PHARMACOLOGICAL STUDY OF PROTECTIVE EFFECT OF THYMOQUINONE IN A MODEL OF LUNG INJURY

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ABSTRACT

Oxidative stress is caused by the imbalance between the production of reactive oxygen species and the body's ability to detoxify them or repair the resulting damage. Current evidence suggests that acute lung injury can lead to pulmonary fibrosis. Pulmonary fibrosis is a progressive and fatal interstitial pneumonitis with high mortality and limited successful treatment. The present study was designed to assess the protective effect of thymoquinone (TQ) and whether it can attenuate the severity of oxidative stress and inflammatory response during bleomycin (BLM) induced pulmonary fibrosis. Male albino rats were treated with either BLM and/or TQ. BLM significantly increased the levels of Lactate dehydrogenase (LDH), total protein and mucin in bronchoalveolar lavage fluid (BALF) and these effects were significantly ameliorated by TQ treatment. BLM also caused a significant elevation in the levels of lipid peroxides and nitric oxide (NO) accompanied with a significant decrease in the antioxidant enzyme activity of superoxide dismutase (SOD). TQ treatment restored these markers toward normal values. Moreover, histopathological examination confirmed the protective effect of TQ.

Key Words: Lung injury – thymoquinone – oxidative stress – inflammation.

INTRODUCTION

The lung is exposed to higher oxygen tension than other tissues. Both endogenous and exogenous stimuli may injure the alveolar epithelium. Several important reactive oxygen species (ROS) are generated endogenously (Kinnula and Crapo, 2003). Lung injury can lead to pulmonary inflammation which if it is not well controlled can then progress to pulmonary fibrosis. Pulmonary fibrosis involves gradual exchange of normal lung parenchyma with fibrotic tissue (Cooper, 2000). This replacement of normal lung with scar tissue causes irreversible decrease in oxygen diffusion capacity. In addition, decreased compliance makes pulmonary fibrosis a restrictive lung disease (Dianne et al., 2008).

Symptoms of pulmonary fibrosis include shortness of breath, coughing and diminished exercise tolerance. Scarring of the alveoli reduces the ability of the lungs to transfer oxygen. The resulting lack of blood oxygen may increase the pressure in the arteries to the lungs, a situation known as pulmonary hypertension. This puts a strain on the right ventricle of the heart, which pumps blood to the lungs and may lead to right heart failure (Altschuler, 2001). Pulmonary fibrosis may be a secondary effect of other diseases. Most of
these are classified as interstitial lung diseases. Examples include autoimmune disorders, viral infections or other microscopic injuries to the lung (El-Medany et al., 2005). However, pulmonary fibrosis can also appear without any known cause. In this case, it is termed "idiopathic". Most idiopathic cases are diagnosed as idiopathic pulmonary fibrosis. This is a diagnosis of exclusion of a characteristic set of histologic/pathologic features known as usual interstitial pneumonia (UIP) (Antoniu, 2006).

Though research trials are ongoing, there is no evidence that any medications can significantly help this condition. Lung transplantation is the only therapeutic option available in severe cases (Banerjee et al., 2012). Since some types of lung fibrosis can respond to corticosteroids (such as Prednisone) and/or other medications that suppress the body's immune system, these types of drugs are sometimes prescribed in an attempt to slow the processes that lead to fibrosis. However, only a minority of patients respond to corticosteroids alone, so additional immunosuppressants, such as cyclophosphamide, azathioprine, methotrexate, penicillamine, and cyclosporine may be used (Ahmed and Al-Zakwani, 2012). Oxygen supplementation improves the quality of life and exercise capacity (Horiuchi et al., 1990).

Bleomycin is an antineoplastic chemotherapeutic. It is active against gram-positive, gram-negative bacteria and against fungi as well, but its cytotoxicity precludes its use as an anti-infective agent (Santos-Silva et al., 2012). There has been a wealth of researches employing BLM for treatment of several malignancies alone or with other drugs. The broad incorporation of BLM in many types of cancers is referred to its capability increasing oxidative stress and thus inducing apoptosis in cancer cells (Cort et al., 2012). BLM is used alone or in combination with other agents in management of many cancers such as head and neck cancers, Hodgkin's lymphoma and cancers of the genital system (Messori et al., 2001).

Thymoquinone is a phytochemical compound found in the plant Nigella sativa. It has antioxidant effects and has been shown to protect against heart, liver and kidney damage in animal studies, as well as having possible anti-cancer effects (Al-Ali et al., 2008). It is an angiogenesis inhibitor. In 2008 in-vitro tests showed it was an HDAC inhibitor and could kill pancreatic cancer cells, affecting important genes such p53, Bax, bcl-2 and p21 (Chehl et al., 2009). TQ has been used as immunomodilative, antibacterial, hypotensive, hepatoprotective and antidiabetic effects (Woo et al., 2012). TQ also has anti-epileptic effects (Akhondian et al., 2011). It inhibits eicosanoid generation in leukocytes and so is used in the treatment of rheumatism and related inflammatory diseases (Ragheb et al., 2009).

The present study aims to investigate the protective effect of thymoquinone in modulating lung injury induced by bleomycin.

Chemicals and drugs:

All chemicals used in this study were analytically pure and purchased from Sigma-Aldrich Co, St. Louis, MO, USA. Bleomycin was obtained as ampoules (Bleocin) (Nippon Kayaku Co. Ltd, Tokyo, Japan).

Animals:

Male albino rats weighing 110-130 g were obtained from the animal house of El Nile Co. for pharmaceutical industries, Cairo, Egypt. The animals were housed in air condition atmosphere at 25±2°C, and fed with a standard laboratory pellet diet and tap water ad libitum.

Experimental design:

Forty-eight adult male albino rats were divided equally into four groups (12 rats each), as following:
The first group was considered as control. The second group was given BLM (15 mg/kg, i.p., three times a week for a total period of 4 weeks) (El-Medany et al., 2005). The third group was given TQ alone (5 mg/kg/day, i.p., for 5 weeks) (Hadjzadeh et al., 2008). The last group was given both BLM (15 mg/kg, i.p., three times a week for a total period of 4 weeks) and TQ (5 mg/kg/day, i.p., one week before and during induction of lung fibrosis).

At the end of 5 weeks the rats were sacrificed, both lungs were rapidly dissected out and washed with ice-cold saline. For six animals, the lung was used for assessment of lung injury markers in bronchoalveolar lavage fluid (BALF) including lactate dehydrogenase activity (LDH), protein concentration and mucin. For the other six animals, one of two lungs was used for assessment of oxidative stress markers including lipid peroxidation, nitric oxide (NO) and the antioxidant enzyme activity of superoxide dismutase (SOD) while the other lung was used for histopathological examination.

Biochemical estimations:

After dissection of lung and trachea, three successive 7 ml 0.9% saline were infused and slowly withdrawn from the lungs through a cannula inserted into the trachea. Recovered BALF volumes were routinely collected. BALF was centrifuged at 500g for 10 minutes at 4 °C. The supernatant was removed and saved at -80 °C for assay of LDH and the content of protein and mucin. LDH was determined using commercial Kit (Stanbio laboratory, Inc., San Antonio, TX, USA) (Stevens et al., 1983). BALF protein content was determined depending on biuret reaction using commercial Kit (Stanbio laboratory, Inc., San Antonio, TX, USA). The colour was measured spectrophotometrically at 550nm (Cundliffe et al., 1974). The mucin content was determined according to the method of Winzler (1955). The method is based on the determination of the hexose content of mucin. The colour product was measured spectrophotometrically at 425 nm.

In 20% lung homogenate, lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA), according to the method of Cragg (1998). SOD activity was estimated by detecting inhibition of pyrogallol autooxidation according to the method of Marklund et al. (1982). Changes in the absorbance at 420 nm were recorded. Total NO content was estimated in lung homogenate spectrophotometrically as formed nitrite according to the method described by Paino et al. (2011). Briefly, the homogenate was added to 100 µl of 1:1 mixture of Greiss reagent I and Greiss reagent II. Then, the absorbance was measured at 540 nm.

Histopathological assessment:

Autopsy samples were taken from the lung of rats in different groups and fixed in 10% formol saline for 24 hrs. Specimens were washed, dehydrated by alcohol, cleared in xylene and embedded in paraffin at 56 degree in hot air oven for twenty four hours. It was stained by hematoxylin and eosin (H&E) for histopathological examination.

Statistical Analysis:

Comparisons between different groups was carried out by one way analysis of variance (ANOVA) followed by Tukey-Kramer as a post-hoc test using Graphpad Prism software version 4. The p values were at p<0.05 which means significance.

RESULTS:

Bleomycin induced a significant increase in LDH activity as well as the levels of total protein and mucin reaching 281%, 246% and 672% respectively as compared to the control group. Concurrent treatment of animals with TQ significantly reduced the LDH activity and
total protein and mucin to the normal level. However, TQ treatment alone didn't show any significant changes in all markers when compared to the control group (Table 1, Figure 1A, 1B, 1C).

Bleomycin-induced oxidative stress was evaluated by assessing lipid peroxides and NO levels as well as the antioxidant enzyme activity of SOD. As shown in (Table 2, Figure 2A, 2B, 2C), BLM induced a significant increase in lung lipid peroxides and NO levels reaching 179.7% and 156%, respectively as compared to the control values. On the other hand, BLM significantly decreased the antioxidant enzyme activity of SOD reaching 14.6% as compared to the control values. Treatment of animals with TQ concomitantly with BLM afforded significant protection against the oxidative stress effect of BLM. Furthermore, animals treated with TQ alone did not show any significant alterations in the oxidative stress markers as compared to the control group.

Lung sections from the control and TQ groups stained with H&E showed normal architecture; neither edema nor alveolar hemorrhage was encountered. On the other hand, lung sections from the BLM treated group showed mild hyperplasia in the lining epithelium of the bronchioles while the air alveoli showed emphysema. It also showed collagen proliferation with fibrosis. Furthermore, there is multiple inflammatory cells infiltration. TQ preserved the normal architecture of the lung when given concurrently with BLM (Figure 3).

Table (1): Effect of thymoquinone on lung injury markers in BALF of rats treated with bleomycin

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>LDH (U/L)</th>
<th>Total protein (g/dl)</th>
<th>Mucin (mg hexose%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>17.6 ± 1.7</td>
<td>1.3 ± 0.08</td>
<td>0.058 ± 0.007</td>
</tr>
<tr>
<td>BLM</td>
<td></td>
<td>49.4 ± 2.4</td>
<td>3.2 ± 0.4</td>
<td>0.39 ± 0.063</td>
</tr>
<tr>
<td>TQ</td>
<td></td>
<td>20.6 ± 0.7</td>
<td>1.3 ± 0.11</td>
<td>0.07 ± 0.006</td>
</tr>
<tr>
<td>TQ + BLM</td>
<td></td>
<td>30.7 ± 2.2</td>
<td>1.3 ± 0.18</td>
<td>0.11 ± 0.02</td>
</tr>
</tbody>
</table>

Data are the mean ±SD, (N=6).
a or b: Significantly different from control or BLM group respectively at P<0.05 using ANOVA followed by Tukey–Kramer as a post-hoc test.

Table (2): Effect of thymoquinone on oxidative stress markers in lung tissues of rats treated with bleomycin

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>MDA (nmol/g wet tissue)</th>
<th>NO (µmol nitrite/g wet tissue)</th>
<th>SOD (U/g protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>13.3 ± 1.2</td>
<td>10.7 ± 1.2</td>
<td>106.9 ± 10.2</td>
</tr>
<tr>
<td>BLM</td>
<td></td>
<td>23.9 ± 2.3</td>
<td>16.7 ± 1.7</td>
<td>15.6 ± 1.7</td>
</tr>
<tr>
<td>TQ</td>
<td></td>
<td>15.3 ± 0.6</td>
<td>12.6 ± 0.8</td>
<td>98.5 ± 8.6</td>
</tr>
<tr>
<td>TQ + BLM</td>
<td></td>
<td>21.13 ± 0.9</td>
<td>11.8 ± 1.4</td>
<td>83.98 ± 8.4</td>
</tr>
</tbody>
</table>

Data are the mean ± SD, (N=6).
a or b: Significantly different from control or BLM group respectively at P<0.05 using ANOVA followed by Tukey–Kramer as a post-hoc test.
Figure (1A): Effect of TQ on Lactate dehydrogenase content in BALF in BLM-treated rats. Values are percentage of control group (n=6) using one-way ANOVA followed by Tukey-Kramer as post hoc test, a or b statistical significant difference from the control and BLM group respectively at P<0.05.

Figure (1B): Effect of TQ on Total protein content in BALF in BLM-treated rats. Values are percentage of control group (n=6) using one-way ANOVA followed by Tukey-Kramer as post hoc test, a or b statistical significant difference from the control and BLM group respectively at P<0.05.
Figure (1C): Effect of TQ on Mucin content in BALF in BLM-treated rats. Values are percentage of control group (n=6) using one-way ANOVA followed by Tukey-Kramer as post hoc test, a or b statistical significant difference from the control and BLM group respectively at P<0.05.

Figure (2A): Effect of TQ on Lipid peroxides content in lung tissues in BLM-treated rats. Values are percentage of control group (n=6) using one-way ANOVA followed by Tukey-Kramer as post hoc test, a or b statistical significant difference from the control and BLM group respectively at P<0.05.
Figure (2B): Effect of TQ on Nitric oxide content in lung tissues in BLM-treated rats. Values are percentage of control group (n=6) using one-way ANOVA followed by Tukey-Kramer as post hoc test, a or b statistical significant difference from the control and BLM group respectively at P<0.05.

Figure (2C): Effect of TQ on Superoxide dismutase content in lung tissues in BLM-treated rats. Values are percentage of control group (n=6) using one-way ANOVA followed by Tukey-Kramer as post hoc test, a or b statistical significant difference from the control and BLM group respectively at P<0.05.
Figure (3): Representative photomicrographs of lung sections stained by H & E (X40). A: Section taken from a lung of control rat. B: Sections taken from lung of rats treated with BLM show mild hyperplasia in the lining epithelium of the bronchioles, emphysema in the air alveoli, collagen proliferation (c) with fibrosis and multiple number of inflammatory cells infiltration (m) in association with lymphoid hyperplastic cells activation surrounding the bronchioles. C: section taken from a lung of TQ treated rat showing normal architecture of bronchioles (b) and alveolar spaces (a). D: section taken from lung treated with both BLM and TQ show few inflammatory cells infiltration in the peribronchiolar tissue.
DISCUSSION:

Thymoquinone has been investigated for its antioxidant and anti-inflammatory activities in both in-vitro and in-vivo models since its extraction in 1960s (Al-Ali et al., 2008). In the present study, we used an experimental model of bleomycin-induced lung injury to assess the potential antifibrotic effect of TQ and elucidated the underlying mechanisms by studying the effect of TQ on different markers of lung injury and oxidative stress. BLM produces pulmonary fibrosis as an adverse effect due to the low levels in lung tissue of the cysteine hydrolase that inactivates BLM (El-Medany et al., 2005).

In the current study, it was found that BLM caused a significant elevation of lung injury markers measured in BALF including LDH, total protein and mucin; all these changes confirm the induction of lung injury. Concurrent treatment of animals with TQ significantly counteracted the inflammatory effect of BLM and retained all the injury markers at the normal levels. Most of the antioxidant agents used in previous studies for the treatment of BLM-induced lung fibrosis models have shown anti-inflammatory effects (Serrano-Mollar et al., 2003; Sriram et al., 2009). Furthermore, Nigella sativa seed extract and TQ prevented many toxic side effects of another anticancer drug; cisplatin when administrated concurrently in previous studies in rats (El Daly, 1998; Nagi and Mansour, 2000).

BLM is binds DNA and Fe$^{2+}$ and forms a complex. This DNA/Fe$^{2+}$/BLM complex undergoes redox cycling and generates ROS (Filderman et al., 1988). Accordingly, different markers of oxidative stress were investigated in the present study. It was found that BLM induced a significant elevation of lipid peroxides and NO accompanied with a significant decrease in the antioxidant enzyme SOD activity as compared to the control group. These changes were significantly ameliorated by TQ concurrent treatment. These results supported the antioxidant effect of TQ (Arslan et al., 2002).

The next step was the histological examination which showed excessive deposition of collagen in lung tissues and the concurrent treatment of TQ protected lung architecture from destruction.

CONCLUSION:

TQ supplementation causes histological and biochemical improvement on BLM induced lung injury. These effects may due to its antioxidative effect.

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دراسة فارماكولوجية للتأثير الوقائي الخاص بالثيموكيتون في نموذج الإصابة بالرئة

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الإجادات التأكسدية ينتج من عدم التوازن بين إنتاج أنواع الأكسجين التفاعلية وقدرة الجسم على إزالة السموم منها أو إصلاح الأضرار الناجمة عن ذلك. الأدلة الحالية تشير إلى أن الإصابة الحادة للرئة يمكن أن تؤدي إلى تليف رئوي.

التليف الرئوي هو التهاب رئوي قاتل مع ارتفاع نسبة الوفيات والعلاج الناجح محدود. وقد تم تصميم هذه الدراسة لتقييم التأثير الوقائي من الثيموكيتون وعما إذا كان يمكن أن يخفف من حدة الأكساء والاستجابة الالتهابية خلال التليف الرئوي الناجم عن بليوميسين. وقد تم حقن ذكور الجرذان بعقار بليوميسين بعد حقنهم صفيحة أيام بالثيموكيتون. البليوميسين أدى إلى زيادة كبيرة في مؤشرات الالتهابات، البروتينات الكلية والأيضية في غشاء القبضات الهوائية و أدى الثيموكيتون إلى التحسن بشكل كبير من هذه الأعراض. تسبب البليوميسين أيضاً في ارتفاع كبير في مؤشرات الدهون الموكسدة وأكسب التربكة مع انخفاض معبر عن نشاط انزيم مضادة للأكسدة بور أكسيد البليوميسين. العلاج بالثيموكيتون أدى إلى اقتراب هذه النتائج إلى القيم الطبيعية. وعلاوة على ذلك، فإن تفسير الآسية أدت التأثير الوقائي للثيموكيتون.

الخلاصة و الاستنتاج:

و بذلك يمكن استنتاج أن هذا العمل يعنى دليلاً على نشاط مادة الثيموكيتون مضادة لاصابة و تليف الرئة المحدث بعقار البليوميسين في الجرذان و يمكن ارجاع هذه الآثار إلى خصائصها مضادة للأكسدة.