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Original Article

Therapeutic effects of quercetin on bleomycin induced pulmonary fibrosis in rats

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Abstract: The present study was performed to evaluate the role of quercetin in bleomycin (BLM)-induced pulmonary fibrosis (PF) in Sprague-Dawley rats. The rats after BLM administration received quercetin and were sacrificed on the days 12, 22 and 36 to measure the total protein content in bronchoalveolar lavage fluid (BALF). In addition, the hydroxyproline (HYP) content, malondialdehyde (MDA), the GSH/GSSG ratio and total anti-oxidative capability (T-AOC) were also evaluated. The results revealed that BLM treatment increased the BALF protein, HYP, MDA and T-AOC contents of the lung tissues and decreased GSH/GSSG ratio after 12 or 24 days. However, quercetin treatment for 24 days reversed the effect produced by BLM. In quercetin treated rats a marked reduction in HYP and total proteins was observed after 24 days. Quercetin prevented reduction in the expression of mRNA for MMP-1 and TIMP-1 at day 24 after treatment which was found to be reduced by BLM in untreated rats. Thus, quercetin exhibits a promising therapeutic effect in the prevention of PF.

Keywords: Pulmonary fibrosis, quercetin, malondialdehyde, bleomycin, antioxidative capability

Introduction

Pulmonary fibrosis (PF) is characterized by severe inflammation due to accumulation of macrophages, neutrophils and lymphocytes in the alveoli and the development of fibrous tissues [1]. It is believed that lung inflammation is induced by the reactive oxygen species, including hydrogen peroxide, peroxynitrite, and hydroxyl radical [2]. Reactive oxygen species generated by the phagocytes cause injury to the pulmonary tissues and inhibit their repair thereby producing PF [1, 3, 4]. The bleomycin (BLM) antibiotic used for the treatment of cancers induces oxidative damage to the DNA and produced PF [5]. It is reported that intra-tracheal administration of BLM into the lungs of various animal species damages alveolar cells and subsequent induces collagen deposition [5]. The BLM treated animal models show similar symptoms as are found in the patients with [6]. The BLM induced PF in rats has been treated by giving animals antioxidants like N-acetylcysteine (NAC) and bilirubin which reverse the effect of ROS [7, 8]. Reports have shown that during inflammatory condition in PF, matrix metalloproteinases (MMPs) along with their specific inhibitors, TIMPs (tissue inhibitor of metalloproteinases) are expressed in higher concentration [9, 10].

Quercetin, a representative member of flavonols comprises 60-75% of the flavonoid intake [11]. It possesses the ability of scavenging the free radicals and chelating the transition metal ions in order to prevent the oxidation of lipoproteins. Therefore, it is believed that quercetin can be a promising agent for the treatment of free radical induced diseases like cancer, chronic inflammation, etc. [12, 13]. Donation of proton of quercetin to radical converts it into a radical which is very low in energy because of resonance stabilization [14]. Our study was aimed to investigate the role of quercetin, an

anti-oxidant in an animal model of PF. The results revealed that quercetin caused a marked suppression of all the markers of lung damage at 20 days of BLM administration. Thus quercetin can be a potential inhibitor for ROS-mediated lung toxicity and PF.

Materials and methods

Chemicals and reagents

Quercetin and bleomycin A5 hydrochloride (BLM) were purchased from Tocris Bioscience (Boston Biochem) and Santa Cruz Biotechnology, Inc., (Dallas, TX, USA), respectively. The total antioxidative capacity (T-AOC) kit was obtained from Jiancheng Bioengineering institute (Nanjing, China) and glutathione (GSH) and other chemicals were purchased from Sigma (St. Louis, MO, USA).

Animals

The male Sprague-Dawley rats (18 week old) were obtained from Beijing Vital River Experimental Animal Technology Co., Ltd. The animals were housed in cages with free access to water and food. All the animal procedures were performed according to SIBS Guide for the Care and Use of Laboratory Animals approved by the Animal Care and Use Committee of the Beijing Institutes for Biological Sciences. The animals were assigned randomly to three groups with 20 each. One group (untreated) was given bleomycin hydrochloride (5 mg/kg body weight), second group (quercetin treated) received bleomycin hydrochloride and quercetin (3 mg/kg body weight) intraperitoneally and the third group (control) was given equal volume of saline. After 12, 24 and 36 days of treatment animals were sacrificed to extract lung tissues.

Histological assessment

The lungs from control, untreated and quercetin treated groups were extracted, fixed in 4% paraformaldehyde and embedded in paraffin on day 12, 24 and 36 following the treatment. The degree of fibrosis in lung sections was examined using haematoxylin and eosin and Masson's trichrome stain. For each lung section, 10 fields were selected and examined under a magnification of ×100. The normal lungs were then assigned 0 grade; alveolar or bronchial fibrous thickening of walls, grade 1; moderate thickening of walls, 2-3 grade; fibrosis with damage to lung architecture, 4-5 grade; severe structural distortion, 6-7 grade; and total fibrosis, 8 grades. The total fibrosis score of the lung section was taken as the mean of all the 10 fields.

Protein content in bronchoalveolar lavage fluid

The animals were sacrificed on the days 12, 24 and 36 and the right lung was washed thrice with saline using tracheal cannula. The bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) was used to determine the total protein concentration in cell-free BALF supernatants. The concentration of the proteins was then expressed as grams of protein per liter of BALF (g/L).

Hydroxyproline measurement

For determination of the hydroxyproline (HYP) content in the lung tissues homogenates was prepared and then hydrolyzed in hydrochloric acid at 110°C followed by addition of sodium hydroxide. The 1 mL aliquots were treated with citrate buffer followed by addition of 1.0 mL chloramines T and 1.0 mL perchloric acid. The samples were incubated for 20 minutes after the addition of dimethylaminobenzaldehyde (100 g/L). The absorbance was measured by a DU530 spectrophotometer (Beckman Instruments, Fullerton, CA) at 565 nm.

Analysis of lung tissue oxidants and antioxidants

The malondialdehyde (MDA) levels in the lung homogenates was quantified using commercially available kits according to the manufacturer's instructions (Nanjing Jiancheng Co Ltd). Results were normalized to the total amount of protein measured by Bradford protein assay (Beyotime Institute of Biotechnology). Reduced glutathione (GSH) was measured fluorometrically by mixing 100 µl aliquots with TCA followed by centrifugation for 20 minutes. To the supernatants 100 µL formaldehyde, 3.6 ml of 0.1 M phosphate buffer (pH 8.3, 5 mM EDTA), 200 µL 0.1% o-phthalaldehyde (OPT) were added and then incubated for 1 h. The mixture was subjected to fluorospectrophotometry at 549 nm. For determination of GSSG content 0.1 M NaOH was used instead of phosphate buffer. Total antioxidant capacity kit (Jiancheng Bioengineering Institute, Nanjing, P. R. China)

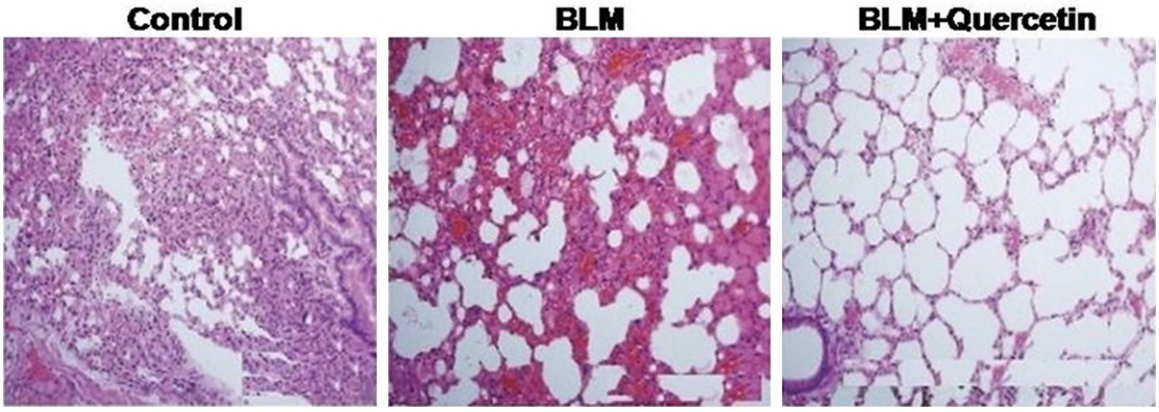

Figure 1. The photomicrographs show lung histology in the control, BLM and BLM+quercetin groups after 24 days of treatment.

Table 1. Grades of pulmonary fibrosis 24 days post-treatment

Groups	Grade of fibrosis
Control	0.95 ± 0.13
Untreated	6.04 ± 0.54
LA-treated	2.44 ± 0.39

was used to determine the antioxidant capacity of plasma and supernatant as per the manual protocol.

RNA preparation and RT-PCR of MMP-1 and TIMP-1

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA from tissue homogenates was isolated using a NucleoSpinRNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to measure the concentration of RNA by measuring the absorbance. AMV reverse transcriptase (Promega) and random primers (Takara Bio Inc., Shiga, Japan) were used to synthesize the complementary DNA.

Immunohistochemical detection of MMP-1 and TIMP-1

The paraffin embedded lung sections were cut into 2 μ thick sections, deparaffinized and exposed to 0.2% hydrogen peroxide in 0.1 M PBS for 45 min. The sections were treated with normal goat serum after PBS, washed again with PBS and incubated with primary antibodies. The primary antibodies used were anti-MMP-1, -TIMP-1, and a negative control antibody (Santa Cruz Biotechnology, Inc.). The sections were then washed again before incubation for 40 minutes with biotinylated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc.). Diaminobenzidine-hydrogen peroxide solution was used to develop the reaction and the sections were stained with haematoxylin and eosin.

Statistical analysis

Two way analysis of variance (ANOVA) was used for the statistical analysis. The results presented are the mean of mean ± SEM. The differences were considered statistically significant at $P < 0.05$.

Results

Histopathology of lungs

The effect of quercetin on BLM-induced pulmonary fibrosis was examined using light microscopy. In quercetin treated rats, lung tissues showed a marked decrease in edema, amount of inflammatory cells both in alveoli and interstitium along with reduced number of damaged endothelia and alveolar epithelial cells compared to that in untreated tissues after 12 days. After 36 days of BLM exposure, large fibrous regions, collapsed alveolar spaces, and traction bronchiectasis in the subpleural and peribronchial regions were clearly visible in untreated group (**Figure 1**). On the other hand, the control rats showed normal lung tissues. The fibrosis scores were significantly higher for untreated group compared to the quercetin

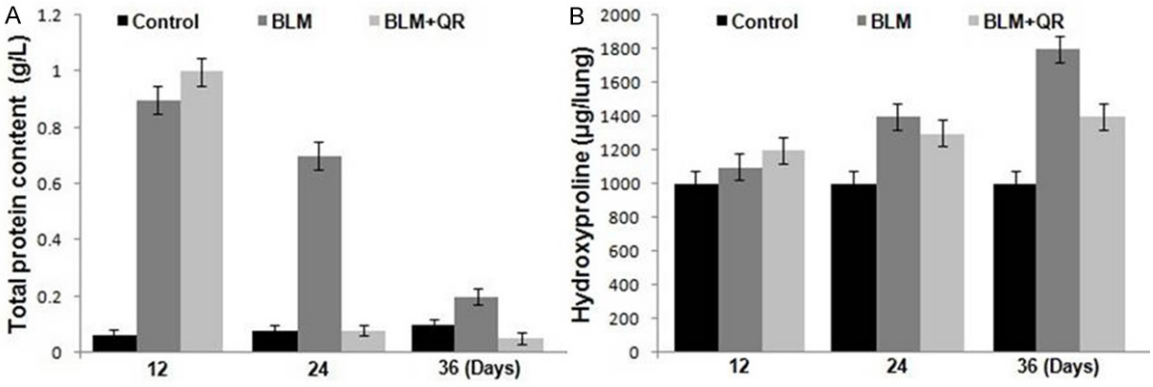

Figure 2. Quercetin suppressed the BLM-induced, (A) total proteins and (B) hydroxyproline contents.

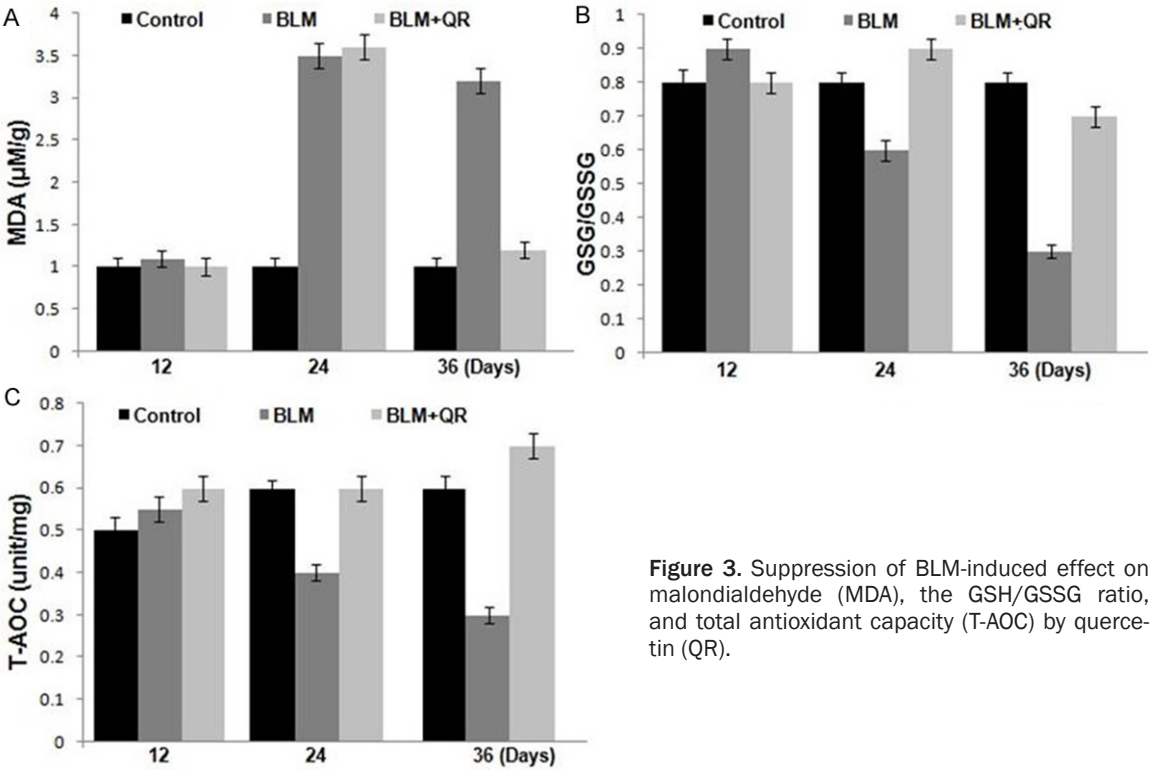

Figure 3. Suppression of BLM-induced effect on malondialdehyde (MDA), the GSH/GSSG ratio, and total antioxidant capacity (T-AOC) by quercetin (QR).

treated group of rats (**Table 1**). These findings suggest that quercetin markedly decreases the lung fibrosis in the BLM-treated rats after day 24.

BALF Protein and lung hydroxyproline contents

Quercetin treatment caused a significant decrease in the level of BALF protein after 24 days compared to the untreated rats. Although reduction in the level of BALF protein started significantly after day 24 and continued at day 36 following the quercetin treatment (**Figure 2A**).

Quantification of the lung HYP concentration revealed that its content decreased significantly by quercetin treatment after 36 days. The level of HYP was higher in untreated group compared to quercetin treated group of rats (**Figure 2B**).

Analysis of oxidative stress markers MDA content

Malondialdehyde (MDA) which is associated with oxidative stress has a vital role in the formation of lung fibrosis. Quercetin treatment exhibited an inhibitory effect on the MDA con-

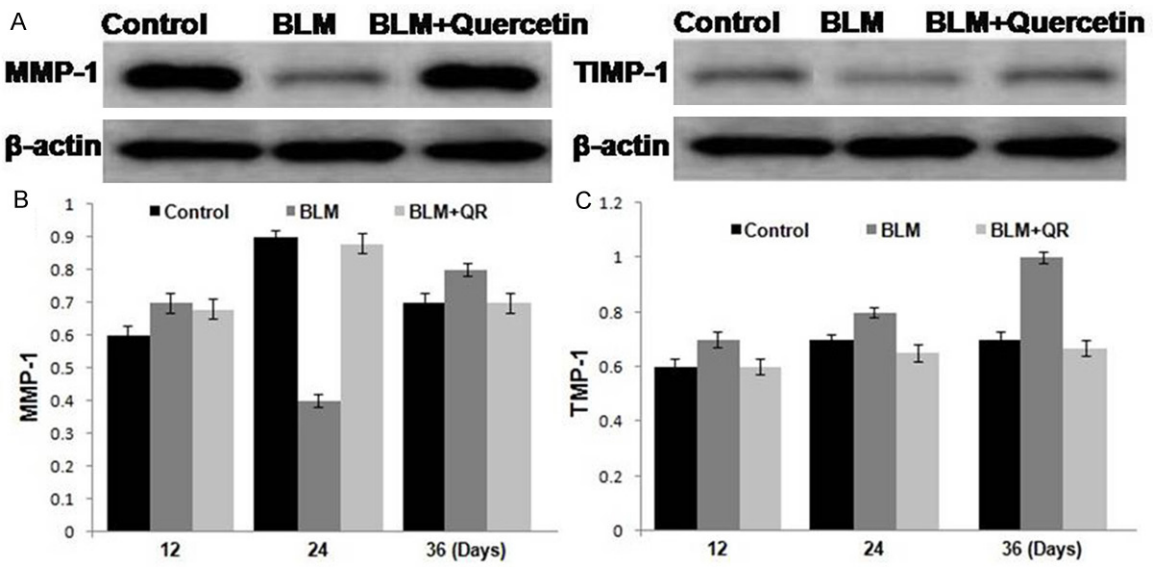

Figure 4. RT-PCR analysis of mRNA expression levels of MMP-1 and TIMP-1. A: Total RNA was isolated from the lung tissue of the control, BLM and BLM+quercetin groups for RT-PCR analysis at day 12, 24 and 36. B: The relative MMP-1 and TIMP-1 mRNA levels, at day 12, 24 and 36 in each group as indicated were estimated using densitometry and were normalized to control β-actin. C: The relative TIMP-1 mRNA levels, at day 12, 24 and 36 in each group as indicated were estimated using densitometry and were normalized to control β-actin.

tent. The lung tissues of untreated rats showed significantly higher MDA content on the day 24 after BLM treatment. However, the content of MDA was found to be markedly lower in the rats treated with quercetin after day 36 (**Figure 3A**). Thus quercetin treatment caused a decrease in the oxidative stress produced by BLM in the rats after day 36. Furthermore, quercetin treatment markedly increased the content of GSH (reduced glutathione) and subsequently decreased the GSSG (oxidized glutathione) in lung tissues after day 24 compared to untreated rats (**Figure 3B**). In addition, quercetin treatment caused a marked enhancement in the total antioxidant capacity (T-AOC) of lung tissues compared to untreated group after day 24 (**Figure 3C**).

MMP-1 and TIMP-1 expression

We also investigated the effect of quercetin treatment on mRNA for metalloproteinases (MMPs) and TIMP-1 by the RT-PCR method. The results showed that quercetin treatment prevented the decrease in the expression of mRNA for MMP-1 and TIMP-1 at day 24 after BLM treatment (**Figure 4**). MMP-1 protein level was decreased by BLM treatment in the rats at day 12 of the treatment. However, quercetin treatment prevented reduction in the MMP-1 level after day 12 and the level was found to be comparable to that of control group of rats.

Discussion

Our study demonstrates the role of quercetin, a flavonol in the prevention of PF in an animal model of BLM-induced PF. Quercetin possesses the ability of scavenging the free radicals and chelating the transition metal ions in order to prevent the oxidation of lipoproteins. Donation of proton of quercetin to radical converts it into a radical which is very low in energy because of resonance stabilization [15]. Our results showed that quercetin decreases lung injury in the rats administered BLM intratracheally. Intraperitoneal administration of quercetin also resulted in reduction of collagen accumulation and enhanced the MMP-1/TIMP-1 ratio.

There are reports that generation of ROS in BLM treated rats cause breakage of DNA chains and oxidation of the cell membrane lipids [16, 17]. The effect of quercetin on PF in BLM treated rats was assessed by investigating malondialdehyde (MDA), the ratio of GSH/GSSG and total antioxidant capacity (T-AOC). It was observed that MDA, a key marker of lipid peroxidation, was increased in BLM-treated rats. However, quercetin treatment significantly reduced the MDA content on day 20 of the

treatment. The content of reduced glutathione (GSH) which plays an important role in protecting the lung cells from oxidative damage [18] was increased in BLM treated rats. Quercetin treatment enhanced the content of GSH on the day 20 following the treatment. The ratio of GSH/GSSG was significantly higher and comparable to control group in quercetin treated rats compared to untreated group after 20 days. In untreated rats the level of T-AOC was markedly lower compared to control group. However, in quercetin treated rats the level of T-AOC was increased and became comparable to that of control group. Therefore, quercetin administration reversed the effect of BLM on MDA content and GSH/GSSG ratio.

It is reported that antioxidants exhibit a protective effect in BLM induced PF in the animal models [19-22]. There is a disturbance in the equilibrium between the formation and decomposition of extracellular matrix (ECM) molecules during the pathogenesis of PF. The decrease of BLM-induced PF in mice by MMP-inhibitor batimastat suggests that MMPs play a vital role in PF [23]. A reduced molar MMP/TIMP ratio is considered to be a hallmark of PF. In humans, increased levels of TIMP mRNA and protein are observed in lungs of patients with IPF, and TIMP expression there exceeds that of MMP. TIMP-1 expression and protein were also increased by BLM administration [15-23]. Our results revealed that both MMP-1 mRNA and protein were increased in BLM treated rats. However, in quercetin treated group MMP-1 mRNA and protein were decreased at day 20. On the other hand, BLM exposure led to a significant increase in TIMP-1 at all time-points, while quercetin lowered its expression at day 20.

In conclusion, quercetin acts as an effective inhibitor of BLM-induced PF and may have promise in the prevention of BLM-induced oxidative lung damage and human PF.

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Disclosure of conflict of interest

None.

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