Evaluation of the antifibrotic effect of serotonin receptor antagonists on bleomycin induced pulmonary fibrosis in rats

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A B S T R A C T

Idiopathic pulmonary fibrosis (IPF) is believed to be an epithelial-fibroblast disease. Activated epithelial cells are thought to release potent fibrogenic molecules and cytokines, such as tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta1 (TGF-β1), which in turn foster the transformation of fibroblasts into myofibroblasts and promote production of extracellular matrix molecules and so collagen deposition. Serotonin (5-hydroxy tryptamine, 5-HT) is an important mediator for lung fibrogenesis, with implication of 5-HT2A/B/C receptors. However, the antifibrotic effects of all 5-HT2 receptor subtypes versus 5-HT2A/C blockade is needing to be explored. So, the present study was conducted to evaluate the antifibrotic effects of mirtazapine (5-HT2A/C receptor blocker) and cyproheptadine (5-HT2A/B/C receptor blocker) on body weight changes, survival rates, the lung hydroxyproline and TGF-β1 levels as well as the histopathological changes of lung fibrosis, in bleomycin-induced rat pulmonary fibrosis. Eighty-eight adult rats were used and subdivided randomly into 11 groups. One normal control, five vehicle control groups and five groups with IPF that induced by intra-tracheal instillation of bleomycin alone (5mg/kg), or bleomycin and treated with either mirtazapine (15 mg/kg/day) or cyproheptadine (5 mg/kg/day) for 7 and 14 days. Oral treatment with either mirtazapine or cyproheptadine, significantly ameliorated losses in body weights, reduction in survival rates, lung hydroxyproline and TGF-β1 levels and the inflammatory effects in lungs induced by bleomycin. The mechanisms underlying these therapeutic effects could be dependent on the reduction of TGF-β1 actions as decreasing lung inflammation and production and deposition of collagen in fibrotic lung tissues.

Key Words: Bleomycin, Cyproheptadine, Idiopathic pulmonary fibrosis, Mirtazapine, Serotonin, TGF-β1.

1. INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common and severe form of idiopathic interstitial pneumonias; it is irreversible, has an unpredictable clinical course and associated with poor prognosis (King et al., 2011).

The exact mechanisms underlying the development of IPF remain unknown. But it is believed that chronic inflammation plays an essential role. This hypothesis is based on the idea that injury/inflammation of the alveolar-capillary constituents and basement membrane leads to the loss of type I epithelial and endothelial cells, the proliferation of type II pneumocytes, the loss of alveolar space integrity, the recruitment and proliferation of stromal cells, and the deposition of the extracellular matrix (Amanda and Daniel, 2016).

Many pro-inflammatory, anti-inflammatory and T lymphocytes producing cytokines e.g., interleukins (IL)-1β, IL-6, IL-8, IL-10, IL-13 and TNF-α, chemokines and pro-fibrotic growth factors, particularly TGF-β1, are known to be associated with development of pulmonary fibrosis (Oku et al., 2008).

In the lung, over-expression of TGF-β1 plays a role in induction of myofibroblasts, collagen synthesis and extracellular matrix deposition (Kulkarni et al., 2011).

Hydroxyproline is one of the amino acids constituting collagen and can be measured in lung homogenates as an index of collagen content (Hutson et al., 2003).

Treatment options for IPF are limited. The clinical management focuses on treatment of
complications, supportive care and in few cases involves lung transplantation. Anti-inflammatory drugs such as prednisone may carry symptomatic relief, but they do not appear to halt progression of fibrosis, cytotoxic drugs have not been shown to improve lung function or life expectancy and may be associated with harmful side effects (Sriram et al., 2009).

Therapeutic strategies include the anti-fibrotic agents and agents targeting specific cytokines including TGF-β1 and chemokines are still being evaluated. The restoration of oxidant balance and inhibition of leukotrienes represent other strategies (Gogali and Wells, 2010). Numerous trials suggest that targeting the fibro-proliferative process, the differentiation of fibroblasts to myofibroblasts and extracellular matrix accumulation may be more effective anti-fibrotic therapies for IPF (Chakraborty et al., 2014). Bleomycin is a chemotherapeutic antibiotic, which induces a well-established model of pulmonary fibrosis in rodents (Zhao et al., 2010).

Serotonin (5-hydroxytryptamine, 5-HT) is known to increase proliferation and collagen synthesis by fibroblasts. Two receptor subtypes, 5-HT2A and 5-HT2B, have been shown to play the most important roles in the lung. It is a novel modulator of alveolar macrophages function through initiating, cytokine secretion and transcriptional regulation. These effects are exclusively driven by the 5-HT2C receptor (Mikulski et al., 2010). Serotonin also stimulates the proliferation and fibrogenic actions of lung myofibroblasts in particular 5-HT2 receptors.

Blocking of 5-HT2A and 5-HT2B receptor subtypes may have a promising role in treatment of pulmonary fibrosis (Fabre et al., 2008; Pytlak et al., 2011).

The therapeutic potential of mirtazapine (5-H2A/C receptor blocker) versus cyproheptadine (5-H2A/B/C receptor blocker) in the regulation of inflammatory/immune-related functions and their effects on TGF-β or on the “fibroproliferative” process and extracellular matrix accumulation in IPF are not well known. So, the present study was designed to investigate the possible anti-fibrotic effects of specific serotonin 5-HT2A/B/C versus 5-HT2A/C receptors antagonists using bleomycin-induced lung fibrosis.

2. MATERIALS AND METHODS

2.1. Drugs and chemicals

Bleomycin sulfate was purchased as vials from Nippon Kayaku, Tokyo, Japan. Each vial contains 15 mg of bleomycin sulfate. Mirtazapine was purchased as white powder from ORGANON, Co. Turkey. Cyproheptadine was purchased as a white powder from KAHIRA, PHARM. & CHEM. IND. CO. All chemicals were supplied from Sigma, St. Louis MO, USA. Rat TGF-β1 Enzyme Linked Immunoassay (ELISA) Kit was supplied from BioVendor LLC, BioVendor Laboratorini Medicine, USA.

2.2. Animals

Eighty-eight adult male albino Wistar rats, weighing 180-200 gm each, were used. Animals were purchased from the National Centre of Research, Cairo, Egypt and housed in polyethylene cages at room temperature (under normal environmental conditions) and were kept with free access to standard rodent chow diet and distilled water ad libitum. Animals were allowed for acclimatization for one week before the start of the study. The experimental protocol was approved by the institutional animal care and use committee at Suez Canal University, which is following the National Institutes of Health guide for the care and use of laboratory animals (Maryland, USA).

2.3. Experimental protocol

2.3.1. Induction of pulmonary fibrosis in rats

Pulmonary fibrosis was induced by bleomycin sulfate solution (5mg/kg in 0.25 ml phosphate buffered saline, PBS, PH 7.4); which was prepared immediately before administration. The bleomycin sulfate solution was given as a single dose of intra-tracheal instillation (Yildirim et al., 2004). The day of bleomycin administration was defined as day (0).

2.3.2. Study groups

Animals were allocated randomly into eleven groups, each comprising of eight rats: control-untreated group, normal rats without induction of pulmonary fibrosis and did not receive any medications; Phosphate buffered saline-control group, normal rats that were received (0.25 ml/kg) phosphate buffered saline by intra-tracheal instillation as a single dose and then received distilled water by oral gavage for 14 days; Bleomycin-induced lung fibrosis group, Rats with bleomycin-induced pulmonary fibrosis that were received 1% distilled water by oral lavage, the day after intra-tracheal bleomycin administration and continue for 14 days; Mirtazapine control group-7 days, rats without pulmonary fibrosis that were given mirtazapine for 7 days; Mirtazapine control group-14 days, rats without pulmonary fibrosis that were given mirtazapine for 14 days; Mirtazapine treated group-7 days, rats with pulmonary fibrosis that were given mirtazapine for 7 days start from the next week after intra-tracheal bleomycin; Mirtazapine treated group-14 days, rats with pulmonary fibrosis that were given mirtazapine for 14 days start from the next week after bleomycin; Cyproheptadine control group-7 days, rats without pulmonary fibrosis that were given cyproheptadine for 7 days; Cyproheptadine control group-14 days, rats without pulmonary fibrosis that were given cyproheptadine for 14 days; Cyproheptadine treated...
group-7 days, rats with pulmonary fibrosis that were given cyproheptadine for 7 days start from the next week after bleomycin; Cyproheptadine treated group-14 days, rats with pulmonary fibrosis that were given cyproheptadine for 14 days start from the next week after bleomycin.

2.4. Body weight assessment

Body weight of each animal was measured immediately before the starting of the experiment (day 0), on the 3rd, 7th, 10th days of the experiment and just before scarification; on the 7th day in groups (4, 6, 8, 10) and on the 14th day in groups (1, 2, 3, 5, 7, 9, 11), then the rats were sacrificed by cervical decapitation (Molina-Molina et al., 2007).

2.5. Lung samples collection and processing

After scarification, the thorax of each rat was opened and the hilum of each lung was ligated. Lungs were dissected free from their bronchi, blood vessels and hilar nodes and then were collected in ice-cold container. Then they were perfused free of blood with ice-cold saline.

Each lung sample was weighed, then left lungs were fixed in 10% neutral buffered formalin for histopathological examination and each right lung was homogenized in (4 ml) of phosphate buffered saline, Using Teflon pestle homogenizer (Glas Col homogenizer system, Vernon hills, US). The homogenate suspension for each lung was divided into four eppendorf tubes equally. Two of the eppendorf tubes were used for measurement of lung hydroxyproline content. The others were centrifuged at 14000 x g for 10 min at 4°C, and the supernatant is kept at -80°C until analysis for TGF-β1 according to the manufactured instructions (Chen et al., 2006).

2.6. Determination of lung hydroxyproline (HYP) content

Lung HYP content was determined as an index of collagen content. Hydroxyproline is an amino acid common to all collagens. The amount of hydroxyproline was determined against a standard curve prepared with the use of known concentrations of hydroxyproline.Trans-4-hydroxy-L-proline (10mg/mL) was used as a standard solution (Liu et al., 2009).

2.7. Determination of total lung TGF-β1

Total TGF-β1 lung contents were measured in lung homogenates by ELISA; using the BioVendor rat TGF-β1 immunoassay.

2.8. Histopathological examination and assessment of lung fibrosis

Left lung samples were fixed in 10% formalin buffer for histological examination. From fixed lungs, paraffin sections 2-4 mm thickness were cut, stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome for visualizing fibrotic lesions and subjected to microscopic observation by an independent blinded observer. For the quantitative histological analysis, a numerical fibrotic scale (Ashcroft score) was used for the fibrotic score (Genovese et al., 2005).

Briefly, the grade of lung fibrosis would be scored on a scale of 0-8 by examination of six randomly chosen fields per sample at a magnification of 100X. The criteria for grading lung fibrosis are as follow: Grade 0: Normal lung, grade 1: Minimal fibrous thickening of alveolar or bronchiolar walls, grade 2-3: Moderate thickening of walls without obvious damage to lung architecture, grade 4-5: Increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses, grade 6-7: Severe distortion of structure and large fibrous areas; (honeycomb lung) was placed in this category, grade 8: Total fibrous obliteration of fields. The mean score of all fields was taken as the fibrosis score of that lung section (Ashcroft et al., 1988).

2.9. Statistical analysis

Results were collected and expressed as the mean ± standard Error (SE). Results were analyzed using The Statistical Package for the Social Sciences, version 20 (SPSS Software, SPSS Inc., Chicago, USA). One-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test was used to test the significance of the difference between quantitative variables. The p-value < 0.05 was considered to be statistically significant.

3. RESULTS

3.1. Effects of mirtazapine and cyproheptadine on changes in body weight

Body weight for each animal was measured in six sessions: immediately before starting of the experiment (day 0), on the 3rd, 7th, 10th, 14th, days of the experiment and just before scarification on the 21th day, as shown in (Table 1A and B). The mean rats’ body weights in PBS control group showed non-significant change compared to the corresponding control-untreated group throughout the experiment. The mean rats’ body weights in bleomycin-induced lung fibrosis group showed a significant decrease compared to the corresponding control-untreated group throughout the experiment. The mean rats’ body weights in mirtazapine-7 days control group showed a significant increase compared to the corresponding control-untreated group throughout the experiment, while the mean rats’ body weights in mirtazapine 14 days control group showed increase although this increase is not statistically significant. The mean rats’ body weights in mirtazapine 7 and 14 days treated groups showed significant increase in 7, 10 and 14 days, as well as 21
days in the latter group, compared to the corresponding lung fibrosis group throughout the experiment. The mean rats’ body weights in cyproheptadine control groups7 and 14 days showed and non-significant decrease compared to the corresponding control-untreated group throughout the experiment. While the mean rats’ body weights in cyproheptadine 7 and 14 days treated groups showed a significant increase in 7, 10 and 14 days, as well as 21 days in the latter group, compared to the corresponding lung fibrosis group throughout the experiment.

3.2. Effects of mirtazapine and cyproheptadine on survival rate among experimental groups

In control untreated group, PBS control group, Mirtazapine control group-7 days, mirtazapine- treated groups for 7 or 14 days, as well as cyproheptadine control or treated groups for 7 and 14 days; no rats were died. Further, the survival rates in normal rats received mirtazapine alone for 14 days was 87.5%. All died animals were died in the first week after treatment. In bleomycin control group, 2 of 8 animals were died in the first week of the experiment before completing the predetermined time of death, yielding a survival rate of 75% which was significantly (p < 0.05) lower than the survival rate of the control untreated animals (100%). None of the rats, previously received bleomycin, which had been treated with either of the mirtazapine and the cyproheptadine were died. Those treated rats had a significant higher survival rates (100%) than bleomycin control group (75%) (p < 0.05, Table 2).

3.3. Effects of mirtazapine and cyproheptadine on lung hydroxyproline content

The mean hydroxyproline concentrations in the control groups was non-significantly changed compared to that in control untreated rats (8.48 ± 1.09 mg/g wet lung), (p < 0.05; Figure 1). The mean lung hydroxyproline content in bleomycin-induced lung fibrosis group (19.71 ± 1.08 mg/g wet lung) was significantly higher than the mean lung hydroxyproline content in the control untreated group (8.48 ± 1.09 mg/g wet lung), (p < 0.05; Figure 1). Treatment with mirtazapine for 7 and 14 days decreased the means of lung hydroxyproline contents to (10.73 ± 1.65 and 10.94 ± 1.2 mg/g wet lung), respectively. On the other hand, treatment with cyproheptadine for 7 and 14 days decreased also the means of lung hydroxyproline contents to (10.36 ± 1.1 and 10.54± 1.01 mg/g wet lung), respectively. Either mirtazapine or cyproheptadine, in both durations, resulted in a significant decrease in the mean of lung hydroxyproline content as compared to bleomycin induced lung fibrosis group (p < 0.05; Figure 1). Of notice, the means lung hydroxyproline content for both cyproheptadine groups were lower that of mirtazapine treated groups, but this difference was non-significant.

3.4. Effects of mirtazapine and cyproheptadine on changes in lung TGF-β1 levels

The mean TGF-β1 concentrations in the control groups was non-significantly changed compared to that in control untreated rats (4.88 ± 1.22ng/ml). The mean lung TGF-β1 level in bleomycin-induced lung fibrosis group was significantly higher (21.7 ± 3.81 ng/ml) than the mean lung TGF-β1 content in the control untreated group. (p < 0.05; Figure 2). Treatment with mirtazapine for 7 days and 14 days decreased the means of lung TGF-β1 contents to (9.44 ± 1.59 and 9.79± 1.80 ng/ml), respectively. Of notice, the means lung TGF-β1 content for both cyproheptadine groups were lower that of mirtazapine treated groups, but this difference was non-significant.

3.5. Effects of mirtazapine and cyproheptadine on histopathological changes

In the current study, histopathological changes were evaluated at the end of the experiment (14 days after bleomycin exposure). In the control untreated group, lungs showed normal alveolar spaces and normal alveolar septum thickening, no inflammation or congestion and normal amount and distribution of collagen as shown in sections stained with H & E and Masson’s trichrome (Figure 3A and 4A). All the previous findings were found in normal rats received PBS (Figure 3B and 4B), mirtazapine (Figure 3D, E and 4 D, E) and cyproheptadine (Figure 3 H, I and 4 H, I), but with very little inflammatory reactions. In the bleomycin-induced lung fibrosis group (Figure 3 C and 4 C), lungs showed collapsed alveoli, marked interalveolar septum thickening, and dense interstitial infiltration by fibroblasts. In addition, there was an excessive amount of collagen deposited around the alveolae. Rats treated with either mirtazapine (Figure 3 F, G and 4 F, G), or cyproheptadine (Figure 3 J, K and 4 J, K), showed marked suppression of the bleomycin induced inflammatory cell infiltration as evidenced by reduced thickening of the interalveolar septa and increased inflation of the alveoli. Furthermore, the amount of collagen deposited in the alveolar septa was markedly reduced compared with that of bleomycin-induced lung fibrosis group.

3.6. Effects of mirtazapine and cyproheptadine on fibrotic score

Table (3); showed that pulmonary fibrotic changes were not-detected in PBS, cyproheptadine and
mirtazapine control groups. The mean pulmonary fibrotic score in bleomycin-induced lung fibrosis group (5.25 ± 0.83) was significantly higher (p < 0.05) than the mean pulmonary fibrotic score in control untreated group. The means pulmonary fibrotic score in mirtazapine treated groups for 7 and 14 days, respectively, was (2.25 ± 0.66), (2.88 ± 0.33), while that in cyproheptadine treated groups for 7 and 14 days, respectively, was (1.25 ± 0.43), (1.38 ± 0.48). These fibrotic score using either mirtazapine or cyproheptadine was significantly lower than that of the bleomycin induced lung fibrosis group (5.25 ± 0.83), (p < 0.05, Table 3). There was a non-significant decrease in the means pulmonary fibrotic score for BLM & cyproheptadine treated group for 7 and 14 days (1.25 ± 0.43 and 1.38 ± 0.48, respectively) as compared to mirtazapine-treated groups for 7 and 14 days (2.25 ± 0.66 and 2.88 ± 0.33, respectively).

Table 1(A): Changes in means body weights after treatment for 7 days of rats in different study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (gm) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>1. Control-untreated group</td>
<td>182.5±1.9</td>
</tr>
<tr>
<td>2. PBS control group</td>
<td>186.5±5.5</td>
</tr>
<tr>
<td>3. Bleomycin-induced lung fibrosis</td>
<td>189.9±5.8</td>
</tr>
<tr>
<td>4. Mirtazapine control group-7 d</td>
<td>192.4±6.3</td>
</tr>
<tr>
<td>5. Bleomycin + mirtazapine-7 d</td>
<td>191.3±3.7</td>
</tr>
<tr>
<td>6. Cyproheptadine control group -7 d</td>
<td>186.9±3.6</td>
</tr>
<tr>
<td>7. Bleomycin + cyproheptadine-7 d</td>
<td>186.3±5.2</td>
</tr>
</tbody>
</table>

* p < 0.05 compared to control-untreated group, # p < 0.05 compared to bleomycin-induced lung fibrosis group. n= 8

Table 1(B): Changes in means body weights after treatment for 14 days of rats in different study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight (gm) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>1. Control-untreated group</td>
<td>182.5±1.9</td>
</tr>
<tr>
<td>2. PBS control group</td>
<td>186.5±5.5</td>
</tr>
<tr>
<td>3. Bleomycin-induced lung fibrosis</td>
<td>189.9±5.8</td>
</tr>
<tr>
<td>4. Mirtazapine control group-14 d</td>
<td>187.8±5.8</td>
</tr>
<tr>
<td>5. Bleomycin + mirtazapine-14 d</td>
<td>188.6±4.3</td>
</tr>
<tr>
<td>6. Cyproheptadine control group-14 d</td>
<td>189.3±5.0</td>
</tr>
<tr>
<td>7. Bleomycin + cyproheptadine-14 d</td>
<td>188.6±4.1</td>
</tr>
</tbody>
</table>

# p < 0.05 compared to control-untreated group, /p < 0.05 compared to bleomycin-induced lung fibrosis group. n= 8
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Table 2: Survival rate among different experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of rats</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control-untreated group</td>
<td>8</td>
<td>100%</td>
</tr>
<tr>
<td>2. PBS control group</td>
<td>8</td>
<td>100%</td>
</tr>
<tr>
<td>3. Bleomycin-induced lung fibrosis</td>
<td>8</td>
<td>75%*</td>
</tr>
<tr>
<td>4. Mirtazapine control group-7d</td>
<td>8</td>
<td>100%</td>
</tr>
<tr>
<td>5. Mirtazapine control group-14d</td>
<td>8</td>
<td>87.5%</td>
</tr>
<tr>
<td>6. Bleomycin + mirtazapine- 7d</td>
<td>8</td>
<td>100%*</td>
</tr>
<tr>
<td>7. Bleomycin + mirtazapine-14d</td>
<td>8</td>
<td>100%*</td>
</tr>
<tr>
<td>8. Cyproheptadine control group-7d</td>
<td>8</td>
<td>100%</td>
</tr>
<tr>
<td>9. Cyproheptadine control group-14d</td>
<td>8</td>
<td>100%</td>
</tr>
<tr>
<td>10. Bleomycin + cyproheptadine 7d</td>
<td>8</td>
<td>100%*</td>
</tr>
<tr>
<td>11. Bleomycin + cyproheptadine 14 d (n=8)</td>
<td>8</td>
<td>100%*</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to control-untreated group. *p < 0.05 compared to bleomycin-induced lung fibrosis group.

Table 3: Means pulmonary fibrotic scores for different study groups.

<table>
<thead>
<tr>
<th>Study Groups</th>
<th>Ashcroft fibrotic score (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control-untreated group (n=8)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2. PBS control group (n=8)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>3. Bleomycin-induced lung fibrosis (n=6)</td>
<td>5.25 ± 0.53*</td>
</tr>
<tr>
<td>4. Mirtazapine control group-7d (n=8)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>5. Mirtazapine control group-14d (n=7)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>6. Bleomycin + mirtazapine-7d (n=8)</td>
<td>2.25 ± 0.26*</td>
</tr>
<tr>
<td>7. Bleomycin + mirtazapine-14d (n=8)</td>
<td>2.88 ± 0.23*</td>
</tr>
<tr>
<td>8. Cyproheptadine control group-7d (n=8)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>9. Cyproheptadine control group-14d (n=8)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>10. Bleomycin + cyproheptadine-7d (n=8)</td>
<td>1.25 ± 0.43*</td>
</tr>
<tr>
<td>11. Bleomycin + cyproheptadine 14 d (n=8)</td>
<td>1.38 ± 0.48*</td>
</tr>
</tbody>
</table>

*p < 0.05 compared to control-untreated group. *p < 0.05 compared to bleomycin-induced lung fibrosis group.

Figure (1): Mean Hydroxyproline contents among the experimental groups. n= 6-8. Group 1: control untreated, Group 2: PBS control group, Group 3: Bleomycin-induced lung fibrosis group, Group 4: Mirtazapine control group-7days, Group 5: Mirtazapine control group-14days, Group 6: Mirtazapine treated group-7days, Group 7: Mirtazapine treated group-14days, Group 8: Cyproheptadine control group-7days, Group 9: Cyproheptadine control group-14days, Group 10: Cyproheptadine treated group-7days, Group 11: Cyproheptadine treated group-14 days. *p < 0.05 when compared to control-untreated group. *p < 0.05 when compared to bleomycin-induced lung fibrosis group.
Figure (2): Mean TGF–β1 among experimental groups. n = 6-8. Group 1: control untreated, Group 2: PBS control group, Group 3: Bleomycin-induced lung fibrosis group, Group 4: Mirtazapine control group-7days, Group 5: Mirtazapine control group-14days, Group 6: Mirtazapine treated group- 7days, Group 7: Mirtazapine treated group-14days, Group 8: Cyproheptadine control group-7days, Group 9: Cyproheptadine control group-14days, Group 10: Cyproheptadine treated group-7 days, Group 11: Cyproheptadine treated group-14 days. *p < 0.05 when compared to control-untreated group, #p < 0.05 when compared to bleomycin-induced lung fibrosis group.

Figure (3): Lung sections of experimental groups (H & E; X100), n = 6-8. Control untreated group (A), PBS control group (B), Bleomycin-induced lung fibrosis group (C), Mirtazapine control group-7 days (D), Mirtazapine control group-14 days (E), mirtazapine treated group-7 days (F), Mirtazapine- treated group-14 days (G), Cyproheptadine control group-7 days (H), Cyproheptadine control group-14 days (I), Cyproheptadine treated group-7 days (J), Cyproheptadine treated group-14 days (K). Moderate inflammation and mild congestion "green arrow" was observed in bleomycin-induced lung fibrosis group. The reversals of these inflammatory changes were obvious after treatment with mirtazapine or cyproheptadine.
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Figure (4): Lung sections of experimental groups (Masson's Trichrome; X100). n= 6-8. Control untreated group (A), PBS control group (B), Bleomycin-induced lung fibrosis group (C), Mirtazapine control group 7days (D), Mirtazapine control group 14days (E), Mirtazapine treated group 7days (F), Mirtazapine treated group 14days (G), Cyproheptadine control group 7days (H), Cyproheptadine control group 14days (I), Cyproheptadine treated group 7days (J), Cyproheptadine treated group 14 days (K). Bleomycin showed severe thickening of walls with obvious damage to lung architecture with increased fibrosis and formation of fibrous bands "black arrow". The reversals of these inflammatory changes were obvious after treatment with mirtazapine or cyproheptadine.
4. DISCUSSION

Idiopathic pulmonary fibrosis is typically characterized by abnormalities of alveolar structure accompanied by myofibroblast accumulation and collagen deposition in the extracellular matrix (Du et al., 2009). Since the available treatment options for IPF are not successful, the development of a potential drug regimen to treat pulmonary fibrosis remains an imperative challenge (Gogali and Wells, 2010; Sriram et al., 2009).

In the current study, lung fibrosis was induced by intratracheal injection of bleomycin. Bleomycin induces single and double-strand DNA breaks and thereby interrupting the cell cycle. This happens by chelation of metal ions, and reaction of the formed pseudoenzyme with oxygen, which leads to production of DNA-cleaving superoxide and hydroxide free radicals, inflammatory response causing pulmonary toxicity, activation of fibroblasts and subsequent fibrosis (Chaudhary et al., 2006). Bleomycin hydrolase, a bleomycin-inactivating enzyme, critically influences the effects of this drug on different tissues. The lungs maintain low levels of the enzyme and therefore are more susceptible to bleomycin-induced tissue injury (Sebti et al., 1989).

Bleomycin exhibited a statistically significant decrease in serial body weight measurements and survival rates compared to the control untreated group. Moreover, the losses in rats’ body weights and the increased mortality rates were higher in the first 7 days after instillation of bleomycin. This could be explained as the bleomycin model of lung fibrosis had an inflammatory phase and a fibrotic phase. The “switch” between inflammation and fibrosis appears to occur around day 9 after bleomycin (Moeller et al., 2008; Molina-Molina et al., 2007).

In accordance, Borzone et al. (2001) stated that rats received bleomycin exhibited higher reductions in their body weights in the first 7 days after instillation and later recovered, without reaching the final body weight of the control animals on the 14th day. Also, Chen et al. (2006) concluded significant higher mortality rate (30%) and higher body weights losses in bleomycin-treated rats within 7 days after administration. Also, Chen (2015) stated that the body weight of the bleomycin-treated animals decreased gradually and reached the lowest level at day 7 after instillation. Also, Fineschi et al. (2008) and Liu et al. (2009) confirmed the significant reductions in body weights of the bleomycin treated mice along the 14 days of their studies. Hydroxyproline is a major component of the protein collagen (Szpak, 2011). Numerous studies stated that bleomycin causes destruction of the alveolar structure, resulting in pulmonary fibrosis that was characterized by an increase in hydroxyproline levels and collagen deposition in the lungs (Chen and Zhao, 2016; Zhao et al., 2010). The current data was in harmony with the above results which confirmed that lung hydroxyproline levels were significantly increased 14 days after bleomycin instillation. In addition, it has been demonstrated that bleomycin itself induces apoptosis of alveolar epithelial cells, which precedes collagen depositions (Li et al., 2003).

Transforming growth factor-β1 is critical to the progression of fibrosis in fibroproliferative acute respiratory distress syndrome (ARDS) and IPF and it is the most potent regulator of inflammation and it induces connective tissue synthesis (Kulkarni et al., 2011). Lung fibrosis due to bleomycin was blocked in mice lacking the TGF-β1-dependent Smad3 signaling pathway (Bonnaud et al., 2004). In addition, it has been established that alveolar macrophages stimulated by bleomycin secrete large quantities of biologically active TGF-β1, which plays a critical role in the development of lung fibrosis in mice and murine (Chen et al., 2009; Zhao et al., 2010). Consistent with the previous reports, we observed high levels of TGF-β1 in the lung homogenates of bleomycin rats.

The histopathological results of the present study demonstrated that bleomycin induced intense cellular infiltrate with severe distortion of lung architecture and excessive deposition of extracellular matrix proteins and large fibrous areas within the pulmonary interstitium. These observations were consistent with previous studies that concluded that intratracheal bleomycin revealed severe epithelial degeneration, inflammatory cell infiltration, fibrosis, excessive collagen deposition, vascular congestion and disturbance of lung architecture (Chen and Zhao, 2016; Yildirim et al., 2010).

Numerous studies stated that mirtazapine is associated with weight gain. Patients taking mirtazapine often report intense cravings for carbohydrates (Laimer et al., 2006; Smit et al., 2016). The current data were in harmony with the above results which confirmed that the body weight in rats treated with mirtazapine was significantly increased compared with that of the normal control rats.

The current data showed a decrease in body weights in rats treated with cyproheptadine compared with that of the normal control rats and that is in contrary to previous studies which showed that has been used for the treatment of anorexia and weight loss and has shown stimulation of appetite (Homnick et al., 2004; Lehrer, 2004).

Also, Najib et al. (2014) stated that significant higher body mass index was observed after 8 weeks intervention with cyproheptadine.

In the current work, the antifibrotic effect observed following treatment with mirtazapine and
cyproheptadine that selectively block (5-HT2A/C and 5-HT2A/B/C receptors respectively) appears to be associated with decrease in lung tissue content of the potent profibrotic factors; TGF-β1 and hydroxyproline. These factors have previously been linked to serotonin, 5-HT2A and 5-HT2B receptor activation. Indeed, upregulation of the TGF-β1 pathway by serotonin via its 5-HT2A receptor has been previously described in cultured mesangial cells (Greene et al., 2000) and also demonstrated in aortic valve interstitial cells (Jian et al., 2002).

Lung serotonin content was increased in bleomycin-induced fibrosis and, in parallel; the expression of the serotonin receptors 5-HT2A and 5-HT2B was increased in the fibrotic lung (Fabre et al., 2008).

Blocking of 5-HT2A and 5-HT2B receptors with specific antagonists promoted an antifibrotic environment in bleomycin-induced lung fibrosis, through the inhibition of key factors involved in lung fibrosis; TGF-β1, connective growth factor and plasminogen activator inhibitor (PAI)-1 (Fabre et al., 2008).

In addition, the expression of 5-HT2A and 5-HT2B receptors in IPF patients was demonstrated previously, and the specific expression of the 5-HT2B receptor was particularly noted in the fibroblastic focus characteristic of usual interstitial pneumonia.

Mast cell numbers are increased in the fibrotic lung and may release serotonin under certain stimuli (Yong, 1997). In addition, the expression of 5-HT2A and 5-HT2B receptors in IPF patients was demonstrated previously (Ito et al., 2002).

The present study concentrated on 5-HT2A and 5-HT2B receptors in lung fibrosis, since these receptors have been shown to play an important role in lung pathophysiology (Shi et al., 1998) and to have profibrotic properties in respiratory (Marcos et al., 2004) and non-respiratory tissues (Ruddell et al., 2006).

Lung vessels, type-II pneumocytes, epithelial bronchial cells, pleural mesothelial cells and lung fibroblasts have also been shown to express 5-HT2A and 5-HT2B receptors in normal and fibrotic lungs. Thus, the serotonin pathway appears to act on many different cell types potentially involved in the pathogenesis of lung fibrosis. The expression of 5-HT2B by fibroblasts in human tissue sampled from IPF lungs, especially in the fibroblastic focus (the site of active fibrosis) puts into perspective the results in the mouse model of lung fibrosis (Fabre et al., 2008).

Lung expression of 5-HT2A and 5-HT2B mRNA was also observed to be markedly increased after bleomycin administration. In addition, immunohistochemical studies indicated that both resident cells and inflammatory cells expressed 5-HT2A and 5-HT2B receptors in the lung (Fabre et al., 2008). In the present study, blocking of 5-HT2A/B/C receptors by mirtazapine and cyproheptadine supported previous published data regarding profibrotic properties of serotonin (Ruddell et al., 2008).

Previous data has suggested that the 5-HTR2A antagonist; ketanserin reduced collagen mRNA and protein levels in the bleomycin-induced mouse model. Accordingly, this evidence demands a detailed characterization of 5-HT receptor expression in IPF and analysis to determine whether 5-HT2A/B/C antagonists represent a therapeutic option in IPF, particularly since 5-HT2 antagonists are already in clinical use for other diseases. Blockade of 5-HT2A/B signaling by terguride reversed lung fibrosis and is thus a promising therapeutic approach for IPF (Königshoff et al., 2010).

5. CONCLUSION

The present study provided evidence that mirtazapine (5-H2A/C receptor blocker) and cyproheptadine (5-H2A/B/C receptor blocker) caused a substantial reduction in lung injury induced by bleomycin which supported the potential use of mirtazapine and cyproheptadine as anti-fibrotic agents in the therapy of lung fibrosis. The mechanisms underlying these therapeutic effects could be dependent on the reduction of TGF-β1 production and deposition of collagen in fibrotic lung tissues.

6. REFERENCES


Evaluation of the antifibrotic effect of serotonin receptor antagonists on bleomycin induced pulmonary fibrosis in rats


