Antifibrosis effects of total glucosides of Danggui–Buxue–Tang in a rat model of bleomycin-induced pulmonary fibrosis

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Aim of the study: The present study examined the antifibrosis effects of DBTG (total glucosides of Danggui–Buxue–Tang) on bleomycin-induced pulmonary injury and fibrosis in rats.

Materials and methods: Animals were randomly divided into six groups: (1) saline control group; (2) Bleomycin group in which rats were endotracheally instillated with bleomycin (5 mg/kg); (3–5) Bleomycin and DBTG group, in which DBTG were given to rats daily (16.32 or 64 mg/kg/day, i.g.) one day after bleomycin injection for 4 weeks until the end of the treatment; (6) Bleomycin and positive control group. Animals were sacrificed at 7, 14, and 28 days post bleomycin administration and lungs were removed. Lung specimens were stained with hematoxylin and eosin (HE) and Masson trichrome for histological evaluation of lung injury and fibrosis by light microscopy. Body weight and lung index from various groups were measured, as well as TNF-α, TGF-β1 and type I collagen concentrations in lung homogenates.

Results: DBTG reduced bleomycin-induced weight loss, decreased the lung index and histological evidence supported the ability of DBTG to attenuate bleomycin-induced lung fibrosis and consolidation. DBTG could partly dose-dependently decrease TNF-α and TGF-β1 activity, as well as it decrease type I collagen expression in lung tissues.

Conclusions: The findings of the present study provide evidence that DBTG may serve as a novel target for potential therapeutic treatment of lung fibrosis.

1. Introduction

Pulmonary fibrosis is characterized by excessive synthesis and deposition of extracellular matrix (ECM) in the distal airspace, and is thought to be initiated by acute or chronic lung injury (Crouch, 1990; Thannickal et al., 2004; Garantziotis et al., 2004).

Bleomycin-induced pulmonary injury and pulmonary fibrosis has been documented in several animal species (Wang et al., 1991; Tzurel et al., 2002). This model has been widely used for studying the mechanisms involved in the progression of human pulmonary fibrosis and the impact of various drugs on this progression (Yara et al., 2001; El-Khatib, 2002).

Danggui–Buxue–Tang (DBT) is a traditional Chinese herbal formula which is a simple combination of two herbs. It was first described in Neiwaishang Bianhuo Lun by Li Dongyuan in China in AD 1247. Li described the DBT formula that included: 10 qian of Radix Astragali (RA), roots of Astragalus membranaceus (Fisch.) Bunge or Astragalus membranaceus (Fisch.) Bunge var. Mongholicus (Bunge) P.K. Hsiao, and two qian of Radix Angelicae Sinensis (Oliv.) Diels. A Qian was the weight unit in ancient China; one qian equals about 3 g. The mixed herbs were boiled in two bowls of water over a moderate heat until the final volume was reduced by half. Traditionally, DBT has been pre-
scribed to women in China as a remedy for menopausal symptoms. These women were directed to drink DBT daily to benefit vital energy raise and promote blood flow.

Pharmacological results indicated that DBT has the abilities to promote hematopoietic functions, stimulate cardiovascular circulation, prevent osteoporosis, increase anti-oxidation activity and stimulate the immune system (Chai et al., 2003; Wang et al., 2004). Although previous studies have demonstrated that the individual herb (RA or RAS) has protective effect on lung fibrosis rats. However, the rationale for having two herbs in DBT has never been explained; the direct effect of total glucosides of DBT on airway fibrosis, one of the features of airway remodeling, has not been assessed. It consequently hinders the development of multi-herb decoctions as disease or disorder remedies. Therefore, in the present study, we focused on the protective effect of DBTG on lung fibrosis rats and investigated its anti-fibrosis mechanisms.

2. Materials and methods

2.1. Animals

Adult male Wistar rats, weighing 180–220 g, 6–7 weeks old, were used in this study. They were obtained from the Experimental Animal Center of Anhui Medical University. All the animals were fed a standard rat chow and water ad libitum and kept in a temperature-controlled environment (20–22 °C) with an alternating cycle of 12 h light and dark. The animal experimental protocol was approved by the University Animal Care and Use Committee.

2.2. Extraction of DBTG and its content determination

The herbs used in this study were purchased from the pharmacy of The First Affiliated Hospital of Anhui Medical University, China, which had been identified by Prof. Wang De-qun (AnHui College of Traditional Chinese Medicine, HeFei, China) as *Astragalus membranaceus* and *Radix Angelicae Sinensis*, respectively. And they were qualified medicinal herbs. All voucher specimens (no. PAHMU: 20080416–20080417) were deposited in the herbarium of School of Pharmacy, Anhui Medical University.

100 portion Radix Astragali and 20 portion Radix Angelicae Sinensis were extracted, which have been added with ethanol of 8 times as great as the two, for three times and 1.5 h every time, all the extracted liquid were put together and filtered, the liquid were decompressed and concentrated (70 °C, vacuum – 0.08 Npa) to the degree at which 1 ml liquid equals 1 g original medical materials, then the liquid were diluted with water to the degree at which 4 ml liquid equals 1 g original medical materials, it was refrigerated and stored for 48 h, then it was filtered again, the liquid was ran through the macroporous resin column, the water-dissolvable impurity was eliminated by rinsing it with water, it was eluted with 80% ethanol, the remaining eluted liquid was collected, then it was filtered again, the ethanol was reclaimed, the liquid was concentrated and the smell of ethanol was eliminated, then 95% ethanol was added to get the liquid with 90% ethanol, it was refrigerated for 24 h, the liquid was filtered and the ethanol was recovered, and finally the liquid was decompressed and concentrated to dry and theuffy powder was collected (Gao et al., 2006, 2007).

The previous study has established a method for content determination of Astragaloside IV in total glucosides, and the HPLC-ELSD analytical method has been developed for the assay of the Astragaloside IV. Kromail C_18_ as the stationary phase of chromatography column and a mixture of acetoneitrile and water (37.5:62.5) as the mobile phase. The flow rate of mobile phase was 1.0 mL min⁻¹. The tube temperature of the detector was 100 °C, the flow rate of N₂ was 2.80 Lmin⁻¹. In addition to the detection of Astragaloside IV, the content of total glucosides in DBTG, was detected according to the references, with an average content of 86% (Gao et al., 2006; Zhao et al., 2007).

2.3. Reagents

The TGF-β₁ and type I collagen ELISA kits were Sigma products and TNF-a radioimmunoassay kit was purchased from Blood Graduate School of Medical College of Soochow University (MCSU) (Suzhou, China).

2.4. Model establishment and division

Rats were randomly divided into six groups (n = 30) as follows: control, model, DBTG (16, 32 or 64 mg/kg), positive control groups (Cortisone 3 mg/kg). Bleomycin at doses of 5 mg/kg body weight was reconstituted in sterile 75 mM NaCl solution and 0.3 mL vol was instilled intratracheally into rats. Control animals received sterile 75 mM NaCl. The drug was orally administrated, one day after bleomycin injection until the end of the experiment for 4 weeks. The drugs were dissolved in 0.5% carboxymethyl cellulose (CMC) and in the control group CMC was intragastrically injected. Animals were sacrificed at 7, 14, and 28 days post bleomycin administration and lungs were removed.

2.5. Preparation of lung tissue for biochemical studies

Rats were anaesthetized with chloral hydrate (i.p. 0.3 ml/100 g), sacrificed by abdominal aorta exsanguination, then the lung lobes were excised, washed in ice-cold saline, and then quickly frozen in liquid nitrogen before being stored at −20 °C. The frozen lung lobes of each animal were thawed and homogenized in isotonic saline. The homogenates were stored at −20 °C until assayed.

2.6. Histopathology studies

Lung specimens were fixed in 4% formaldehyde for 24 h, dehydrated in ethyl alcohol, and embedded in paraffin. They were stained with hematoxylin and eosin (HE) and Masson trichrome products and TNF-a radioimmunoassay kit was purchased from Blood Graduate School of Medical College of Soochow University (MCSU) (Suzhou, China).

2.7. Effect of DBTG on weight of lung fibrosis rats

The animals in different groups were weighed at the beginning, through and the end of experiments. The changes in body weight were determined.

2.8. Effect of DBTG on lung index

The lungs were removed, trimmed of extraneous tissue, rinsed and weighed lung index was determined by the equation:

\[
\text{lung index} = \frac{\text{lung weight}}{\text{body weight}} \times 100\%
\]
Fig. 1. Histopathological abnormalities in lungs on day 7, 14, 28 after BLM injection were showed in (a–c). Upper line showed the HE staining (A–D) and lower line showed the Masson trichrome staining (E–H) of lung issue. Magnification for all photomicrographs is 200×. A: control (HE staining); B: model (HE staining); C: DBTG (32 mg/kg) group (HE staining); D: cortisone (3 mg/kg) group (HE staining); E: control (Masson trichrome staining); F: model (Masson trichrome staining); G: DBTG (32 mg/kg) group (Masson trichrome staining); H: cortisone (3 mg/kg) group (Masson trichrome staining).
2.9. Effect of DBTG on TNF-α, TGF-β1 and type I collagen expressions in lung homogenates

TGF-β1 and type I collagen concentrations in lung homogenates from various groups were measured by ELISA kits. TNF-α level in the supernatants was measured by radioimmunoassay according to instruction.

2.10. Data analysis and statistics

All data were expressed as mean ± SD. All statistical analyses were performed by SPSS 10.01 software. One-way ANOVA analysis was used for assessing statistical significance between drug groups and various related control groups. The post Hoc Multiple Comparisons of ANOVA tests named the LSD type was then used. A \( P < 0.05 \) was considered to be significant.

3. Results

3.1. Effect of DBTG on BLM-induced histopathological abnormalities in lungs

Histopathological abnormalities in lungs here were detected on day 7, 14 and 28 using HE staining (A–D) and Masson’s trichrome staining (E–H) as shown in (Fig. 1(a)–(c)). Lungs of control rats showed normal alveolar spaces and normal thickening of alveolar septa (Fig. 1(a)–(c) A and E). The BLM treatment led to significant interstitial infiltration by inflammatory cells mainly lymphocytes and neutrophils (Fig. 1(a) B and F). The inflammatory reaction upregulated as early as on day 7 and reached a maximum on day 14 (Fig. 1(b) B and F). In addition, dense pulmonary fibrotic changes including thickening of the alveolar/bronchial walls, collapse of alveolar spaces, proliferation of fibroblasts, and deposition of ECM were strongly induced by bleomycin (Fig. 1(b) and (c) B and F). These changes began on day 7, became more severe and reached a maximum on day 28. On the other hand, rats treated with both BLM and DBTG showed marked suppression of the bleomycin-induced inflammatory cellular infiltration as evidenced by reduced thickening of the interalveolar septa and more inflation of the alveoli (Fig. 1(a)–(c) C). Furthermore, the amount of collagen deposited in the alveolar septa was markedly reduced in DBTG (32 mg/kg) group (Fig. 1(a)–(c) C). And rats treated with both BLM and Cortisone (3 mg/kg) showed similar appearances as those in DBTG (Fig. 1(a)–(c) D and H) group.

3.2. Effect of DBTG on body weight

Treatment of rats with bleomycin resulted in a marked decrease in their body weight as compared to the saline treated control group on 7, 14, 28 day (\( P < 0.01 \)). Administration of DBTG (16, 32, 64 mg/kg) and Cortisone (3 mg/kg) led to a significant increase in body weight as compared to the bleomycin group (\( P < 0.05 \) or \( P < 0.01 \)) (Fig. 2). On the other hand, as shown in Fig. 3, treatment of rats with bleomycin resulted in a marked increase in lung index as compared to control group on 7, 14, 28 day (\( P < 0.01 \)). DBTG (16, 32, 64 mg/kg) and Cortisone (3 mg/kg) administration led to a significant decrease in lung index compared with the bleomycin group \( P < 0.05 \) or \( P < 0.01 \).

3.3. Effect of DBTG on type I collagen concentration in lung homogenates

Time course of type I collagen expression level in lungs on day 7, 14 and 28 were assessed (Fig. 4). Type I collagen concentration was significantly upregulated as early as day 7 after BLM treatment, reached its maximal value at day 28. DBTG (16, 32, 64 mg/kg)-administration significantly inhibited the BLM-induced upregulation of type I collagen. Compared with model group, type I collagen release in Cortisone group was also evidently reduced (\( P < 0.01 \)).

3.4. Effect of DBTG on TNF-α and TGF-β1 concentrations in lung homogenates

As shown in Figs. 5 and 6, TNF-α and TGF-β1 concentrations in the BLM group were significantly increased compared with the saline-treated control group at different time point (\( P < 0.01 \)). TNF-α and TGF-β1 concentrations were significantly upregulated as early as on day 7 and reached a maximum on day 14. In comparison with the bleomycin-treated group, DBTG dose-dependently decreased TNF-α and TGF-β1 concentrations at the 7, 14 and 28 days points.
The identification of TNF-α as a key player in the pathogenesis of inflammation is supported by the effects of various therapeutic strategies aimed at blocking TNF-α activity. A variety of drugs used to treat human inflammatory diseases had effects on TNF-α (Tak and Firestein, 2001; Huang et al., 2006). It has been shown that during the process of bleomycin-induced pulmonary fibrosis several inflammatory responses are produced leading to an increase in the number of macrophages, which induce inflammatory cells in the injured tissue to produce cytokines (Phan and Kunkel, 1992; Kuroki et al., 2003; Huang et al., 2006, 2007, 2009). These cytokines have been linked to airway fibrosis because of their ability to regulate fibroblast and matrix production (Elías et al., 1999).

In the present study, co-administration of DBTG with bleomycin reduced bleomycin induced TNF-α elevation in lung. These results are in accordance with that of Underwood (Underwood et al., 2000), who showed that inhibition of inflammatory cytokines attenuated bleomycin-induced pulmonary fibrosis.

It is likely that dysregulations in the balance of some growth factors play major roles in determining the differences between normal and pathologic tissue repair. Among these, transforming growth factor TGF-β is one of the key cytokines involved in the pathogenesis of pulmonary fibrosis (Leask et al., 2004). Most publications investigating tissue fibrosis have focused on the most prominent isoform, TGF-β1, demonstrating an array of profibrotic functions. It is well established that TGF-β1 promotes differentiation of fibroblasts into activated myofibroblasts, enhances collagen synthesis, and reduces collagen degradation by downregulation of proteases and upregulation of protease inhibitors (Kelly et al., 2003).

There is a compelling need for improved strategies to limit the action of TGF-β1 in fibrotic diseases. In the present study, we detected the effect of DBTG on TGF-β1 expression in lung fibrosis rats. We found that co-administration of DBTG with bleomycin reduced bleomycin-induced elevation in lung TGF-β1 activity. One concern about targeting this molecule is that TGF-β1 has many essential roles, including immune regulation, cancer surveillance, and wound healing. With regard to the kidney, it has been proposed that TGF-β1 is part of a glomerular self-defense system that limits injury from inflammation. Therefore, anti-TGF-β1 therapies may have to be carefully tailored to be tissue-specific and cell-type-specific, and the duration of therapy carefully selected to suppress fibrosis without compromising normal defense and repair functions.

An interesting and potentially important aspect of the DBTG inhibition of both TNF-α and TGF-β1 expression is that the inhibition appears to only involve the increment induced by the bleomycin injury, and not baseline levels. This effect may be very important as we turn to trials of DBTG therapy of human diseases of excessive fibrosis and inflammation. For example anti-TNF-α antibodies, used in therapy of rheumatoid arthritis and Crohn’s disease, have been discovered to reactivate latent tuberculosis (Shanahan and St Clair, 2002), perhaps because they inhibit not only disease stimulated activity but the normal steady state activity. Similarly, excessive inhibition of TGF-β by antibodies, as exemplified by mice with mutations that knockout TGF-β expression (Wahl et al., 2000; Hahm et al., 2000), may increase inflammation as a side effect, even while inhibiting fibrosis. Use of DBTG in this situation, by not inhibiting normal levels of TGF-β1, might not evoke this reaction.

In conclusion, the data reported here reveal that DBTG can attenuate bleomycin-induced lung fibrosis and the mechanisms underlying this protective action may be attributed to either the decline in neutrophil trafficking and activation in lung via reduction of pro-inflammatory cytokines or the decrease of ECM deposition. These findings suggest that DBTG may be a promising candidate to prevent bleomycin-induced lung damage or other interstitial pul-

![Figure 5. Effects of DBTG on TNF-α concentration in lung homogenates. Data were expressed as mean of OD values ± SD of 10 separated rats in each groups. *P < 0.05, **ΔP < 0.01, compared with control group; *P < 0.05, **P < 0.01, compared with model group.](image)

![Figure 6. Effects of DBTG on TGF-β1 concentration in lung homogenates. Data were expressed as mean of OD values ± SD of 10 separated rats in each groups. *P < 0.05, **ΔP < 0.01, compared with control group; *P < 0.05, **P < 0.01, compared with model group.](image)

4. Discussion

The present study examined the effect of DBTG on bleomycin-induced pulmonary injury and fibrosis in rats. Histopathology changes revealed that bleomycin-induced lung injury typically consisted of two overlapping stages; an early inflammatory phase characterized by leukocyte infiltration and injury to alveolar epithelial cells, and a subsequent fibroproliferative phase with matrix remodeling and fibrosis. On the other hand, bleomycin-induced lung injury was evident biochemically characterized by increased TNF-α and TGF-β1 activity, augmentation of ECM including type I collagen expression in lung tissues, a significant weight index increase, which was in agreement with Brewer (Brewer et al., 2003). Administration of DBTG resulted in a significant reduction in the number of macrophages, which induce inflammatory cells in the injured tissue to produce cytokines (Phan and Kunkel, 1992; Kuroki et al., 2003; Huang et al., 2006, 2007, 2009). These cytokines have been linked to airway fibrosis because of their ability to regulate fibroblast and matrix production (Elías et al., 1999).

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monary fibrosis, which hints DBTG might be one of the major active constituent in antifibrosis effects of DBT. However, more detailed work is required to completely clarify detailed mechanisms of the antifibrosis effects of DBTG.

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