Protective Effect of Genistein on Lipopolysaccharide-induced Acute Lung Injury in Rats

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Summary: To investigate the protective effect of genistein on endotoxin-induced acute lung injury in rats, and explore the underlying mechanisms, 32 male Sprague-Dawley rats were randomly divided into 4 experimental groups: saline control, genistein alone, lipopolysaccaride alone, and genistein pretreatment. Each treatment group consisted of eight animals. Animals were observed for 6 h after LPS challenge, and the wet/dry (W/D) weight ratio of the lung and bronchoalveolar lavage fluid (BALF) protein content were used as a measure of lung injury. Neutrophil recruitment and activation were evaluated by BALF cellularity and myeloperoxidase (MPO) activity. RT-PCR analysis was performed in lung tissue to assess gene expression of ICAM-1. The histopathological changes were also observed using the HE staining of lung tissue. Our results showed that lung injury parameters, including the wet/dry weight ratio and protein content in BALF, were significantly higher in the LPS alone group than in the saline control group ($P<0.01$). In the LPS alone group, a larger number of neutrophils and greater MPO activity in cell-free BAL and lung homogenates were observed when compared with the saline control group ($P<0.01$). There was a significant increase in lung ICAM-1 mRNA in response to LPS challenge ($P<0.01$, group L versus group S). Genistein pretreatment significantly attenuated LPS-induced changes in these indices. LPS caused extensive lung damage, which was also lessened after genistein pretreatment. All above-mentioned parameters in the genistein alone group were not significantly different from those of the saline control group. It is concluded that genistein pretreatment attenuated LPS-induced lung injury in rats. This beneficial effect of genistein may involves, in part, an inhibition of neutrophilic recruitment and activity, possibly through an inhibition of lung ICAM-1 expression.

Key words: acute lung injury; lipopolysaccharide; neutrophils; ICAM-1

Acute lung injury (ALI) is a syndrome characterized by increased alveolar-capillary permeability and hypoxemia. Both ALI and its most severe form, the acute respiratory distress syndrome (ARDS), are associated with an extensive and heterogeneous list of pulmonary and extrapulmonary insults. What these inciting factors have in common is the ability to elicit an acute inflammatory reaction in the lung, which culminates in parenchymal injury[1]. Despite advances in supportive care, the mortality rate of patients with ARDS remains high, frequently in excess of 50 %[2]. Genistein is a naturally plant-derived oestrogen-like compound. Recently, it has been shown to possess an anti-inflammatory propertiy[3]. In this study, experiments were designed to evaluate the effects of genistein on acute lung injury induced by intravenously administered LPS in rats and to explore its underlying mechanism.

1 MATERIALS AND METHODS

1.1 Animal Experimental Protocol

Thirty-two Sprague-Dawley rats (male, 190 g −210 g), supplied by Center of Experimental Animals of Tongji Medical College, China, were randomly divided into 4 experimental groups: (1) saline control, (2) genistein alone group, (3) lipopolysaccaride (LPS) alone group, and (4) genistein + LPS group. The saline control, genistein alone, and LPS-alone groups were injected with 0.5 mL of saline (iv), 50 mg/kg genistein (Sigma, USA) in 0.5 mL of saline (ip), and 5 mg/kg LPS (*E. Coli*, 055:B5, Sigma, USA) in 0.5 mL of saline (iv), respectively. The genistein + LPS group received a 50 mg/kg bolus of genistein 2 h before the injection of 5 mg/kg of LPS. For injection, rats were treated with soluble pentobarbitone anesthesia. Each treatment group consisted of 8 animals. Animals were sacrificed with sodium pentobarbitone (200 mg/kg, ip) 6h after saline treatment or LPS challenge.

1.2 Bronchoalveolar Lavage (BAL)

Six h after saline or LPS challenge, animals were euthanized, the trachea was cannulated, the thoracic cavity was opened, and the light helium was ligated. BAL was performed on the right lung. The lung was lavaged with ice-cold saline (5 mL/kg) delivered through the tracheal cannula and removed after a 30-s interval. This procedure was done three times and samples were then pooled for each animal. BAL fluid was centrifuged at 500 g for 5 min at 4 ℃, and the supernatants were stored

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at −20 ℃. The total cell count and the percentage of neutrophils were measured under a light microscope. The protein content of the supernatant was assayed according to the method of Lorry *et al*.

1.3 Lung Wet-to-weight (W/D) Ratio

The left upper lobe was removed after the experiment, then weighted (wet weight) and dried in a 70 ℃ oven until weight is constant (dry weight). The rate of wet/dry weight of the lung was calculated.

1.4 Histopathological Examination

Immediately after the rats were killed, a piece of lung tissue taken from the left lower lobe was sampled, fixed in 10 % formaldehyde, embedded in paraffin and sectioned. Sections were stained with hematoxylin-eosin and examined under light microscope.

1.5 Neutrophil Myoloperoxidase (MPO) Assay

MPO activities in cell-free BAL fluid and lung homogenates were determined according to the method described by Luo and Guo[11].

1.6 Lung IACM-1 mRNA Expression

Total cellular RNA from rat lung recovered 6 h after the challenge was isolated according to the instructions of the total RNA extracting kit (Promega, USA). First strand cDNA was synthesized using reverse transcriptase with oligo (dT). Total RNA was reversely transcribed (RT) for each sample. Specific primers were designed for PCR: IACM-1, sense: 5′-cagggtgctttcctcaaaag-3′, antisense: 5′-gggcatgagactccattgtt-3′; β-actin, sense: 5′-acccagatcatgtttgagacct-3′, antisense: 5′-ggatgtcaacgtcacacttcat-3′. The expected sizes of amplication was 249 bp for IACM-1 and 500 bp for β-actin. After an initial denaturation at 94 ℃ for 5 min, amplification was conducted through 28 cycles of denaturation at 94 ℃ for 30 s, annealing at 64 ℃ for 45 s and extension at 72 ℃ for 50 s. Final extension was at 72 ℃ for 7 min followed by a final hold at 4 ℃.

The PCR products were separated by electrophoresis using 1.5 % agarose gels stained with ehtidium bromide. The optical density (OD) for each band were quantified by molecular image system (Bio-Rad, USA). The level of gene expression of the transcript was normalized to that of the housekeeping gene β-actin.

1.7 Statistical Analysis

Values were expressed as $\bar{x}\pm s$ of n independent observations. Statistical comparisons were made using one-way ANOVA. All statistical analyses were performed by using SPSS for windows version 11.0 (SPSS Inc., USA). A P value <0.05 was considered to be statistically significant.

2 RESULTS

2.1 Lung W/D-weight Ratio and BAL Protein Content

The W/D-weight ratio and BAL protein content were significantly higher in the LPS alone ($P<0.01$) and LPS + genistein groups ($P<0.05$) than those in the saline control group. Pretreatment with genistein significantly inhibited LPS-induced the W/D-weight ratio and BAL protein content by 71 % and 18 %, respectively (table 1).

Table 1 Changes in the W/D-weight ratio and BAL protein content ($\bar{x}\pm s$)

Groups	Lung W/D ratio	BAL protein content (g/L)
Saline control	4.12±0.15	0.17±0.02
Genistein alone	4.13±0.16	0.18±0.03
LPS alone	5.53±0.23**	0.82±0.04**
LPS+genistein	4.51±0.41*▲▲	0.23±0.03*▲▲

* $P<0.05$ and ** $P<0.01$ versus the saline group; ▲▲ $P<0.01$ versus the LPS alone group

2.2 MPO Activities and Neutrophils in BAL Fluid

The number of neutrophils in the BAL fluid and MPO activities in lung homogenates and cell-free BAL were significantly higher in the LPS alone group than those in the saline control group ($P<0.01$). Pretreatment with genistein significantly lowered LPS-induced increases in these variables (table 2).

Table 2 Changes in the number of neutrophils in the BAL fluid and MPO activities in lung homogenates and cell-free BAL ($\bar{x}\pm s$)

Groups	Neutrophils (10^9/L)	MPO activity in lung homogenates (U/mL)	MPO activity in cell-free BAL (U/g)
Saline control	2.97±0.58	1.410±0.018	1.232±0.411
Genistein alone	3.11±0.63	1.511±0.020	1.107±0.372
LPS alone	9.52±0.90*	11.200±0.023*	15.123±0.509*
Genistein + LPS	6.50±0.73*▲	5.314±0.016*▲	5.661±0.476*▲

* $P<0.01$ versus the saline group; ▲ $P<0.01$ versus the LPS alone group

2.3 Lung ICAM-1 Expression

There is a constitutive expression of IACM-1 mRNA in rat lungs, which was not significantly changed by ginistein alone. A significant increase in lung ICAM-1 expression was observed in LPS alone group ($P<0.01$ as compared with saline control). Pretreatment with genistein was able to significantly inhibited the increase in IACM-1 mRNA

expression induced by LPS (fig. 1).

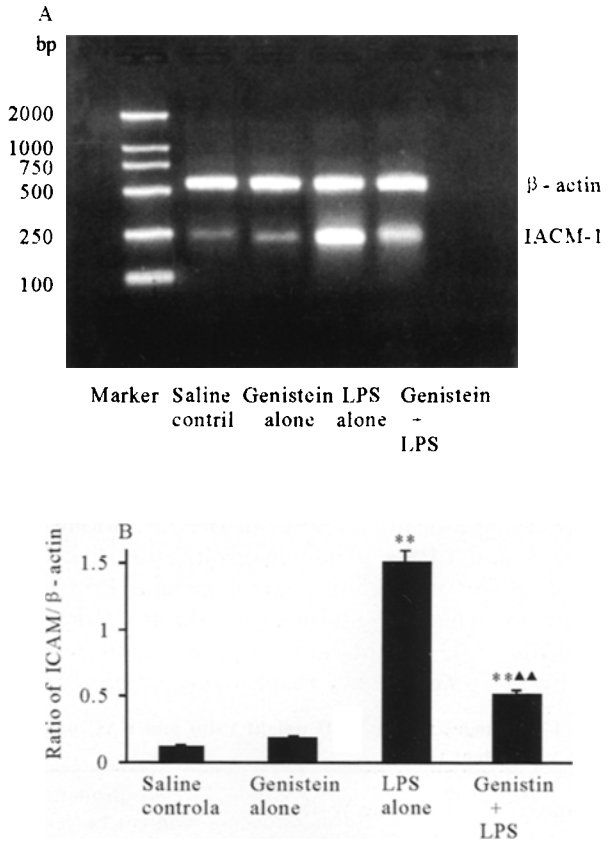

Fig. 1 Gel electrophoresis of cytokine specific mRNA for IACM

2.4 Histopathological Fingdings

Light microscopic examination showed that the morphology of lung tissues was basically normal in the saline group (fig. 2A), similar to that in the genistein alone group, and that edema, hemorrhage, increased thickness of alveolar wall, and infiltration of inflammatory cells into lung tissues were observed in the LPS alone group (fig. 2B). These changes caused by LPS were less pronounced in the LPS + genistein group (fig. 2C).

3 DISCUSSION

Genistein, the principal isoflavone found in soy is structurally similar to 17β estradiol and diethylbestrol, binds to oestrogen receptors and exhibits oestrogenic properties in some tissue. It has been shown to reduce the inflammatory response, thereby causing a marked cardioprotection in myocardial ischemia with reperfusion[3].

In this study, LPS induced acute lung injury as assessed by the W/D ratio, albumin levels in the BALF and the pathological changes 6 h after LPS challenge. Pretreatment with genistein significantly not only attenuated the LPS-induced increases in the biological makers of the lung injury, but also lessened the extensive lung damage caused by LPS. These data demonstrated that genistein provided a significant protection against LPS-induced acute lung injury.

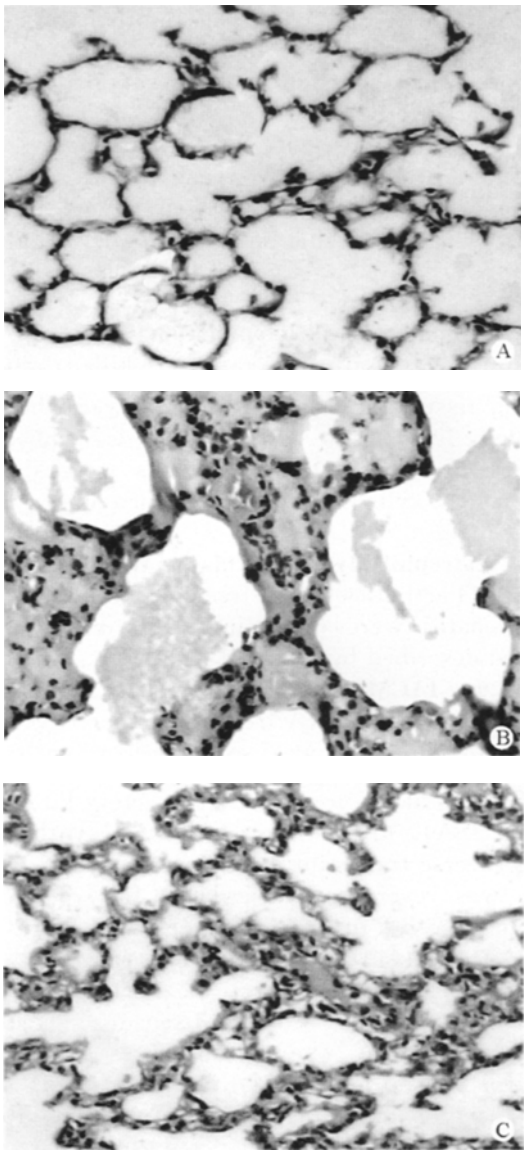

Fig. 2 Histological section of rat lung (A) saline control group; (B) LPS alone group; (C). LPS + genistein group (HE×200)

To explore the underlying mechanisms by which genistein achieves its beneficial effects, we investigated its effects on neutrophil influx and activation. Neutrophils are considered to be central to the pathogenesis of most forms of acute lung injury (ALI)[4]. LPS has been reported to prime neutrophils. The activated neutrophils recruited into vascular, interstitial, and alveolar spaces are thought to damage the delicate structure of the lung by generating reactive oxygen species and releasing proteolytic enzymes. It has previously been demonstrated that MPO activity is directly related to neutrophil number and can be used as a neutrophil marker. Therefore, we used the MPO assay to assess the extent of neutrophil recruitment in lung tissue. LPS injection induced a marked increase in MPO content in lung homogenates, which was significantly reduced by genistein pretreatment, consistent with the BAL neutrophil deferential cell

count. MPO activity in cell-free BAL is also quantified as an index of neutrophil degranulation and activation[5]. LPS-induced MPO activity in cell-free BAL was significantly inhibited by genistein. Our data suggest that the inhibitory effect of genistein on acute lung injury might be, at least in part, due to the inhibition of neutrophil recruitment and activation in the lung.

IACM-1 is an adhesion molecule on the cellular surface and a member of immunoglobulin superfamily. Its ligands are lymphocyte function related antigen-1 (LFA-1) (CD11a/CD18) and macrophage antigen complex-1 (MAC-1) (CD11b/CD18) that are members of integrins and may express on the surface of lymphocytes and PMNs. In an inflammatory response, IACM-1 plays a central role in the course of cells getting into contact cells. ICAM-1 has previously been shown to be a requirement for neutrophil recruitment after LPS challenge with peak levels of expression associated with maximum leukocyte adherence. Studies have shown protective effects of anti-IACM-1 mAbs in models of acute lung injury, which was attributed to inhibition of PMN sequestration in the lungs[6,7]. In agreement with published data, we found that lung IACM-1 expression was up-regulated by LPS exposure. Pretreatment with genistein significantly diminished IACM-1 mRNA expression. Therefore, it is possible that reduced IACM-1 expression by genistein may have contributed to impaired neutrophil recruitment. However, we cannot preclude any additional effects that genistein may have on PMN-endothelial cell interactions apart from the effect on IACM-1 expression as demonstrated in the present studies. Indeed, genistein may have additional effects on other endothelial cell adhesion molecules involved in lung leukocyte sequestration, and these possibilities require further investigations.

In summary, the present findings suggest that genistein has a prophylactic effect on endotoxin-induced acute lung injury, and that this effect of genistein is related to an inhibition of neutrophil influx and activation, in part, through an inhibition of lung IACM-1 expression. Genistein has a very low toxicity in animal studies[8]. Therefore, we propose that genistein may have potential as a treatment in the early stages of lung injury.

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