

Ameliorative Potential of Biguanides on Experimentally-induced Lung Fibrosis

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Keywords

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Abstract

Lung fibrosis is a disease that carries poor prognosis and high mortality rate. The mechanisms of fibrosis may include disordered wound healing, infiltration with inflammatory cells and fibroblasts and release of reactive oxygen species and growth factors. The aim of this study was to assess the effect of metformin (Biguanide) on lung fibrosis induced by bleomycin and to clarify the molecular mechanisms of this effect. Sixty male Wistar rats were divided into 6 equal groups as follows: control group; bleomycin for 4 weeks group; metformin prophylactic group; bleomycin for 6 weeks group; metformin therapeutic group and metformin alone group. The weight of rats was recorded. Bronchoalveolar lavage (BAL) was analyzed for total and differential leukocyte count, tumor necrosis factor alpha (TNF- α) and transforming growth factor beta 1 (TGF- β 1). Lung tissue hydroxyproline, malondialdehyde and superoxide dismutase were measured. Also, parts of the lungs were subjected to histopathological and immunohistochemical examination for nuclear factor kappa B (NF- κ B). Metformin used prophylactically improved the histopathological picture and NF- κ B immunostaining and decreased the oxidative stress, TGF- β 1, TNF- α and BAL cellularity. When used therapeutically, metformin decreased oxidative stress and TGF- β 1 but didn't improve TNF- α , the histopathological picture and NF- κ B immunostaining. In conclusion, metformin has ameliorative effect on bleomycin-induced lung fibrosis when used prophylactically better than when used therapeutically..

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INTRODUCTION

Idiopathic lung fibrosis is an interstitial lung disease which is characterized by replacement of the normal lung tissue with fibrous tissue due to imbalance between matrix production and degradation that ultimately ends in respiratory failure and death. It carries poor prognosis, high mortality rate and limited therapeutic options due to complex pathogenesis [1].

Up till now, no exact mechanism fully explains the pathogenesis of the fibrotic process encountered in bleomycin-induced pulmonary fibrosis. The old theory of inflammatory alveolitis followed by fibrosis had been criticized due to failure of anti-inflammatory drugs to attenuate fibrosis and unfortunately exacerbated the ongoing fibrosis [2]. The new paradigm states that multiple factors may play a role in initiation and progression of fibrotic process. It is now considered a state of impaired wound healing due to repetitive micro injuries which result in altered microenvironment of alveolar epithelial, endothelial and mesenchymal cells through various growth factors, cytokines, and inflammatory cells, which leads to activation of fibroblasts and resistance to apoptosis, so healing process becomes disrupted and fibrosis ensues [3].

Bleomycin is an anti-cancer drug which is effective against many types of tumors, but this effectiveness is limited due to development of dose dependent lung fibrosis, which occurs due to enhanced oxidative stress that leads to inflammatory response and activation of fibroblasts through various cytokines and growth factors. Bleomycin is the most widely used model for studying lung fibrosis mechanisms and evaluating therapy; as it is well characterized, clinically

relevant and develops fibrosis more rapid than other models [4].

Many studies have evaluated the protective effect of drugs in bleomycin model, but this may be misleading; as it doesn't lead to clinically therapeutic benefit, so to gain the utmost benefit of the tested drug, it must be given after establishment of fibrosis that becomes evident after inflammation subsides, and this occurs at day 15 after bleomycin administration [5].

Metformin is one of biguanides that are recommended as first line oral therapy of type 2 diabetes mellitus. In addition, it has a therapeutic potential in diabetic nephropathy, cardiovascular diseases, nonalcoholic fatty liver disease, inflammatory disorders and the prevention or treatment of cancer [6,7]. Recently, metformin has been studied for its efficacy against tissue remodeling in the lung [8]. Currently, few studies have been conducted to elucidate the preventive role of metformin in attenuating lung fibrosis [9,10], while no studies were conducted to test the therapeutic potential of metformin. The aim of this study was to assess the effect of metformin (Biguanide) on lung fibrosis induced by bleomycin and to clarify the molecular mechanisms of this effect.

1. Materials & Methods

All the experiments in this study were conducted in accordance with the U.S. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. This study was approved by the Research Ethics Committee of faculty of medicine, Tanta University (Approval code 2066/9/13). Animal handling was followed according to Helsinki declaration of animal ethics.

2.1. Drugs used

Bleomycin 15 U/vial of (Cipla, India) equivalent to 15mg/vial white powder freely soluble in saline prepared by reconstitution of powder with 3ml saline to a final concentration of 5mg/ml. Metformin (Glucophage®) of Merck 500 mg/tablet was prepared by dissolving tablet in distilled water to reach a final concentration of 100 mg/ml.

2.2. Animal model

Sixty male Wistar rats, weighing initially 90-120 gm were used in this study. They were maintained on a standard laboratory diet and tap water and exposed to a 12/12 h light dark cycle. The animals were randomly divided into 6 equal groups as follows: Group 1 (control group), Group 2 BLE4w (Bleomycin for 4 weeks), Group 3 MET (Prophylactic) received metformin one week before and with bleomycin for 4 weeks, Group 4 BLE6w (Bleomycin for 6 weeks) and was considered as a positive control group for group 5 MET (Therapeutic) group which received metformin after 2 weeks of bleomycin administration and continued for further 4 weeks, and the last group 6 received metformin only. Rats were injected intraperitoneally by bleomycin at a dose 15 mg/kg/day 3 times per week [11] and metformin was given in dose 300 mg/kg/day by oral gavage [12].

2.3. Experimental protocol

Follow up of weight changes was recorded across the period of experiment and growth rates of different groups were compared. At the end of experiment, rats were killed by intraperitoneal injection of high dose of thiopental (30 mg/kg).

Bronchoalveolar lavage fluid (BALF) was done for total and differential leukocytic count and

assay of cytokines. After opening the chest, the left bronchus was ligated by silk suture for BAL. The left lung was dissected for histopathological examination and immunohistochemical staining NF-kB (p65) and the right lung was dissected for biochemical assay.

2.4. Bronchoalveolar lavage (BAL)

BAL was performed 4 times by applying a tracheal polyethylene catheter 7 Fr. to the right lung with 6mL of saline, with the left main bronchus ligated. Approximately, 5.5 mL (91%) of BAL fluid was recovered from each rat examined. BAL fluid was centrifuged at 1000 g for 10 minutes. Supernatants were stored at -20°C for analysis of transforming growth factor beta 1 (TGF-β1) and tumor necrosis factor alpha (TNF-α). Cell pellets were resuspended in 1 mL saline and total leukocytic count was determined by the hemocytometer (Marienfeld, nebular improved) after dilution with Turk solution and cell differentiation was determined for 200 cells performed on smeared preparation stained with Giemsa stain.

2.5. Assessment of BAL TGF-β1 and TNF-α

Transforming growth factor beta-1 (TGF-β1) was determined by using BOSTER Immunoleader ELISA kit (Catalog No. EK0514) of BOSTER BIOLOGICAL TECHNOLOGY according to the manufacturer's instructions, Tumor necrosis factor alpha was determined by using Ray Bio® Rat TNF-alpha ELISA Kit of Ray Biotech, Inc. according to manufacturer's instructions.

2.6. Biochemical assay for tissue hydroxyproline, malondialdehyde (MDA) and superoxide dismutase (SOD)

After BAL, the right lung was dissected and washed by ice cold saline and stored at -20°C for determination of tissue hydroxyproline according to the method of Reddy and Enwemeka [13], malondialdehyde (MDA) by using lipid peroxide kit obtained from BIODIAGNOSTIC co according to the method of Ohkawa et al. [14] (1979), tissue superoxide dismutase (SOD) by using superoxide dismutase kits obtained from BIODIAGNOSTIC co according to the method of Nishikimi et al. [15].

2.7. Histopathological examination

The left lungs were immediately fixed in 10% neutral buffered formalin. Paraffin sections were done and stained with hematoxylin and eosin stain as well as Mallory trichrome stain for detection of fibrosis and examined by light microscope for histopathological changes. The sections were graded according to the method of Hubner et al. [16].

2.8. Assessment of NF- κ B (p65) immunostaining

The sections were immunostained with primary antibody polyclonal IgG to rat NF- κ B p65 (Thermo scientific Lab vision, Cat No. RB9034-R7) and the slides were visualized under light microscope. The NF- κ B was assessed by detecting the activated subunit p65 in lung tissue and calculation of percentage of positive nuclear staining via IHC profiler tool in image J software (1.49v) national institute of health, USA [17].

2.9. Statistical analysis

Statistical analysis was carried out using Graph Pad Prism version 5. Data were expressed as mean \pm standard error of mean (SEM), while non-parametric data were presented as median (min-max). Multiple comparisons were performed using one-way ANOVA followed by Tukey–Kramer as a

post hoc test. For histology scoring and total leukocytic count, Mann Whitney test was used. P-values less than 0.05 were considered statistically significant.

2. Results

3.1. Effect of different treatments on weight gain rate

The results of weight gain rate are shown in (figure 1), the weight of rats at the start of study was identical in all groups. Administration of bleomycin caused significant decrease in growth rate of weight across 4 weeks in comparison with the control group ($p < 0.01$) (figure. 1A). Administration of metformin at first week before administration of bleomycin caused non-significant change in growth rate of weight in comparison with group 2 ($p > 0.05$) (figure 1B). Administration of bleomycin for 6 weeks caused significant decrease in growth rate of weight in comparison with control group ($p < 0.001$) (Figure 1C). Administration of metformin after 2 weeks of administration of bleomycin resulted in significant increase in growth rate of weight in comparison with group 4 ($p < 0.01$) (figure 1D). Administration of metformin alone for 4 weeks didn't cause significant changes in growth rate of weight in comparison with the control group ($p > 0.05$) (Figure 1E).

3.2. Effect of different treatments on total and differential cell count in BALF

Administration of bleomycin either for 4 or 6 weeks led to significant increase in total leukocytic count (TLC), prophylactic metformin caused significant reduction in TLC, while metformin used therapeutically did not lead to significant reduction as shown in (figure2). Results of differential percentage of cells are shown in

(table1) there was significant increase in neutrophils and lymphocytes and significant reduction in macrophage percentage in both

bleomycin groups 2 and 4, while metformin caused significant reduction of neutrophils and elevation of macrophage in both treatment groups 3 and 5.

Table 1: Percentage of differential cell count in different studied groups

| Cell type | Macrophage% | Neutrophils % | Lymphocytes % |
|----------------------------------|----------------|---------------|---------------|
| Control (group1) | 91.56±0.67 | 1.03±0.22 | 8.43±0.53 |
| BLE4w(group2) | 78.02±1.45 *** | 6.23±0.37*** | 15.43±1.37*** |
| BLE4w+MET(prophylactic) (group3) | 84.15±0.64 ### | 1.02±0.14### | 12.98±0.68 |
| BLE6w(group4) | 79.51±0.85*** | 3.69±0.19*** | 18.85±0.75*** |
| BLE6w+MET(therapeutic) (group5) | 83.52±1.144 Δ | 2.52±0.32 ΔΔ | 15.03±0.93 |
| Metformin (group6) | 94.65±0.53 | 1.07±0.18 | 5.64±0.66 |

Data were expressed as mean±SEM

*= compared to control group *₂=P<0.05, **₂=P<0.01, ***₂=P<0.001 #= compared to BLE4w group #₂=P<0.05, ##₂=P<0.01, ###₂=P<0.001 Δ= compared to BLE6w group Δ₂=P<0.05, ΔΔ₂=P<0.01, ΔΔΔ₂=P<0.001

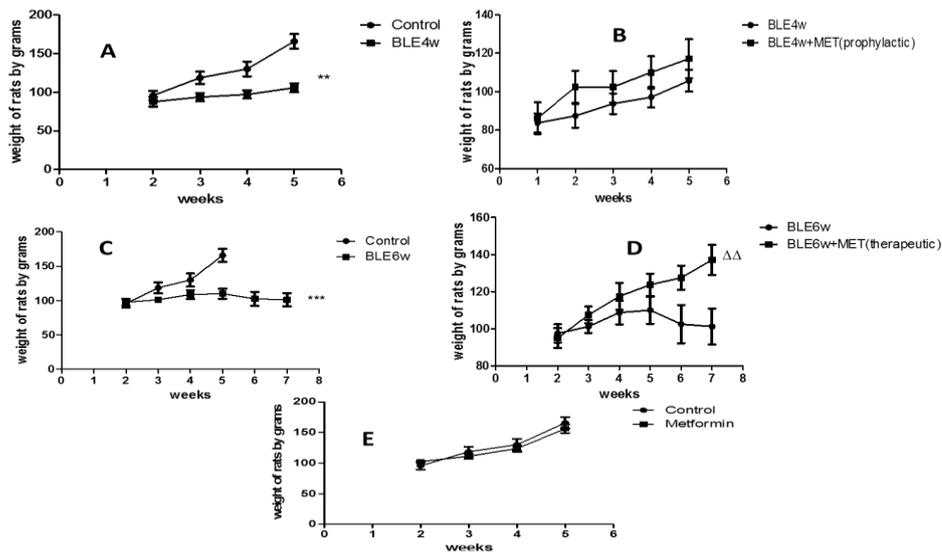


Fig.1. Weight changes comparison of different studied groups A: control vs. BLE4w, B: BLE4vs. MET (prophylactic), C: control vs. BLE6w, D: BLE6w vs. MET (therapeutic), E: control vs. Metformin*₂= compared to control group *₂=P<0.05, **₂=P<0.01, ***₂=P<0.001,Δ₂= compared to BLE6w group Δ₂=P<0.05, ΔΔ₂=P<0.01, ΔΔΔ₂=P<0.001.

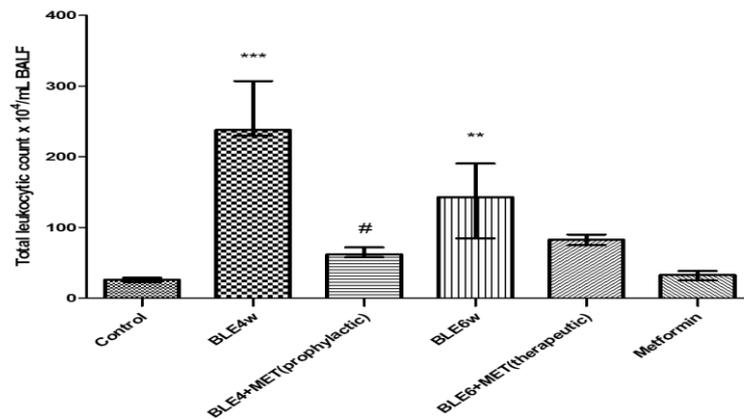


Fig.2. Total leukocytic count x10⁴/mL BALF in different studied groups, the result of different studied groups are presented as median and range (min-max),*₂= compared to control group *₂=P<0.05, **₂=P<0.01, ***₂=P<0.001 ,#₂=compared to BLE4w group #₂=P<0.05, ##₂=P<0.01, ###₂=P<0.001,Δ₂= compared to BLE6w group Δ₂=P<0.05, ΔΔ₂=P<0.01, ΔΔΔ₂=P<0.001

3.3. Effect of drug treatment on BALF TGF- β , TNF- α

There was significant increase in TGF- β 1 level vs. Control group ($P<0.05$) and ($P<0.01$) for both bleomycin groups 2 and 4 respectively, while administration of metformin caused significant reduction of TGF- β 1 level vs. group 2 and 4 for both metformin groups 3 and 5 ($P<0.05$) and ($P<0.01$) respectively. Tumor necrosis factor alpha (TNF- α) was significantly increased in both bleomycin groups 2 and 4 vs. Control ($P<0.001$) and ($P<0.001$) respectively, but in group 4 there was significant decrease vs. Group 2 ($P<0.001$), administration of prophylactic metformin resulted in significant decrease of TNF- α vs. group 2 ($P<0.01$), while metformin therapeutic caused significant increase in TNF- α vs. group 4 ($P<0.001$) (Figure 3 A and B).

3.4. Effect of different treatments on lung tissue hydroxyproline, MDA and SOD

Administration of bleomycin led to significant increase in hydroxyproline and MDA and significant reduction of SOD vs. control group in both bleomycin groups 2 and 4. Administration of metformin caused significant reduction in hydroxyproline, MDA and significant elevation in SOD in both groups 3, 5 vs. group 2, 4 respectively (Figure 4A-C).

3.5. Histopathological results

In group 2, there was significant increase fibrosis score (median 5) (Figure 5B) compared to control group (median 0) (Figure 5A) ($p<0.01$). In group 3, there was significant decrease in fibrosis score (median 2) (Figure 5C) compared to group 2 ($p<0.01$). In group 4, there was significant increase in fibrosis score (median 6) (Figure 5D) compared to control group (median 0) ($p<0.01$). In group 5, there was non-significant change in fibrotic score (median 4) (Figure 5E) compared to group 4 ($p>0.05$). In group 6, there was non-significant change in fibrosis score (median 0.5) (Figure 5F) compared to the control group (median 0) ($p>0.05$).

3.6. Effect of different treatments on NF- κ B (p65) immunostaining

Administration of bleomycin in group 2 results in significant increase in positive nuclear staining vs. control ($P<0.001$), while bleomycin in group 4 results in non-significant results vs. control ($p>0.05$). Administration of metformin prophylactically resulted in significant decrease immunostaining vs. group 2 ($P<0.05$), while metformin therapeutically resulted in significant increase vs. group 4 ($P<0.001$).

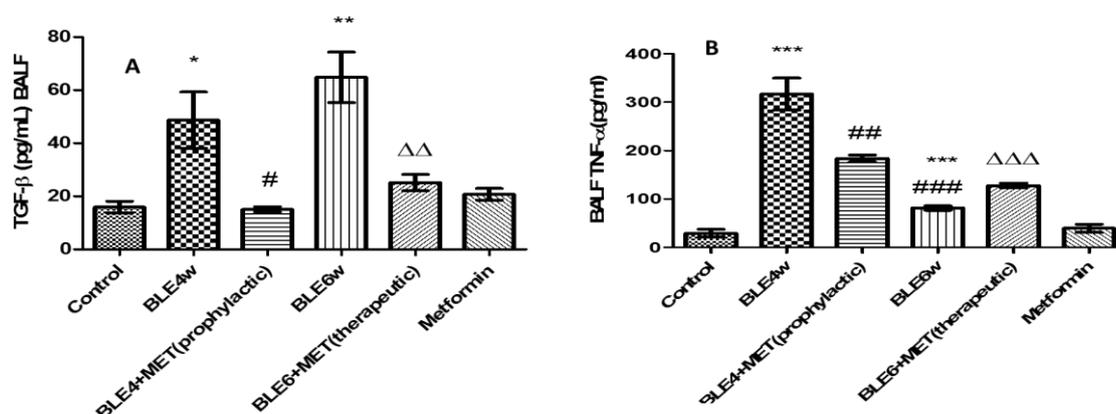


Fig. 3. BALF level of A: TGF- β 1, B: TNF- α in different studied groups, data are presented as mean \pm SEM, *= compared to control group $*=P<0.05$, $**=P<0.01$, $***=P<0.001$, #=compared to BLE4w group $\#=P<0.05$, $\##=P<0.01$, $\###=P<0.001$, Δ = compared to BLE6w group $\Delta=P<0.05$, $\Delta\Delta=P<0.01$, $\Delta\Delta\Delta=P<0.001$

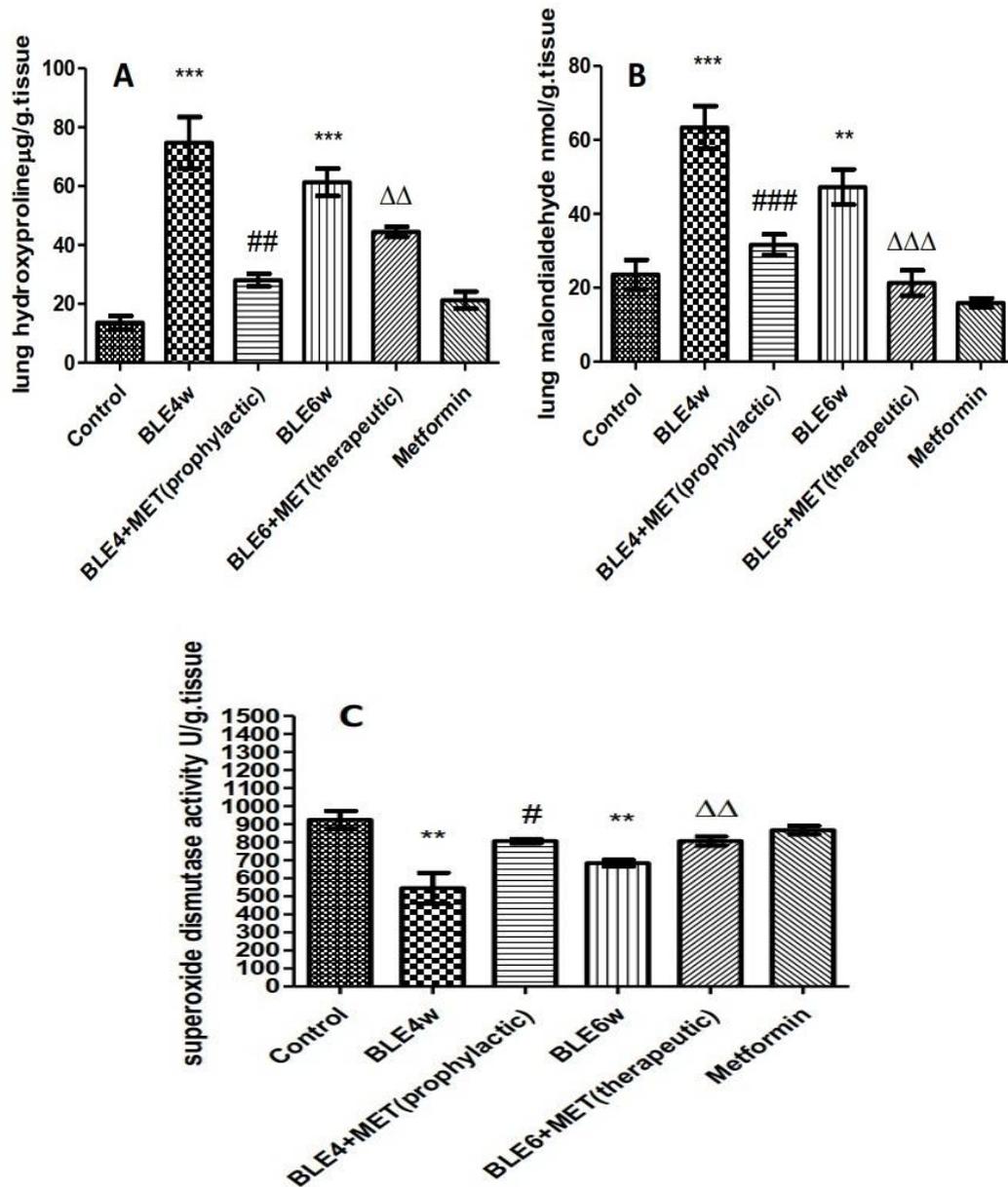


Fig.4. Lung tissue levels of A: hydroxyproline, B: malondialdehyde, C: superoxide dismutase, data are presented as mean±SEM, *= compared to control group * $P<0.05$, ** $P<0.01$, *** $P<0.001$, #=compared to BLE4w group # $P<0.05$, ## $P<0.01$, ### $P<0.001$, Δ= compared to BLE6w group Δ $P<0.05$, ΔΔ $P<0.01$, ΔΔΔ $P<0.001$

3. Discussion

In the present study, bleomycin was injected by intraperitoneal route in a repetitive continuous manner to overcome the problem of reversibility of this model which occurs with intratracheal single dose, also to mimic the pulmonary fibrosis pattern in humans which is sub-pleural lesions as reported by Gauldie and Kolb [18] and Mutsaers et al. [19]. Administration of

bleomycin either for 4 or 6 weeks caused significant reduction of weight and increased TLC compared to the control group. The inflammatory cells might create a microenvironment that promotes fibrosis by secreting growth factors, chemokines, cytokines and reactive oxygen species (ROS) [20] which was demonstrated in the present study by significant elevation of TGF- β , MDA and

reduction of SOD, also increased hydroxyproline content of lungs with elevation of fibrotic score.

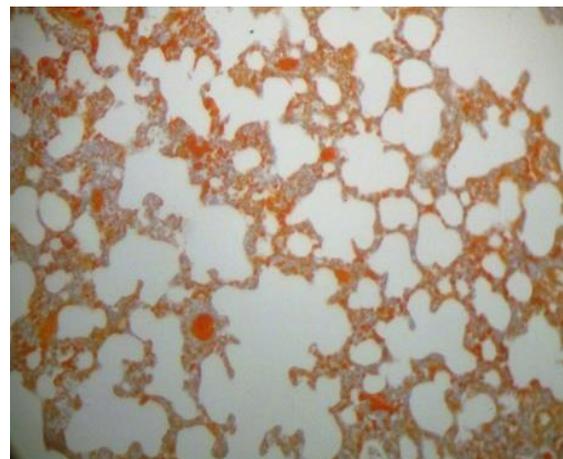
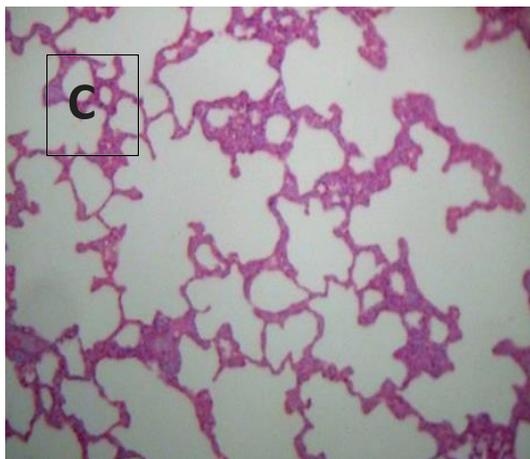
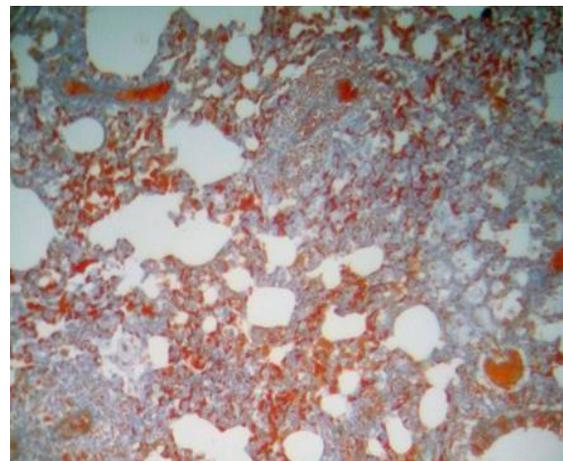
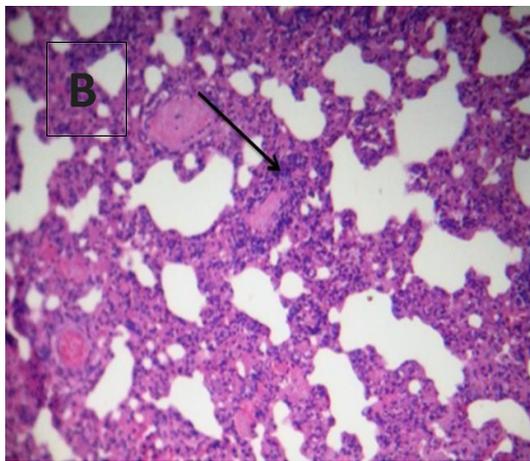
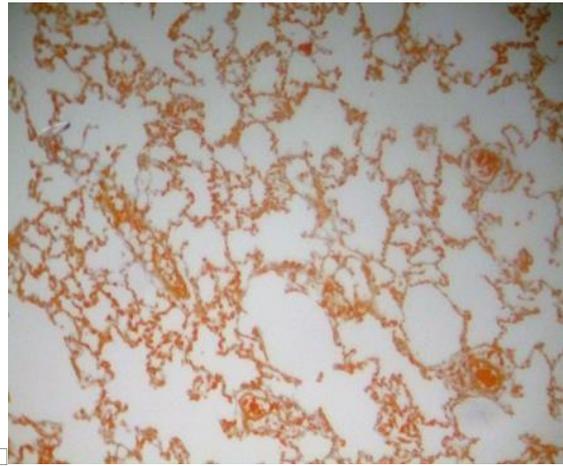
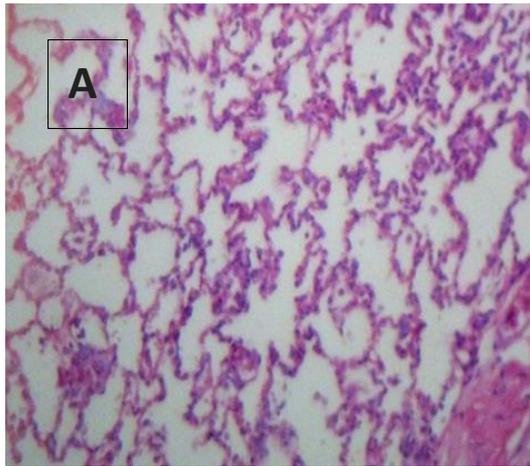
For resolution of inflammation to be efficient there must be an inhibition of neutrophils influx and promotion of monocyte recruitment to engulf neutrophil and regenerate disrupted tissue structure, beside promotion of apoptosis of neutrophils by inflammatory microenvironment. One of the factors that may induce apoptosis in neutrophils is TNF- α , and it has a complex effect on neutrophils ; as if it does not induce apoptosis, it may promote survival of them [21], while bleomycin administered for 4 weeks in this study led to increased TNF- α in BALF which was associated with increased neutrophils, the TNF- α has markedly decreased in the group which was given bleomycin for 6 weeks while still there are neutrophils, and this may denote that absence of TNF- α in this phase led to survival of neutrophils as there is no apoptosis.

It was found that NF- κ B (p65) may promote survival of neutrophils and inhibits their apoptosis [21]. In the present study administration of bleomycin for 4 weeks had increased positive active nuclear subunit of NF- κ B (p65), and this may explain the neutrophils' increased percentage, while administration of bleomycin for 6 weeks led to very mild immunostaining of NF- κ B(p65) comparable with control group, and because it is known that TNF- α is the main inducer of NF- κ B. This may explain that loss of TNF- α led to survival of neutrophils in this group denoting complex actions of TNF- α in pathogenesis of pulmonary fibrosis.

There is controversy about the role of TNF- α in fibrosis. As a component of the inflammatory response, TNF- α may be important

in the early stages of the disease, while rarely seen after that as inflammation subsides and fibrosis ensues [22]. TNF- α may be profibrotic as it may induce neutrophils and lymphocytes influx, depletes antioxidant defense, and promotes various inflammatory molecules such as adhesion molecules, also stimulates collagen synthesis, induces TGF- β ,and stimulates fibroblast proliferation [23]. On the other hand, it may act as antifibrotic; as it is expressed by macrophages during resolution of fibrosis, it also suppresses collagen production induced by TGF- β , promotes degradation of extracellular matrix (ECM) , beside inducing NF- κ B which induces smad7 ,so interferes with smad phosphorylation and inhibits fibrosis [22,24]. NF- κ B is expressed in cytoplasm of all cells and its activity is controlled by I κ B- α which undergo degradation via TNF- α to release NF- κ B (p65) which enters the nucleus and increases the expression of chemokines, inflammation and cell survival genes [25].

Administration of bleomycin for 6 weeks resulted in markedly decreased TNF- α , while still significantly increased more than control, but it is markedly decreased relative to group of bleomycin 4weeks accompanied by more fibrotic score and this is in agreement with Fujita et al. [26], as they explained in their study that low level of TNF- α may contribute to more fibrotic change than sustained high level. Also, bleomycin for 6 weeks led to very mild immunostaining of NF- κ B (p65) which indicates that there was shut down of its activation as I κ B- α not only localize it in cytoplasm but also shuttle the nuclear active part to get back to cytoplasm and this occurs as a negative feedback inhibition through induction of I κ B- α by NF- κ B (p65) itself [27].



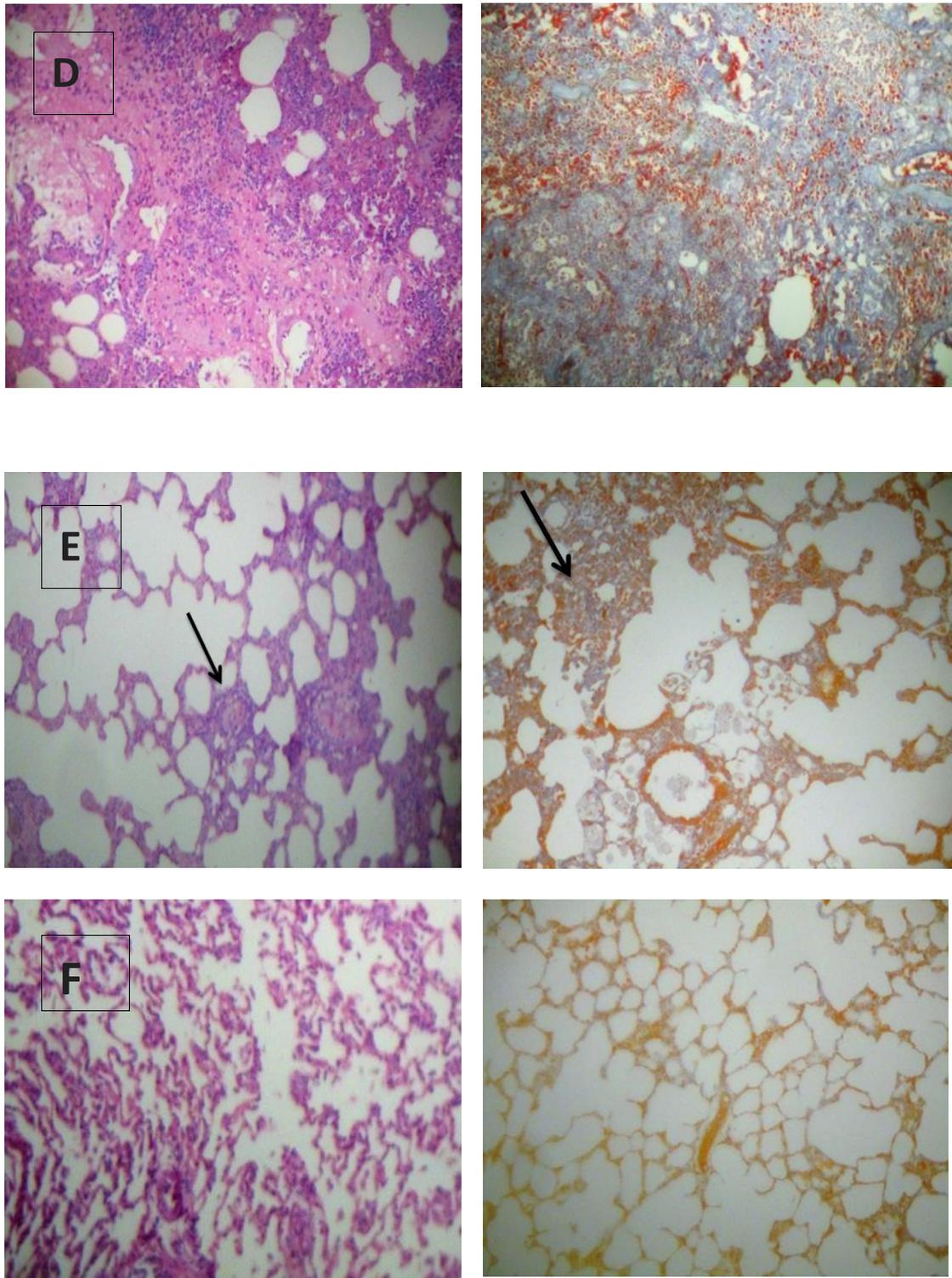


Fig.5. Section of the lung (H&E, 200X on the left & Malory trichrome, 200X on the right) from A) control group with normal alveoli and lung structure; B) group 2 (BLE4w) showing confluent fibrotic masses >10% and <50 % lung structure is severely damaged also section shows inflammatory infiltrate (arrow); C) group 3 (BLE4w+MET prophylactic) showing mildly thickened alveolar septa with knot-like formation but not connected to each other, Alveoli partly enlarged and rarefied, but no fibrotic masses; D) group 4 (BLE6w) showing lost alveolar septa and lung structure with confluent fibrotic mass >50% of field with inflammatory infiltrate; E) group 5 (BLE6w+MET (therapeutic) showing fibrotic masses (arrow) but <10% of field; F) group 6 (Metformin) showing normal alveolar septa and lung structure .

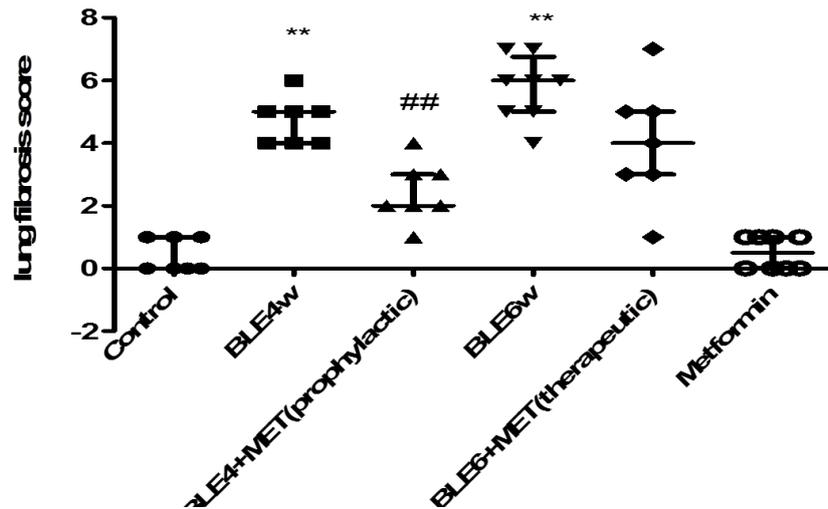


Fig.6: Fibrosis score of different studied groups ,data were expressed by median,*= compared to control group *=P<0.05, **=P<0.01, ***=P<0.001,#=compared to BLE4w group #=P<0.05, ##=P<0.01, ###=P<0.001, Δ= compared to BLE6w group Δ=P<0.05, ΔΔ=P<0.01, ΔΔΔ = P<0.001

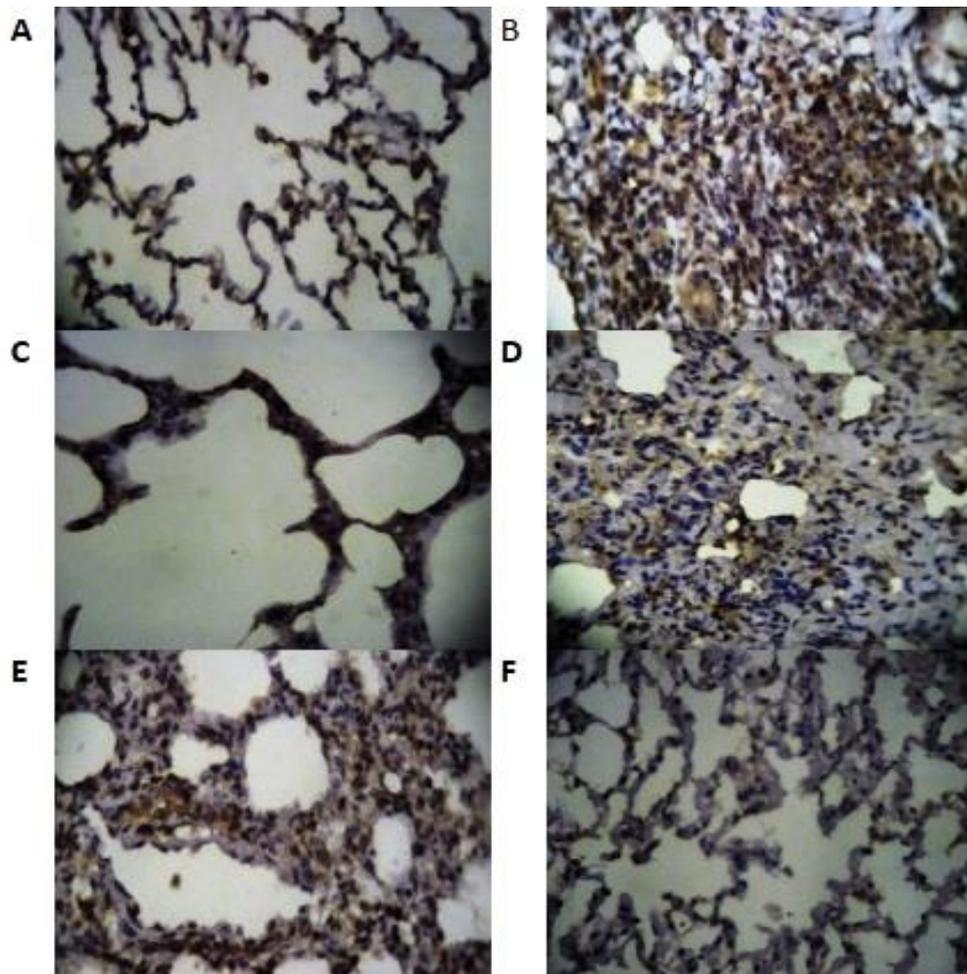


Fig.7: Lung sections of immunohistochemical staining of NF-κB (p65) of A) control group x400 showing mild positive staining for NF-κB (p65); B) BLE4w group x400 showing increased positive staining for NF-κB (p65); C) BLE4w+MET (prophylactic) x400 showing decreased positive staining for NF-κB (p65); D) BLE6w x400 group showing mild positive staining for NF-κB (p65); E) BLE6w+MET (therapeutic) x400 showing increased positive staining for NF-κB (p65); F) Metformin group x400 showing mild positive staining for NF-κB (p65)

Although metformin is known to prevent weight gain beside causing reduction of food intake and promoting weight loss owing to increased glucagon like polypeptide -1 (GLP-1) as denoted by Hu et al. [28] and Yasuda et al. [29]. Yet in the present study, it didn't affect weight gain when given alone to rats.

Therapeutic not prophylactic metformin led to prevention of weight loss induced by bleomycin. Administration of prophylactic not therapeutic metformin decreased total cell count. However, at differential level there was no difference between the two groups as both decreased neutrophils and elevated macrophages, but no effect was observed on elevated lymphocytes and this is in agreement with the notion that lymphocytosis may indicate good prognosis and response to therapy [30]. Zmijewski et al. [31] reported that metformin inhibited mitochondrial respiratory complex I which resulted in intracellular degradation of I κ B- α , that acts as inhibitor of NF- κ B activation. This led to reduction of neutrophil activation and reduced lung injury. Also, metformin enhanced phagocytosis of neutrophils by macrophage, and that explains the protective effect of metformin when used prophylactically in the present study as there was significant decrease in the immunostaining of NF- κ B (p65). In the present study, metformin has increased percentage of macrophage with resolution of fibrosis and this denotes that macrophages may have antifibrotic role as they secrete matrix metalloproteinases which lead to proteolysis and degradation of ECM and aid in apoptosis of myofibroblasts [32,33].

TGF- β 1 is considered the cornerstone mediator of lung fibrosis; it is secreted by platelets,

apoptotic epithelial cells and myofibroblasts, it activates gene transcription of fibrotic molecules as collagen I and fibronectin via smad effector proteins, in addition to induction of epithelial cells apoptosis and activation of myofibroblasts, it aids in crosstalk between epithelium and mesenchyme to mediate fibrosis [34]. Administration of metformin either prophylactically or therapeutically led to decrease in TGF- β 1 in BAL and this is in agreement with several studies that reported that metformin inhibits TGF- β 1 and its smad signaling in cardiac fibrosis [35] and recently lung fibrosis [36,37]. Also, Park et al. [8] reported that metformin attenuates airway remodeling in a murine model of bronchial asthma via reduced expression of TGF- β 1, but these studies evaluate the prophylactic role of metformin only.

Administration of metformin prophylactically decreased TNF- α level associated with improvement of fibrosis score and decreased NF- κ B(p65) staining and this in agreement with Arai et al. [38] as they reported that metformin decreased release of TNF- α from monocytes. Also, Hyun et al. [39] reported that metformin decreased TNF- α and suppressed NF- κ B in macrophage stimulated by lipopolysaccharide, while administration of metformin as therapeutic increased TNF- α and NF- κ B (p65) immunostaining, and did not improve fibrosis score. This may be explained in this study by that metformin promotes macrophages increase as denoted previously, so lead to release of TNF- α and NF- κ B activation. Kuroki et al. [40] found that endogenous TNF- α was important to help resolution of inflammatory cells as it induces apoptosis in inflammatory cells especially

neutrophils which was decreased in this group of the present study.

Administration of metformin resulted in decrease of tissue hydroxyproline which is considered as a specific marker of collagen synthesis and degradation, in addition to its abundance in collagen relative to other proteins in ECM [41]. This was associated with improvement in fibrosis score of prophylactic metformin while no improvement in therapeutic group.

ROS are important for initiation and progression of pulmonary fibrosis, they are produced by macrophages and neutrophils and cause epithelial cell injury, and also activated myofibroblasts produce H₂O₂ which induce epithelial cell apoptosis. TGF- β induces ROS production by suppressing antioxidants defenses such as catalase, reduced glutathione, SOD and activates oxidases in cell membranes leading to release of H₂O₂ to the extracellular space [42,43].

Administration of metformin either prophylactically or therapeutically led to decreased MDA which is a marker of lipid peroxidation by ROS. SOD is the only enzyme system that decomposes superoxide radicals to H₂O₂ and plays a significant role in protecting the lung from oxidants. It is expressed mainly by alveolar macrophages, vascular endothelium, ECM and epithelial cells, it protect the lung specifically by preventing oxidative degradation of matrix by binding to collagen I and IV [42]. Metformin either prophylactically or therapeutically led to elevation of SOD in lung tissue.

4. Conclusion

Metformin when used prophylactically is more beneficial in reduction of bleomycin-induced

lung fibrosis than when used therapeutically. This effect may be due to reduction of NF- κ B, TNF- α and TGF- β 1 together with amelioration of the oxidative stress and reduction of the inflammatory cells.

Conflicts of interest

The authors declare that there is no conflict of interest.

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