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THERAPEUTIC EFFECTS OF DEXAMETHASONE AND /OR CURCUMIN AGAINST PULMONARY FIBROSIS INDUCED BY BLEOMYCIN IN MALE C57BL/6 MICE

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ABSTRACT

Pulmonary fibrosis mouse model was constructed by intratracheal instillation of 1 mg/kg body weight of bleomycin to C57BL/6 mice. This caused alveolar epithelial cell damage and developed inflammation followed by fibrotic alveolar changes**.** Dexamethasone plays an important role in attenuating bleomycin induced lung fibrosis in mice. Also curcumin, is a naturally occurring compound that has anti-inflammatory and antioxidant effects. In this study, 70 male mice were divided into seven groups: the first group served as normal control receiving distilled water. The second group represented the dexamethasone group receiving 0.45 mg/kg/body weight. The third group served as curcumin group receiving 100 mg/kg body weight. The fourth group represented the bleomycin group and received single intratracheal dose (1 mg/kg body weight). The fifth group served as the bleomycin / dexamethasone, the sixth group represented the bleomycin / curcumin group and the seventh group represented the bleomycin / dexamethasone + curcumin group. Hydroxyproline was analyzed in lung tissues and lactate dehydrogenase was assessed in serum samples. Histological changes in the lung were evaluated by hematoxylin and eosin stain. Masson's trichrome staining was investigated for collagenous fibers deposition. The results elucidated that either dexamethasone or curcumin decreased lung fibrosis. In addition, combined treatments of dexamethasone and curcumin revealed better therapeutic effects on bleomycin induced lung fibrosis than the single treatment of either dexamethasone or curcumin alone. Thus, curcumin may act as an additive

therapeutic strategy scavenging free radicals to improve the outcome of dexamethasone treatment in bleomycin- induced lung fibrosis mice model.

KEYWORDS: Bleomycin, Curcumin, Dexamethasone, Hydroxyproline, Histopathology, Pulmonary fibrosis.

INTRODUCTION

Pulmonary fibrosis is a chronic, progressive fatal lung disease which is characterized by fibroblast proliferation and extracellular matrix remodeling**.** Multiple cycles of lung injuries lead to destruction of epithelial alveolar cells that in turn cause the migration, proliferation and activation of mesenchymal cells and the exaggerated accumulation of fibroblasts and myofibroblasts. This causes excessive collagen deposition within the lung interstitium and alveolar space causing abnormal wound repair.[1,2]

Bleomycin (BLM) is a glycosylated linear non-ribosomal peptide antibiotic produced by the bacterium Streptomyces verticillus.[3] Bleomycin has potent tumor killing properties, which gave it an important role in cancer chemotherapy. Nevertheless, the major limitation of bleomycin therapy is pulmonary toxicity which can be life threatening in up to 10% of the patients receiving this drug. Based on these evidences, bleomycin is the most widely used drug for the induction of experimental pulmonary fibrosis.[4,5]

The bleomycin lung mice model is represented mainly by the inflammatory stage that develops within the first 2 weeks after the injury which then subsides to the fibrotic phase.[6] The assessment tools used in the bleomycin model are based on histology of lung tissue and quantitative assessment hydroxyproline levels and lung collagen content.[7, 8]

Dexamethasone (DEX) is a synthetic glucocorticoid with therapeutic action in pulmonary diseases. Dexamethasone possesses anti-inflammatory and immunosuppressive properties which influence and body functions.[9] Dexamethasone plays an important role in attenuating bleomycin -induced lung fibrosis in mice. Also Dexamethasone is widely used as an antifibrotic agent due to its protection of the lungs against fibrosis by inhibiting the production of inflammatory mediators.[10]

Dexamethasone stabilizes lysosomal membrane and prevents the release of protolytic enzymes released during the inflammatory process. It also decreases edema and reduces chemotaxis at inflammatory areas.[11] However, dexamethasone has significant metabolic and

immunological side effects. Immune suppression and hyperglycemia are the cardinal concerns that may contribute to an increased risk of infection with the concurrent use of dexamethasone.[12] Furthermore, dexamethasone stimulates lipolysis and increase protein catabolism. It has catabolic effects on lymphoid tissues, connective tissue, muscle and skin. Also DEX treatment produces many side effects, such as growth retardation, hypertension, myocardial hypertrophy, gastrointestinal perforation, and neurological impairment.[13]

In addition, DEX is known to reduce collagen synthesis and steady state levels of procollagen mRNA by lung fibroblasts.[14]

Curcumin (CURC), a yellow curry pigment from turmeric (*Curcuma longa*) which has been shown to have a broad range of antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, anti-proliferative, and pro-apoptotic properties. Curcumin has also been reported to have anti-fibrotic capabilities in lung fibrosis models.[15, 16]

In particular, curcumin has been shown to inhibit processes essential to development of lung and pancreatic fibrosis. These processes include proliferation and differentiation of pulmonary and pancreatic stellate cells as well as profibrotic expression of extracellular matrix genes and collagen deposition. These data suggest that curcumin may be an effective treatment for pulmonary fibrosis.[17, 18]

So, the aim of the present study is to assess the effect of either dexamethasone and /or curcumin treatments in attenuating lung fibrosis induced by bleomycin for 14 days.

2- MATERIAL AND METHODS

2.1. Experimental Animals

Male mice of the strain C57BL/6 weighing average weight of 18±30 grams. They were housed in wire mesh laboratory animal cages in the vivarium of the Animal house of Faculty of Agriculture, Alexandria University at constant room temperature (24°C) with natural day/night light cycle, food and water ad libitum. Mice were adapted to laboratory conditions for a week before the beginning of the study. All animal procedures were performed in accordance with the guidelines for the care and use of experimental animals established by the Committee of the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) protocol approved by Alexandria University.

2.2. Experimental Chemicals and Dosage

A- Bleomycin (BLM)

Bleomycin sulphate powder ($C_{55}H_{84}N_{17}O_{21}S_3$), Sigma-Aldrich.USA is an antineoplastic antibiotic isolated from Streptomyces verticillus. Bleomycin was administered as a single intra-tracheal dose of 1 mg/ kg per animal dissolved in 0.09% saline solution according to Kimura et al.[19]

B- Dexamethasone (DEX)

Dexamethasone ($C_{22}H_{29}FO_5$) of molecular weight 293 was purchased as a powder from Sigma-Aldrich- USA. Dexamethasone was dissolved 0.09% saline and injected intraperitoneally to mice at dose of 0.45 mg/kg body weight/ day for 14 days according to Shi et al.[9]

C- Curcumin (CURC)

Curcumin ($C_{21}H_{20}O_6$) was purchased as powder formula from Sigma-Aldrich- USA, It was dissolved in distilled water and given orally to mice at a dose of 100 mg/kg body weight /day for 2 weeks according to Smith et al.[20]

2.3. Experimental Design

In the present investigation mice were divided into seven groups, 10 mice each. The first group served as normal control receiving tap water, the second group represented the DEX group, the third group represented the CURC group and the fourth group represented the BLM group. On the second day intratracheal instillation of bleomycin, BLM group was further divided into BLM / DEX, BLM / CURC group and BLM / DEX+CURC. Mice were subjected to different experimental treatments for 14 days.

2.4. Biochemical Analyses

At the end of study period, all experimental animals were sacrificed by ether inhalation anesthesia. Blood samples were withdrawn by heart acupuncture from all groups in dry clean sample tubes containing EDTA for the determination of Lactate dehydrogenase (LDH) activity in serum. Dissected right lungs from all animals were washed with saline and homogenated in a phosphate buffer solution at pH 7.4 for the measurement of collagen.

2.4.1. Lactate Dehydrogenase (LDH)

Lactate dehydrogenase (LDH) activity in serum was assessed spectrophotometrically by

monitoring the reduction of nicotinamide adenine dinucleotide (NAD^+) at 340 nm in the presence of lactate. LDH was determined by the method of Caraway, (1976). Commercial kit was purchased from Randox, U.K.

2.4.2. Collagen Assay

Mice Collagen content was estimated by measuring hydroxyproline content in lung homogenate tissue by Eliza Kit which was purchased from MyBio Source Company.

2.5. Histological investigations

Left lung lobe from mice in all groups was carefully dissected. They were subsequently placed in 10 % buffered formalin, dehydrated, cleared with xylene, infiltrated with paraffin wax at 60° C then embedded. Paraffin blocks were cut at 6 microns and affixed to slides then stained with Haematoxylin and Eosin for general histological examination [21] and Masson's trichrome stain[22] for collagen deposition.

2.6. Statistical Analysis

The recorded data were analyzed using the Statistical Processor System Support (SPSS) version 10 computer program. The significance of differences between means of normal control group and all treated mice were represented by (a). The significance of differences between means of bleomycin group and bleomycin treated mice were represented by (b). All data were analyzed using one-way analysis of variance (ANOVA) test.

3- RESULTS

3.1. Lactate dehydrogenase (LDH) content in serum

The mean values of lactate dehydrogenase of control and experimental groups during 14 days of study were shown in table (**1**). The mean values of either BLM, or BLM treated with DEX, CURC or both DEX and CURC showed a significant increase in LDH levels after 14 days. This elevation recorded mean values of (470.64±1.25, 324.42±3.78, 311.23±6.00 and 183.89±5.6) respectively when compared (129.98±7.41) of normal control animals. On the contrary, in relation to BLM group, a significant ($P< 0.05$) decrease in in the mean values of (BLM / DEX and BLM / CURC or both BLM /DEX +CURC) after 14 days to record a 0.68 fold, 0.66 fold and 0.39 fold decrease in these groups respectively.

Table (1): Mean values of LDH and HYP of control and experimental groups at 14 days.

Parameters	Groups	NC	DEX	CURC	BLM	BLM/ DEX	BLM/ CURC	Bleo/DEX+ CURC
LDH (U/l)	Mean±SE	129.99$^{\#}$±7.40	153.9$^{\#}$±7.24	148$^{\#}$±5.96	470.64*±12.5	324.42*$^{\#}$±3.78	311.23*$^{\#}$±6	183.89*$^{\#}$±5.6
HYP (ng/ml)	Mean±SE	4.75$^{\#}$±0.05	3.63$^{\#}$±0.05	3.68$^{\#}$±0.03	16.55*±1.07	13.4*$^{\#}$±0.22	15.02*$^{\#}$±0.63	9.93*$^{\#}$±0.38

Values are expressed as mean ± SE compared with the control and bleomycin group

*$p < 0.05$ (Significant) compared with control group.

# $p < 0.05$ (Significant) compared with bleomycin group.

3.2. Collagen Assay

Hydroxyproline content in lung tissue

The mean values of hydroxyproline contents of control and experimental groups at 14 days of study were shown in table (**1**). The mean values of hydroxyproline contents of either BLM group alone or BLM treated with different experimental treatments of DEX , CURC and both elucidated a significant increase in their mean values after 14 days to record (16.55±1.07, 13.41±0.22, 15.02±0.63 and 9.93±0.38) respectively in the pre-mentioned groups when compared to (4.75±0.05) of normal control animals.

On the other hand, applying different treatments to BLM induced lung fibrotic animals in BLM / DEX and BLM / CURC and BLM / DEX + CURC manifested significant decreases of 0.8 fold, 0.9 fold and 0.6 fold decrease respectively after 14 days compared to BLM group.

3.3. Histological Investigation with H&E

The histological investigation of lung sections obtained from normal control group after 14 days of experimental study showed normal architecture, spongy appearance of the lung with thin alveolar septa, (**Fig. 1-a**).

Also the microscopic observation of lung mice treated with DEX alone group for 14 days of experimental study showed normal architecture with simple rupture of alveolar sacs. (**Fig. 1-b**).

Similarly lung mice treated with CURC alone for 14 days manifested normal lung structure similar morphology with that of control animals (**Fig. 1-c**) showing clear alveolar cavities and normal alveolar ducts. The interalveolar septa were formed from the epithelial tissue lining of the alveoli and loose connective tissue contained extensive capillary network around the

alveoli.

Histological examination of lung sections of BLM treated mice after14 days showed extensive damage lung tissue with distorted lung morphologies. In addition, there were collapsed alveolar spaces with inflammatory exudates, wider and thickened interalveolar septa, loss of normal alveolar architecture. The majority of the alveolar walls were occupied by collagenous fibers with diffused cellular infiltration (increased macrophages, neutrophils and lymphocytes) (**Fig. 1-d**).

Moreover, this study clarified that lung sections from animals treated with BLM / DEX after 14 days ratified restored normal lung tissue sections with slight thickening and space expansion. Also, mild edema of the interstitium and alveolar spaces with clear terminal bronchioles which may had minimum drained lesions were also prominent. Nevertheless, still congested interalveolar septa and scattered inflammatory cells were also found (**Fig. 1-e**). Also, H&E stained sections of BLM / CURC treated lung mice showed increased alveolar wall rupture with inflammatory infiltrates and lessened alveolar thickening (**Fig. 1-f**).

H&E stained sections of BLM / DEX + CURC treated lung mice showed more improvement in walls of alveolar sacs returning back to their normal structure with spongy lung appearance with thin alveolar septa, clear alveolar cavities and normal alveolar ducts (**Fig. 1-g**).

Collagenous fibers deposition with Masson's Trichrome

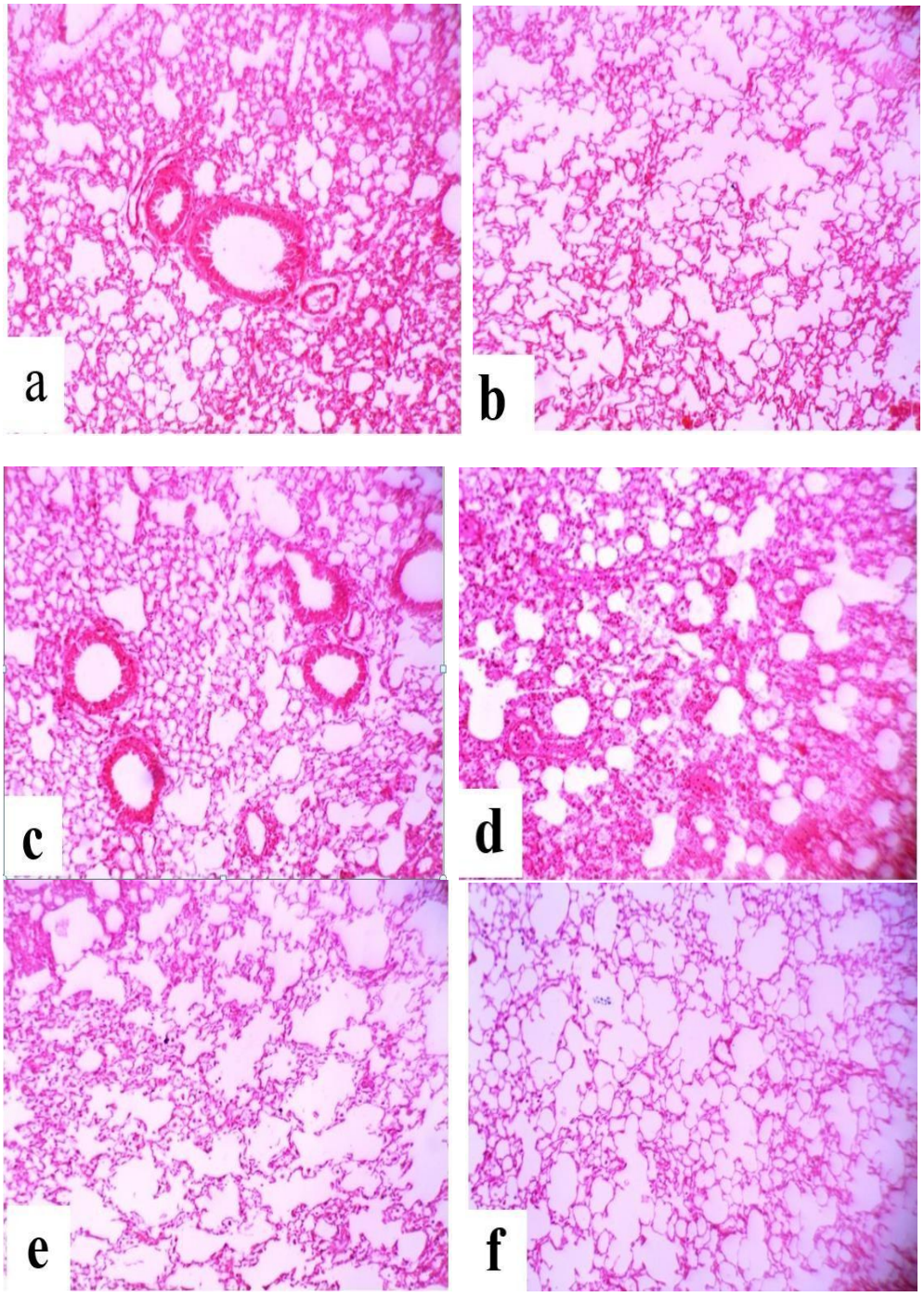
Lung tissue sections of normal control mice after14 days showed normal distribution of collagenous fibers, alveolar septa with no burden at the most flimsy small fibers in some alveolar walls. (**Fig. 2-a**).

Also lung tissue sections stained with masson's trichrome stain and treated with DEX alone for 14 days demonstrated moderate thickening in alveolar walls without obvious damage to lung architecture (**Fig. 2.b**). The group of animals treated with CURC alone for14 days showed minimal fibrous thickening of alveolar or bronchiolar walls (**Fig. 2-c**).

On the other hand, animals treated with BLM for 14 days showed severe distortion of lung structure and increased lung fibrotic areas with deposition of collagenous fibers (**Fig. 2-d**). Furthermore, lung sections manifested preserved collagenous fibers architecture after 14 days of BLM and DEX treatments (**Fig. 2.e**).

Moreover, lung tissue of BLM / CURC group after 14 days demonstrated decreased collagenous fibers in lung architecture (**Fig. 2-f**).

Also, lung tissue of BLM treated with both DEX and CURC group after 14 days showed slight collagenous fibers in lung section (**Fig. 2-g**).



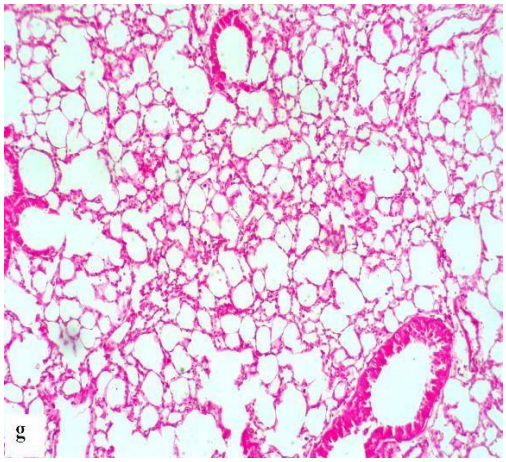
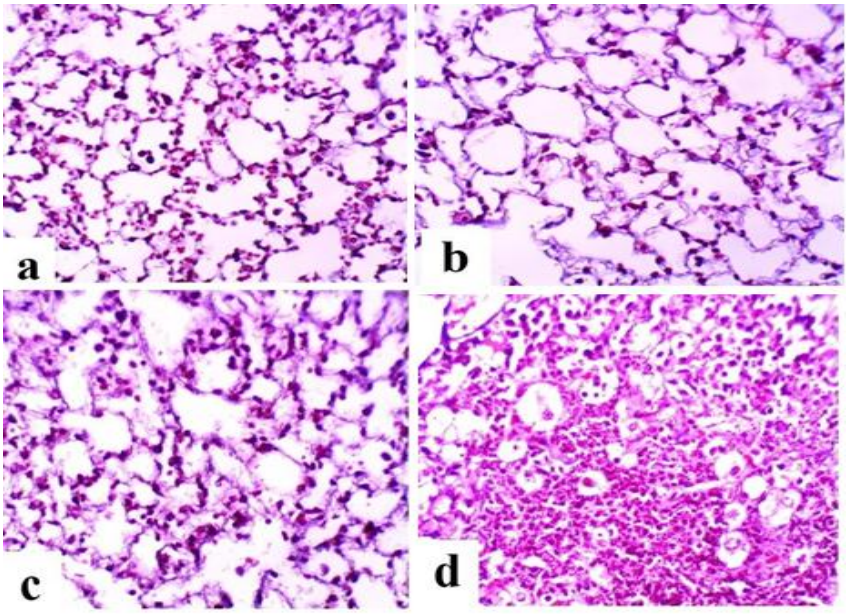


Fig. (1): Photomicrograph of lung section stained by Hematoxylin and Eosin:

a) **Control mice showing normal lung architecture (H &E; x100).**
b) **DEX group after 14 days showing simple rupture of alveolar sacs. (H &E; x100).**
c) **CURC group after 14 days showing a normal lung structure similar morphology with that of control (H &E; x100).**
d) **14 days post treatment of BLM showing extensive damage of the lung tissue (H &E; x100).**
e) **14 days post treatment of BLM / DEX showing restored normal tissue, slight thickening (H &E; x100).**
f) **BLM / CURC group after 14 days showing increased alveolar wall rupture with inflammatory infiltrates (H &E; x100).**
g) **BLM / DEX+ CURC group after 14 days showing improvement alveolar walls, clear alveolar cavities and normal alveolar ducts (H &E; x100).**



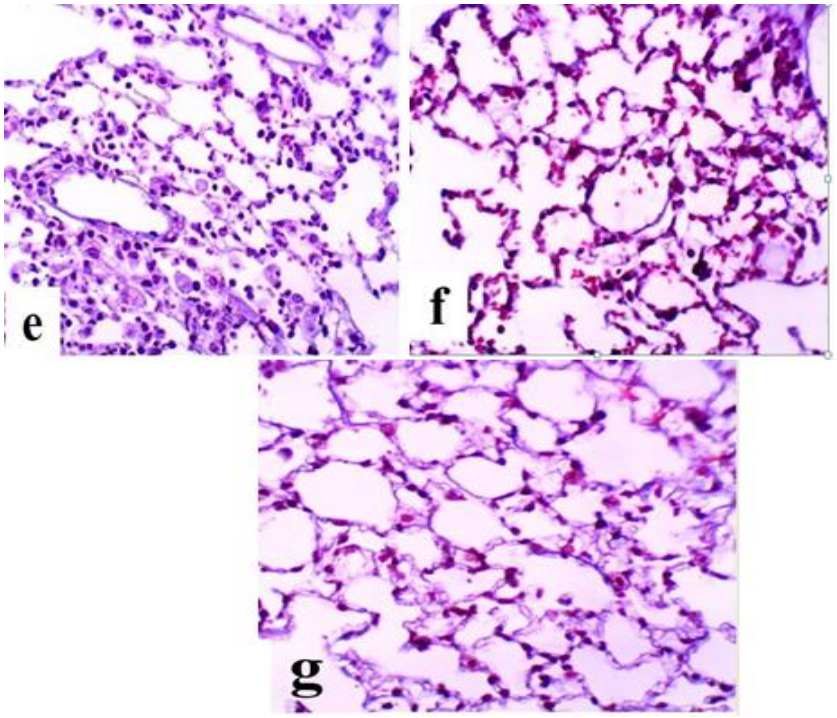


Fig. (2): Photomicrograph of lung section stained by Masson's trichrome:

a) **Normal distribution of collagenous fibers in normal control mice (Masson's trichrome; x400)**
b) **DEX group after 14 days showing slight increase in collagenous fibers (Masson's trichrome; x400).**
c) **CURC group after 14 days showing mild collagenous fibers (Masson's trichrome ;x400).**
d) **BLM group after 14 days showing excess increase in collagenous fibers surrounding alveolar wall (Masson's trichrome; x400).**
e) **14 days post treatment with BLM / DEX showing normal distribution of collagenous fibers (Masson'strichrome; x400).**
f) **14 days post BLM / CURC treatment showing preserved collagenous fibers (Masson'strichrome; x400).**
g) **14 days post BLM / DEX+ CURC treatment showing slight collagenous fibers (Masson'strichrome; x400).**

4- DISCUSSION

The results of the present investigation elucidated that lung tissue hydroxyproline content was elevated significantly after 14 days of intratreacheal bleomycin instillation. On the other hand, the percentage of increase of lung hydroxyproline decreased significantly in BLM/ DEX, BLM/CURC and BLM/ DEX+CURC.

The increase in hydroxyproline content in lung tissue of BLM group was explained by the involvement of more events of inflammation leading to pulmonary fibrosis. Hydroxyproline is an amino acid present in the composition of collagen which induced pro-fibrotic proteins synthesized by transdifferentiation of quiescent fibroblasts into myofibroblasts.[23, 24, 25]

The deleterious effect of bleomycin instillation to lung tissue was also witnessed by the elevated activities of serum LDH in this study .LDH can be located in the cells of body tissues, including lungs. Elevated LDH activity is thought to be a marker for interstitial lung fibrosis.[26] The increased activities of LDH might be attributed to increased lipid peroxidation in cellular membranes that produced marked alterations in molecular organization of lipids resulting in increased membrane permeability and leakage of cytoplasmic markers into circulation.[27]

The histological examination of lung sections of BLM group stained by H&E of the present study supported the biochemical results where various changes were observed in lung tissue sections of mice under BLM installation such as collapsed alveoli. Other alveoli were dilated and ruptured. The bronchiole was lined by epithelial cells with deeply stained nuclei and its lumen was full of exfoliated epithelial cells. Heavy mononuclear cellular infiltration surrounding the bronchioles and in the interalveolar septa was observed with dilated and congested blood vessels. Moreover, masson trichrome stained lung sections from BLM group demonstrated significant increase in the amount of collagen and elastic fibers around the walls of the alveoli.[28, 29, 30] Also, there was diffused mononuclear cellular infiltration surrounding the bronchioles with diffused thickening of the interalveolar septa.[31] Intratracheal BLM installtion stimulated endothelial cells, macrophages and fibroblasst to induce inflammatory mediators which in turn stimulated proinflammatory cytokines, transforming growth factor-β (TGF-β). All these initiated apoptosis process with the release of free radicals and the secretion of pathologic extracellular matrix, leading to fibrosis that interfere with the normal lung architecture and disable gas exchange in the lungs.[32]

On the other hand, applying DEX treatment for 14 days to BLM group reduced hydroxyproline content suggesting the role of dexamethasone in delaying pulmonary fibrosis induced by BLM treatment via inhibition of collagen synthesis.[33] Dexamethasone reduced lung inflammation by reducing inflammatory cell migration and proliferation.[34]

In the current study, the use of dexamethasone largely protected the pulmonary tissue from

the injurious effect by BLM which was clearly observed in histological sections of lung tissue stained with H &E. Lung tissue sections of mice treated with dexamethasone concomitantly with BLM for 14 days revealed minimal damage in most specimens with preservation of normal alveolar pattern. Alveolar cell injury and inflammatory cells infiltration were much less encountered in BLM mice treated with DEX than in mice treated with BLM alone. Besides, masson trichrome lung tissue sections of this group elucidated minimal thickening of the walls of the interalveolar septa, bronchiolar and blood vessels. This confirmed that dexamethasone suppressed BLM induced lung damage.[14, 35, 36] Dexamethasone treatment for 14 days directly inhibited lung fibrosis by direct suppression of fibroblasts and transcription of type1 procollagen mRNA in the fibroblast, thus suppressing collagen synthesis.[37]

Based on the results of the present study, bleomycin instillation also increased lactate dehydrogenase levels (LDH) which is a marker of lung tissue injury and cell damage. Dexamethasone decreased LDH levels indicating the prevention of cell injury and hence preventing pulmonary fibrosis.[38] Bleomycin treatment has been shown to generate toxic free radicals. There is a direct relationship between the involvements of free radicals in the formation of hydroxylated proline.[39]

Curcumin is a natural antioxidant which not only can effectively scavenge free radicals and endogenous oxidizing active substances but also act as a regulator of antioxidant enzymes.[40,41] Curcumin has been studied as a potential drug for the treatment of lung fibrosis. In addition, curcumin has also been shown to modulate collagen metabolism.[42] It lowered hydroxyproline and caused a reduction in collagen deposition in lung tissue thus preventing the progression of pulmonary damage due to its antioxidant properties.[20]

The mechanism of blocking fibrosis by curcumin is related to decreased collagen accumulation in the lung.[20] It also acts as antifibrotic agent in BLM induced pulmonary fibrosis in animal models by blocking the production of free radicals.[42]

It can also increase intracellular glutathione levels by maintaining the activity of histone acetyltransferase in monocytes, thereby mitigating damage during oxidative stress.[43] The core mechanisms by which curcumin exerts its protective effect is promoting the the expression of phase II detoxification enzymes and antioxidant enzymes.[41]

The histological examination of lung sections of BLM group treated with curcumin of the present study revealed much improvement than BLM group. This was demonstrated as a decrease in collapsed alveoli. Few mononuclear cellular infiltration surrounding the bronchioles and in the interalveolar septa, as well as the few red blood cells and congested blood vessels were seen. Moreover, lung tissue section stained with masson trichrome stain verified a significant decrease of collagen and elastic fibers around alveoli, within the interalveolar septa and around bronchioles as compared to BLM group.

These results were in agreement with previous researchers,[44] who stated that curcumin inhibited neutrophil infiltration, suppressed proinflammatory cytokines in alveolar macrophages, and prevented the formation of reactive oxygen species (ROS).[45] Additionally curcumin has the capability to scavenge free radicals and enhance the activities of superoxide dismutase in animal models.[46] The most ameliorated results in this study were observed in BLM/ DEX+CURC group where combined treatments of DEX and CURC to BLM toxicated mice attenuated inflammation by reducing collagen deposition and scavenging free radicals.

CONCLUSION

The present study confirmed that combined treatments of DEX and CURC have better therapeutic effects on BLM induced lung fibrosis than the single treatment of either DEX or CURC alone. Thus, curcumin may act as an additive therapeutic strategy scavenging free radicals to improve the outcome of DEX treatment in bleomycin- induced lung fibrosis mice model.

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