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Original Article	**The Possible Protective Effect of Losartan on Bleomycin-Induced Pulmonary Fibrosis in Albino Rats: A Histological And Immunohistochemical Study**

Soheir A. Filobbos, Dina H. Mohammed, Lamiaa I. Abd-Elfattah and Marwa M. Sabry

Histology Department, Faculty of Medicine, Cairo University.

ABSTRACT

The possible protective effect of Losartan, an angiotensin II receptor 1 antagonist, on bleomycin-induced rat lung fibrosis was evaluated using histological and immunohistochemical techniques. Twenty adult male albino rats were divided into 4 groups each of five rats: Group I (control) given saline both I.V and orally, Group II given bleomycin 10mg/Kg/day I.V., Group III given losartan 10 mg/Kg/day orally and Group IV given both bleomycin and losartan. Lung sections were taken at the 28th day of the experiment, stained with H&E, Masson's trichrome, Orcein and immunohistochemical stain for α-SMA. This was followed by morphometric measurements and statistical analysis.

The present study showed that bleomycin induced fibrotic changes in the lung in the form of thickened interalveolar septa filled with proliferated type II pneumocytes, fibroblasts and inflammatory cells with significant increase in collagen and elastic fibres deposition and in α-SMA immunoreactivity.

It was found that concomitant treatment with losartan (Group IV) showed significant reduction in these fibrotic changes. The changes in α-SMA immunoreactivity in different groups showed the same pattern as those of collagen and elastic fibres. Thus, it could be concluded that myofibroblasts might play a pivotal role in induction of lung fibrosis and they could be a target of future therapeutic strategies.

Key Words: Pulmonary fibrosis- bleomycin- losartan- immunohistochemistry

Corresponding Author: Dr. Dina Helmy, Mobile:01118077724, Email: dinahelmy2000@yahoo.com

INTRODUCTION

Fibrotic lung diseases are heterogenous groups of chronic lung disorders that might be produced by a variety of toxic agents, infections, immunomediated disorders or may be idiopathic *(Zhang et al., 2007).*

Bleomycin is an antineoplastic drug that is used as palliative treatment either as a single agent or in combination as in squamous cell carcinoma, Hodgkin and non Hodgkin lymphomas, testicular carcinoma and malignant pleural effusion *(Hoshino et al., 2009).*

Bleomycin is widely used in experimental models of lung fibrosis that resemble those of human pulmonary fibrosis *(Molina-Molina et al., 2006),* as one of its serious side effects is lung toxicity, pneumonitis and fibrosis *(Takimoto & Calvo, 2008).*

Apoptotic alveolar epithelial cells have been found in areas of normal alveoli, suggesting that apoptosis in Alveolar Epithelial Cells (AEC) was a primary event in the pathogenesis of Idiopathic Pulmonary Fibrosis (IPF) prior to the onset of fibrosis *(Barbas-Filho et al., 2001).* It has been also reported that AEC apoptosis in response to various stimuli required the production of Angiotensin II and subsequent activation of Angiotensin II receptors *(Li et al., 2003-a).*

Losartan, used as antihypertensive drug, an angiotensin II type I receptor blocker (ARB) is one of the drugs that provide a site-specific blockage of the effects of angiotensin II, which is the most important peptide of the renin-angiotensin system *(Marshall et al., 2004).*

Therefore, the aim of this study was to demonstrate the bleomycin-induced histological changes in the lung and the possible protective effect of losartan against these changes.

MATERIALS AND METHODS

1. Drugs:

- **Bleomycin hydrocloride:** (Nippon Kayaku, Japan), available as 15 mg powder per vial. It was given as a single daily intravenous dose of 10 mg/kg/day dissolved in sterile saline for 5 consecutive days *(Kakugawa et al., 2004)* in the tail's vein and the dose was adjusted according to the body weight of the animal species.

- **Losartan potassium: "Cozaar"** (E. I. du Pont de Nemours Company, Wilmington, Delaware, USA) available as 100 mg tablets which were crushed. It was given as a single daily oral dose of 10 mg/kg/day dissolved in sterile saline using oral gastric tube *(Safaeian et al., 2009).*

2. Animals and experimental design:

Twenty adult male albino rats (200-220g) were used and divided equally into four groups. Rats were fed *ad libitum* and allowed free water supply. Rats of each group were kept in a separate cage under good hygienic conditions according to guidelines of Animal Ethics Committee.

- **Group I:** Control group (5 rats).

Rats were given both intravenous and oral saline, as a control for bleomycin and Losartan.

- **Group II:** Bleomycin-treated group (5 rats).

- **Group III:** Losartan-treated group (5 rats).

- **Group IV:** Combined Bleomycin and Losartan-treated concomitantly (5 rats).

Rats of all groups were sacrificed using overdose of chloroform on the 28th day of the experiment. Right sided lung specimens were fixed in 10% formol saline. Paraffin blocks were prepared, sectioned and stained with H&E, Masson's trichrome and orcein stains. Immunohistochemical staining using anti-αSMA antibody was performed *(Willis et al., 2005),* supplied by NEOMARKER labvision (USA) as ready to use mouse monoclonal antibody.

3. Morphometric study:

Using "Leica Qwin 500C" image analyzer sysytem (Cambridge, England), the area percent of collagen in Masson's trichrome stained sections, of elastic fibers in Orcein-stained sections and of αSMA immunopositive cells in immunostained sections were measured. Measurements were done in ten non-overlapping low power fieldsX100 in all sections except for collagen it was measured in high power fields X400. The standard measuring frame was (116964.91um^2) on using magnification X100 and was (7286.783 um^2) on magnification X400.

4. Statistical analysis of the obtained data was performed using analysis of variance ANOVA test , followed by post Hock test using the Soft ware "SPSS for windows version 9"**.** Differences were considered significant when "P" was ≤0.05 *(Armitage & Berry, 1994)*.

RESULTS

Histological examination of rat lung sections with H&E staining, in the control group (group I), revealed normal lung architecture with thin interalveolar septa in between. Bronchioles were seen lined with simple columnar ciliated epithelium, surrounded with circularly arranged smooth muscles with no cartilage or glands (Fig. 1).

In group II, there was segmental affection of the lung with patchy areas of collapse of the alveoli and thickened interalveolar septa with inflammatory cellular infiltration as well as intrabronchiolar infiltration (Fig. 2). Many fibroblasts and Type II pneumocytes were seen within the thickened septa (Fig. 3). Proliferating pneumocytes type II with their rounded outline and vesicular nuclei, with acinar formation, were commonly seen (Fig. 4).

Meanwhile, in group III, the lung showed more or less normal architecture with thin interalveolar septa in most areas and slightly thickened septa in other areas. Some vascular and lymphatic congestion was observed (Fig. 5).

In group IV, there were focal areas of mild thickening of interalveolar septa with congested blood vessels (Fig.6).

In Masson's trichrome stain, in the control group, there was minimal amount of collagen fibers within the lung interstitium (Fig. 7). In group II, there was marked thickening of the alveolar wall with marked collagen fibers deposition (Fig. 8). However, in group III, the inter-

stitium of the lung showed minimal amount of collagen fibers (Fig. 9). In group IV, there was diffuse and moderate collagen deposition within the lung interstitium (Fig. 10).

In orcein-stained sections, there was normal structure of the lung with thin interalveolar septa with few elastic fibers, in the control group (Fig. 11). In group II, marked thickening of the alveolar wall with increased elastic fiber deposition was detected (Fig. 12). However, in group III, there was almost thin walled septa and minimal amount of elastic fibers (Fig. 13). In group IV, moderate amount of elastic fibers within the moderately thickened interstitium was observed (Fig. 14).

Examination of lung sections of the control group, immunoexpression of anti-α smooth muscle actin antibody revealed moderate localized immunoreactivity that was detected in the smooth muscle around the bronchioles, blood vessels and in knobs of alveolar ducts (Fig. 15).

In group II, wide distribution of the positive αSMA immunoreactivity in the thickened interstitium and in smooth muscle cells around blood vessels and bronchioles (Fig. 16). Positive αSMA immunoreactivity was observed within the cytoplasm of pneumocyte type II cells and within the cytoplasm of fibroblasts (Fig. 17).

In group III, localized αSMA immunoreactivity was detected in the muscle cells of the bronchioles and blood vessels (Fig. 18).

On the other hand, in group IV, there were areas of diffuse αSMA immunoreactivity seen in lung interstitium and in the smooth muscle cells of the bronchioles (Fig. 19).

The values of the mean area percent of collagen and elastic fibres and the αSMA immunopositivity in the different groups together with their statistical significance are summarized in the following, histogram, graph and table.

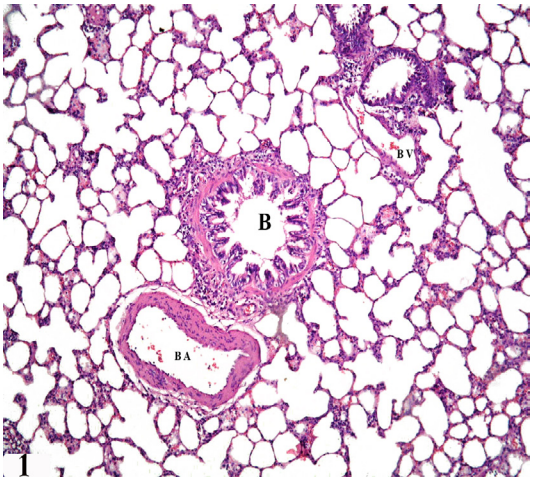


Fig. 1: A photomicrograph of a section in the lung of a control group (group I) rat showing structure of the lung with thin interalveolar septa. Note bronchiole with their simple columnar ciliated epithelial lining and circularly arranged smooth muscles (B), branches of bronchial artery (BA) and bronchial vein (BV). Hx & E; X100

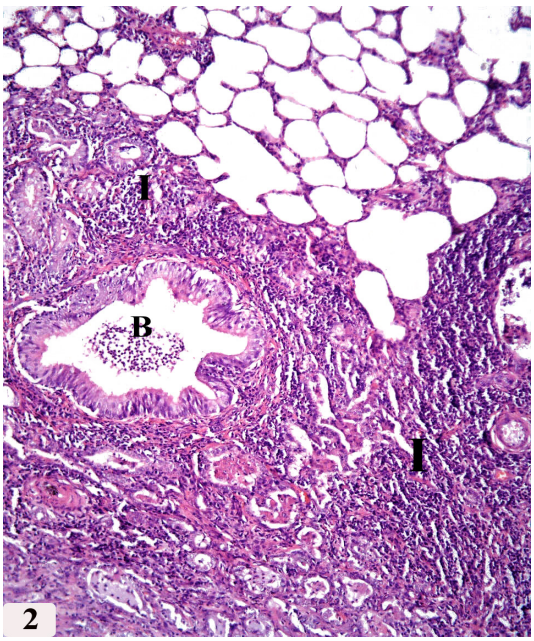


Fig. 2: A photomicrograph of a section in the lung of a group II rat showing areas of complete obliteration of air spaces with inflammatory cellular infiltrate within the markedly thickened septa (I) as well as intrabronchiolar (B). Hx& E; X100

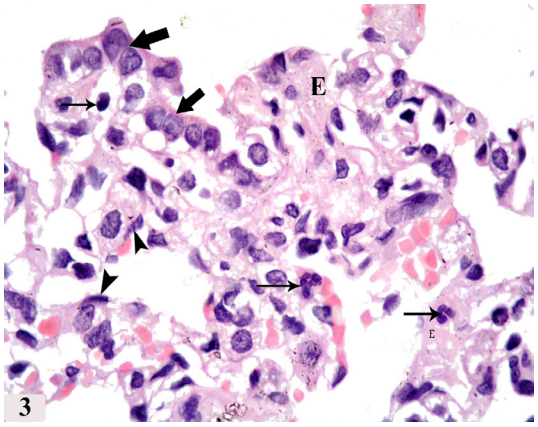


Fig. 3: A photomicrograph of a section in the lung of a group II rat showing fluid exudates (E), dividing pneumocytes type II with rounded vesicular nuclei (thick arrow), fibroblasts with large oval nuclei (arrowheads) and many inflammatory cells (thin arrow). Hx & E; X1000

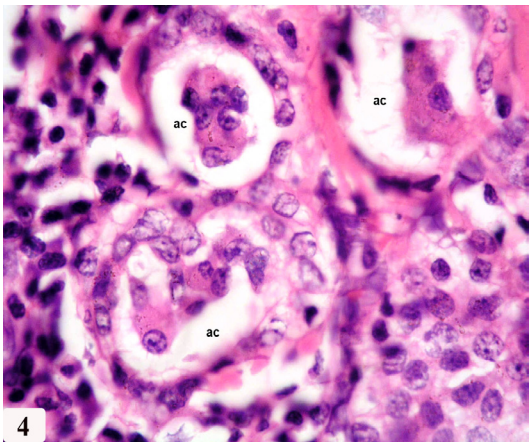


Fig. 4: A photomicrograph of a section in the lung of a group II rat showing acinar formation (ac) by pneumocyte type II cells. H & E; X1000

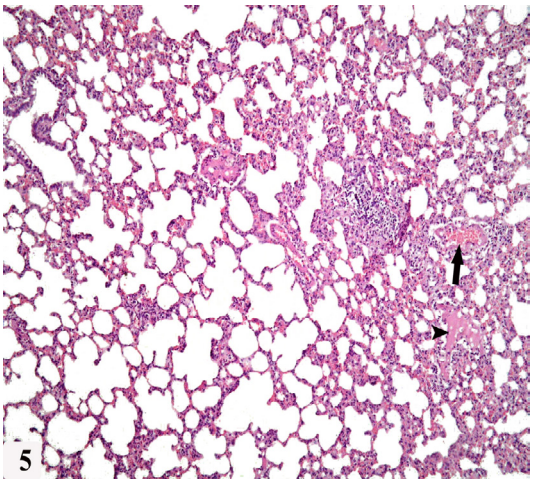


Fig. 5: A photomicrograph of a section in the lung of a group III rat showing normal architecture of the lung with some vascular blood congestion (arrow) and lymphatic congestion (arrowhead). Small areas exhibits slightly thickened septa. Hx & E; X100

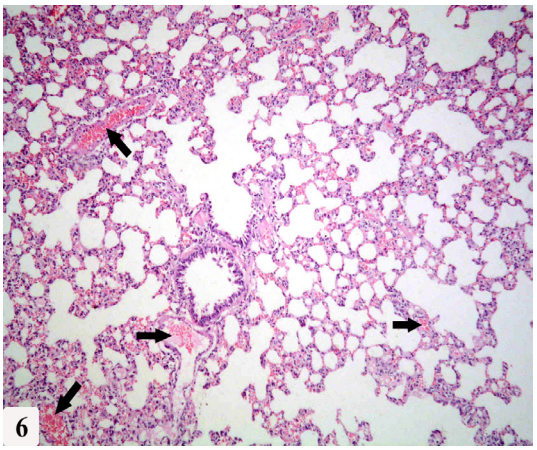


Fig. 6: A photomicrograph of a section in the lung of a group IV rat showing focal areas of mild thickening of interalveolar septa and some vascular congestion (arrow). Hx & E; X100

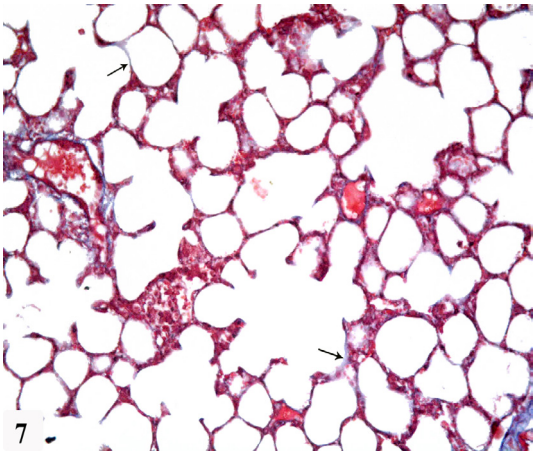


Fig. 7: A photomicrograph of a section in the lung of a control group (group I) rat showing minimal amount of collagen in the lung interstitium (arrow). .Masson's trichrome; X200

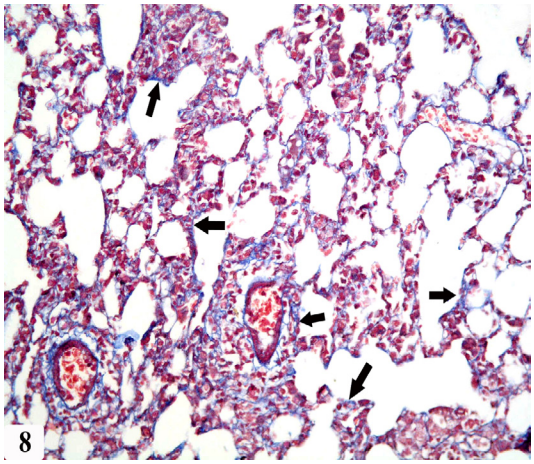


Fig. 8: A photomicrograph of a section in the lung of a group II rat showing marked thickening of the alveolar wall septa with extensive collagen deposition in the lung interstitium (arrow). Masson's trichrome; X200

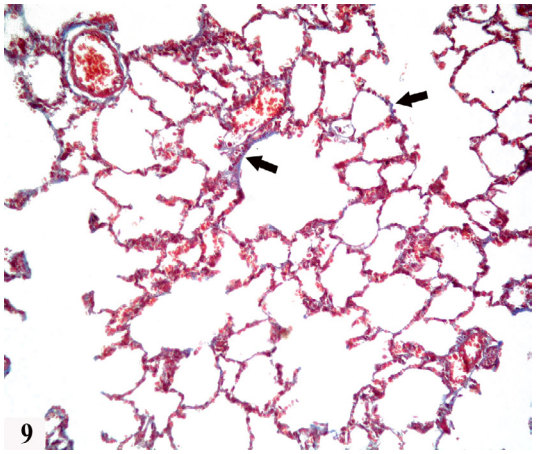


Fig. 9: A photomicrograph of a section in the lung of a group III rat showing minimal amount of collagen in the lung interstitium (arrow). Masson's trichrome; X200

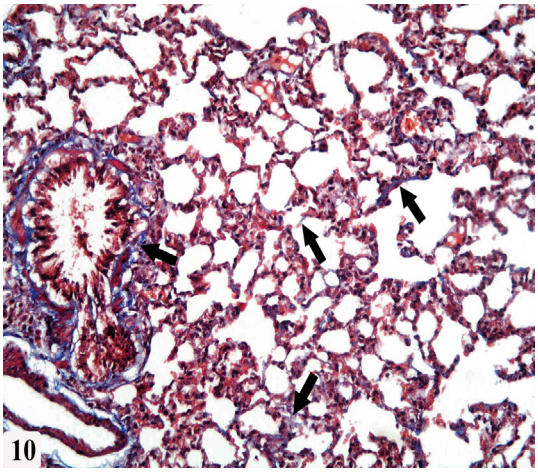


Fig. 10: A photomicrograph of a section in the lung of a group IV rat showing diffuse and moderate increase of collagen deposition (arrow). Masson's trichrome; X200

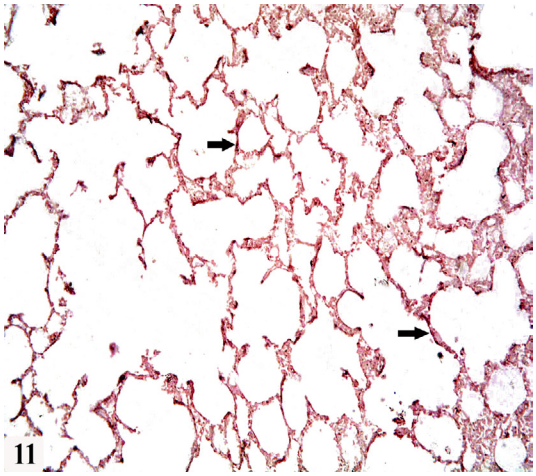


Fig. 11: A photomicrograph of a section in the lung of a control group (group I) rat showing normal structure of the lung. Elastic fibers are seen within the lung interstitium (arrow). Orcein; X200

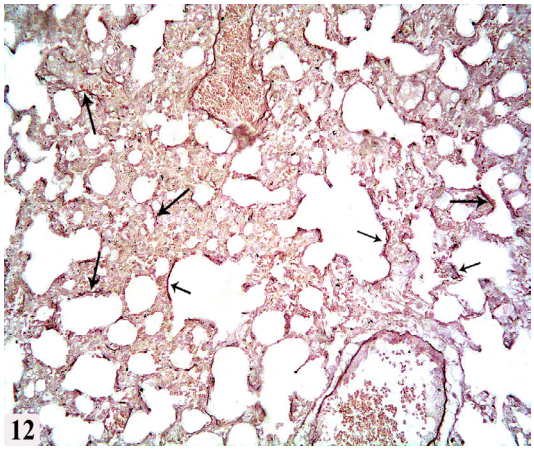


Fig. 12: A photomicrograph of a section in the lung of a group II rat showing marked thickening of the alveolar wall with increased elastic fibers deposition within the lung interstitium (arrow). Orcein; X200

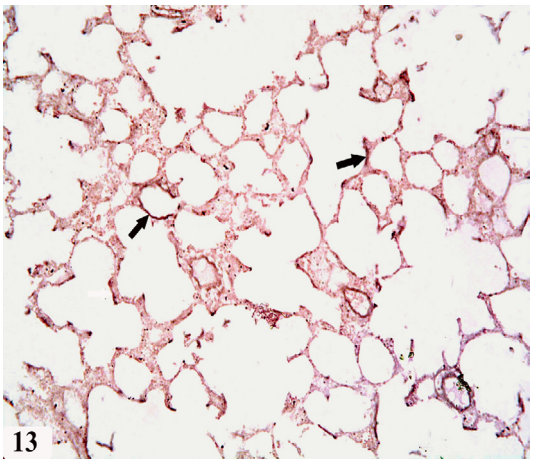


Fig. 13: A photomicrograph of a section in the lung of a group III rat showing almost thin walled septa and minimal amount of elastic fibers within the lung interstitium (arrow). Orcein; X200

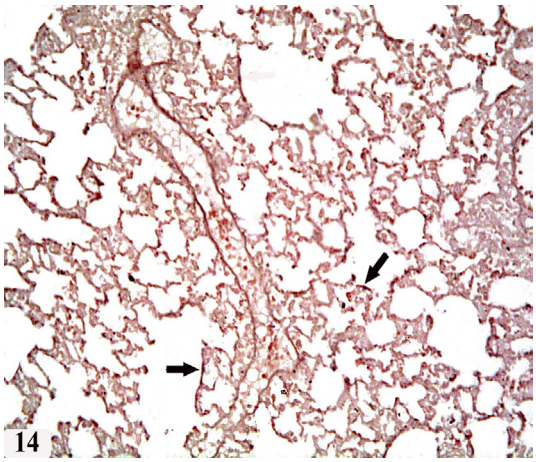


Fig. 14: A photomicrograph of a section in the lung of a group IV rat showing elastic fibers within the moderately thickened interstitium (arrow). Orcein; X200

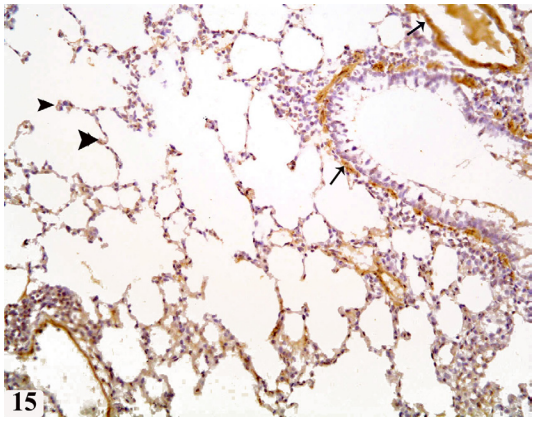


Fig. 15: A photomicrograph of a section in the lung of a Control group rat showing positive α-smooth muscle actin immunoreactivity detected in the muscle cells of the bronchioles and blood vessels (arrow) and in knobs of alveolar ducts (arrowhead). α-SMA; X200

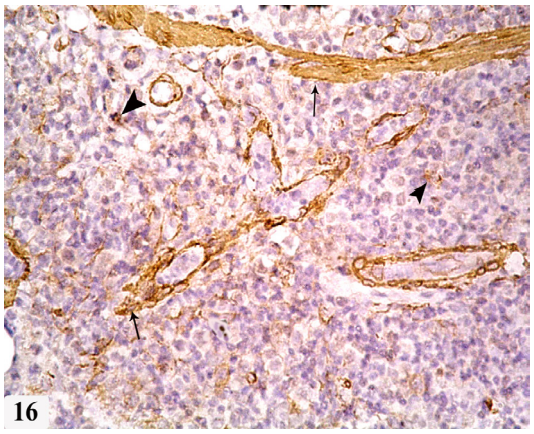


Fig. 16: A photomicrograph of a section in the lung of a group II rat showing wide distribution of the positive α-smooth muscle actin immunoreactivity in the thickened interstitum (arrowhead) and in smooth musle cells around blood vessels and bronchioles (arrow). α-SMA; X400

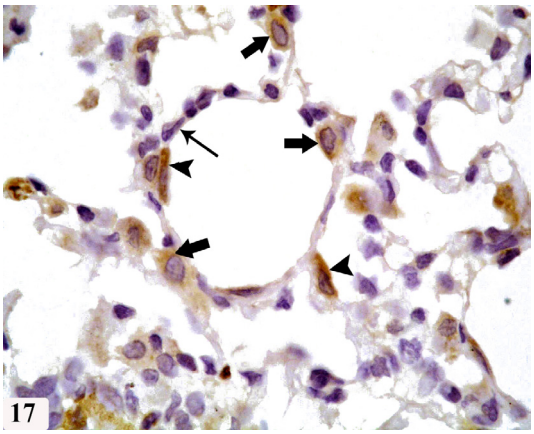


Fig. 17: A photomicrograph of a section in the lung of a group II rat showing positive α-smooth muscle actin immunoreactivity within the cytoplasm of pneumocyte type II cells (thick arrow) and within the cytoplasm of fibroblasts (arrowhead). Note that type I pneumocytes (thin arrow) show negative immunoreactivity. α-SMA; X400

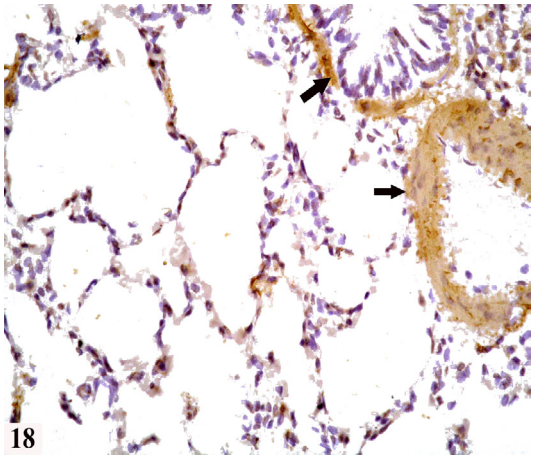


Fig. 18: A photomicrograph of a section in the lung of a group III rat showing localized positive α-smooth muscle actin immunoreactivity detected in the muscle cells of the bronchioles and blood vessels (arrow). α-SMA; X400

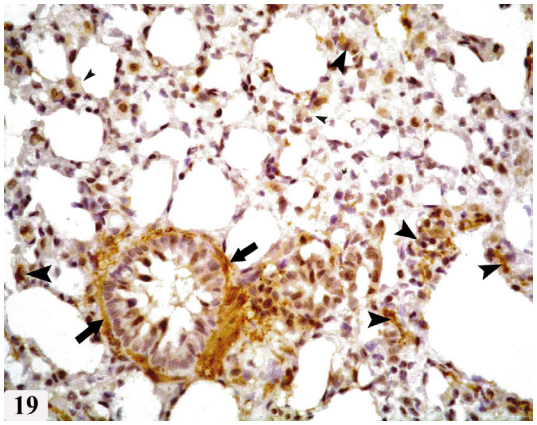
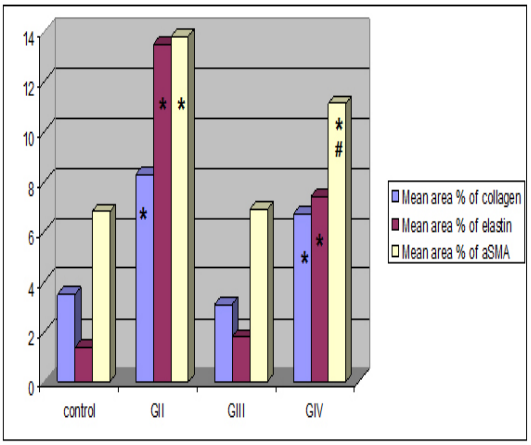


Fig. 19: A photomicrograph of a section in the lung of a group IV rat showing diffuse areas of positive α-smooth muscle actin immunoreactivity detected in lung interstitium (arrowhead) and in the muscle cells of the bronchioles (arrow). α-SMA; X400

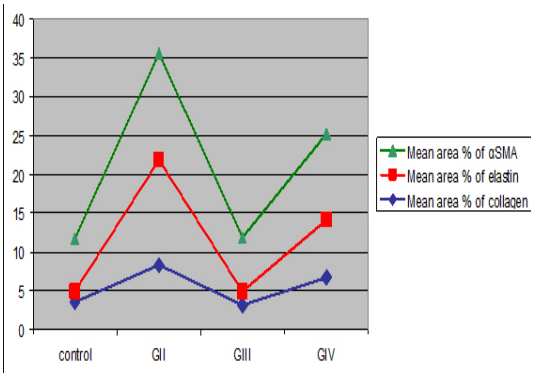


Histogram: showing the mean area percent of collagen and elastic fibres and α–SMA immunopositive cells in control and experimental groups.
* = Statistically significant compared to control
# = Statistically significant compared to group II

Table: Showing the mean area percent±Standard Deviation (SD) of collagen and elastic fibres and α–SMA immunopositive cells in control and experimental groups.

Group	Mean area %±SD			
	Group I (Control)	Group II	Group III	Group IV
collagen fibers	3.5±0.84	8.3*±1.7	3.1±0.64	6.7*±1.0
elastic fibers	1.4±0.52	13.5*±1.1	1.8±0.37	7.4*±1.1
α–SMA immunopositive cells.	6.8±0.45	13.8*±1.1	6.9±0.83	11.1*°±0.76

* = Statistically significant compared to control
°= Statistically significant compared to group II



Graph: showing the linear relation between the mean area percent of collagen and elastic fibres and α–SMA immunopositive cells in control and experimental groups.

DISCUSSION

Idiopathic Pulmonary Fibrosis (IPF) is a specific form of chronic interstitial lung disease which is associated with the histological appearance of usual interstitial pneumonia. The poor prognosis of IPF patients, with a mean survival period of 2–4 years, underlines the need for new therapeutic strategies *(Molina-Molina et al., 2007).*

In this study, an attempt was done to detect histologically the possible protective effect of losartan as a combined treatment on bleomycin-induced pulmonary fibrosis.

Bleomycin model in rodents is the most commonly used in induction of pulmonary fibrosis as it is easy to perform, widely accessible and reproducible *(Moller et al., 2008).*

In the present study, histological examination of group II (bleomycin treated) showed an inflammatory reaction in the form of inflammatory cellular infiltration in the markedly thickened interalveolar septa. This is concomitant with *Hay et al. (1991)* who reported that the lung injury seen following bleomycin- comprised an interstitial vascular congestion with an influx of inflammatory and immune cells.

This could be explained by *Chaudhary et al. (2006)* who reported that bleomycin treatment led to overproduction of reactive oxygen species that can lead to an inflammatory response causing pulmonary toxicity, activation of fibroblasts and subsequent fibrosis.

Moreover, in the present study many proliferating type II pneumocytes within the thickened interalveolar septa with acinar formation were observed, in group II. This increase in number with acini-like appearance might be considered as an attempt of repair by these cells. This is in accordance with *Pecquet et al. (1987)* who found that treatment with bleomycin resulted in increased number of type II pneumocytes. This is also in agreement with *Hollande et al. (2004)* who stated that Alveolar type II pneumocytes are thought to be progenitor cells capable of self-renewal and differentiation into type I pneumocytes after exposure to injury.

Also, these cells might be a source of the fibroblasts as they are found mainly in contact with the fibroblasts. This explanation could be supported by the work of *Condeelis and Segall (2003)* and *Kalluri and Neilson (2003)* who reported that epithelial–mesenchymal transition (EMT) is the process by which an epithelial cell becomes a more motile mesenchymal cell. Furthermore**,** *Li et al. (2003-b)* reported that during EMT, cell–cell junctions are altered, with de novo expression of the mesenchymal markers vimentin and α-SMA and loss of the epithelial marker E-cadherin.

In group II, the present work showed focal areas of marked lung fibrosis with a significant increase in the mean area percent of collagen and elastic fibers. This could be attributed to the production of inflammatory and fibrotic mediators leading to increased collagen and elastic fibres deposition. This assumption was based on the report of *Chaudhary et al. (2006)* who reported elevation of pro-inflammatory cytokines (interleukin-1 and tumor necrosis factor-α) followed by increased expression of pro-fibrotic markers (transforming growth factor-β1 (TGF- β1) and procollagen-1) after bleomycin administration.

The detected fibrosis is in accordance with *Kakugawa et al. (2004)* who suggested that increased type II pneumocytes, in addition to myofibroblasts, might contribute to the fibrotic process through the production of type I procollagen.

The current study also showed a significant increase in the mean area percent of α-SMA immunoreactivity inboth type II pneumocytes and fibroblasts, in bleomycin group, which might be explained by the ability of bleomycin to increase the profibrotic marker TGF- β leading to EMT of type II pneumocytes into myofibroblasts.

This suggestion hinged upon the report of *Yao et al. (2004)* who reported that TGF- β induced EMT in freshly isolated type II alveolar epithelial (AECs II) cells, with morphological transformation to myofibroblasts. This is further supported by *Willis et al. (2005)* who observed that cells co-expressing epithelial markers and α-SMA were abundant in lung tissue from IPF patients.

These immunohistochemical results are also in accordance with *Kakugawa et al. (2004)* who found that bleomycin treatment significantly increased the mean number of α-SMA positive myofibroblast (MF). They also emphasized that MF are also the principal cells responsible for deposition of collagen and extracellular matrix in lung fibrosis.

Losartan is a selective angiotensin receptor antagonist *(Simpson & McClellen, 2000)*. Also, *Molina-Molina et al. (2006)* reported that the angiotensin system had an important role in the pathogenesis of pulmonary fibrosis. This was also based on the findings of *Marshall et al. (2004)* found that angiotensin II, through stimulation of AT1 receptors, led to upregulation of TGF-β which is a potent profibrotic mediator.

Furthermore, the present work showed a decrease in the mean area percent of collagen and elastic fibers in the group IV (concomitantly treated with losartan and bleomycin) as compared to the bleomycin treated group.

Hand in hand with the current findings, *Li et al. (2003-a)* reported that simultaneous administration of losartan with bleomycin had reduced the collagen content of the lung significantly. Also, this is in accordance with *Meng and Nan Fang (2008)* who reported that concomitant treatment with losartan significantly reduced the bleomycin-induced pulmonary fibrosis score and hydroxyl-proline content of the lung.

The present work also showed significant decrease in the mean area percent of α-SMA immunopositive cells in group IV compared to group II. The mechanism of the pulmonary antifibrotic effect of losartan in the present study could be attributed to its inhibitory effect on α-SMA positive myofibroblasts, which were reported by *Kakugawa et al. (2004)* to be the principal cells responsible for accumulation and deposition of extracellular matrix seen in pulmonary fibrosis.

Moreover, *Yao et al. (2006)* reported that the antifibrotic effect of Losartan in the context of pulmonary fibrosis might be associated with antioxidant activity and reduction in TGF-β1 levels which is a mediator of myofibroblast differentiation.

Accordingly, it could be concluded that, although the actual mechanism of induction of pulmonary fibrosis is still unknown, pneumocytes type II cells may play an important role through transformation into myofibroblast which could be the key cells in induction of such fibrosis. Thus, it could be suggested that these cells might represent a suitable target for future therapeutic strategies designed for pulmonary fibrosis.

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التأثيرالوقائى المحتمل لعقار اللوسارتان على تليف الرئة المحدث بعقار البليوميسين : دراسة هستولوجية و هستوكيميائية مناعية

سهير أسعد فيلبس - دينا حلمى محمد - لمياء ابراهيم عبد الفتاح - مروة محمد صبرى

قسم الهستولوجيا - كلية الطب - جامعة القاهرة

ملخص البحث

في هذا العمل تم دراسة التأثير الوقائي المحتمل لعقار اللوسارتان (المغلق للمستقبل ١ للأنجيوتينسين ٢) علي تليف الرئة المحدث بعقار البليوميسين باستخدام الوسائل الهستولوجية والهستوكيميائية المناعية.

أجريت هذه الدراسة على عشرين من ذكور الفئران البيضاء والتي قسمت إلى أربع مجموعات، خمسة فئران لكل منها: المجموعة الاولى (الضابطة) اعطيت محلول ملح بالحقن الوريدى وبالفم، المجموعة الثانية اعطيت عقار البليوميسين ١٠ملجم \كجم \يوم بالحقن الوريدى، المجموعة الثالثة اعطيت عقار اللوسارتان ١٠ملجم \كجم \يوم بالفم والمجموعة الرابعة اعطيت كلا من البليوميسين و اللوسارتان .أخذت عينات الرئة بعد ٢٨ يوم من بداية التجربة. وقد تم صبغ الشرائح بالهيماتوكسيلين والإيوسين، وصبغة ماسون ثلاثي الألوان وصبغة الأورسين والصبغة الهستوكيميائية المناعية ضد ألفا اكتين العضلات الملساء (ألفا س م أ). ثم أجريت بعض القياسات المترية وتلاها تحليل النتائج احصائيا.

أظهرت الدراسة أن عقار البليوميسين أدي الي حدوث تغيرات تليفية في الرئة في صورة زيادة في سمك الحواجز ما بين الحويصلات وانقسام للخلايا الرئوية من النوع الثانى والخلايا المنتجة للألياف والخلايا الالتهابية مع زيادة ذات دلالة إحصائية في الألياف الغروية والمرنة والمناعة الايجابية لألفا س م أ.

وقد وجد أن تزامن العلاج بعقار اللوسارتان (المجموعة الرابعة) أدي الي تحسن ذو دلالة إحصائية في هذه التغيرات التليفية. وأن التغيرات الايجابية المناعية لألفا س م أ في المجموعات المختلفة كانت لها نفس النمط الذى حدث في نسبة الألياف الغروية والمرنة ولهذا يمكن أن نستنتج أن خلايا الميوفايبروبلاست تلعب دورا محوريا في احداث تليف الرئة وأنها يمكن ان تستخدم كهدف للنظم العلاجية لهذا المرض في المستقبل.