Efficacy study of edaravone and acetylcysteine towards bleomycin-induced rat pulmonary fibrosis

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Received March 11, 2015; Accepted April 28, 2015; Epub June 15, 2015; Published June 30, 2015

Abstract: The aim of this study was to investigate the interventional effects of Edaravone (EDA) and Acetylcysteine (NAC) towards the Bleomycin (BLM)-induced pulmonary fibrosis. 48 Wistar rats were divided into the control group, the BLM group, the hormone group, the EDA group, the NAC group and the combination group. After performing the BLM intratracheal injection to prepare the pulmonary fibrosis model, the rats were administrated EDA, dexamethasone (DEX), NAC and EDA+NAC combined intervention, the lung HRCT examination was performed on the 7th, 21st and 31st day. On the 31st day, the rats were killed for the detection of serum malondialdehyde (MDA) and superoxide dismutase (SOD) contents; the lung tissues were performed the HE and Masson staining and determined the hydroxyproline content. The rats of the intervention group exhibited mild hypoxic phenomenon, with less ground-glass shadow and consolidated shadow than the BLM group, the MDA content decreased while the SOD content increased, and the degrees of alveolar inflammatory cell infiltration and fibrosis were low. The results of the EDA group and the NAC group were similar, and those of the combination group were better. EDA could inhibit the BLM-induced pulmonary fibrosis through adjusting the oxidant/antioxidant imbalance, with the effect similar to NAC, and the combined application of these 2 drugs were much more effective.

Keywords: Pulmonary fibrosis, bleomycin, acetylcysteine, edaravone

Introduction

The idiopathic pulmonary interstitial fibrosis (IPF) was a special type of chronic fibrotic interstitial pneumonia that was limited to lung, and its cause was still unknown so far, with only 2.8 to 4.2 median survival period after the diagnosis, and the 5-year survival rate was only 20% [1]. Unfortunately, though various drugs had been used in the treatment of IPF, except for pirfenidone, which had been confirmed the effects of delaying the deterioration of lung functions to a certain extent, improving the exercise endurance, prolonging the progression-free survival [2]. Thus obtained the permission in Japan, EU and China for the IPF treatment, and confirmed by the BIBF 1120 II clinical trial that it could delay the FVC declining, as well as the disease progress [3]. There was no medical evidence that could prove that any other kind of drugs could significantly reverse or change the course of IPF, thus improving the prognosis. Therefore, based on the understanding of the IPF pathogenesis, more effective and inexpensive drugs were urgently needed.

The basic cause of IPF was still unclear, but like many other chronic diseases, there was evidence that proved there existed the oxidant/antioxidant imbalance and ROS-product increasing in the IPF lung tissues [4], the clinical and animal studies strongly suggested that the oxidative stress might play a key role in the IPF pathogenesis. The levels of ROS, H$_2$O$_2$ and free radical products, which could mediate the lipid peroxidation, in the condensate of IPF patients’ exhaled gas were increased. On the other hand, the levels of anti-oxidants, such as glutathione, in the bronchoalveolar lavage fluid and epithelial lining fluid of IPF patients were decreased [5]. Also, in the BLM-, asbestos-induced lung fibrosis animal models, there also existed the phenomena of increased ROS level and decreased antioxidants, such as superoxide dismutase, and in the animal models. After the application of NAC, the overexpressions of phospholipids superoxide dismutase or extra-
Edaravone and acetylcysteine towards bleomycin-induced cellular superoxide dismutase could inhibit the above substances-induced lung inflammation and fibrosis [6]. Based on the above hypothesis of oxidant/antioxidant imbalance, IFIGENIA study applied the conventional immune suppression therapy towards 20 IPF patients, accompanied with the oral administration of NAC (600 mg, 3 times/day) for 12 weeks, the clinical symptoms and examination indexes had no changes. However, the Diffusion Capacity for Carbon Monoxide of the Lung (DLCO) was significantly increased [7], suggesting that the antioxidant therapy might exist a pivotal position in the treatment of pulmonary fibrosis.

EDA was a potent new oxygen free radical scavenger, it could eliminate the free radicals, reduce the free radical-induced inflammation waterfall cascade, prevent the lipid peroxidation, reduce the cellular oxidative damage, thus playing certain effects in such neurological diseases as cerebrovascular disease, cerebral hemorrhage and cerebral vascular dementia, as well as in the diseases of cardiovascular, digestive and endocrine systems [8]. Based on its important antioxidant effects, we believed that EDA could adjust the oxidant/antioxidant imbalance, and play the therapeutic role towards the pulmonary fibrosis. Therefore, in the present study, we observed the intervention of EDA and NAC towards the BLM-induced pulmonary fibrosis, meanwhile, aimed to evaluate its impacts towards the redox state, our findings suggested that both EDA and NAC could inhibit the BLM-induced MDA generation, increase the level of SOD, thus significantly inhibited the pulmonary fibrosis, and the combined application exhibited the synergetic effects, indicating that EDA might become a potential drug towards the pulmonary fibrosis treatment.

Materials and methods

Animal grouping and establishment of pulmonary fibrosis model

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experiment protocol was approved by the Animal Ethics Committee of the Affiliated Hospital, Medical College of Qingdao University. 48 males Wistar rats (Animal Center, Shandong Provincial Institute for food and Drug Control, Qingdao, weighed 200±10 g) were randomly divided into six groups: the control group, the BLM group, the hormone group, the EDA group, and NAC group and the combination group (NAC and EDA combined intervention). 10% chloral hydrate (Affiliated Hospital of Medical College, Qingdao University) was used to anesthetize the rats, the BLM A5 (Japan Ltd. 5 mg/kg, with the concentration as 4 mg/ml) was then intratracheally injected to produce the Wistar rat pulmonary fibrosis model. The rats in the control group used the same method, while with saline instead of BLM for the intratracheal injection. The BLM group was performed the intraperitoneal injection of dexamethasone (DEX, Zhengzhou Zhuoeng Pharmaceutical) 0.5 mg/kg and EDA (Nanjing Simcere Dongyuan Pharmaceutical Co., Ltd.) 6 mg/kg, together with the intragastric administration of saline, on the first day after the modeling, with the intragastric administration of saline, once/day; the hormone group and the EDA group were performed the intraperitoneal injection of dexamethasone (DEX, Zhengzhou Zhuoeng Pharmaceutical) 0.5 mg/kg and EDA (Nanjing Simcere Dongyuan Pharmaceutical Co., Ltd.) 6 mg/kg, together with the intragastric administration of saline, on the first day after the modeling, once/day. The NAC group (NAC, Zambon, Italy) was performed the intragastric administration of NAC 250 mg/kg on the first day after the modeling, together with the intraperitoneal injection of saline, once/day; the combination group was performed the intragastric administration of NAC 250 mg/kg and intraperitoneal injection of EDA 6 mg/kg on the first day after the modeling, once/day.

Lung imaging examination: the rats of each group were performed the lung CT scan on the 7th, 21st and 31st day after the modeling, respectively. The bone algorithm (high spatial frequency algorithm) and standard algorithm were used for the reconstruction. The window width was set as 1000 HU and the bed was set as 700 HU for the observation.

Specimen collection: after the 31st day lung CT examination, all the rats were killed and collected the specimens. The abdominal aortas and veins were taken for the simultaneous determination. The lung tissue was fixed in 10% neutral formalin, then stained according to the HE staining and Masson staining procedures, followed by the histopathological examination. The other part of lung tissue was placed in -70°C refrigerator for the HYP determined.
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Figure 1. CT on the 7th day. A: Hormone group; B: BLM group; C: Control group; D: combination group; E: EDA group; F: NAC group.
Scoring of pulmonary inflammation and fibrosis

After the HE staining and Masson staining, the rat lung sections were scored towards the degrees of alveolitis and fibrosis according to the Szapiel classification scheme [9].

Determination of lung tissue HYP, serum SOD and MDA

The HYP content in the preserved lung tissue was detected by the lye method, the SOD content was detected by the xanthine method, the MDA content was detected by the thiobarbituric acid (TBA) method, all the procedures were according to the kit instructions (Nanjing Jiansheng Bioengineering Institute).

Statistical analysis

The SPSS17.0 statistical software was sued to analyze the experimental results. All data were expressed as $\bar{x} \pm s$, and the intergroup difference was analyzed with the single factor analysis of variance and the homogeneity of variance test, with $P < 0.05$ considered as the statistical difference.

Results

Impacts of EDA towards the mortality of experimental rats

The chloral hydrate was used to anesthetize the rats, and during the anesthesia, 2, 1, 2, 2, 2 and 3 rats in the normal group, the BLM group, the hormone group, the EDA group, the NAC group and the combination group, respectively, died. During the experiment, 2 rats in the BLM group died of hypoxia, and 3 rats in the hormone group died of infection.

Impacts of EDA towards rat lung imaging experiment

On the post-modeling 7th, 21st and 31st day, the CT scanning was performed and the results were as follows: the 7th day CT imaging was shown in Figure 1, compared with the control group, the BLM group showed the ground glass shadow in partial regions, with minor consolidated shadow, which mainly focused in the both lower lungs. The hormone group, the EDA group and the NAC group exhibited different degrees of ground glass shadow, while the range was smaller than the BLM group. The combination group had no significant change in imaging; on the 21st day, it could be seen in Figure 2 that the pulmonary images of the BLM group exhibited the most serious fibrosis, the combination group and the control group exhibited the similar imaging results; the fibrotic degrees of the hormone group, the NAC group and the EDA group were less than the BLM group; the NAC group and the EDA group exhibited a little ground glass shadow, while there was no significant difference; the combination group showed no abnormalities. On the 31st day, it could be seen in the Figure 3 that the lung field of the control group could be fully displayed, the lung tissue exhibited the good expansion and good contrast, without high density shadow appeared; the BLM group exhibited the high density shadow, and the upper, middle and lower lungs exhibited the consolidated shadows. In the intervention group, the consolidated shadow inside the lung field was less than the BLM group, without the large area of consolidated shadow, and mainly as the ground-glass shadow. The images of the EDA group and the NAC group had no significant difference; the lung images of the combination group and the control group were similar.

The impacts of EDA towards lung tissue inflammation and fibrosis scoring

The HE staining revealed that the BLM group existed large range of inflammatory cell infiltration, as well as the maximum degree of alveolar structural damages; the degrees of the drug intervention groups were relatively lighter; and the combination group had the lightest degree (Figure 5). As for the alveolitis scoring, the BLM group had the severe inflammatory score, the EDA group, the NAC group and the hormone group were mild to moderate, respectively. The comparison among the EDA group, the NAC group and the hormone group revealed that the inflammatory cell infiltration were similar, while the control group and the combination group showed no significant infiltration. Compared with the EDA group, the NAC group and the hormone group, the degree of the combination group was significantly reduced, $P < 0.05$ (Table 1).

MASSON staining and fibrosis scoring

The BLM group exhibited the severe alveolar damage, the alveolar wall thickened, with loss
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Figure 2. Lung CT image on the 21st day. A: Control group; B: BLM group; C: Combination group; D: Hormone group; E: NAC group; F: EDA group.
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of partial alveolar structures, and severe fibrosis. The drug intervention groups exhibited the relatively intact alveolar structures, the alveolar wall was thicker than the control group, and the degree of fibrosis was mild. The combination group exhibited the minimum alveolar structural damage (Figure 4). The comparison of fibrosis scores among the groups revealed that there was no significant difference among the EDA group, the NAC group and the hormone group, the pulmonary fibrosis score of the combination groups was not significantly different from the control group, \( P > 0.05 \). Compared with the EDA group, the NAC group and the hormone group, the fibrosis degree of the combination group was significantly reduced, \( P < 0.05 \).

The impacts of EDA towards lung tissue HYP content: the HYP content of the BLM group was significantly higher than the control group and the other intervention groups, \( P < 0.05 \); and those of the EDA group, the hormone group and the NAC group were significantly lower than the BLM group, while significantly higher than the control group, \( P < 0.05 \). Compared with the hormone group, the HYP contents of the NAC group and the EDA group showed no significant difference, \( P > 0.05 \); the HYP content of the combination group was the lowest (Table 2).

Impacts of EDA towards serum MDA and SOD contents

The BLM group exhibited the highest MDA level, \( P < 0.05 \); compared with the hormone group, the EDA group and the NAC group exhibited the significant reduction of MDA content, \( P < 0.05 \). While there was no significant difference between these 2 groups, \( P > 0.05 \); the MDA content in the combined group was the least, \( P < 0.05 \). The SOD content of the BLM group was significantly lower than each treatment group and the control group, \( P < 0.01 \); compared with the hormone group, the SOD contents of the EDA group and the NAC group were significantly higher, \( P < 0.05 \).

Discussion

The previous studies had demonstrated that within various reasons-caused animals model of lung injury, the injection of EDA injection might lead to the alleviation of alveolar hemorrhage extent, the MDA content decreased,
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While the SOD activity increased; meanwhile, the lung injury would be significantly reduced [10-13]. The clinical studies had found that the addition of EDA into the conventional treatment towards the silicosis might increase the efficacy. Also, in the present study, we found that in the BLM-induced animal pulmonary fibrosis model, after the early intervention of EDA, the rats of the intervention group had the good mental state, and the initial imaging confirmed that the intervention group had the less ground glass shadow and consolidated shadow than the control group. The further serum detection revealed that that the MDA content of the EDA group was reduced, while the SOD activity was increased, the inflammatory cell infiltration and the HYP content were reduced, the pulmonary fibrosis degree was reduced, suggesting that EDA might play the anti-inflammatory and anti-fibrotic effects through regulating the oxidant/antioxidant imbalance.

The lung imaging had an important role for the diagnosis of pulmonary fibrosis, fibrosis degree and treatment evaluation. It was reported in the literature [14] that the HRCT imaging was used to diagnose the BLM-induced rabbit pulmonary fibrosis model, and the results were consistent with the radiographic study results of pulmonary fibrosis, the imaging could be used as an important means to evaluate whether the animal model was successfully prepared, as well as the degrees of pulmonary fibrosis. The imaging of rabbit pulmonary fibrosis mainly exhibited as: ground-glass shadow, nodule shadow, lung consolidation, partial honeycomb-like lung. In the rat fibrosis model, we also found the similar imaging appearance, with the ground-glass shadow as the main images in the early stage, while the consolidated shadow in the late stage. After the drug intervention, the images of different groups were improved by various degrees.

The study had confirmed that BLM could induce the production of a variety of oxidizing substances within the body [15], among which MDA was one of the important membrane lipid peroxidation products. The detection of MDA content could indirectly understand the extent

Figure 4. MASSON staining (x200). A: Control group; B: BLM group; C: EDA group; D: Hormone group; E: NAC group; F: Combination group.
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The previous studies had demonstrated that the antioxidants such as acetylcysteine, resveratrol, and the reductase such as exogenous superoxide dismutase could play a good role in protecting the lung injury diseases [16-18]. This study also found that after the BLM modeling, the SOD content was decreased, while the MDA content was increased, causing the oxidative damages, and leading to the pulmonary fibrosis. When administrated the reducing agents, either NAC or EDA, could reduce the serum MDA, while increase the SOD level, thus reducing the extent of pulmonary fibrosis accordingly. Under the similar anti-fibrotic effects, the SOD contents of the NAC group and the EDA group were higher than the hormone group, while the MDA content was lower than the hormone group, and the effects of combined NAC and EDA application were much better.

Table 1. Alveolar inflammation and fibrosis scores

<table>
<thead>
<tr>
<th>Group</th>
<th>Alveolitis</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>0.00±0.000</td>
<td>0.00±0.000</td>
</tr>
<tr>
<td>BLM group</td>
<td>2.80±0.447*</td>
<td>2.80±0.447*</td>
</tr>
<tr>
<td>Hormone group</td>
<td>1.25±0.957</td>
<td>2.00±0.816</td>
</tr>
<tr>
<td>EDA group</td>
<td>1.67±0.516Δ,▲,★</td>
<td>1.50±0.548Δ,▲,★</td>
</tr>
<tr>
<td>NAC group</td>
<td>1.50±0.548#,☆</td>
<td>1.50±0.548#,☆</td>
</tr>
<tr>
<td>Combination group</td>
<td>0.20±0.447○,●</td>
<td>0.20±0.447○,●</td>
</tr>
</tbody>
</table>

*Compared with other groups, P < 0.05; ΔCompared with the control group, P < 0.05; ▲Compared with the NAC group, P < 0.05; ★Compared with the hormone group P > 0.05; #Compared with the hormone group P > 0.05; ☆Compared with the normal group, P < 0.05; ○Compared with the intervention groups < 0.05.

Figure 5. HE staining. A: Normal group; B: model group; C: EDA group; D: Hormone group; E: NAC group; F: Combination group.

of membrane lipid peroxidation, reflect the production amount of free radicals. Meanwhile, BLM could also inhibit the activity of antioxidant enzymes, and SOD was one of the important reductases, which could scavenge the oxidation products such as oxygen free radicals and others. The compensation of exogenous antioxidant enzymes or antioxidants, as well as scavenging the oxygen free radicals, could possibly make the damages or injuries attenuated towards the lung tissues.
Table 2. Comparison of serum MDA and SOD contents (X ±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>MDA (nmol/ml)</th>
<th>SOD (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>6</td>
<td>2.3670.339</td>
<td>180.49±15.0</td>
</tr>
<tr>
<td>BLM group</td>
<td>5</td>
<td>4.985±0.498</td>
<td>3.364.62*</td>
</tr>
<tr>
<td>Hormone group</td>
<td>4</td>
<td>3.999±0.122</td>
<td>110.86±12.1</td>
</tr>
<tr>
<td>EDA group</td>
<td>6</td>
<td>2.817±0.808</td>
<td>△,▲,★</td>
</tr>
<tr>
<td>NAC group</td>
<td>6</td>
<td>3.158±0.217</td>
<td>☆</td>
</tr>
<tr>
<td>Combination</td>
<td>5</td>
<td>1.449±0.335</td>
<td>●,▲,★</td>
</tr>
</tbody>
</table>

*Compared with the other groups P < 0.05; △Compared with the control group, P < 0.05; ▲Compared with the NAC group, P < 0.05; ★Compared with the hormone group P < 0.05; ☆Compared with the hormone group P < 0.05; □Compared with the control group, P < 0.05; ●Compared with the normal group, P < 0.05; •Compared with other intervention groups P < 0.05.

The BLM intratracheal injection-induced pulmonary fibrosis was the easy and classical pulmonary fibrosis preparation method [19]. In our experiment, the single dropwise of BLM into the trachea successfully prepared the fibrosis model, and on the first post-modeling day, EDA, NAC, prednisone or the co-administration of the above 3 were performed. But the lung HRCT on the post-modeling 7th day indicated that except for the control group, the lung images of other groups showed the ground-glass shadow, suggesting it was still in the inflammatory phase, consistent with the previous studies which suggested that the BLM-induced fibrotic changes happened nearly on the 9th day of BLM administration [20]. While, in fact, when IPF was clinically diagnosed, it would have been in the fibrosis stage. Therefore, in the present study, we actually intervened the inflammatory stage before the fibrosis. If the intervention was performed after the fibrosis was formed, namely like most IPF patients clinically, administrated on or after the 9th day, whether EDA, NAC, prednisone, or the combination of the above 3 would still play the significant role in anti-fibrosis still needed the further study.

In summary, our study showed that EDA could adjust the oxidant/antioxidant imbalance, thus inhibit the BLM-induced rat pulmonary fibrosis, its efficacy was similar to NAC, while the combination of these 2 exhibited the much more pronounced anti-fibrosis effect. In the IPF therapy, its effects of antioxidant therapy were better than the hormone therapy, while the side effects were small, but whether EDA would exhibit the similar anti-fibrotic role in the IPF patients still needed the further clinical studies to confirm.

Disclosure of conflict of interest

None.

References


[4] Kinnula VL and Mylläriemi M. Oxidant-antioxidant imbalance as a potential contribu-
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