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Combination therapy of metformin and atorvastatin against benzopyrene-induced lung cancer *via* inflammatory signaling pathway

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Abstract. The most prevalent cause of lung cancer is smoking tobacco, but exposure to second hand smoke, air pollution, and certain chemicals and substances at work can also raise the risk of disease. In this study, we scrutinized the chemoprotective effect of the metformin and atorvastatin combination against benzo[a]pyrene (BaP)-induced lung cancer in mice of Swiss albino. BaP (50 mg/kg) was used for induction of lung cancer and mice were treated with metformin, atorvastatin or their combination. Metformin + atorvastatin combination significantly ($p < 0.001$) improved the body weight, liver weight, suppressed the lung weight and tumor incidence and altered the levels of immunocompetent cells, polyamines, lung tumor markers, lung parameters and antioxidant parameters, respectively. Metformin + atorvastatin combination also suppressed cytokines levels, inflammatory parameters and caspase parameters. On the basis of the results, we can conclude that metformin + atorvastatin combination remarkably suppressed lung cancer *via* the inflammatory pathway.

Key words: Metformin — Atorvastatin — Lung cancer — Polyamine — Inflammation

Abbreviations: 5'NT, 5'-nucleotidase; BPDE, benzo(a)pyrene-7,8-diol-9,10-epoxide; BaP, benzopyrene; CEA, carcinoembryonic antigen; CAT, catalase; CAV1, caveolin-1; CEA, carcinoembryonic antigen; CP, cisplatin; COX2, cyclooxygenase-2; EGFR, cpidermal growth factor receptor; GLUT3, glucose transporter 3; GPx, glutathione peroxidase; GSH, reduced glutathione; IL, interleukin; LDH, lactate dehydrogenase; MMPs, matrix metalloproteinases; NAD+, nicotinamide adenine dinucleotide; NF-κB, nuclear kappa B factor; NSCLC, non-small cell lung cancer; NSE, neuron-specific enolase; PGE_2, prostaglandin; SOD, superoxide dismutase; TKIs, tyrosine kinase inhibitors; TNF-α, tumor necrosis factor α.

Introduction

Respiratory diseases constitute leading causes of mortality globally, encompassing nations such as the USA, India, China, and Saudi Arabia (Barta et al. 2019). Tobacco smoking stands as a primary contributor to the development of lung-related pathologies. According to the World Health Organization (WHO), 1 in 10 deaths worldwide is caused by tobacco use, and each year almost 7 million deaths occur. If these consumption patterns remain the same, by 2030, al-

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most 8 million people will die from this habit (Behera 2012; Mao et al. 2016; Xia et al. 2022).

Non-small cell lung cancer (NSCLC) is often diagnosed at an advanced stage, and despite the available treatments such as chemotherapy, surgery, and radiotherapy, the survival rate for patients with advanced NSCLC remains poor (Ruiz-Cordero and Devine 2020; Wu and Lin 2022). The development of resistance to chemotherapy is a major challenge in the treatment of NSCLC (Liu et al. 2016). One approach to addressing this challenge is to develop new targeted therapies that are more specific to the molecular characteristics of the cancer cells. An illustrative instance is the discovery of mutations within the epidermal growth factor receptor (EGFR) gene, which has subsequently paved the way for the creation of EGFR tyrosine kinase inhibitors (TKIs). These inhibitors have demonstrated effectiveness in individuals afflicted by NSCLC characterized by EGFR mutations (Noor et al. 2020; Ye et al. 2021). Another approach is to combine different treatments to increase their effectiveness. For example, combining chemotherapy with immunotherapy has shown promising results in treating advanced NSCLC (Guo et al. 2022). Additionally, advances in precision medicine and the use of biomarkers to guide treatment decisions are also improving outcomes for NSCLC patients (Ruiz-Cordero and Devine 2020; Wu and Lin 2022). Prevention is also an important aspect of reducing the incidence of lung cancer. Strategies to reduce smoking rates, promote healthy lifestyles, and minimize exposure to environmental factors such as air pollution and radon can help prevent lung cancer. Resistance to chemotherapy and metastasis are major challenges in the treatment of cancer, and they can lead to treatment failure and poor outcomes for patients (Zappa and Mousa 2016; Ye et al. 2021). Early diagnosis is crucial for successful treatment, as it allows for earlier intervention when the cancer is more responsive to treatment and may also reduce the risk of metastasis. In addition to early diagnosis, developing new therapies that are more effective and have fewer side effects is also important. Targeted therapies that are designed to specifically target the molecular characteristics of cancer cells are showing promising results in many types of cancer. Immunotherapy, a strategy that harnesses the immune system's capabilities to combat cancer, has exhibited substantial advantages in treating specific cancer types (Noor et al. 2020; Chhouri et al. 2023; Xiao et al. 2023).

Inflammation is a normal and necessary response of the immune system to injury, infection, or tissue damage (Conway et al. 2016). It involves a complex series of events that bring immune cells, such as macrophages and neutrophils, to the site of injury or infection, where they release pro-inflammatory mediators such as cytokines, chemokines, and prostaglandins (Conway et al. 2016; Tan et al. 2021). Nonetheless, should the inflammatory response become chronic and unregulated, it has the potential to induce tissue damage and play a role in the onset and advancement of various chronic conditions. These encompass autoimmune diseases, metabolic disorders like obesity and type 2 diabetes, cardiovascular ailments, and specific forms of cancer (Nasim et al. 2019; Bade and Dela Cruz 2020). Infections, environmental toxins, stress, and lifestyle factors like a poor diet and insufficient exercise are just a few of the causes of chronic inflammation. Managing chronic inflammation is an important strategy for preventing and treating many chronic diseases (Mao et al. 2016; Ruiz-Cordero and Devine 2020). This can be achieved through lifestyle changes such as adopting a healthy diet and regular exercise, reducing stress, avoiding exposure to environmental toxins, and getting adequate sleep. In some cases, medications or other interventions may also be necessary to control inflammation and prevent further damage to tissues and organs (Magesh et al. 2009; Barta et al. 2019a).

Benzo[a]pyrene (BaP) is a potent carcinogen that has been shown to induce lung cancer in both animal and human studies (Du et al. 2021). Inhaled cigarette smoke is a major source of BaP exposure, and smokers have been found to have higher levels of BaP in their lungs than nonsmokers. It is estimated that smoking is responsible for approximately 85% of all lung cancers, with BaP being one of the key carcinogens in tobacco smoke (Velli et al. 2019; Yang et al. 2020). Smoking also damages the respiratory system and can lead to a variety of other health problems, including chronic obstructive pulmonary disease (COPD), emphysema, bronchitis, and asthma. Other sources of BaP exposure include environmental pollution, such as exhaust from diesel engines and industrial emissions. However, the level of exposure to BaP from these sources is typically much lower than from smoking. When BaP is metabolized in the body, it forms a reactive metabolite called benzo(a) pyrene-7,8-diol-9,10-epoxide (BPDE), which can bind to DNA and cause mutations that can contribute to the development of cancer (Anandakumar et al. 2012; Velli et al. 2019; Yang et al. 2020). During BaP induced lung cancer, an increased level of ROS can cause damage to DNA and other cellular components, leading to mutations that can contribute to the development of cancer. In addition to lung cancer, smoking is also a risk factor for many other types of cancer, including cancers of the mouth, throat, bladder, pancreas, and kidney (Velli et al. 2019; Islam et al. 2022; Nithya et al. 2023).

Atorvastatin falls within the category of medications known as statins. Its primary purpose lies in diminishing cholesterol levels within the bloodstream, subsequently diminishing the likelihood of cardiovascular incidents such as heart attacks and strokes (Du et al. 2021). Atorvastatin is

a lipid-lowering medication that has been found to have potential anticancer properties in different cell lines, including melanoma cells, myeloma cells, and B and T lymphoblastoid cell lines. Atorvastatin has also been shown to reduce the viability of myeloma cells and B and T lymphoblastoid cell lines *in vitro*. It is thought that this effect may be due to the ability of atorvastatin to induce apoptosis, or programmed cell death, in these cell lines (Vlachopoulos et al. 2007; Bełtowski et al. 2011; Du et al. 2021). Atorvastatin has been found to target the CAV1-GLUT3 signaling pathway in NSCLC. Caveolin-1 (CAV1) is a protein that plays a role in cellular processes, including signal transduction. Glucose transporter 3 (GLUT3) is responsible for the uptake of glucose into cells. Studies have suggested that atorvastatin may influence this pathway, potentially affecting glucose metabolism in cancer cells. This could have implications for cancer growth and survival, as cancer cells often rely on increased glucose uptake for their energy needs. TIMP-1 is a protein that plays a role in controlling the activity of matrix metalloproteinases (MMPs), enzymes that are involved in tissue remodeling and extracellular matrix degradation. In the context of NSCLC, studies suggest that upregulation of TIMP-1 might influence the sensitivity of cancer cells to certain treatments, such as chemotherapy with cisplatin. Modulation of TIMP-1 levels could impact the tumor microenvironment and potentially affect the response to therapy.

Metformin is a widely prescribed medication employed in the management of type 2 diabetes. Notably, emerging research has indicated that metformin might possess protective attributes against cancer, marking a noteworthy development in recent years (Afzal et al. 2012). There are several ways in which metformin may exert its protective effects against cancer. Metformin exhibits the ability to regulate cellular metabolism. Cancer cells often have an altered metabolism compared to normal cells, and metformin has been shown to inhibit certain metabolic pathways that are important for cancer cell growth and survival (Sun et al. 2020). Furthermore, metformin has demonstrated anti-inflammatory properties that hold the potential to mitigate the persistent inflammation implicated in cancer genesis. Epidemiological investigations have yielded indications of metformin's safeguarding influence against a spectrum of cancers, encompassing breast, colon, and prostate cancers (Alimova et al. 2009; Afzal et al. 2012; Sun et al. 2020). However, the evidence is not yet conclusive, and more research is needed to better understand the potential benefits of metformin in cancer prevention and treatment. In this experimental study, we try to explore the chemoprotective effect of atorvastatin and metformin against BaP-induced lung cancer and the possible mechanisms.

Material and Methods

Animals

Swiss albino mice (sex: male; weight: 20 ± 5 g aged 7–8 weeks) were used in this experimental study. The mice were kept in the standard laboratory conditions (22 ± 5°C temperature; 65% relative humidity; 12/12 h dark/light cycle). The mice received the standard diet and water *ad libitum*. The whole study was approved by the institutional animal ethics committee.

Experimental protocol

After acclimatization, the mice were divided into 5 different groups (n = 6 in each). 1. Control group, control normal mice received corn oil (1 ml); 2. LC group, lung cancer group, mice received BaP (50 mg/kg) in corn oil *via* oral gavage (Wang et al. 2021); 3. LC+Met group, LC mice received the oral administration of 5 mg/kg metformin (Afzal et al. 2012); 4. LC+Ator group, LC mice received the oral administration of 2.5 mg/kg atorvastin (Du et al. 2021); 5. LC+Met+Ator group, LC mice received the oral administration of 5 mg/kg metformin and 2.5 mg/kg atorvastin. The mice received the oral administration of metformin and atorvastin for 6 weeks. The body weight of all group mice was estimated at regular time (initial weight and final weight).

Upon the culmination of the experimental study, blood samples from all groups of mice were collected by puncturing the retro-orbital plexus. These samples were subsequently stored in anticoagulant test tubes to facilitate the assessment of immunocompetent cells. Additionally, coagulated blood was taken and used to estimate the level of cytokines and immunoglobulins (IgG, IgA, and IgM). Subsequently, the mice were euthanized *via* cervical dislocation. Swiftly thereafter, lung tissues were extracted and thoroughly rinsed with an ice-cold saline solution. These tissues were then gently dried on filter paper and subjected to re-weighing. The lung tissues underwent homogenization utilizing a potassium phosphate buffer (0.1 M) and were subsequently centrifuged at 5000 rpm for 10 min to facilitate serum separation for the determination of biochemical parameters. The isolated serum was diligently stored at −80°C for subsequent utilization.

Lung marker enzymes

5'-nucleotidase (5'-NT)

5'-NT was identified using a previously described technique (Sotil and Jensen 2004; Du et al. 2021). Using glycerophosphate as the substrate, non-specific phosphatase activity was

subtracted to get the serum 5'-NT activity. The serum sample was mixed properly with 0.3 ml water and buffer substrate (pH = 9.3) and incubated for 2 h at 37°C. The test tube was successfully removed upon incubation, and trichloroacetic acid (30%) was added. The mixture was left for 2 min before filtering and left for 15 min for developing the color and the final absorbance was measured at 660 nm.

Aryl hydrocarbon hydroxylase

The previously reported procedure was employed to estimate aryl hydrocarbon hydroxylase (Du et al. 2021)**.** The serum sample was mixed with the NAD (0.43 mM), glucose-6-phosphate (2.5 mM), NADP (0.37 mM), glucose-6-phosphate dehydrogenase (1 IU/ml), bovine serum albumin (0.8 mg/ml), Tris buffer (50 mM), $MgCl_2$ (5 mM) and BaP (0.08 mM) were mixed in the acetone (20 µl) and incubated at room temperature.

Adenosine deaminase

Spectrophotometry method was used for the determination of adenosine deaminase. In brief, the serum sample was combined with phosphate buffer (pH) and incubated for 15 min at 37°C. Using a spectrophotometry approach, the absorbance at 265 nm was determined to determine the conversion of adenosine into inosine (Du et al. 2021).

Lactate dehydrogenase (LDH)

Based on its capacity to convert lactate to pyruvate in the presence of the coenzyme nicotinamide adenine dinucleotide (NAD+), LDH activity was measured in units *per* litre (U/l) of serum (Rajendran et al. 2008; Kamaraj et al. 2009).

Determination of hexosamine and hexose

Briefly, the weighted amount of defatted tissues was added to the HCl (4 M) solution in 2 ml and refluxed for 4 h at 100°C in a test tube, which was completely closed with the suitable marble lid. Sodium hydroxide was used to neutralise the hydrolysate. The neutralised samples were divided into aliquots for examination. The level of hexose was calculated using the previously reported method (Niebes 1972). The level of hexosamine was determined using the previously reported method with minor modification (Wagner 1979). To release sialic acid connected to the proteins, the weighed quantity of defatted tissues was hydrolysed with 1.0 ml of 0.1 M sulfuric acid at 80°C for 60 min. Sodium hydroxide was used to neutralise the solution. The method of using the sialic acid level was determined (Warren 1963).

Polyamines determination

The previously reported method was used for the determination of polyamines with minor modification (Endo 1978; Magesh et al. 2009).

Carcinoembryonic antigen (CEA) and neuron specific enolase (NSE)

The level of CFA and NSE were determine by the using the chemiluminescent immunoassay (Bayer Chemiluminescence system, Thermo Fisher Scientific, USA).

Determination of enzymatic and non-enzymatic antioxidant

Previous reported methods were used for the estimation of antioxidant parameters like reduced glutathione (Rahman et al. 2007), glutathione peroxidase (GPx) (Rahman et al. 2007; Du et al. 2021), superoxide dismutase (SOD) (Ukeda et al. 1997), catalase (CAT) (Sinha 1972). The level of vitamin C and vitamin E were estimated using the previous reported method of Roe and Kuether (1943) and Du et al. (2021).

Phase I and II enzymes

Cytochrome P450

Briefly, 0.1 M Tris buffer was combined with the serum sample before being incubated at 37°C for 15–20 min at 4°C. After filtering the material, collecting the filtrate, adding sodium deoxycholate, and centrifuging it at 15,000 × *g* for 1 h, the wavelength was calculated to be 450 nm (Omura and Sato 1964).

Cytochrome b5

A previously established procedure with some modest adjustments was used to measure the cytochrome b5 activity (Phillips and Langdon 1962). The serum sample was combined with 0.25 M sucrose and centrifuged at 6,000 × *g* for 10 min. At pH 7.6, Tris buffer (0.5 M) was added, and absorbance was measured at 550 nm.

NADPH cytochrome C reductase

The activity of nucleotide-cytochrome C reductase was measured using a slightly modified version of the previously reported methodology (Omura and Sato 1964; Du et al. 2021). The serum sample was combined with phosphate buffer (0.33 M) and 2,6-dichlorophenolindophenol and

incubated at 37°C for 60 min before the absorbance was measured at 550 nm.

Glutathione S-transferase

The guanidine hydrochloride (6 M), which contains 2-mercaptoethanol (10 mM), was combined with the serum sample. Then, at pH 6.7, potassium phosphate (50 mM) was added. After 24 h at room temperature, absorbance was measured at 650 nm (Habig et al. 1976).

UDP-glucuronyltransferase

UDP-glucuronyltransferase activity was estimated using the prior approach with a few slight adjustments (Du et al. 2021). Briefly, the absorbance was measured using UV spectroscopy after the UDPGA (1.5 mM) and morphine (15 mM) were added.

Pro-inflammatory cytokines, inflammatory mediators and apoptosis

ELISA kits were used for the determination of cytokines like interleukin-1β (IL-1β), IL-6, IL-10 and tumor necrosis factor α (TNF-α) *via* following the manufacture protocol (Xitang Bio-Technology, Shanghai, China).

The inflammatory parameters like prostaglandin (PGE_2), cyclooxygenase-2 (COX-2) and nuclear kappa B factor (NF-κB) *via* following the manufacture protocol (Xitang Bio-Technology, Shanghai, China).

The apoptosis parameters like caspase-3 and 9 were determined using the available kits *via* following the manufacture protocol (Xitang Bio-Technology, Shanghai, China).

Histopathology

The lung tissues were removed and immersed in paraformaldehyde for 48 h. In accordance with the H&E staining protocol, a blade was used to remove the tissue situated between the optic chiasm and the transverse fissure of the lung. Subsequently, a series of steps including gradient dehydration, paraffin embedding, and sequential coronal sectioning were carried out. The tissue underwent dehydration using a gradient of xylene and ethanol.

Statistical analysis

The whole data obtained from the all-experimental groups was presented as standard error mean (SEM). GraphPad Prism version 8 (St. Louis, USA) was used to scrutinise the differences between the various groups. To determine whether there were any statistically significant differences, one-way analysis of variance (ANOVA) was used, and $p < 0.05$ was considered significant.

Result

Body weight, organ weight and tumor incidence

During the cancer disease, reduction in the body weight is commonly observed. BaP-induced mice exhibited the reduction in the body weight and suggest the confirmation

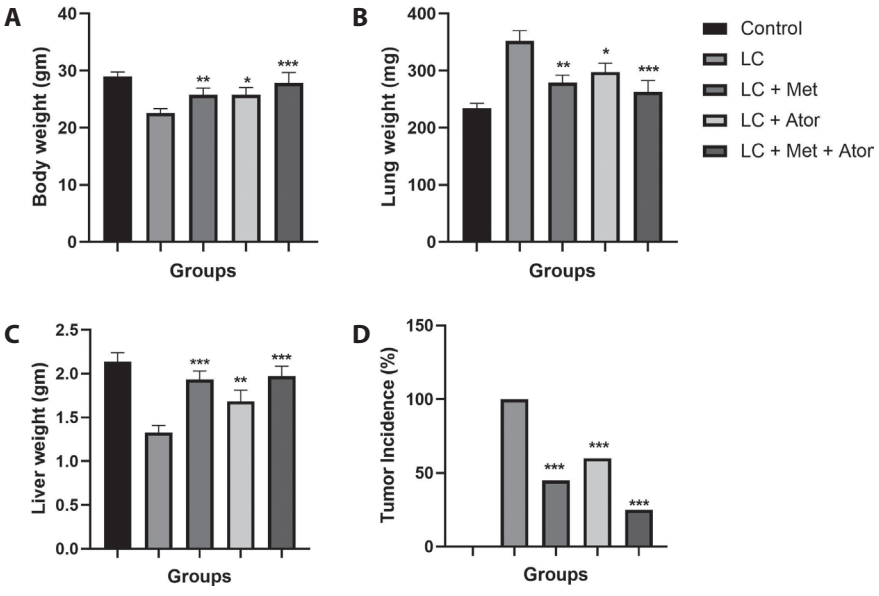

Figure 1. Effects of metformin and atorvastatin, either alone or in combination, against BaP-induced lung cancer on the body weight (**A**) and organ weight, lung (**B**) and liver (**C**), and to tumor incidence (**D**) of different groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* LC group were considered as the significant. Control, normal mice received corn oil (1 ml); LC, lung cancer mice received BaP (50 mg/kg) in corn oil; LC+Met, LC mice received the oral administration of 5 mg/kg metformin; LC+Ator, LC mice received the oral administration of 2.5 mg/kg atorvastin; LC+Met+Ator, LC mice received the oral administration of 5 mg/kg metformin and 2.5 mg/kg atorvastin.

of cancer in the mice. Metformin- and atorvastatin-treated mice exhibited the increased body weight as compared to control mice. The combination treatment of metformin and atorvastatin significantly ($p < 0.001$) enhanced the body weight (Fig. 1A).

BaP exhibited increased lung weight (Fig. 1B) and suppressed liver weight (Fig. 1C) and the metformin and atorvastatin individual treatments altered lung and liver weight. The combination treatment of metformin and atorvastatin significantly ($p < 0.001$) suppressed the lung weight and improved the liver weight.

Figure 1D shows the tumor incidence of different groups. BaP exhibited a 100% tumor incidence; metformin treatment exhibited a 45% and atorvastatin treatment showed a 60% tumor incidence. The combination treatment of metformin and atorvastatin remarkably suppressed the tumor incidence (25%).

Immunocompetent cells

Immunocompetent cells, also known as immune cells, play an essential role in the development and progression of lung cancer. BaP group mice (LC group) exhibited alteration in immunocompetent cells such as total leucocyte count, lymphocytes, neutrophils, absolute lymphocyte count, absolute neutrophil count, phagocytic index, avidity index, NBT reduction and serum immune complexes (Fig. 2). Metformin, atorvastatin and their combination treatment remarkably restored the level of immunocompetent cells.

CEA and NSE

CEA and NSE are considered tumor markers, and during cancer disease, enhance the levels of CEA and NSE. BaP-

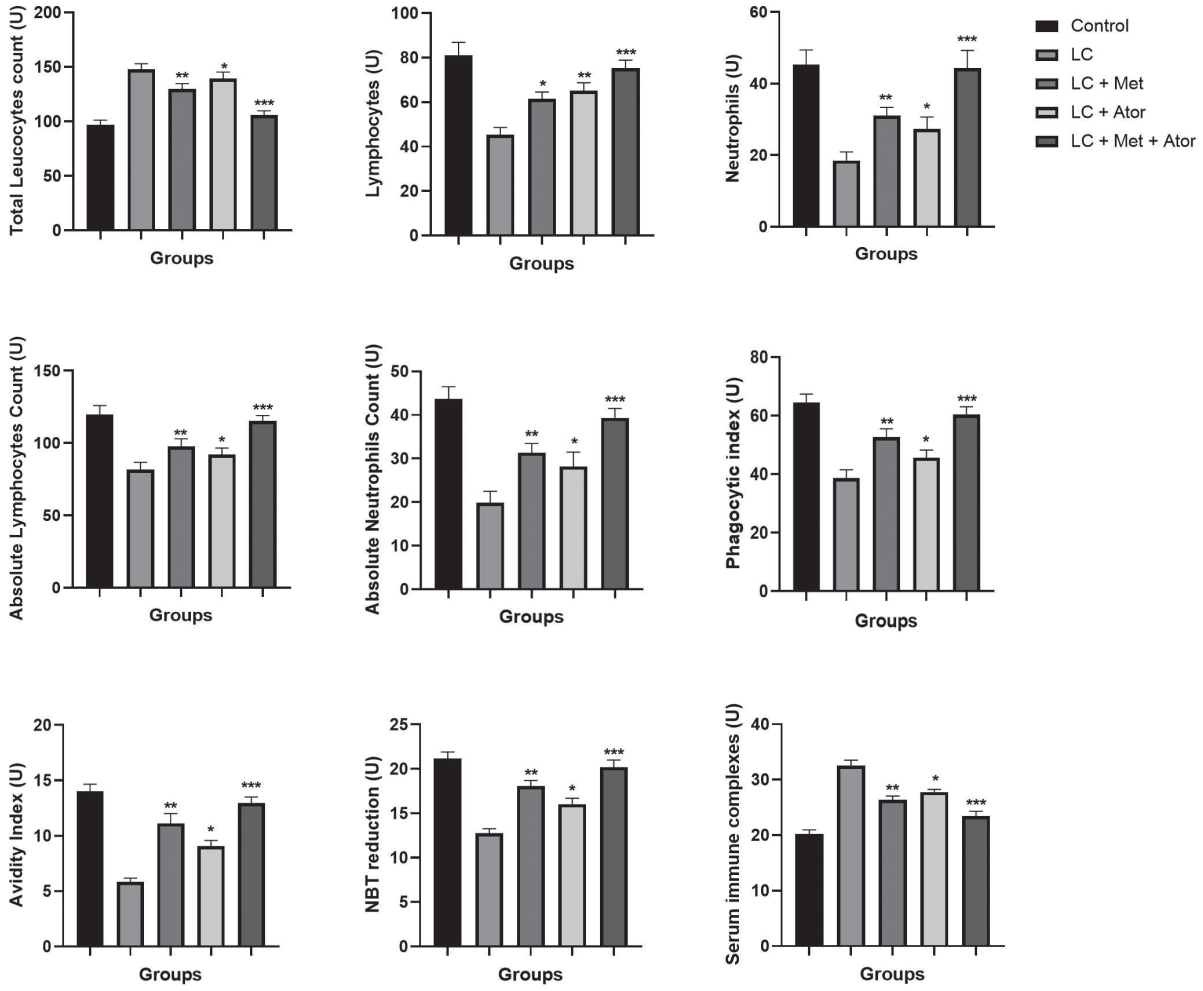

Figure 2. Effects of metformin and atorvastatin, either alone or in combination, against BaP-induced lung cancer on immunocompetent cells: leucocytes, lymphocytes and neutrophils levels; and on phagocytic index, avidity index, NBT reduction and serum immune complexes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* LC group were considered as the significant. For abbreviations, see Figure 1.

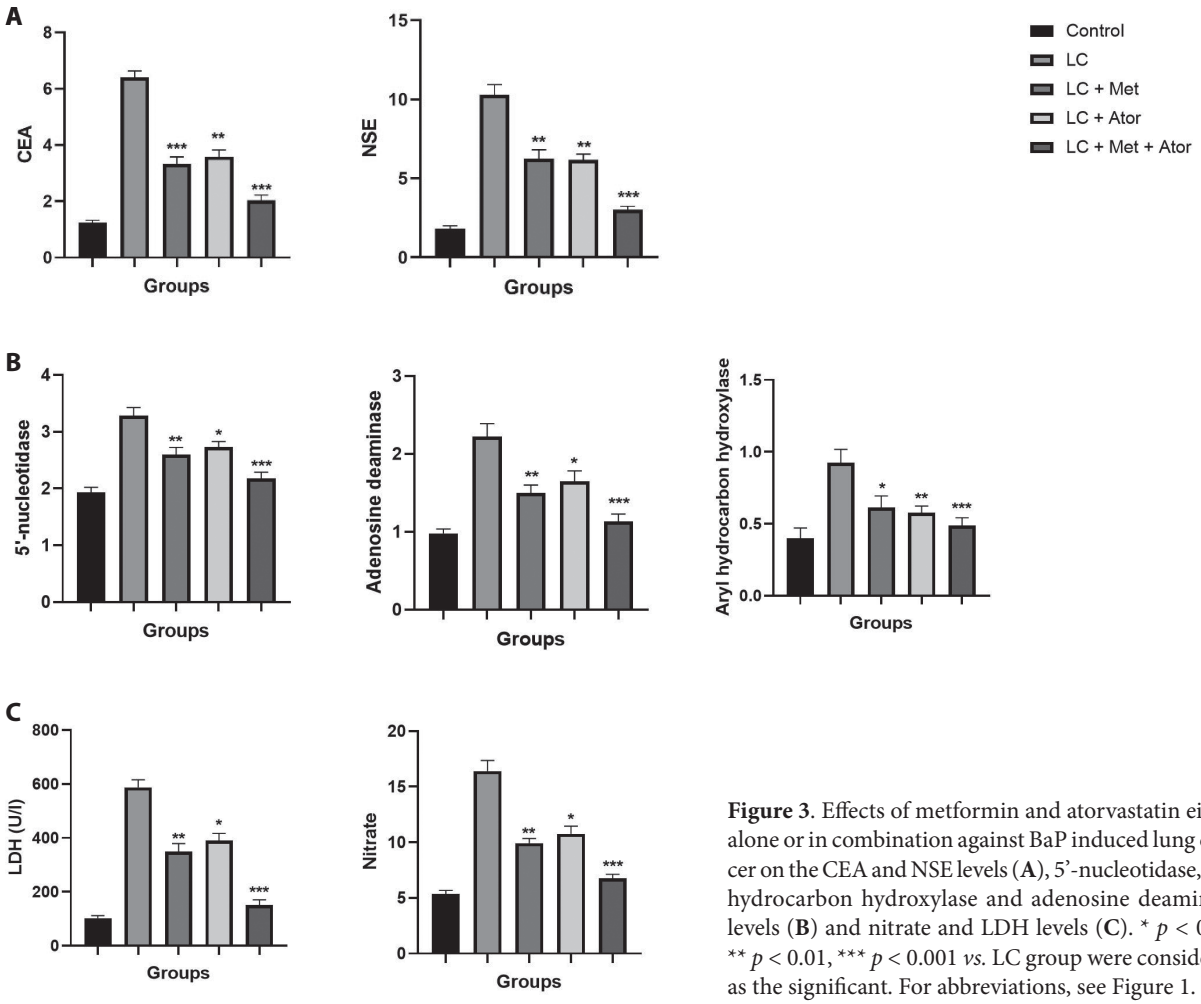

Figure 3. Effects of metformin and atorvastatin either alone or in combination against BaP induced lung cancer on the CEA and NSE levels (**A**), 5'-nucleotidase, aryl hydrocarbon hydroxylase and adenosine deaminase levels (**B**) and nitrate and LDH levels (**C**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* LC group were considered as the significant. For abbreviations, see Figure 1.

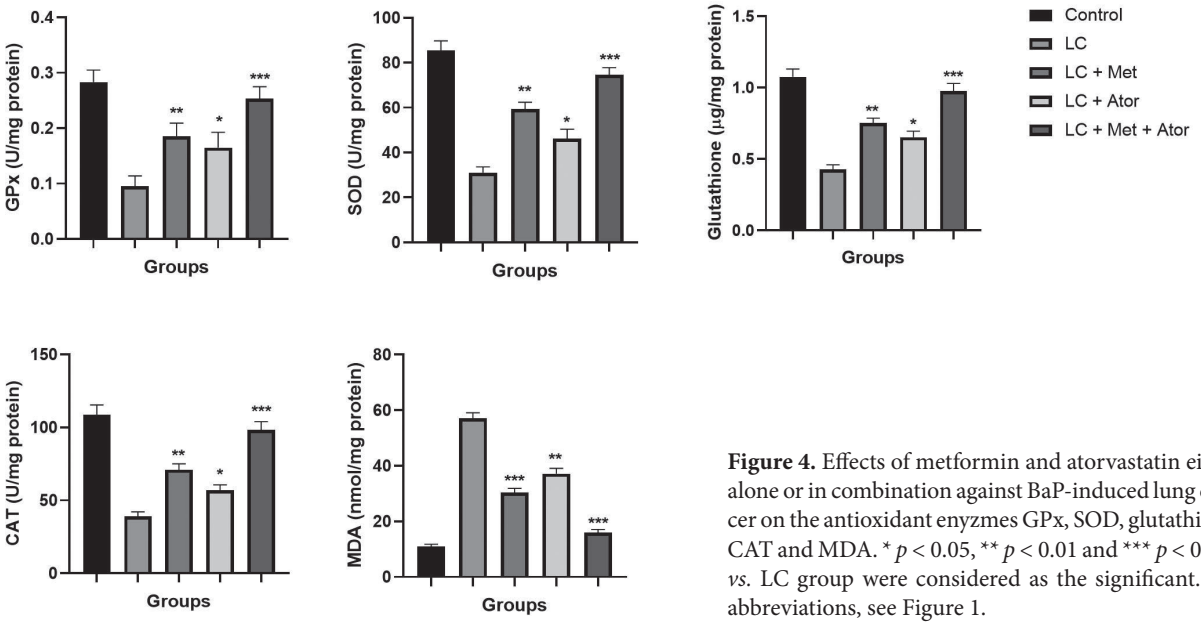

Figure 4. Effects of metformin and atorvastatin either alone or in combination against BaP-induced lung cancer on the antioxidant enyzmes GPx, SOD, glutathione, CAT and MDA. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* LC group were considered as the significant. For abbreviations, see Figure 1.

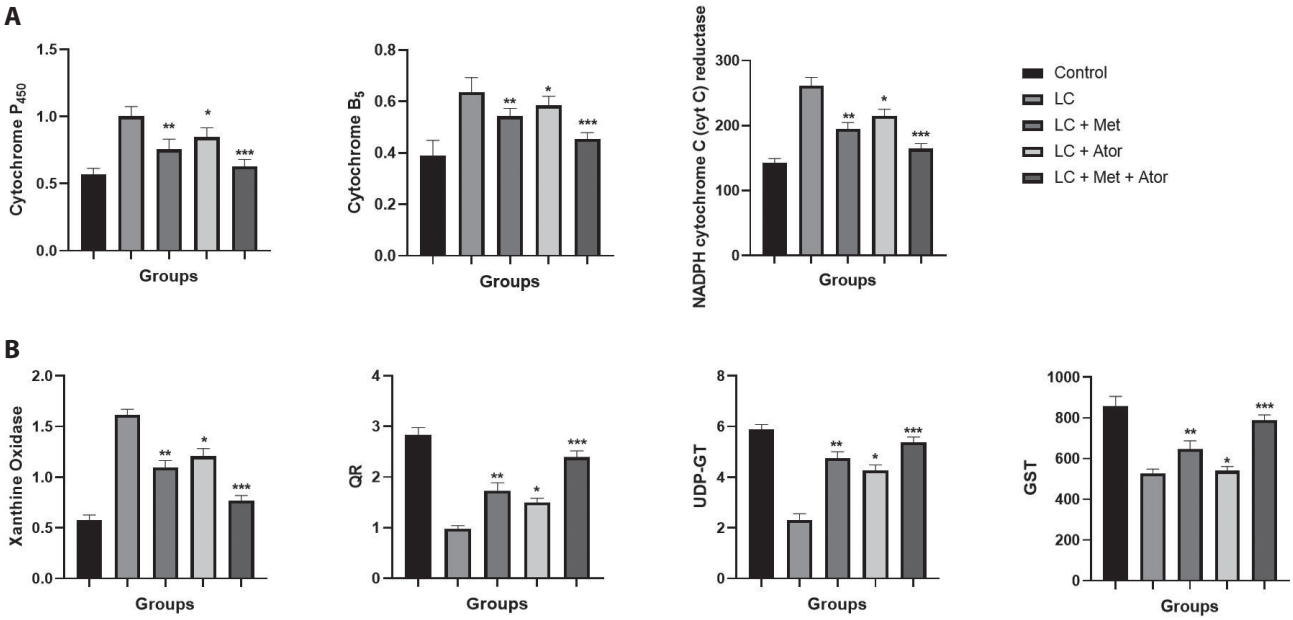

Figure 5. Effects of metformin and atorvastatin either alone or in combination against BaP-induced lung cancer on the phase I enzymes: cytochrome P450, cytochrome B5 and NADPH cytochrome C reductase (**A**) and phase II enzymes: xanthine oxidase, QR, UDP-GT and GST (**B**). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* LC group were considered as the significant. For abbreviations, see Figure 1.

induced lung cancer mice (LC group) exhibited the boosted level of CEA and NSE (Fig. 3A) and metformin, atorvastatin and their combination treatment remarkably ($p < 0.001$) suppressed these levels.

Lung marker enzyme

BaP-induced lung cancer mice exhibited a boosted level of 5'-NT, aryl hydrocarbon hydroxylase and adenosine deaminase (Fig. 3B). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) suppressed these levels.

Nitrate and LDH

BaP-induced lung cancer mice exhibited a boosted level of nitrate and LDH (Fig. 3C). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) suppressed these levels.

Antioxidant enzymes

Oxidative stress plays a crucial role in the expansion of lung cancer. BaP-induced lung cancer mice exhibited suppressed levels of GPx, SOD, glutathione, CAT and a boosted level of MDA (Fig. 4). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) altered the level of antioxidant parameters.

Phase I and phase II

Figure 5 shows the levels of phase I and phase II enzymes. BaP-induced group lung cancer mice showed that altered levels of cytochrome P_{450}, cytochrome B_5, NADPH cy-

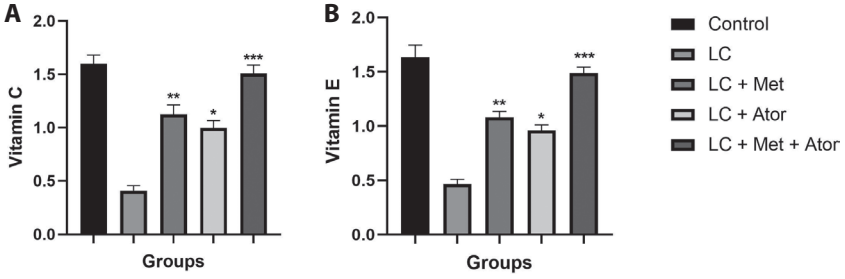

Figure 6. Effects of metformin and atorvastatin either alone or in combination against BaP-induced lung cancer on the vitamin C (**A**) and vitamin E (**B**) levels. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* LC group were considered as the significant. For abbreviations, see Figure 1.

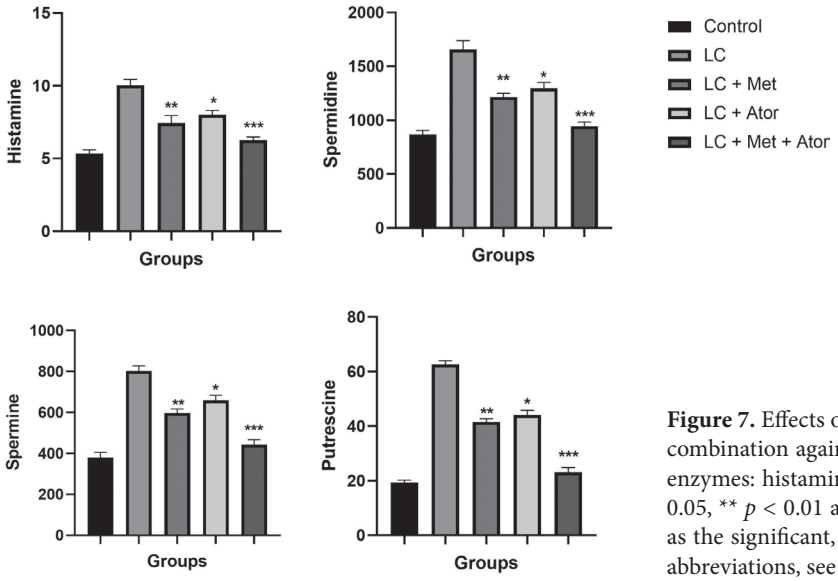

Figure 7. Effects of metformin and atorvastatin either alone or in combination against BaP-induced lung cancer on the polyamine enzymes: histamine, spermidine, spermine and putrescine. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* LC group were considered as the significant, more and extreme significant respectively. For abbreviations, see Figure 1.

tochrome C reductase (Fig. 5A) and xanthine oxidase, QR, UDP-GT and GST (Fig. 5B). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) altered level of phase I and phase II parameters.

Vitamin level

BaP-induced lung cancer mice exhibited the suppressed level of vitamin C and vitamin E (Fig. 6). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) boosted the level of vitamins.

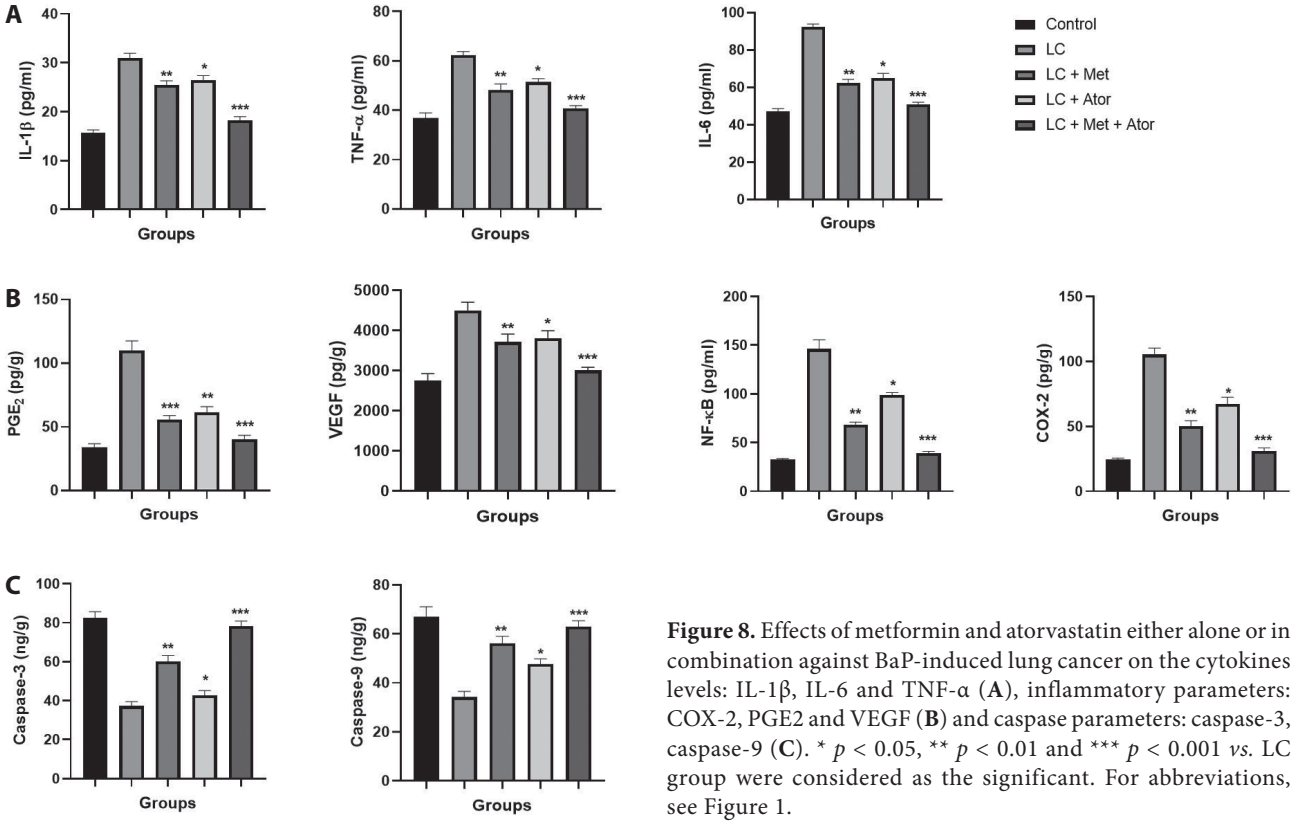

Figure 8. Effects of metformin and atorvastatin either alone or in combination against BaP-induced lung cancer on the cytokines levels: IL-1β, IL-6 and TNF-α (**A**), inflammatory parameters: COX-2, PGE2 and VEGF (**B**) and caspase parameters: caspase-3, caspase-9 (**C**). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* LC group were considered as the significant. For abbreviations, see Figure 1.

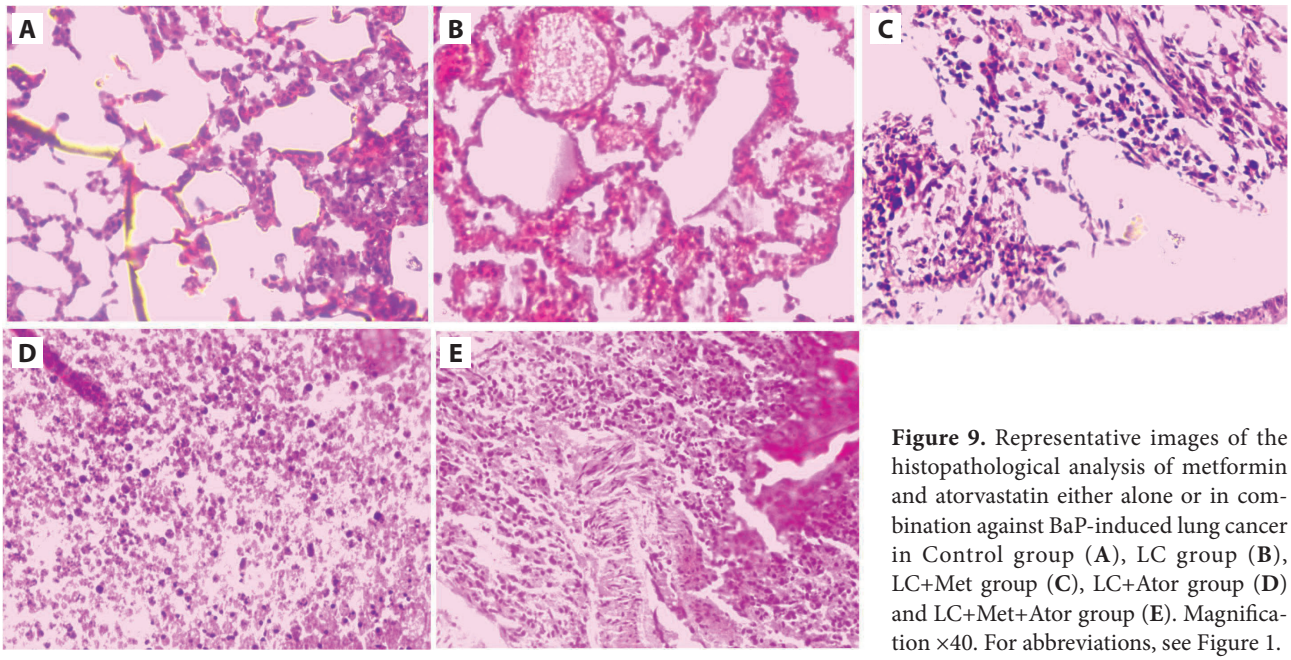

Figure 9. Representative images of the histopathological analysis of metformin and atorvastatin either alone or in combination against BaP-induced lung cancer in Control group (**A**), LC group (**B**), LC+Met group (**C**), LC+Ator group (**D**) and LC+Met+Ator group (**E**). Magnification ×40. For abbreviations, see Figure 1.

Polyamine enzymes

BaP-induced lung cancer mice exhibited a boosted level of histamine, spermidine, spermine and putrescine (Fig. 7). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) reduced the level of polyamine enzymes.

Cytokines

It is well known that cytokines play a crucial role in the expansion of cancer. In this study, we observed a boosted level of IL-1β, Il-6 and TNF-α (Fig. 8A). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) suppressed the level of cytokines.

Inflammatory parameters

Inflammatory reactions play an important role in the expansion of cancer. BaP-induced lung cancer mice exhibited the boosted level of COX-2, PGE2, VEGF, NF-κB (Fig. 8B). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) suppressed the level of inflammatory parameters.

Caspase parameters

BaP-induced lung cancer mice exhibited a boosted level of caspase-3, caspase-9 (Fig. 8C). Metformin, atorvastatin and their combination treatment significantly ($p < 0.001$) suppressed the level of caspase parameters.

Histopathology

Figure 9 showed the histopathology of different groups. No changes observed in Control and LC group exhibited the alteration in the lung histopathology. LC+Met+Ator group restored the alteration.

Discussion

Metformin is a frequently prescribed medication employed in the management of type 2 diabetes. Its mechanism of action involves curtailing the liver's glucose production while concurrently augmenting insulin sensitivity. This combined effect results in the reduction of blood glucose levels (Afzal et al. 2012). Beyond its capacity to lower blood glucose levels, research has unveiled that metformin possesses the ability to mitigate the risk of particular cancer types, such as breast, colorectal, and pancreatic cancer (Zhao et al. 2019; Sun et al. 2020). Atorvastatin is a medication frequently employed to effectively diminish cholesterol levels within the bloodstream and thereby mitigate the likelihood of cardiovascular disease (Vlachopoulos et al. 2007). In recent years, there has been increasing interest in the potential role of atorvastatin in the prevention and treatment of cancer, including lung cancer (Du et al. 2021). In this experimental study, we used the combination of metformin and atorvastatin for the treatment of BaP-induced lung cancer in mice.

During lung cancer, changes in body weight and organ weight can occur due to various factors related to the disease,

such as altered metabolism, inflammation, and cachexia. Cachexia is a distinctive syndrome marked by pronounced weight loss, degradation of muscle tissue, and a decline in physical strength. This syndrome can manifest in patients who are in advanced stages of cancer (Du et al. 2021). In lung cancer, cachexia is a common complication and can result in significant reductions in body weight and muscle mass. In addition, changes in organ weight can also occur during lung cancer. The lungs may become enlarged or heavier due to the presence of tumors or inflammation, while the liver may become suppressed due to changes in metabolism and nutrient utilization. Changes in body weight and organ weight can have important implications for the prognosis and treatment of lung cancer (Wang et al. 2021). BaP-induced group mice exhibited the reduction in the body weight, liver weight and enhancement in the lung tissue weight, which suggest the induction of lung cancer and combination therapy of metformin and atorvastatin remarkably improved the body weight, liver weight and suppressed the lung tissue weight.

Tumor markers are substances that are produced by cancer cells or in response to cancer growth, and they can be measured in the blood, urine, or tissue samples of cancer patients. Tumor markers can serve as useful diagnostic and prognostic tools in cancer management, as well as targets for cancer therapy (Grunnet and Sorensen 2012). CEA is a glycoprotein that is often overexpressed in cancer cells, and its levels in the blood can serve as a prognostic indicator of lung cancer. High levels of CEA are associated with more advanced stages of the disease, and monitoring CEA levels during treatment can help to assess treatment response and disease progression (Niho and Shinkai 2001; Grunnet and Sorensen 2012). NSE is an intracellular enzyme that is released into the bloodstream when cells are damaged or destroyed, such as during cancer growth. Elevated levels of NSE in the blood have been associated with a poorer prognosis in lung cancer patients, and NSE levels can be used to monitor treatment response and disease progression (Niho and Shinkai 2001; Wu et al. 2020). The similar result was observed in the BaP group mice and combination treatment of metformin and atorvastatin remarkably restored the level of tumor markers.

Lung cancer and other types of neoplasms can be associated with changes in the levels of various enzymes and biomarkers, including adenosine deaminase, aryl hydrocarbon hydroxylase, and 5'-NT. Adenosine deaminase is an enzyme that plays a role in the metabolism of purine nucleotides. In some types of cancer, including lung cancer, the levels of adenosine deaminase can be increased. Elevated levels of adenosine deaminase have been associated with a poorer prognosis in lung cancer patients, and monitoring adenosine deaminase levels may help to predict treatment response and disease progression (Du et al. 2021). Aryl hydrocarbon hydroxylase is a cell surface enzyme that is involved in the metabolism of xenobiotics, including carcinogens. In lung cancer, aryl hydrocarbon hydroxylase levels have been found to be increased, and the enzyme can be used as a marker for the early detection of lung cancer. Aryl hydrocarbon hydroxylase has also been implicated in the development of lung cancer by promoting the formation of DNA adducts, which can lead to mutations and genetic instability. 5'-NT is an enzyme that hydrolyses nucleotides and nucleotide analogs (Diggs et al. 2011; Elfaki et al. 2018). Enhanced activity of this enzyme has been observed in proliferating tumor cells, including lung tumors. Increased levels of 5'-NT have also been found in lung tumors, suggesting that this enzyme may be involved in the growth and progression of lung cancer. BaP-induced group mice exhibited the increased level of adenosine deaminase, aryl hydrocarbon hydroxylase and 5'-NT and combination therapy of metformin and atorvastatin remarkably restored the level almost near to the normal level.

Antioxidant enzymes and phase I and phase II enzymes play a role in the expansion of lung cancer through their involvement in cellular detoxification and oxidative stress regulation. Antioxidant enzymes such as SOD, CAT and GPx play a key role in regulating oxidative stress in cells (Alzohairy et al. 2021). In lung cancer, the expression and activity of these enzymes can be altered, resulting in increased oxidative stress and DNA damage, which can promote tumor growth and progression (Velli et al. 2019; Alzohairy et al. 2021). Phase I and phase II enzymes are involved in cellular detoxification processes, including the metabolism and elimination of xenobiotics, drugs, and carcinogens. In lung cancer, alterations in the expression and activity of these enzymes can result in increased exposure to carcinogens and other toxic compounds, contributing to the development and progression of the disease (Du et al. 2021). The role of antioxidant enzymes, phase I, and phase II enzymes in the expansion of lung cancer highlights the importance of cellular detoxification and oxidative stress regulation in cancer development and progression. Targeting these pathways may represent a promising approach for the prevention and treatment of lung cancer (Velli et al. 2019; Du et al. 2021).

Cytokines play critical roles in the progression and expansion of lung cancer by promoting tumor growth, metastasis, and immune suppression. Targeting these cytokines and their associated signaling pathways may represent a promising approach for the treatment of lung cancer (Pian et al. 2022; Song et al. 2022). Cytokines play important roles in the progression and expansion of lung cancer. TNF-α is a proinflammatory cytokine that is produced by immune cells in response to infection or injury. In lung cancer, TNF-α can promote tumor growth and progression by stimulating angiogenesis, suppressing immune function, and inducing apoptosis in normal cells (Li et al. 2018). TNF-α can also

activate signaling pathways that promote the proliferation and survival of tumor cells, and has been associated with poor prognosis in lung cancer patients. IL-1β is another proinflammatory cytokine that is produced by immune cells in response to injury or infection. In lung cancer, IL-1β can promote tumor growth and metastasis by stimulating angiogenesis and inducing the expression of matrix metalloproteinases (MMPs), which can degrade extracellular matrix and promote tumor invasion (Li et al. 2018; Du et al. 2021). IL-1β can also activate signaling pathways that promote the proliferation and survival of tumor cells, and has been associated with poor prognosis in lung cancer patients. IL-6 is a cytokine that is produced by immune cells and other cells in response to inflammation and infection (Zhang and Veeramachaneni 2022). In lung cancer, IL-6 can promote tumor growth and progression by stimulating angiogenesis, suppressing immune function, and inducing the expression of MMPs. IL-6 can also activate signaling pathways that promote the proliferation and survival of tumor cells, and has been associated with poor prognosis in lung cancer patients (Ritzmann et al. 2022; Zhang and Veeramachaneni 2022).

COX-2 is an enzyme that is upregulated in many types of cancer, including lung cancer. COX-2 plays an important role in the production of PGE2, a lipid mediator that has been shown to promote tumor growth and metastasis. In lung cancer, COX-2 expression and PGE2 levels have been found to be increased, and have been associated with poor prognosis (Pang et al. 2016). COX-2/PGE2 signaling can promote tumor growth and metastasis by stimulating angiogenesis, immune suppression, and promoting invasion and migration of tumor cells. VEGF is a signaling protein that is critical for the formation of new blood vessels (angiogenesis) (Subbaramaiah et al. 2008). In lung cancer, VEGF expression is upregulated and has been associated with poor prognosis. VEGF can promote tumor growth and metastasis by stimulating angiogenesis and promoting invasion and migration of tumor cells. Targeting VEGF and its associated signaling pathways has been explored as a potential therapeutic strategy in lung cancer. COX-2/PGE2 signaling and VEGF play important roles in the expansion and progression of lung cancer by promoting angiogenesis, immune suppression, and invasion and migration of tumor cells. Targeting these pathways may represent a promising approach for the treatment of lung cancer (Alevizakos et al. 2013; Lin et al. 2015).

Caspases are a family of enzymes that play a critical role in the execution of apoptosis, or programmed cell death. Caspases are activated in response to a variety of signals, including DNA damage, oxidative stress, and cytokine signaling, and they are responsible for cleaving key cellular proteins and promoting the dismantling of the cell. Caspase-3, in particular, is a key executioner caspase that is responsible for cleaving a variety of substrates during the late stages of apoptosis (Samarghandian et al. 2013; Kim et al. 2022). The cleavage of these substrates triggers the distinctive morphological alterations linked with apoptosis. These changes encompass DNA fragmentation, cellular contraction, and the creation of apoptotic bodies. Caspase-9 is another important member of the caspase family of enzymes, which plays a critical role in the initiation of apoptosis, or programmed cell death. In lung cancer, the balance between cell death and cell division is disrupted, leading to the uncontrolled growth and proliferation of cancer cells. Research has shown that the levels of caspases, including caspase-3, are often decreased in lung cancer cells, which may contribute to the resistance of these cells to apoptosis. Several drugs have been developed that target the apoptotic pathway and increase the activation of caspases, including caspase-3, in cancer cells. These drugs, known as apoptosis inducers, have shown promise in preclinical and clinical studies as potential treatments for lung cancer and other types of cancer (Imran et al. 2020; Li et al. 2022). By promoting the activation of caspases and inducing apoptosis in cancer cells, these drugs may help to restore the balance between cell death and cell division and slow the progression of the disease. Caspase-9 is activated in response to a variety of signals, including DNA damage, oxidative stress, and cytokine signaling, and it is responsible for cleaving and activating downstream caspases, including caspase-3, which ultimately leads to the dismantling of the cell. In lung cancer, the balance between cell death and cell division is disrupted, leading to the uncontrolled growth and proliferation of cancer cells. Research has shown that the levels of caspase-9 are often decreased in lung cancer cells, which may contribute to the resistance of these cells to apoptosis (Huang et al. 2022; Li et al. 2022). BaP induced lung cancer mice exhibited suppressed level of caspase-3 and caspase-9 and metformin and atorvastatin combination treatment remarkably restored the level. These findings suggest that targeting caspase-3 and caspase-9 may be a promising strategy for the treatment of lung cancer.

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