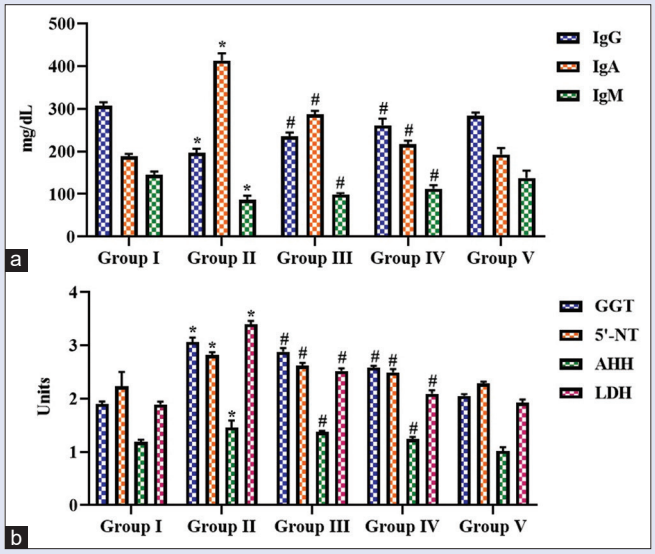

Figure 1: Effect of Daidzein on immunoglobulin levels and tissue maker enzymes in the serum of BaP-induced experimental animals. (a) Immunoglobulin levels (b) tissue marker enzymes. Results are expressed as mean ± standard deviation for six mice in each group. Statistical significance at $P < 0.05$. * indicates compared with control group (Group I); # indicates compared with Group II

Table 1: Effect of daidzein on body weight, lung weight, and tumor incidence in benzo(a)pyrene induced experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
Body weight (g)	41.18±11.61	28.25±6.52	33.03±8.92	36.35±9.37	43.53±13.56
Lung weight (mg)	294.74±88.73	358.07±112.5*	333.91±110.41	315.89±98.5#	300.10±94.39#
Tumor incidence	0	6±0.47*	0	2±0.07#	4±0.38#
Total number of tumor (per mice)	0	7±0.53*	0	1±0.03#	2±0.27#
Inhibition (%)	0	0	0	80±19.38#	60±21.87#

*Indicates compared with control group (Group I); #Indicates compared with Group II. Statistical significance at $P<0.05$. Results are expressed as mean±SD for six mice in each group. SD: Standard deviation

Impact of daidzein on Phase I and II enzymes in the lungs

Figure 2a and b depicts the expression level of Phase I and II enzymes in the lungs of the control and treated mice. The expression levels of cytochromes P450 and b5, NADPH, and cytochrome P450 reductase were found to be ($P < 0.05$) increased in the B(a)P-treated animals, and their levels were decreased upon treatment with DAZ. Other enzymes such as UDP glucuronosyltransferase (UDP-GT), GST, and quinone reductase (QR), the expression levels were significantly decreased in the B(a)P-induced mice. DAZ elevated the expressions of these enzymes in the treated animals. Both the control groups and DAZ alone treated groups did not show any deviations in both the phase of the enzymes.

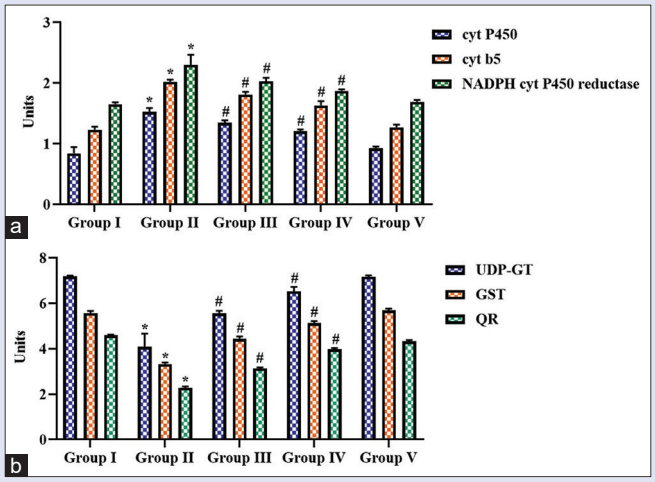

Figure 2: Effect of Daidzein on phase I and phase II enzymes in the lung of BaP-induced experimental animals. (a) Phase I enzymes (b) Phase II enzymes. Results are expressed as mean ± standard deviation for six mice in each group. Statistical significance at $P < 0.05$. * indicates compared with the control group (Group I); # indicates compared with Group II

Impact of daidzein on tumor markers and cytokines in serum

Figure 3a and b shows the results obtained with respect to tumor markers such as CEA and CYFRA 21-1. The expression of both these markers was significantly ($P < 0.05$) upregulated in B(a)P-challenged mice. Upon pre- and postadministration with DAZ, the expression levels were downregulated in the serum samples. In addition, the postinitiation group with DAZ showed decreased expression compared with preinitiation group with DAZ. Figure 3c shows the data for the estimation of TNF-α, IL-6, and IL-1β in all the groups. Our results show that the expression levels were significantly upregulated in the B(a)P-challenged mice. Furthermore, upon DAZ initiation, the level of all the aforementioned cytokines was decreased. No changes were found in the control and DAZ alone supplemented mice.

Effect of daidzein on mitochondrial enzymes in the lungs

Figure 4a-d shows the expression levels of the key mitochondrial enzymes: KDH, ICDH, SDH, and MDH, which were estimated in the lungs of the treated and control animals. According to the results, the mitochondrial enzymes in the B(a)P-challenged animals were found to be significantly downregulated ($P < 0.05$). According to our results, DAZ increased the activity of these mitochondrial enzymes. We also found that no changes were noted in both control and DAZ alone treated mice.

Impact of daidzein on electron transport chain complex and adenosine-5-triphosphate levels in the lungs

Figure 5a shows the effect of DAZ in modulating the electron chain transport chain complex, and Figure 5b shows the ATP status in the lungs of the animals. According to our results, all four complexes (I, II, III, and IV) and ATP levels were significantly ($P < 0.05$) downregulated in the B(a)P-treated animals. Pre- and postinitiation of DAZ showed an upregulation of all the complexes and ATP levels. The complexes and ATP levels were not changed in the control and DAZ alone supplemented mice.

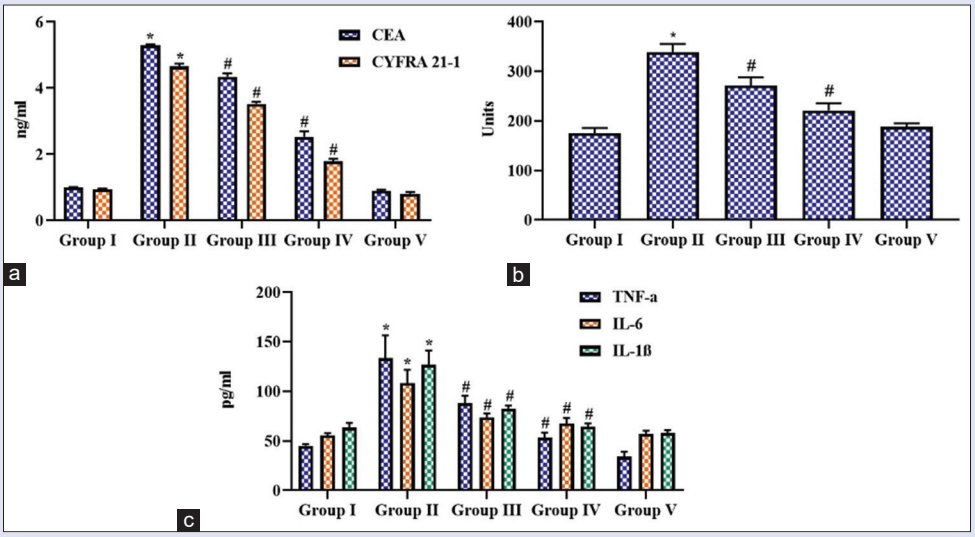

Figure 3: Effect of Daidzein on the levels of tumor markers and cytokines in the serum of BaP induced experimental animals. (a) tumor markers CEA and CYFRA21; (b) tissue maker enzymes adenosine deaminase; (c) pro inflammatory cytokines. The results are expressed as mean ± standard deviation for six mice in each group. Statistical significance at $P < 0.05$. * indicates compared with control group (Group I); # indicates compared with Group II

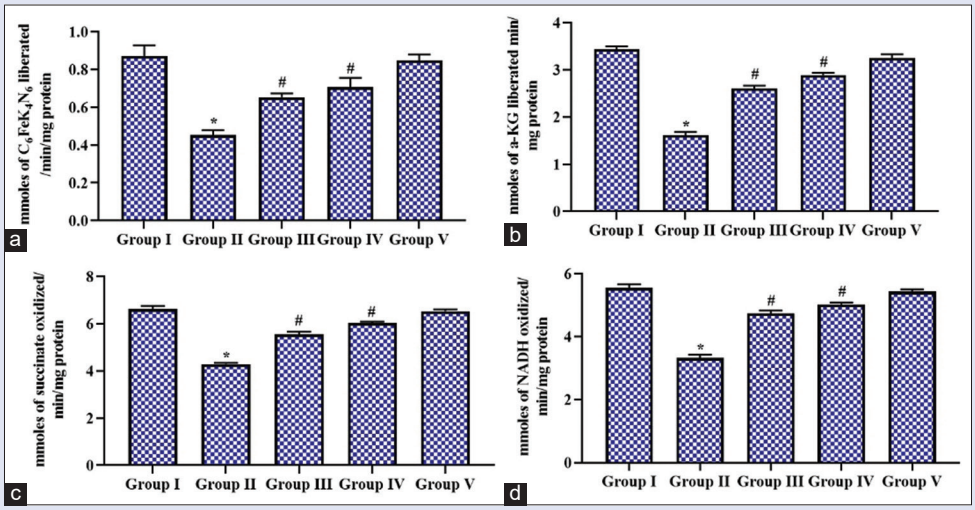

Figure 4: Effect of Daidzein on the activity of mitochondrial enzymes in the lung of BaP-induced experimental animals. (a) KDH, (b) ICDH, (c) SDH; (d) MDH Results are expressed as mean ± S.D. for six mice in each group. Statistical significance at $P < 0.05$. * indicates compared with control group (Gr standard deviation oup I); # indicates compared with Group II.

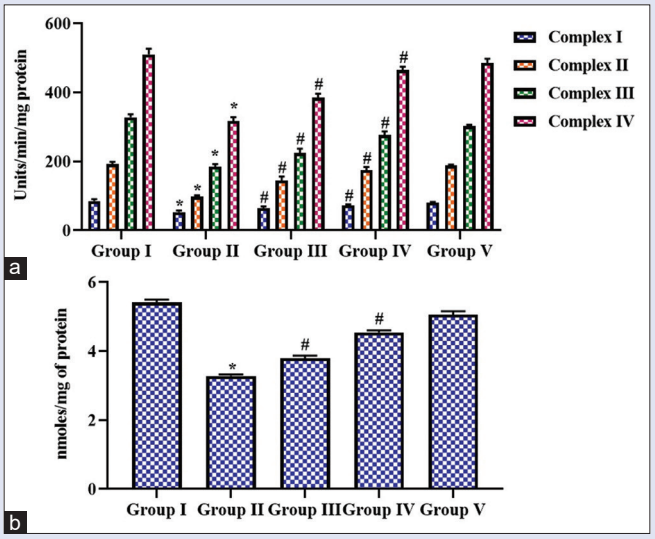

Figure 5: Effect of Daidzein on the activity of electron transport chain complex of the lung in the BaP-induced experimental animals. (a) electron transport chain complex; (b) The levels of ATP in the lung mitochondria. The results are expressed as mean ± standard deviation for six mice in each group. Statistical significance at $P < 0.05$. * indicates compared with the control group (Group I); # indicates compared with Group II

Effect of proliferating cell nuclear antigen, NF-κB, CYP1A1, and NRF2 in lungs

Figure 7 represents the levels of the key markers including PCNA, NF-κB, CYP1A1, and NRF2 in the lungs of the animals using RT-qPCR analysis. According to the results, the expression levels of markers such as PCNA, NF-κB, and CYP1A1 were significantly upregulated and NRF2 status were significantly downregulated ($P < 0.05$) in the B(a)P-challenged mice. The expression levels of these markers were modulated in pre- and postinitiation of DAZ in the animals. All the four markers did not show any change in their levels in control and DAZ alone treated animals.

Effect of daidzein in histological changes in the lung

Figure 6 shows the effect of DAZ in histological findings of the lung from control and treated animals. It is observed that control and DAZ alone treated group showed normal histological structures with small nuclei. The B(a)P-induced animals showed lesions exerting proliferations with focal bronchial and hyperplasia in alveolar epithelium. The animals which had pre- and postinitiation of DAZ showed reduced injury in the alveolar region with near-normal arrangements.

DISCUSSION

Most of the lung-related problems are caused due to tobacco smoking.[47] In this study, we aimed to understand the immunomodulatory effect of DAZ in the B(a)P-provoked animals. B(a)P-activated mice develop lung cancer in the treated animals. Previous studies suggest that B(a)P induction leads to loss in BW, and it further enhances the process of carcinogenesis in pulmonary region which is known to be the common sign of tumorigenesis process. One of the major reasons for this weight loss is tumor anorexia cachexia, which is the key player in the inhibition of skeletal muscle and adipose tissue of the animals.[48,49] Another key factor for this reduction in BW is the higher occurrence of inflammatory nodules. In our study, we found that postinitiation of DAZ helped in maintaining the BW by arresting the TI. We observed that the number of tumors were significantly lower in the DAZ postinitiation group. These results demonstrate that DAZ possess a strong defensive ability and has the potential to arrest the proliferation, tumor growth, and inflammation.

Recent studies suggest that immunomodulation is an efficient alternative strategy for the cancer treatment, which can be achieved by using the bioactive components from plant origin or synthetic chemicals.[50] Our results show that the expression level of IgG and IgM was diminished in cancer, which is a clear indicator of low humoral immune response. These Ig play a key role in neutralizing the toxins, complement activation, and opsonization.[51] The levels of the aforementioned Ig in the serum of patients with cancer are known to be expressed diversely. Studies suggest that factors involved in the hepatic injury may lead to the IgA leakage in serum samples.[52] B(a)P showed immunosuppressive activity in the treated animals and thereby compromising immune system of the

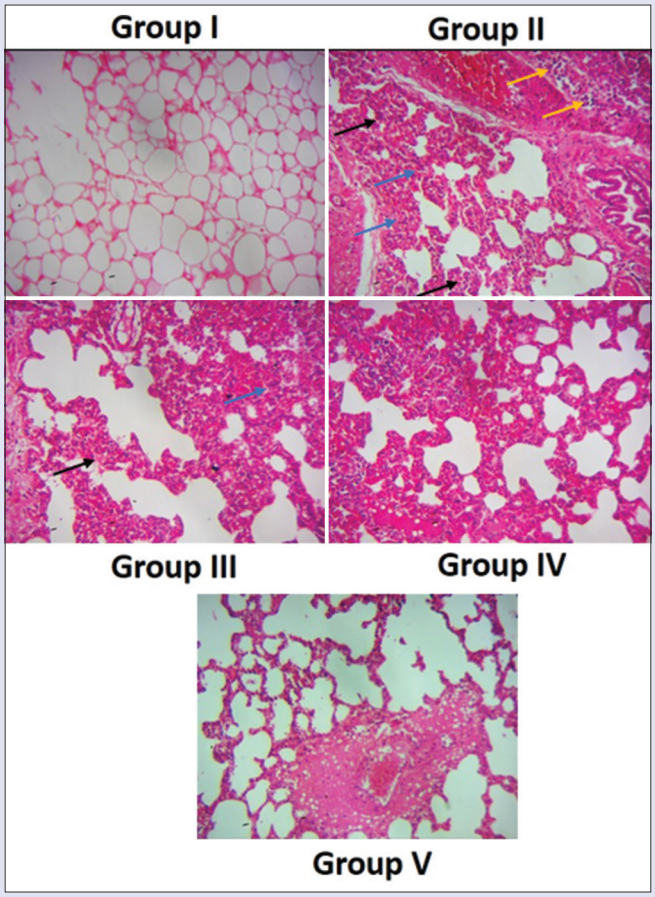

Figure 6: Effect of Daidzein on the histopathological changes of the lung on BaP-induced experimental animals. Control (Group I) and Daidzein alone (Group V) treated group showed normal histomorphological structures without changes. The B(a)P treated animals (Group II) showed lesions (black arrows) exerting abnormal proliferations (blue arrows) with focal bronchial and hyperplasia (yellow arrows) in alveolar epithelium. The animals pre- and postinitiation of Daidzein (Group III and IV) showed diminished injury in the alveolar region with near normal arrangements

animals. After treatment with DAZ, the levels of IgM and IgG were found to be higher and IgA levels were lower than that of control animals. This shows that DAZ possess ameliorative effect.

AHH is known to be cytochrome P-450-dependent tumor-activating enzyme and a key marker for diagnosing the lung cancer. Its activity and expression level are activated by PAHs.[53] A previous study showed that AHH levels were higher in both serum and tissues of B(a)P-provoked animals.[54] LDH is known to be a promising prognostic marker. Previous studies report that there is an increase in the level of LDH in the serum samples obtained from patients with cancer.[55] It plays a key role in the glycolytic pathway, which helps in the production of energy for the tumor to grow.[56] It is also important for tumor survival and growth.[57] Other important tumor-specific markers such as GGT, 5′-NT, and ADA are known to have ability in predicting the process of tumorigenesis. In this study, we found an increase in the cytochromes P450 and b5, NADPH, cytochrome P450 reductase, AHH, LDH, GGT, 5′-NT, and ADA levels in B(a)P-provoked mice, and their levels decreased after DAZ administration.

In general, the level of GST in plasma was elevated due to the hepatic injury and leakage of the cytosolic enzymes that is excreted into the extracellular region.[58-61] Another important phase II enzyme called UDPGTs is distributed broadly in both hepatic and extrahepatic injury.[62,63] In this study, we found that these phase II enzymes were restored to their normal levels after DAZ initiation in the treated animals.

There several studies conducted on the inflammatory markers such as CEA, CYFRA 21-1, TNF-α, IL-6, and IL-1β with respect to the development and progression of cancer.[64,65] In this study, DAZ reduced the levels of these aforementioned markers close to normal, which shows the promising immunomodulatory effects of DAZ.

Enzymes having thiol groups act as receptor sites for the carcinogen to bind, which causes imbalance in the cellular activities.[65] In this study, we found that the enzymes of Trichloroacetic acid (TCA) cycle such as ICDH, MDH, KDH, and SDH were diminished in a B(a)P-challenged mice. The main reason behind this is the changes in the cell morphology and mitochondrial changes happening in the cancer cells.[66] DAZ can balance this situation by increasing the levels of antioxidants.

The electron transport system is known to be the important endogenous source of reactive oxygen species (ROS).[67] ROS has the potential to affect various cellular process which in turn can lead to changes that compromise the integrity and function of normal cells leading to induction of cancer.[68,69] Our results confirm that DAZ protected the key TCA cycle enzymes and electron transport complexes, thereby the ATP levels were also increased with DAZ initiation.

During the process of tumorigenesis, various transcription factors either get induced or suppressed. NF-κB is the most studied target as it is present in each and every cell performing key functions.[70] Previous studies have shown that B(a)P is functionally activated by CYP1A1 which eventually leads to the formation of DNA adducts, which further increases cancer formation.[71] According to the literature, PCNA is a 33 kDa nuclear protein and is a key player in the proliferation and progression of cancer cells. Our results show that all the key markers were elevated in B(a)P-treated animals which suggest its protective role against cancer conditions. The data from the histopathological analysis of the lung tissue strongly suggest that DAZ initiation reduces the induction of the tumor and ameliorates the factors involved with immuneregulatory action, which might be helpful in the management of lung cancer.

CONCLUSION

In conclusion, B(a)P is responsible for the changes in the tissue and serum of the animals with lung cancer. According to our results, it is evident that DAZ initiation in B(a)P-activated lung cancer modulates the antioxidant activities and immune responses of the animals. DAZ might alter the responses of immune cells, either by scavenging or inhibiting the formation of ROS. Overall DAZ possess an immunomodulatory action in B(a)P-induced lung cancer.

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Conflicts of interest

There are no conflicts of interest.

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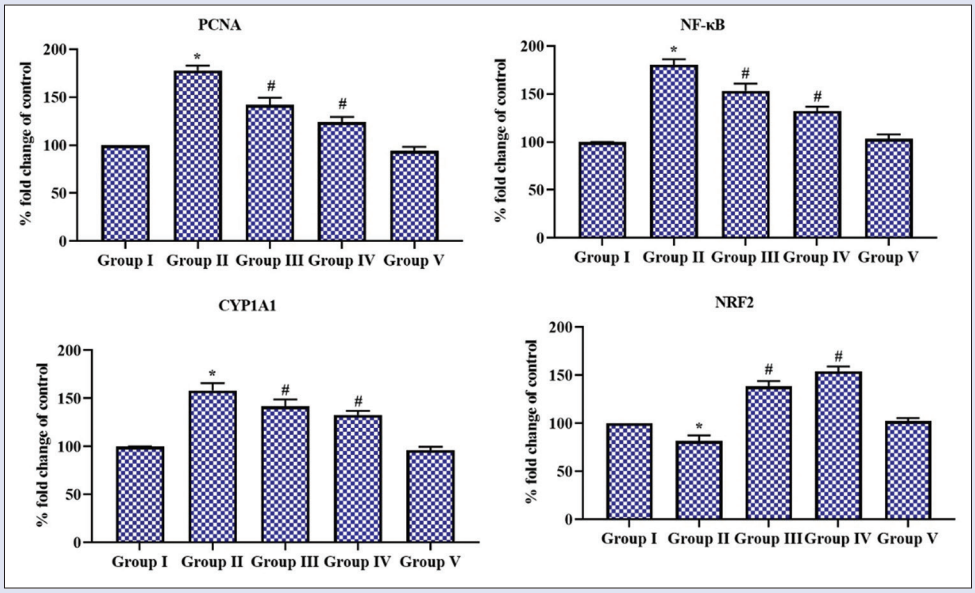

Figure 7: Effects of Daidzein on proliferating cell nuclear antigen, NF-kB, CYP1AI and NRF-2 mRNA gene expression in benzo(a)pyrene-induced lung cancer. Results are expressed as mean ± standard deviation for six mice in each group. Statistical significance at $P < 0.05$. * indicates compared with control group (Group I); # indicates compared with Group II

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