Original Article

Histological and Immunohistochemical Study to Evaluate the Effects of Metformin versus Green Tea Extracts on Bleomycin Induced Lung Injury In Adult Male Albino Rats

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ABSTRACT

Introduction: Bleomycin is a widely used chemotherapy which is used in the treatment of many tumors. Lung injury especially pulmonary fibrosis is considered as one of the most common side effects of it.

Aim of the work: We aimed to evaluate the possible effect of Green tea versus Metformin on Bleomycin induced pulmonary injury in adult male albino rats.

Materials and Methods: 48 adult male albino rats were used in this study. The rats were divided into six groups of eight rats each. Control group, Bleomycin group, Bleomycin with Green tea group, Bleomycin with Metformin group, green tea group and Metformin group. Bleomycin was administered intra-tracheally to ensure induction of fibrosis. All animals were sacrificed after 14 days of starting the experiment. The lung sections were prepared and stained with H&E stain, Mallory′s trichrome stain and immunohistochemical staining for alpha smooth muscle actin (α –SMA). Morphometric measurements and statistical analysis were performed.

Results: There was marked multifocal distortion of the lung architecture with significant increase of area % of collagen deposition and α –SMA staining in Bleomycin group compared to control group. There was noticeable partial preservation of normal lung structure in combined Bleomycin with either Metformin or Green tea groups compared to Bleomycin group. The effect of Green tea was more obvious than Metformin which still showed more areas of congestion and hemorrahge in the lung. In addition to significant decrease in area % of collagen deposition and α-SMA in both combined Bleomycin with either Green tea or Metformin groups compared to Bleomycin group. While both Metformin and Green tea groups appeared as the control.

Conclusion: We can conclude that administration of either Green tea or Metformin can attenuate Bleomycin induced lung injury. Green tea produced more protective effect than Metformin.

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Key Words: α –SMA, bleomycin, green tea, lung, metformin.

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INTRODUCTION

Bleomycin (BLM) which is an antibiotic produced by the bacterium "Streptomyces Verticillus"[1], is one of the first described chemotherapeutic agents that has been used for cancer treatment. Chemotherapy using bleomycin is often complicated by interstitial pulmonary fibrosis[2,3].

Green tea (GT) is a natural agent produced from Camellia sinensis plant[4]. It is one of the most commonly consumed drinks in the world that has a potent antioxidant, anti-inflammatory, antiproliferative, anticarcinogenic and antimicrobial properties[5]. The combination of GT catechins and anticancer drugs is a treatment strategy that has been widely accepted by cancer researchers[6].

Metformin is the most commonly prescribed drug for type 2 diabetes mellitus. Metformin has a protective role against BLM induced pulmonary fibrosis through its antioxidant, anti-inflammatory and antifibrotic properties[7].

So in this study we aimed to evaluate the effects of green tea (GT) versus metformin on bleomycin induced pulmonary injury in adult male albino rats.

MATERIALS AND METHODS

Forty eight adult male albino rats of 200-225 gm body weight were used in this study. the animals were bred at the animal house of Faculty of Science Fayoum University in hygienic stainless steel cages and in a clean well ventilated room, temperature from 22-28c. Standard laboratory chow and tap water were available ad libitum. The experimental design was approved by the ethics committee Faculty of Medicine Fayoum University.

We used Bleomycin vials containing 15 international units (IU) (Celon Laboratories Reg. No. 14/RR/AP/2008/F/cc, India) which was dissolved in 1.5 ml phosphate buffered saline (PBS).

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Green tea extract: we used tablets containing 200 mg extract (pharmaceutical industries, El-Obour city. Reg. No. 2958-2002). Each tablet is crushed and dissolved in 10 ml distilled water (DW).

Metformin: we used metformin 500 mg tablets. (Minapharm. Reg. No. 23436-2004). Each tablet was crushed and dissolved in 50 ml DW.

Rats were divided into six groups of 8 rats each

- ***Group I: serving as a control group***

Control group was further subdivided into 3 subgroups corresponding to different experimental groups. 3 rats were injected intratracheally with a single dose of 0.1 ml PBS (solvent of bleomycin). 3 rats were injected intratracheally with a single dose of 0.1 ml PBS (solvent of bleomycin) and received 1.5 ml DW daily for 14 days orally by intragastric gavage tube (solvent of green tea and Metformin). 2 rats received 1.5 ml DW daily for 14 days orally by intragastric gavage tube (solvent of green tea and Metformin).

- ***Group II: (BLM group)***

Each rat was injected intratracheally with a single dose of (5 IU/kg) bleomycin dissolved in 0.1 ml sterile PBS at the first day of experiment and sacrificed at day 14. This dose was used in other experimental studies to confirm induction of lung fibrosis[8].

- ***Group III: (BLM with Green tea group)***

Each rat was injected intratracheally with a single dose of (5 IU/kg) bleomycin dissolved in 0.1 ml sterile PBS. From the first day of injection, each rat received 1.5 ml green tea extract dissolved in DW (150mg/kg) daily orally by intragastric gavage tube for 14 days. We used a higher dose than other studies as in our pilot study we found that the low dose was less effective and this higher dose was still considered as a safe dose[9].

- ***Group IV: (BLM with Metformin group)***

Each rat was injected intratracheally with a single dose of (5 IU/kg) bleomycin dissolved in 0.1 ml sterile PBS. From the first day of injection, each rat received 1ml metformin dissolved in DW (50mg/kg) daily for 14 day orally by intragastric gavage tube[10].

- ***Group V:***

Each rat received 1.5 ml green tea extract dissolved in DW (150mg/kg) daily for 14 days orally by intragastric gavage tube[9].

- ***Group VI:***

Each rat received 1 ml metformin dissolved in DW (50mg/kg) daily for 14 day orally by intragastric gavage tube[10].

At the end of experimental period (14 days), all rats were anaesthesized with intraperitoneal injection of 50 mg/kg thiopental sodium and a midline incision was done in each animal then the lungs were resected. Specimens were fixed immediately in 10% buffered formalin, and then processed for paraffin technique.

Paraffin embbeded tissues were cut into serial sections of 5 µm thickness and were subjected to Hematoxylin and Eosin H&E staining for routine histological examination, Mallory's trichrome staining to demonstrate collagen fibers and Immunohistochemical staining using of monoclonal antibodies against alpha smooth muscle actin (α –SMA) (Lab vision neomarkers USA, Catalog NO.1-GE002-07) as a marker of activated myofibroblasts showing brown cytoplasmic deposits[11]. We applied positive control on sections of colon showing positive cytoplasmic deposits staining smooth muscle cells. Negative control specimen of the lungs was processed in the same way omitting the steps of primary antibodiy.

Quantitative Morphometric Study

The data were obtained by using "Top view" image analyzer computer system (China). The image analyzer consists of a coloured video camera, coloured monitor, hard disc of IBM personal computer connected to the microscope and controlled by "Top view" software. We made this morphometric study in Histology department Faculty of Medicine Fayoum University. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. Digitized images were captured from 10 randomly chosen non-overlapping fields from each section. We used 5 sections from each group.

The following parameters were measured

1) Area percent of collagen

Sections from all groups were measured using an objective lens of magnification 4, i.e. a total magnification of 40 in 10 non-overlapping fields from each section.

2) Area percent of α–SMA

Sections from all groups were measured using an objective lens of magnification 10, i.e. a total magnification of 100 in 10 non-overlapping fields from each section.

3) Number of pneumocytes type II

Counting pneumocytes type II (cubic in shape with central rounded nuclei), the magnification used for this parameter was 1000. Counting was performed in 10 non-overlapping fields from each section.

Statistical Methods

Statistical analysis was performed using the arithmetic mean, standard deviation (S.D.), analysis of variance (one way ANOVA) and comparison between each two groups using post Hoc test. All statistical analyses were done on an IBM personal computer using the statistical software "SPSS for Windows" Version 19. Results were considered significant when probability (p) was < 0.05.

RESULTS

As regard the mortality; it occurred at the time of Bleomycin intratracheal injection in 3 rats of bleomycin group and 2 rats in each of Bleomycin with each of GT or Metformine groups just at the time of injection. Otherwise no mortality was detected during the time of experiment.

As regard the body weight

As compared to the control group, the body weight of the bleomycin treated group decreased gradually and reached the lowest level at day 14 after bleomycin injection. While bleomycin combined with either GT or metformine treatment kept the body weight of rats comparable to that of the control group with no remarkable loss.

Lung index (wet lung weight per body weight) is a parameter to evaluate lung edema. In contrast to body weight loss, the lung index of the bleomycin treated rats increased due to lung weight gain as measured at the time of sacrifice.

Both GT and Metformin treatment inhibited the rise of the lung index when administered with bleomycin.

The gross appearance of lungs from animals receiving saline instillation was normal with a pink appearance and normal lung architecture. Gross examination of lungs from bleomycin-treated rats showed multifocal lesions with normally appearing areas of the lung. These foci appeared variable in size, involving multiple lobes in both sides of the lungs. The lungs in groups of bleomycin combined with either GT or metformin showed more or less normal lung architecture with only few focal lesions especially in bleomycin with metformin group. While that of bleomycin with green tea group appeared comparable to that of the control group.

Hematoxylin & Eosin stained lung sections

Group I (control group) showed normal architecture of the lung; formed of many bronchi, bronchioles, alveolar ducts, sacs, and many alveoli. Bronchi were lined by pseudostratified columnar ciliated epithelium with goblet cells. The bronchioles were lined by simple columnar epithelium (Figure 1 A, B, D).

Alveolar ducts arise from a respiratory bronchiole branches, they are linear arrangements of alveoli, the resultant alveolar ducts usually ends in two or more small clusters of alveoli called alveolar sacs. Alveoli form the primary structural and functional unit of the respiratory system. Alveoli are the predominant feature in the lung, they are found in large number frequently pressed against each other, with a thin connective tissue interstitium between them. The region between adjacent alveoli is known as the interalveolar septum, rich in elastic and collagen fibers and few macrophage cells are present in these septa. It is occupied by an extensive capillary bed composed of continuous capillaries. Walls of alveoli are composed of two types of cells: type I pneumocytes and type II pneumocytes Approximately 95% of the alveolar surface is composed of simple squamous epithelium, called type I pneumocytes. In addition to that, type II pneumocytes occupy only about 5% of the alveolar surface. These cuboidal cells are present among type I pneumocytes. Their dome-shaped apical surface protrude inside the lumen of the alveoli (Figure 1A, B, C, E). The visceral pleura was lined by simple squamous methothelium (Figure 1 E).

Group II (BLM group): Most of the lung passages were highly affected. Many bronchi are lined by apparently thick pseudostratified ciliated columnar eithlium with thickened smooth muscle layer and surrounded by dilated congested blood vessels (Figure 2E). Epithelial damage and separation is observed in many bronchioles. Most of inter-alveolar septa appeared thickened with areas of hemorrhage and congested blood vessels with Marked interstitial mononuclear cellular infiltrations (Figure 2B). Many alveoli appeared distorted showing marked narrowing and collapse of many alveoli with dilatation of few adjacent ones. There was large number of pneumocytes type II with their rounded outline and vesicular nuclei that were aggregated in many areas after alveolar collapse. While pneumocytes type I appeared mostly destroyed with alveolar collapse (Figure 2A, C, D). In the sub-pleural regions; acidophilic hyaline exudates, hemorrhagic areas and inflammatory cells infiltration could be observed (Figure 2F, G).

Group III (BLM with GT group): there was marked protection of the lung as compared to that of Bleomycin group with few inflammatory cellular infiltrations which were limited and surrounded only some bronchioles and blood vessels. Most of the bronchi and bronchioles restored their lining epithelium compared to group II and become comparable to that of control group (Figure 3B, D). Most of the inter-alveolar septa were thin except in a few areas comparable to that of control group. Type I pneumocytes appeared more predominant with relative decrease in number of type II pneumocytes to be more or less as that of control group (Figure 3A, C). The sub-pleural area showed thin inter-alveolar septa (Figure 3E).

Group IV (BLM with metformin group): there was remarkable protection of the lung exhibited normal architecture with normally appearing alveoli with thin inter-alveolar septa, with only few cellular infiltration (Figure 4 A, C). Normal lining epithelium of the bronchi and normal bronchiolar structure were observed (Figure 4 B, D). The interstitial hemorrhage was still observed. Sub-pleural area showed thin inter-alveolar septa (Figure 4E)

Both group V (green tea group) and group VI (metformin group) showed a picture more or less similar to that of the control (Figure 5 A,B,C,D).

Mallory's trichrome stain

Mallory's trichrome staining of lung sections in group I (control group) revealed minimal amount of collagen fibers around bronchioles and blood vessels (Figure 6A) whereas marked collagen fibers deposition was detected in group II (BLM group). This deposition was obvious in the interstitium and around both blood vessels and bronchioles (Figure 6 B).

In group III (GT treated group); obvious decrease of collagen fiber deposition in the interstitium and around bronchioles and blood vessels was observed (Figure 6 C) as compared to that in the BLM group. In group IV (metformin treated group), collagen fibers deposition exhibited an obvious decrease in lung tissue (Figure 6 D) compared to BLM group. Group V (GT group) and group VI (metformin group) showed the same findings as that of the control group (Figure 6 E and F).

α-SMA immunostaining

α-SMA immunostaining in group I (control group) is only seen in the smooth muscle layers of the bronchioles and blood vessels and in the knobs of alveolar duct with the absence of the immunoreactivity within the lining cells of the alveoli (Figure 7A,7B) meanwhile in group II (BLM group) there was an obvious increase in α-SMA immunoreactivity, compared to that in the control group, in the smooth muscle layer of the bronchioles and blood vessels in addition to positive immunoreactivity in the cytoplasm of pneumocytes type II and within the cytoplasm of myofibroblasts in the interalveolar septa with the absence of type I pneumocytes immunoreactivity (Figure 7C,7D).

Group III (BLM and GT group) showed an obvious decrease in α-SMA immunoreactivity, compared to BLM group, in the smooth muscle layer of bronchioles, knobs of alveolar ducts, cytoplasm of myofibroblasts and pneumocytes type II (Figure 7E,7F), another correlated findings were obvious in group IV (BLM and metformin group) in which an obvious decrease in α-SMA immunoreactivity, compared to BLM group, in the smooth muscle layers of a bronchioles, blood vessels, cytoplasm of myofibroblasts and pneumocytes type II was observed (Figure 7G,7H).

Group V (GT group) (Figure 7I) and group VI (metformin group) (Figure 7J) were comparable to that of control group regarding α-SMA immunoreactivity that was confined to the smooth muscle layer of bronchioles and blood vessels. In addition to that the negative control group omitting the steps of primary antibody showed negative immunoreaction (Figure 7 K)

Morphometric results

Mean area % of collagen fibers, α- SMA distribution and mean number of pneumocytes type II

There was statistical significant increase in Mean area % of collagen fibers, α- SMA distribution and mean number of pneumocytes type II in group II (BLM group) compared to that of group I (control group), group III (BLM and GT), group IV (BLM and metformin), group V (GT) and group VI (metformin). *There was no statistical significant difference between group I, group III, group IV, group V and group VI. So green tea and metformin, as regard the measured parameters, have similar protective effect against Bleomycin induced lung injury.

The values of the mean area percent of collagen fibers, α- SMA distribution and mean number of pneumocytes type II in the different groups together with their statistical significance are summarized in the following (Table1 and Histograms 1,2).

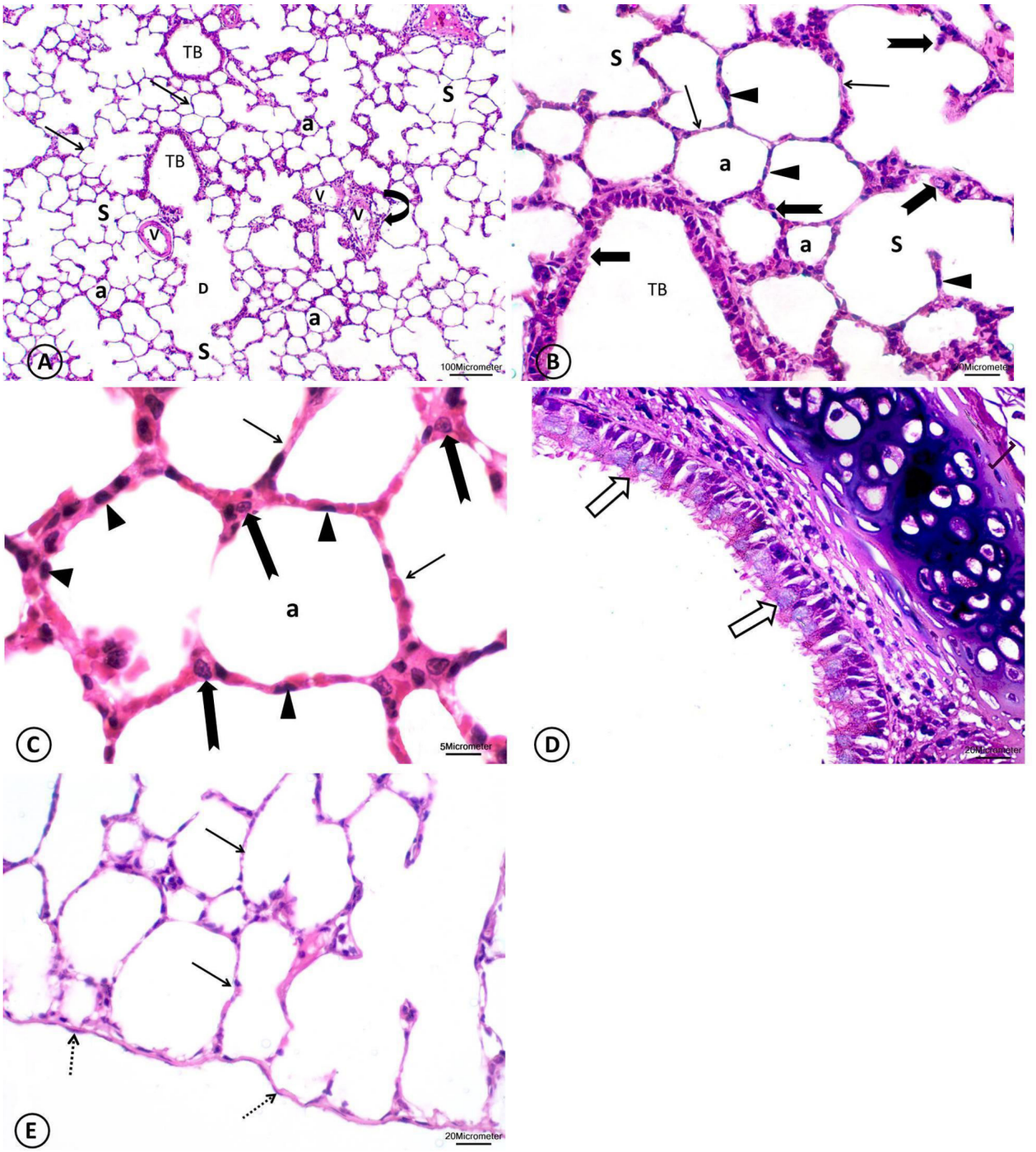


Fig. 1: Photomicrographs of lung sections of adult rats from group I (control group) stained with H&E. Showing thin inter-alveolar septa (thin arrows), normal patent alveolar lumina (a) and blood vessels (V) with few perivascular inflammatory cells (curved arrow). Notice alveolar duct (D) and alveolar sacs (S) lined by pneumocytes and terminal bronchioles (TB) lined by intact simple columnar epithelium (thick arrow). The alveoli are lined by flat type I pneumocytes (arrow heads) and few rounded type II pneumocytes (notched arrows) (1A, 1 B and 1C). Bronchi are lined by pseudostratified columnar ciliated epithelium with goblet cells (hollow arrows) notice the muscle layer (brace) (1D). Visceral pleura are lined by simple squamous methothelium (dotted arrows). Sub-pleural region with inter-alveolar septa (thin arrows)is observed (1E) H&E staining. Scale bar=100µm (1A), 20µm (1B, 1D, 1E), 5 µm (1C).

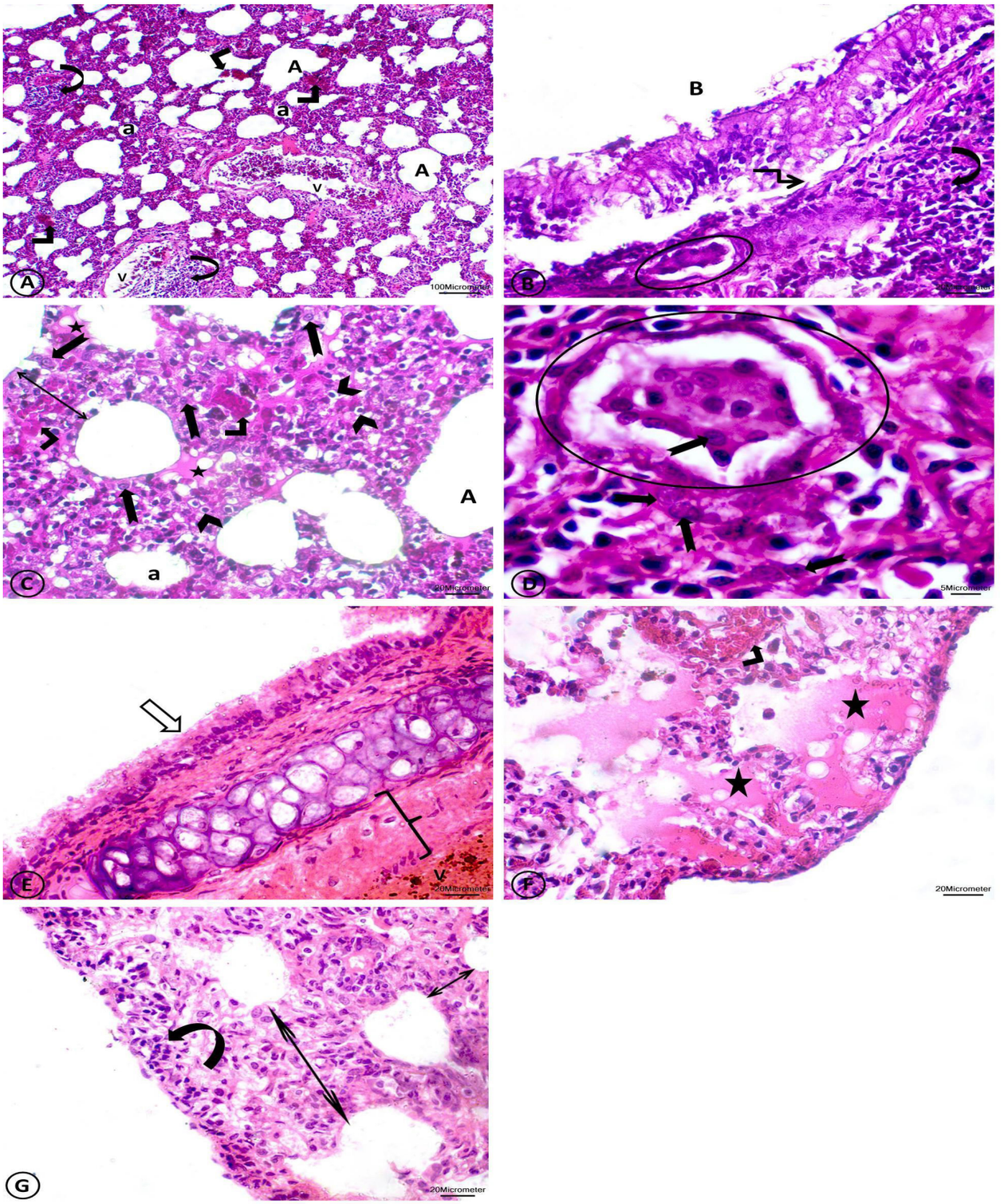


Fig 2: Photomicrographs of lung sections of adult rats from group II (BLM group) stained with H&E. Showing thickening of the inter-alveolar septa, areas of hemorrhage (right angled arrows), narrowing of many alveoli (a) and dilatation of adjacent ones (A). Notice the large number of pneumocytes type II with their rounded outline and vesicular nuclei (notched arrows) that are aggregated in many areas (encircled). Congested blood vessels (V), marked aggregation of inflammatory cells (curved arrow) are prominent findings. Epithelial damage and separation (broken arrows) is observed in many bronchioles (B) (2A, 2B, 2C, 2D). Bronchi are lined by thick pseudostratified columnar eithlium (hollow arrow) and surrounded by apparent congestion (V) with thickened smooth muscle layer (brace) (2E). In the sub-pleural regions; acidophilic hyaline exudate (stars), hemorrhagic areas (right angled arrow), inflammatory cells infiltration (curved arrow) and thickening of the inter-alveolar septa (double head arrows) are noticed (2F&2G). H&E staining. Scale bar=100µm (2A), 20µm (2B, 2C, 2E, 2F and 2G), 5 µm (2D).

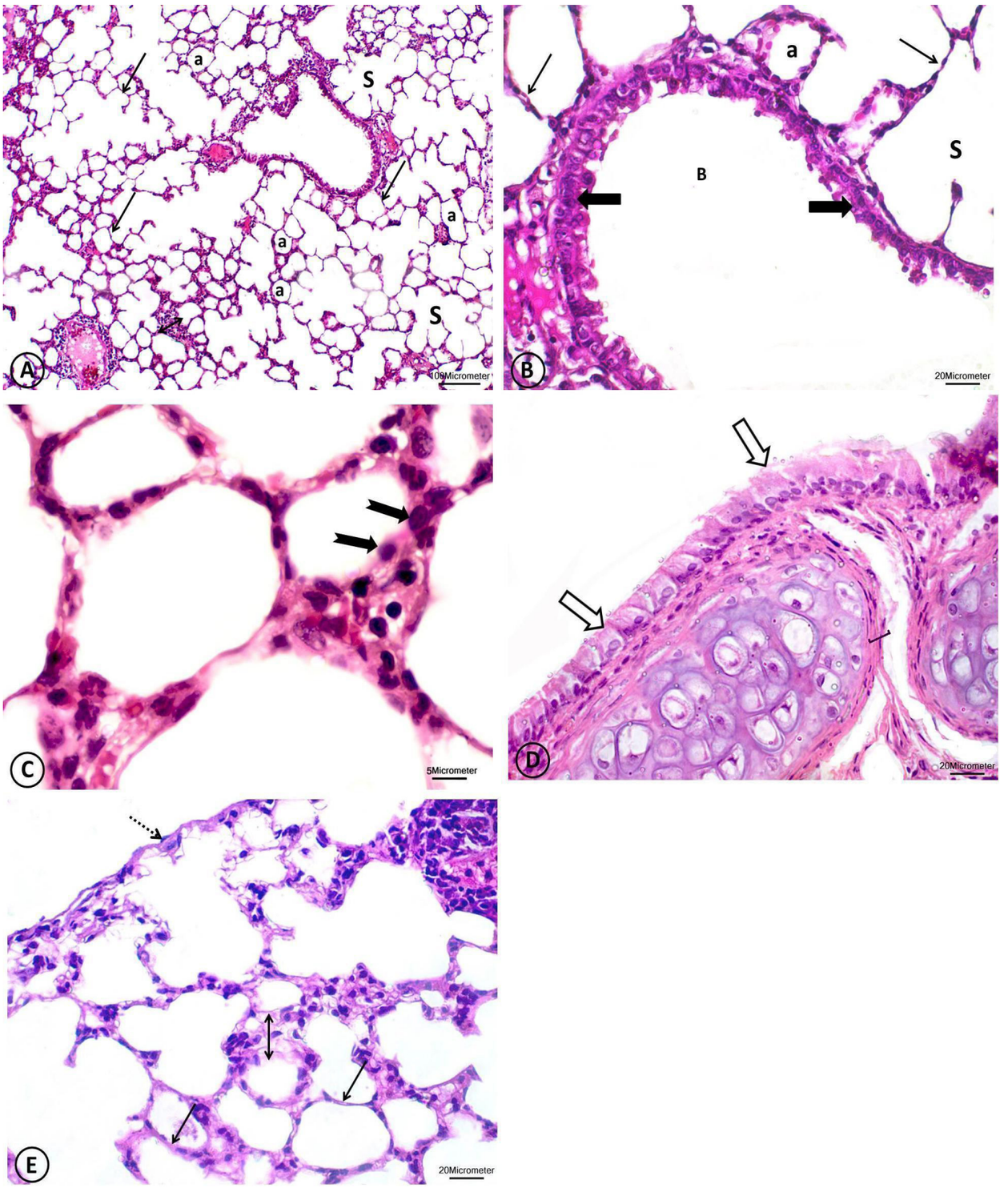


Fig. 3: Photomicrograph of lung sections of adult rats from group III (BLM and GT) stained with H&E. Showing that most of inter alveolar septa are thin (thin arrows) with few thickened septa (double head arrows). Notice normal structure of alveolar sacs (S) and alveoli (a). Bronchioles (B) are lined by intact simple columnar epithelium (thick arrows) (3A&3B). Notice few pneumocytes type II (notched arrows) (3C). Bronchi are lined by intact respiratory epithelium (pseudostratified columnar ciliated with goblet cells) (hollow arrows) and surrounded by thin smooth muscle layer (brace) (3D). Visceral pleura are lined by simple squamous methothelium (dotted arrow) and the sub-pleural regions are exhibiting thin inter-alveolar septa (thin arrows) with few thickened septa (double head arrow) (3E). H&E staining. Scale bar=100µm (3A), 20µm (3B, 3D and 3E), 5 µm (3C).

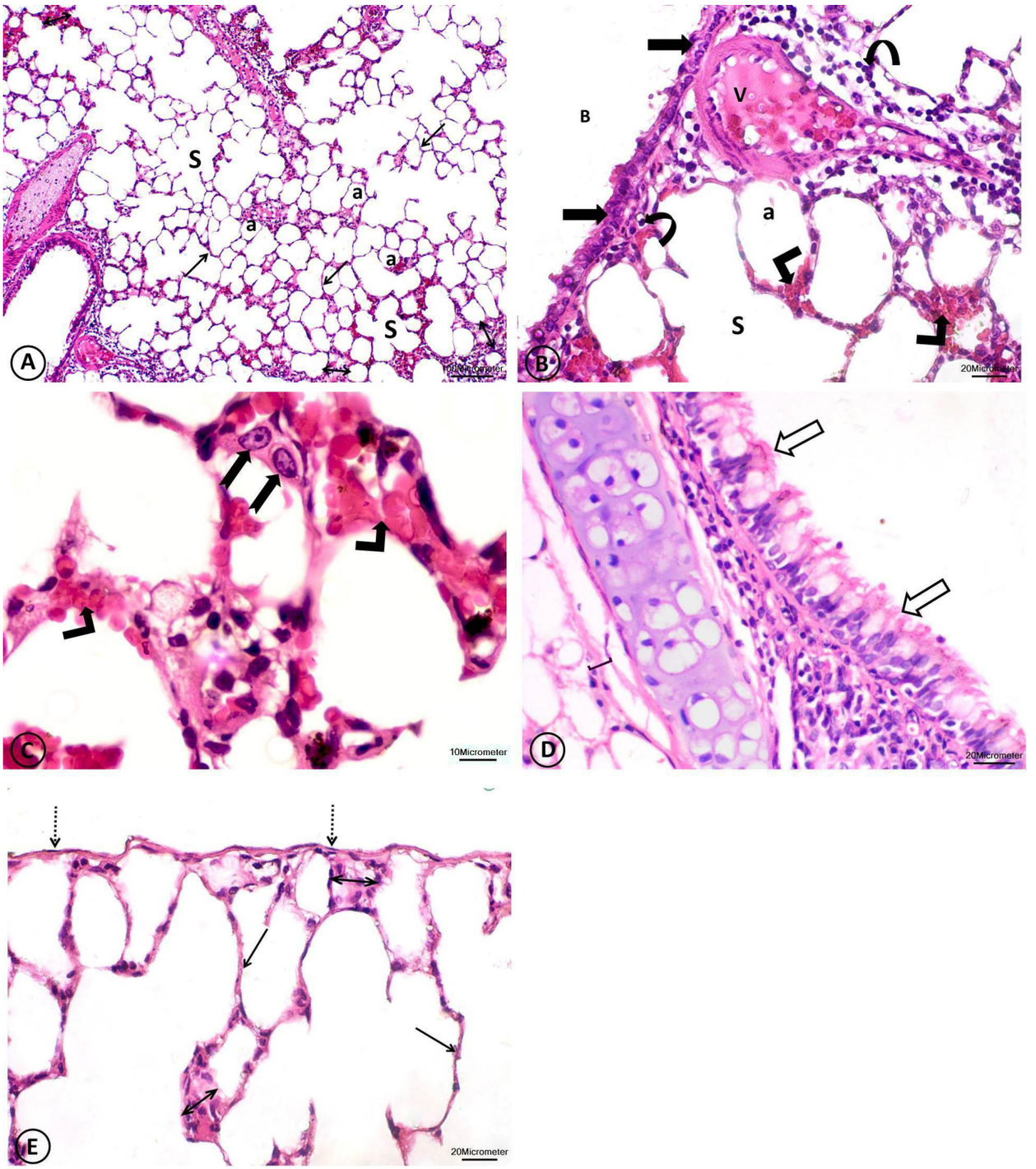


Fig. 4: Photomicrograph of lung sections of adult rats from group IV (BLM and metformin group) stained with H&E. Showing that most of inter-alveolar septa are thin (thin arrows) with few thickened septa (double head arrows). Notice normal structure of alveolar sacs (S) and patent alveoli (a). Residual hemorrhage (right angled arrows) could be detected. Bronchioles (B) are lined by intact simple columnar epithelium (thick arrows). Few mononuclear cellular infiltrations (curved arrows) are detected surrounding both bronchiole (B) and congested blood vessels (V). Few pneumocytes type II (notched arrows) are observed (4A, 4 B, 4C). Bronchi are lined by intact respiratory epithelium (hollow arrows) and surrounded by thin smooth muscle layer (brace) (4D). Visceral pleura are lined by simple squamous methothelium (dotted arrows In the sub-pleural region; The majority of inter-alveolar septa are thin (thin arrows) with few remaining thick septa (double end arrows) (4E). H&E staining. Scale bar=100µm (A), 20µm (B, D and E), 5 µm (C).

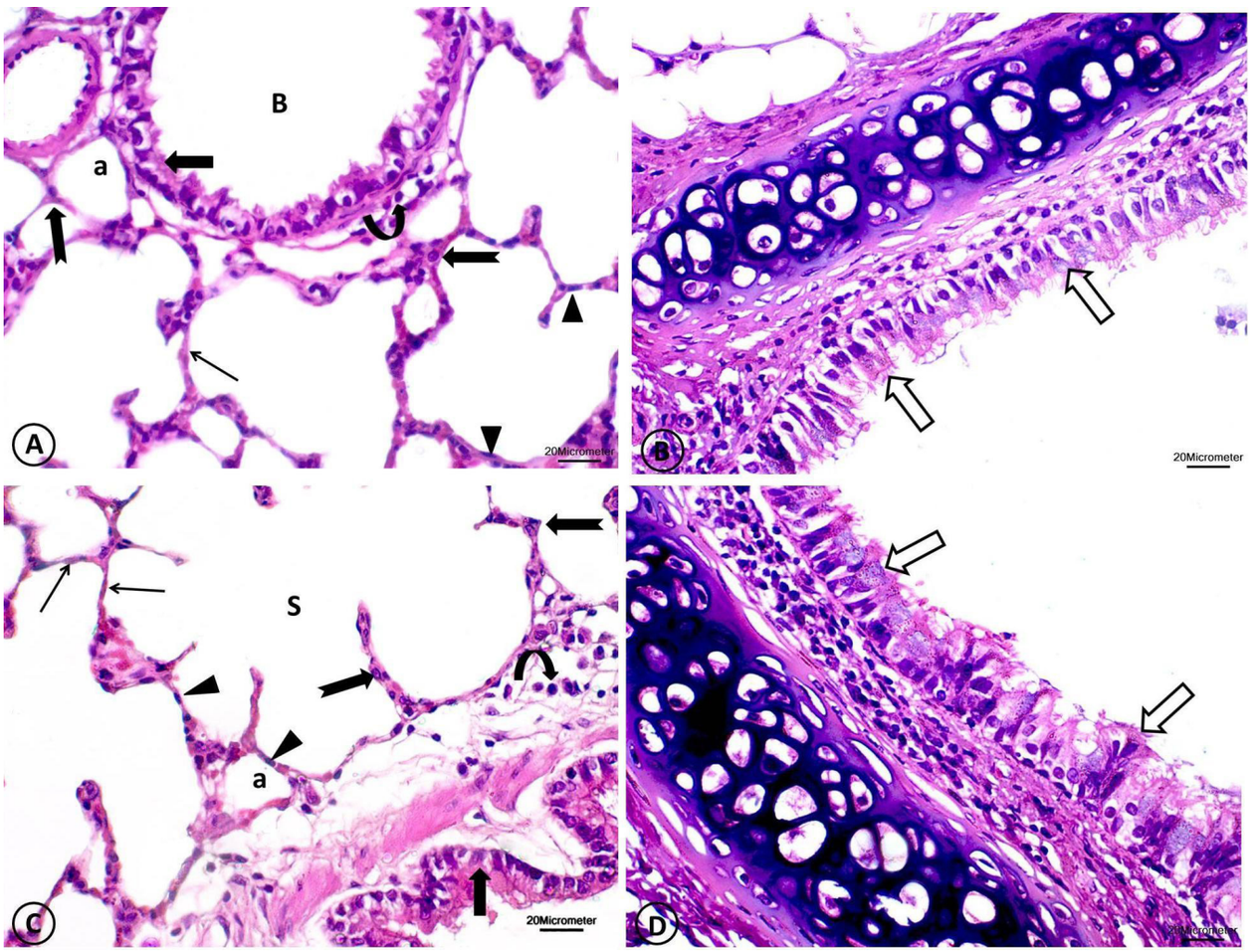


Fig 5: Photomicrographs of lung sections of adult rats from group V (GT tea group) (5A&5B) and group VI (metformin group) (5C&5D); stained with H&E. They are showing normal lung structure with thin interalveolar septa (thin arrows), normal patent alveolar lumina (a) The alveoli are lined by flat type I pneumocytes (arrow heads) and few rounded type II pneumocytes, alveolar sacs (S).normal histological structure of bronchioles(B)that are lined by intact simple columnar epithelium (thick arrows)and bronchi whose epithelial lining is intact pseudostratified ciliated with goblet cells (hollow arrows) could be observed with thin muscle layer. H&E staining. Scale bar=20µm.

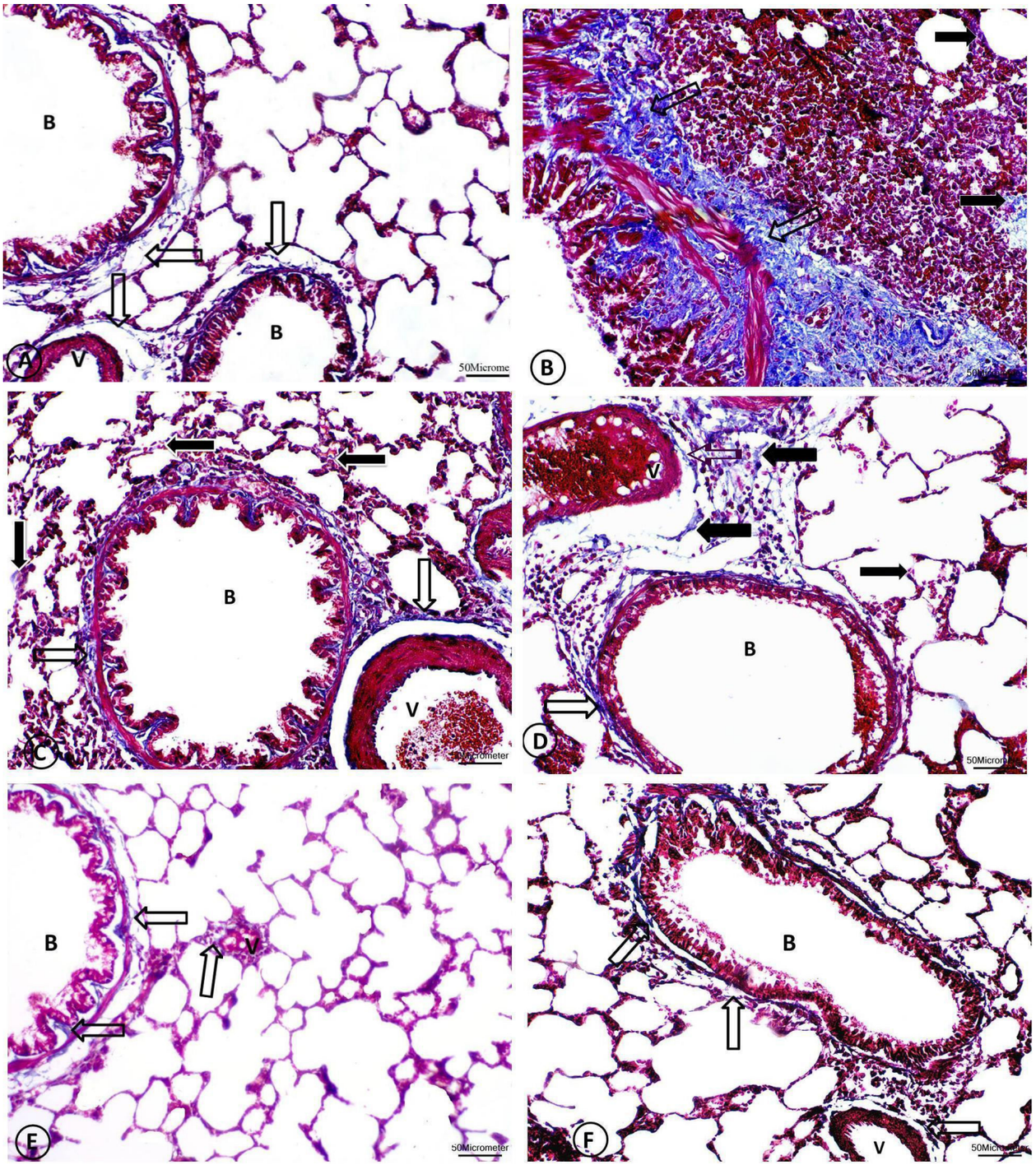


Fig. 6: Photomicrographs of lung sections of adult rats from all groups stained with Mallory's trichrome stain: **Group I (control group)** (6A); showing fine collagen fibers (hollow arrows) around bronchioles (B) and blood vessels (V). **Group II (BLM group)** (6B); showing marked collagen fibers deposition around bronchioles (hollow arrow) and in the interstitium (thick arrows). **Group III (BLM and GT)** (6C); showing moderate collagen fibers deposition (hollow arrows) around both of bronchioles (B) and blood vessels (V) and minimal deposition in the interstitium (thick arrows). **Group IV (BLM and metformin group)** (6D); showing moderate collagen fibers deposition (hollow arrows) around both of bronchioles (B) and blood vessels (V) and obvious deposition in the interstitium (thick arrows). **Group V (GT group)** (6E) and **group VI (metformin group)** (6F); showing fine collagen fibers (hollow arrows) around bronchioles (B) and blood vessels (V). Mallory's trichrome stain, Scale bar=50µm.

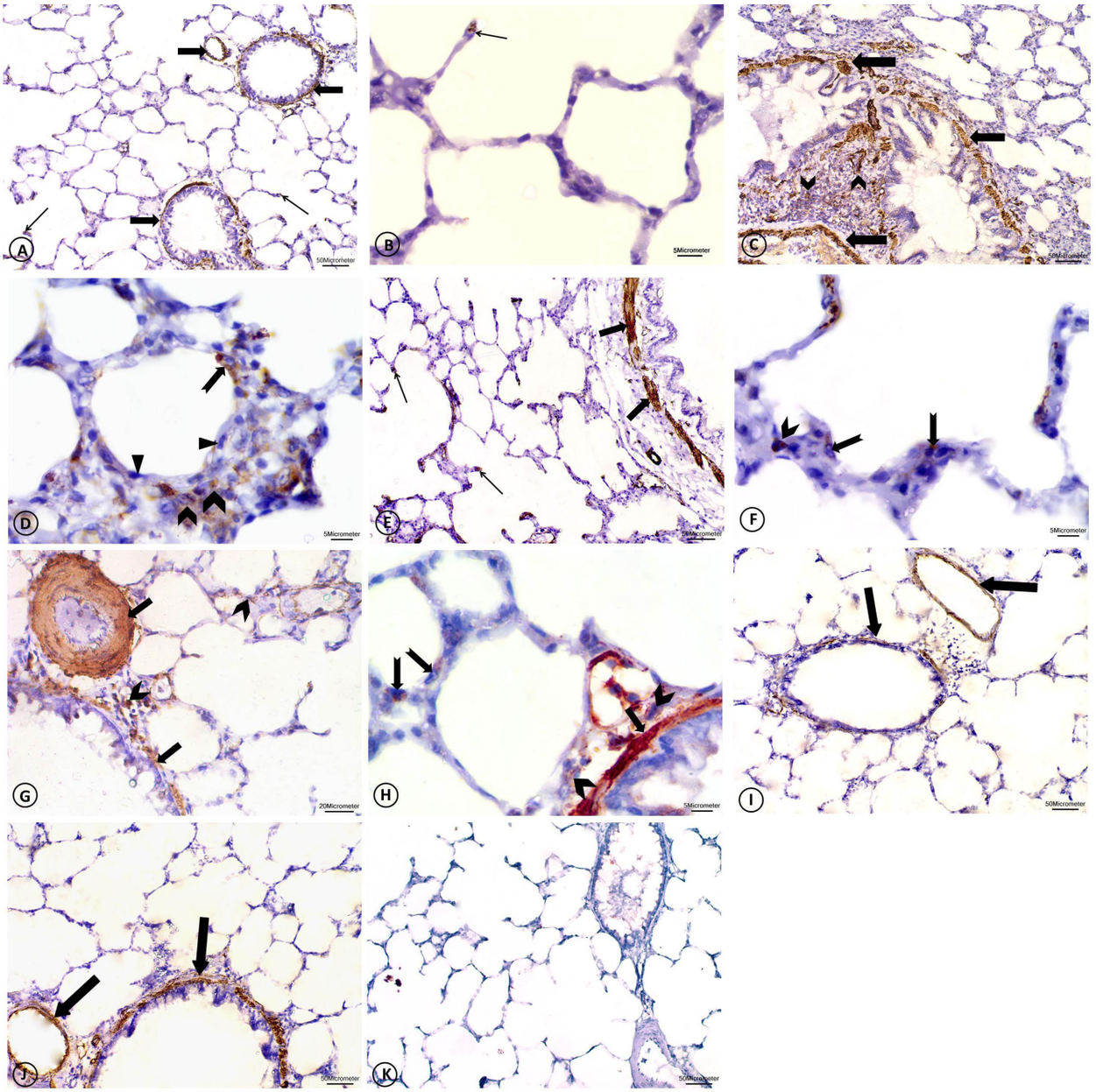


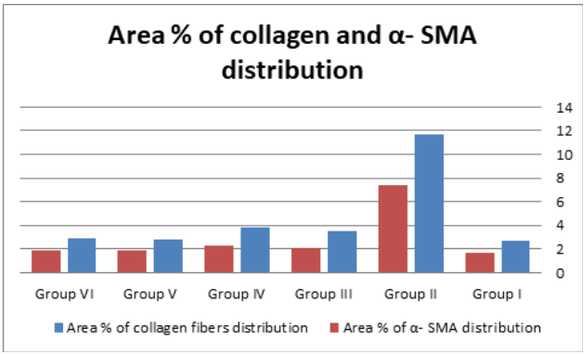
Fig. 7: Photomicrographs of lung sections of adult rats from all groups stained with α-SMA immunostain: **Group I (control group)** (7A&7B); showing α-SMA immunoreaction is only seen in the smooth muscle layer of the bronchioles and blood vessels (thick arrows) and in the knobs of alveolar duct (thin arrows)with the absence of α-SMA immunoreactivity within the lining cells of the alveoli. **Group II (bleomycin group)** (7C&7D); showing positive α-SMA immunoreactivity in the smooth muscle layer of bronchioles and blood vessels (thick arrows). There is also positive immunoreactivity in the cytoplasm of pneumocyte type II cells (notched arrows) and within the cytoplasm of myofibroblasts (arrowheads) in the thickened interalveolar septa. Note that type I pneumocytes (triangles) show negative immunoreactivity. **Group III (bleomycin and green tea group)** (7E&7F); showing positive α-SMA immunoreactivity in the smooth muscle layer of bronchioles (thick arrows), knobs of alveolar ducts (thin arrows), cytoplasm of myofibroblasts (arrowheads) and pneumocytes type II cells (notched arrow). **Group IV (bleomycin and metformin group)** (7G&7H); showing positive α-SMA immunoreactivity in the smooth muscle layer of a bronchioles, blood vessels(thick arrows), cytoplasm of myofibroblasts (arrowheads) and pneumocytes type II cells (notched arrow). **Group V (green tea group)** (7I) and group VI (metformin group) (7J); showing positive α-SMA immunoreactivity in the smooth muscle layer of bronchioles and blood vessels (thick arrows). α-SMA immunostain. Scale bar=50µm (7A, 7C, 7E, 7G, 7I and 7J) and 5µm (7B, 7D, 7F and 7H) Negative control (7K); lung section that was processed omitting the steps of primary antibody and it is showing negative immunoreaction. Scale bar=50µm

Table 1: mean area percent ± Standard Deviation (SD) of collagen fibers, α- SMA distribution and mean number of pneumocytes type II

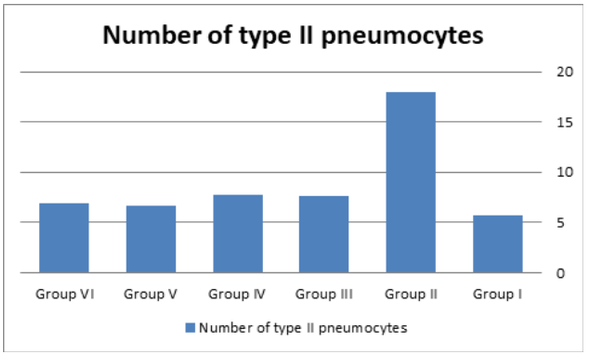
	Group I	Group II	Group III	Group IV	Group V	Group VI
Area % of collagen fibers distribution	2.69 ± 0.34[a]	11.68 ± 1.19[b]	3.55 ± 0.44[a]	3.81 ± 0.83[a]	2.76 ± 0.61[a]	2.96 ± 0.93[a]
Area % of α- SMA distribution	1.73 ± 0.26[a]	7.4 ± 0.47[b]	2.14 ± 0.41[a]	2.32 ± 0.58[a]	1.88 ± 0.41[a]	1.89 ± 0.22[a]
Number of type II pneumocytes	5.75 ± 0.85[a]	18 ± 0.93[b]	7.62 ± 1.3[a]	7.75 ± 1.83[a]	6.62 ± 1.41[a]	6.88 ± 1.5[a]

Significance at *P-value* < 0.05

Different superscript letter in the same row indicate statistically significant difference.



Histogram 1: Showing the mean area percent of collagen fibers, α- SMA distribution



Histogram 2: Showing mean number of pneumocytes type II

DISCUSSION

This work aimed to evaluate the effects of metformin versus green tea on Bleomycin induced lung injury of male albino rats.

In this work, male adults albino rats were chosen because of advantages as their stable genetic backgrounds, ease of handling[12], exclude the antioxidant effect of estrogen[13] and avoid the effect of certain X-linked genes[14]. Although some differences between rat and human lung structure but still it is the most well studied and accepted small animal model to simulate the human lung[15].

Bleomycin may induce lung fibrosis as a common side effect when administered by intra venous route which is the most common route of administration in clinical use[2]. In this study we used the intra tracheal route which is not applied in clinical practice just to ensure induction of lung fibrosis to study the effect of green tea and metformin on this experimentally induced lung fibrosis. This route of administration is the commonest route to induce and mimic idiopathic lung fibrosis in many studies with fewer systemic manifestations[16].

As regard the mortality occurred in this study; rats only died at the time of intra tracheal Bleomycin administration even in the groups assigned for combination with green tea or metformin which could be due to allergic reaction or faulty administration or perforation and they were excluded from the study. Other rats which passed this step showed no mortality during experimental period. While other studies showed mortality with prolongation of experiment for longer periods than we performed in the current study[16].

The body weight of the bleomycin treated group decreased gradually, while lung index as a parameter of lung edema was elevated in bleomycin treated rats due to lung weight gain with reduced body weight, similar results were previously reported and explained by occurrence of inflammation, congestion and interstitial edema[8].

In the present study; Bleomycin combined with either GT or metformin treatment kept the body weight of rats comparable to control with no remarkable loss.In addition to that; both GT and Metformin treatment inhibited the rise of the lung index when administered with bleomycin which means remarkable protection against occurance of lung edema with no remarkable differences between GT and Metformin effects.

In the present study, there were no apparent gross or histological differences between rats of control subgroup and that of green tea or metformin groups as regard the lung. Gross examination of lungs from bleomycin-treated rats showed variable sized multifocal lesions in both sides of the lung. While the lungs in groups of Bleomycin combined with either GT or metformine showed more or less normal lung architecture with only few focal lesions especially in Bleomycin with Metformin group. While that of bleomycin with green tea group appeared comparable to that of control group. This could demonstrate the protective effects of green tea and to lesser extent Metformin in preventing hazardous effects of Bleomycin on the lungs.

H&E sections of group II (BLM group) showed extensive alveolar affection as inter-alveolar septal thickening which may be due to severe mono nuclear cellular infiltration, increase in the number of pneumocytes type II which proliferate at time of respiratory distress increasing surfactant secretion, alveolar exudates due to secretions and remnants of dead cells as pneumocytes and macrophages. Diffuse hemorrhagic area due to marked congestion and loss

of elasticity in blood vessels, activation of myofibroblasts and increased collagen secretion leading to fibrosis and collapsed alveolar spaces. These histological changes coincide with other studies who used bleomycin to induce lung fibrosis[17,18,19].

Since the key steps of bleomycin-induced pulmonary fibrosis included oxidative stress. So one of the most acceptable theories in bleomycin induced toxicity is that starting cellular damage induced the secretion of oxygen free radicals or the so called reactive oxygen species ROS which play important roles in progression of such tissue damaging effect[20].

The previous findings were further explained by that bleomycin oxidative stress cytotoxicity may lead to single and double stranded breaks in DNA and rapid DNA fragmentation[21]. In addition to that, BLM is inactivated by bleomycin hydrolase, an enzyme present in most tissues except for the lungs and skin so it can affect the lung if administerd parentrally or direct intra-tracheally which was more effective in inducing fibrosis[21].

In the present study, Mallory's trichrome staining of Bleomycin group lung sections showed increased collagen fibers deposition. histological evidence of lung injury as thickening of alveolar walls, swelling and fragmentation of alveolar epithelial cells, cellular infiltrates. These changes could be due to alterations of the alveolar capillary barrier in addition to inflammatory responses as increase in the inflammatory markers IL-1b, IL-6, and monocyte chemoattractant protein[22,23,24]. The collagen area % in bleomycin group significantly increased compared to the control group and this was in agreement with the resuls of other studies explaining fibrosis by BLM induced epithelial injury[25], these mediators exert their profibrotic activities through the activation and proliferation of fibroblasts. In addition, bleomycin significantly led to upregulation of pro-fibrotic genes[26]. It was found also that TGF- β induces the synthesis of fibronectin and type I collagen which accounts for increased extracellular matrix (ECM) deposition in the interstitium[7].

In present study collagen fibers deposition in the subpleural area was explained by the differentiation of pleural mesothelial cells (PMCs) into myofibroblasts in response to transforming growth factor (TGF-β1)[27,28].

The previous results were confirmed by statistically significant increase in the mean area percent % of α-SMA of bleomycin group compared to control group. This was in agreement with other studies showing that in addition to bronchial and vascular smooth muscles, myofibroblasts expressed α -SMA with a strong stain density in the pulmonary interstitium in the bleomycin induced fibrosis group[29].

As examination of the lung of group III (BLM with GT group) showed nearly normal architecture with apparently thin interalveolar septa, few cellular infiltration, normal bronchiolar structure, mild interstitial hemorrhage and less congested blood vessels. Alveoli appeared lined by peumocytes type I and II without remarkable proliferation or any remnants of dead cells. The septa between alveoli appeared thin. Given these results, we demonstrated that green tea extract treatment significantly ameliorates Bleomycin-induced pulmonary fibrosis. Other studies supported our results and proved that green tea extract attenuates bleomycin induced pulmonary fibrosis[30].

The oxidant/antioxidant balance in the lung was affected with Bleomycin administration leading to severe tissue damage leading to fibrosis. Therefore, any therapeutic intervention that may prevent or attenuate oxidant insults may be used to treat pulmonary fibrosis. Due to its potent antioxidant activity, green tea content of polyphenols

epigallocatechin-3-gallate (EGCE), which is the main active ingredient in green tea extract, can protect the lung tissue from the unbalanced elevated ROS activity in many conditions as with bleomycin which induced lung fibrosis[31].

The effect of GT could be due to its anti-oxidant property. GT retains the highest level of (EGCG)[32]. Moreover, polyphenols EGCG suppressed ROS formation, scavenging of ROS, upregulation and protection of antioxidant defenses. In addition to that, that EGCG reduces the production of inflammatory cytokines (IL-6, IL-10, and TNF-α) and inhibits Pneumocytes type II cell dysplasia by suppressing the secretion of TGF-β1[9].

The EGCG also reduced the expression of proapoptotic genes and inducing the anti-apoptotic genes expression. GT extract contains minerals that function as co-factors for antioxidant enzymes. Zinc traces present in GT could be considered as a selective inhibitor of apoptosis. So it can inhibit some cell mechanisms in epithelial cells[33].

GT catechins reduce vascular permeability and vasodilation through the inhibition of cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). So reducing edema, inflammation and pain[34]. GT polyphenols reduce level of vascular endothelial growth factor (VEGF) that lead to a decrease in angiogenesis[35].

As regard Collagen area % of group III there was statistically high significant decrease as compared to group II (BLM group). The reducing effect of GTof collagen fibers deposition might be explained by the inhibitory effect of EGCG on TGF-b1 by suppressing various sources of TGF-b1 activators such as inflammatory cytokines, MMPs, macrophages and other inflammatory cells. In addition, EGCG also influences downstream transcription factors of TGF-b1 like downregulation of Smad3 and upregulation of Smad7[30].

The current study of group III showed a significant decrease in the mean area percent % of α-SMA immunoreaction as compared to BLM group. This was explained by EGCG interruption of TGF-B signalling in myofibroblasts by blocking Smad activation and suppressing α -SMA expression[30].

In the current work, (group IV) when we used metformin with BLM as another modality line of treatment of BLM induced lung injury. H & E sections showed less cellular infiltration, minimal blood vessels congestion, less extravasated RBCs, decreased bronchiolar and blood vessel wall thickness compared to BLM group.

Metformin increased the systemic production of antioxidant proteins such as reduced glutathione (GSH) and superoxide dismutase (SOD) and decreased lipid peroxidation in cases of diabetic nephropathy; so it may have similar mechanism as antioxidant in protecting against Bleomycin induced lung injury[36].

Metformin has the ability to augment 5' adenosine monophosphate-activated protein kinase (AMPK) which is a negative regulator of protein synthesis resulting in the inhibition of cellular proliferation, protein and DNA synthesis. Moreover, AMPK is a suppressor of NADPH oxidases that generate oxidative stress[37,38].

Collagen area % in the present study revealed statistically significant decrease in group IV (BLM and metformin group) compared to BLM group. This could be suggested that antifibrotic effect of metformin in inhibiting the expression of fibrotic markers such as Collagen type I, alpha 1 (col1 α 1) and Collagen Type III Alpha 1 (col3 α 1) is by inhibition of TGF-β[39].

Metformin inhibits the profibrotic effect of TGFβ1 in lung fibroblasts via AMKP activation. In addition to that, AMPK can alleviate inflammation-related fibrosis in the lung[40].

The present study showed a significant decrease in the mean area % of α-SMA (myofibroblast marker) immunoreaction in lung fibroblasts in group IV (BLM and metformin) compared to BLM group. Metformin-induced AMPK activation suppresses TGF-β-induced NADPH oxidase 4 (NOX4) expression and lung myofibroblast differentiation which blocks the synthesis of collagen and α-SMA and attenuates bleomycin-induced pulmonary fibrosis[41].

So in our opinion both green tea extract and Metformin act as antioxidants by different mechanisms and pathways. Both of them may have a protective effect against Bleomycin-induced lung fibrosis. There are better results obtained with green tea extract use especially as regard congestion and haemorrhage but still both of them may be beneficial and can be used according to different cases especially when applied to human patients with multiple systematic diseases.

CONCLUSION

Bleomycin has profound cytotoxic effects on lung tissue. Green tea as a natural antioxidant with anti-inflammatory and antifibrotic effects, improved bleomycin induced lung injury. Metformin as an oral antidiabetic drug with anti-inflammatory and anti-tissue remodeling effects, had also the same effect as green tea in the protection against bleomycin induced lung injury except for minimal amount of hemorrhage and congested blood vessels. So we can conclude that green tea has a better protective effect than metformin as regard bleomycin induced lung injury.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسةهستولوجية و هستوكيميائية مناعية لتقييم تأثيرات عقار الميتفورمين مقارنة بمستخلص الشاى الأخضرعلى إصابة الرئة المستحثة بعقار البليوميسين فى ذكور الفئران البيضاء البالغة

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مقدمة: بليوميسين هو علاج كيميائي يستخدم على نطاق واسع في علاج العديد من الأورام. وقد لوحظ أن التليف الرئوي يعد من أشهرو أخطر الآثار الجانبية له.

الهدف من الدراسة: معرفة التأثير المحتمل للشاي الأخضر مقارنة بالميتفورمين على الإصابات الرئوية التي يسببها البليوميسين لذكور الفئران البيضاء البالغة.

المواد والطرق المستخدمة: تم استخدام ٤٨ من ذكور الفئران البيضاء البالغة في هذه الدراسة. وقد تم تقسيمها إلى ٦ مجموعات كل واحدة تشمل ٨ فئران. المجموعة الضابطة ، مجموعة بليوميسين ، مجموعة بليوميسين مع الشاى الأخضر، مجموعة بليوميسين مع الميتفورمين، مجموعة الشاى الأخضر ومجموعة الميتفورمين. وقد تم إعطاء البليوميسين عن طريق القصبة الهوائية لتأكيد إحداث التليف. تم التضحية بجميع الفئران بعد ١٤ يومًا من بدء التجربة. تم تحضير مقاطع الرئة وصبغها بصبغة هيماتوكسلين وإيوسين وبصبغة مالورى ثلاثية الألوان والصبغة الهستوكيميائية المناعية للألفا أكتين الخاص بالعضلات الملساء. وتم عمل القياسات والتحليلات الإحصائية للمجموعات المختلفة.

النتائج: كان هناك تشويه ملحوظ في بنية الرئة مع زيادة كبيرة في نسبة مساحة ترسب الكولاجين وللألفا أكتين الخاص بالعضلات الملساء في مجموعة بليوميسين مقارنة بالمجموعة الضابطة. كان هناك وقاية لبنية الرئة في مجموعات بليوميسين مع الميتفورمين، بليوميسين مع الشاى الأخضر مقارنة مع مجموعة بليوميسين. وقد كان تأثير الشاى الأخضر أفضل كعامل وقائى فعال أكثر من الميتفورمين الذى وجد معه بعض الإحتقان والنزيف. مع تراجع نسبة الكولاجين ولألفا أكتين الخاص بالعضلات الملساء فى كل المجموعات مقارنة بمجموعة البليوميسين.

الخلاصة: يمكننا أن نستنتج أن تناول الميتفورمين والشاى الأخضر يمكن أن يخفف من إصابة الرئة الناجمة عن البليوميسين. مع وجود أفضلية للشاى الأخضر عن الميتفورمين كعامل وقائى.