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Ellagic Acid Protects Against LPS-Induced Acute Lung Injury Through Inhibition of Nuclear Factor Kappa B, Proinflammatory Cytokines and Enhancement of Interleukin-10

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ABSTRACT

Ellagic acid is a naturally occurring polyphenolic compound which is found in many fruits, nut galls and plant extracts. In the present study, we explored the ability of ellagic acid to modulate LPS response using macrophage-mediated inflammatory conditions and acute lung injury (ALI).

Methods and Results: First, we found that ellagic acid reduced tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and interleukin-1 β (IL-1 β) secretions, enhance interleukin-6 (IL-10) production by lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages *in vitro*. These results inspired us to further study the effects of ellagic acid *in vivo*. In murine ALI model, mice were treated with ellagic acid prior to LPS challenge. The data showed that ellagic acid possess a protective effect on LPS-induced ALI in mice. In addition, We further investigated how ellagic acid worked on signal transduction by western-blot and immunocytochemical analysis. The data showed that ellagic acid efficiently blunted nuclear factor-kappa B (NF- κ B) activation by inhibiting the degradation and phosphorylation of I κ B α , as well as the translocation of p65 from cytoplasm to the nucleus.

Conclusions: These results indicate that ellagic acid could be highly effective in inhibiting ALI, and underlying mechanism may be through shocking the NF- κ B pathway to attenuate the nonspecific pulmonary inflammation induced by LPS administration.

INTRODUCTION

Acute lung injury (ALI) or its more severe form, acute respiratory distress syndrome (ARDS) is an acute life-threatening form of hypoxemic respiratory failure, characterized by lung tissue edema and injury, inflammatory responses, following macrophage activation, increased vascular permeability, parenchymal injury and impaired gas exchange. During the past several decades, the incidence of ALI and ARDS in the USA has been reported to be 79 and 59 per 100,000 people per year, respectively [1-5]. Based on the exponential growth of the population, the incidence will likely double in the next 25 years [4]. The highest incidence of ALI is seen during sepsis, with approximately 25% of all ARDS cases stemming from severe sepsis and 7% of intensive care patients eventually developing ALI/ARDS [6,7]. The mechanisms of ALI are gradually becoming clear. LPS endotoxin is the most important pathogen that leads to the development of ALI/ARDS and multiple organ dysfunction syndromes. During the process,

LPS-activated inflammatory cells could produce a large number of mediators in the early stage of the disease, resulting in injury to both the vascular endothelium and the alveolar epithelium [8,9]. Injury to the alveolar capillary barrier increases permeability of the alveolar wall, increases accumulation of inflammatory cells and cytokines, and impairs alveolar fluid clearance, resulting in accumulation of protein-rich edema in the alveolar space and lung tissue injury [9]. Despite recent evidence-based advances in clinical management and extensive investigations into new strategies for treatment, ALI/ARDS is still associated with significant mortality. At present, popular methods to deal with LPS induced inflammation and its associated symptoms involve the use of steroidal or non-steroidal anti-inflammatory drugs, however the use of these drugs are associated with severe side effects. Therefore, investigations concerned with natural methods of inflammatory control are warranted. Overproduction proinflammatory cytokines is one of important reasons of ALI/ARDS. So, inhibitors of these proinflammatory molecules may become a candidate way to reduce mortality of ALI/ARDS.

Ellagic acid, the structure as shown in **Figure 1**, is a naturally occurring polyphenolic compound which is found in many fruits, nut galls and plant extracts such as raspberries, strawberries, grapes, pomegranate, black currants, camu-camu, mango, guava, walnuts, almonds, longan seeds and green tea [10-12]. Ellagic acid has been reported to have a wide range of biological effects, mainly including ant carcinogenic [13], antibacterial [14], and antiviral [15], anti-inflammatory [16], anti-anxiety [17] and neuroprotective [18]. However, little study has studied regulatory effects of Ellagic acid on LPS response. The aim of the present study was to investigate effects of Ellagic acid on LPS induced ALI and associated mechanisms. Our results would be beneficial for Ellagic acid to be further used as a potential treatment choice of ALI.

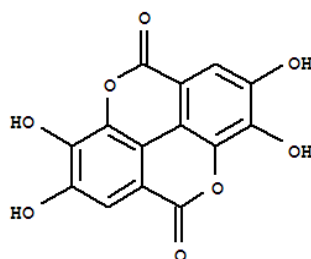


Figure 1. The structure of Ellagic acid.

MATERIALS AND METHODS

Materials

Ellagic acid (purity >98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICBP, China). LPS (*Escherichia coli* O55:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dexamethasone (DEX) was purchased from Changle Pharmaceutical Co. (Xinxiang, Henan, China). Mouse myeloperoxidase (MPO) ELISA kits were purchased from USCN Life Co. (Missouri City, TX). TNF- α , IL-1 β , IL-6 and IL-10 ELISA kits were purchased from Biolegend. Dulbeccos modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY). Phospho-specific antibodies for I κ B as well as antibodies against I κ B and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cy3-conjugated sheep anti-rabbit IgG, Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H + L) were purchased from PTG (Chicago, IL, USA).

Experimental Animals

Balb/c male mice weighing 18-22 g were purchased from Jilin University Experimental Animal Center and acclimatized for 2 week before use. Rodent laboratory chow and tap water were provided ad libitum and maintained under controlled conditions with a temperature of $24 \pm 1^\circ\text{C}$, humidity of 40-80%, and a 12-h light/12-h dark cycle. All of the procedures were in strict accordance with the guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Cell Culture

The RAW 264.7 murine macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS at 37°C under a humidified atmosphere of 5% CO_2 .

Cytotoxicity Assay

Cytotoxicity studies induced by ellagic acid were performed by MTT assay. RAW 264.7 cells were mechanically scraped, plated at a density of 4×10^5 cells/mL onto 96-well plates (Costar USA) containing 100 μL of DMEM medium. After overnight incubation, the cells were treated with various concentrations of ellagic acid (0-10 $\mu\text{g}/\text{mL}$) according to the experimental design. After 20 h, 20 μL of MTT was added to each well and the cells were further incubated for 4 h at 37°C with 5% CO_2 . MTT was removed and cells were lysed with 150 $\mu\text{L}/\text{well}$ DMSO. The optical density (OD) values were measured at 570 nm on a microplate reader (TECAN, Austria).

Cytokine assays *in vitro*

To determine the effects of ellagic acid on cytokine responses from LPS-induced cells, RAW 264.7 cells were plated onto 24-well plates (4×10^5 cells/mL), and incubated in the presence of either 1 $\mu\text{g}/\text{mL}$ LPS alone, or LPS plus ellagic acid 1, 2, 4 $\mu\text{g}/\text{mL}$ for 24 h at 37 °C with 5% CO_2 . Cell-free supernatants were collected and stored at -20 °C until assayed for cytokines. The concentrations of cytokine TNF- α , IL-1 β , IL-6 and IL-10 in the supernatants of RAW 264.7 cells culture were measured by ELISA using commercially available reagents according to the manufacturer's instructions (BioLegend, Inc. Camino Santa Fe, Suite E San Diego, CA, USA).

RT-PCR Assay for TNF- α , IL-6, IL-10 and IL-1 β mRNA Expression

RAW264.7 cells (4×10^5 cells/mL) were incubated in the presence of either 1 $\mu\text{g}/\text{mL}$ LPS alone or LPS plus ellagic acid 1, 2, 4 $\mu\text{g}/\text{mL}$ for 24 h at 37 °C with 5% CO_2 . Total RNA was isolated using easy-BLUETM kits according to the manufacturer's instructions and stored at -70 °C until use. Briefly, Integrity of RNA was confirmed by agarose gel electrophoresis, and RNA was quantified by spectrophotometric analysis. The PCR mixture was prepared according to the manufacturer's instructions using the following primers: mouse TNF- α , forward 5'-AATGAGGCTGGATAAGAT -3' and reverse 5'-AGAGGTTTCAGTGATGTAG -3', 387 bp; mouse IL-6, forward 5'-ACAAGAAAGACAAAGCCAGAGT -3' and reverse 5'-TGCCGAGTAGATCTCAAAGTG-3', 229 bp; IL-1 β , forward 5'-CCTCGTGCTGTCGGACCCAT-3' and reverse 5'-CAGGCTTGTGCTCTGCTTGTGA -3', 344 bp; IL-10 forward 5'-AAGACCAAGGTGTCTACAAGGCCA-3' and reverse 5'-TGAAAGGACACCATAGCAAAGGGC-3', 488 bp; β -actin forward 5'-GACTACCTCATGAAGATCCT-3' and reverse 5'-CCACATCTGCTGGAAGGTGG-3', 510 bp. Each reaction was performed using 2 μg of total RNA, and the thermocycler was programmed for reverse transcription at 60 °C for 45 min, 95 °C for 5 min, and 5 °C for 5 min. The initial denaturation of the cDNA was accomplished at 94 °C for 5 min, followed by 30 amplification cycles, each of which consisted of denaturation at 94 °C for 30 s, 30 s of annealing (51 °C, 53.5 °C, and 54 °C for TNF- α , IL-6, IL-10, and IL-1 β , respectively), and extension at 72 °C for 1 min 30 s. These were followed by a final extension at 72 °C for 10 min. Amplified PCR products were electrophoresed on a 1.5% TAE agarose gel.

LPS-induced ALI model

Based on the *vivo* experiment, we choose 5 mg/kg bodyweight with intraperitoneally injected in the experiment. After the BALB/c mice were diethyl ether-anaesthetized, 10 μg of LPS was instilled intranasal (i.n.) in 50 μL PBS to induce lung injury. Control mice were given 50 μL PBS i.n. without LPS. Ellagic acid (5 mg/kg bodyweight) or vehicle (PBS) was intraperitoneally injected 1 h prior to LPS administration. 5 mg/kg DEX was given by intraperitoneal injection as a positive control. After 12 h of LPS treatment, collection of bronchoalveolar lavage fluid (BALF) was performed three times through a tracheal cannula with 0.5 ml of autoclaved PBS, instilled up to a total volume of 1.5 mL. The fluid recovered from each sample was centrifuged (4 °C, 3000 rpm, 10 min) to pellet the cells. The supernatants of BALF were stored at -70 °C until required for determination of cytokine levels protein content and cell infiltration.

Assays for Cytokines

The levels of the proinflammatory cytokines TNF- α , IL-6, IL-1 β and IL-10 in the BALF were measured by a sandwich ELISA kit.

Protein Analysis

Protein concentrations in the supernatant of the BALF were quantified using the bicinchoninic acid (BCA) method to evaluate vascular permeability in the airways.

Myeloperoxidase Assay

Neutrophil and macrophage parenchymal infiltration, as reflected by MPO activity, was measured. Mice under diethyl ether anesthesia were killed 12 h after LPS administration. The right lung was excised and 100 mg were homogenized in 50 mM hydroxyethyl piperazine ethanesulfonic acid (HEPES) (pH 8.0) containing 0.5% cetyltrimethyl ammonium bromide (CTAB) and subjected to three freeze-thaw cycles. The homogenate was centrifuged at $13000 \times g$ for 30 min at 4 °C, and the cell-free extracts were stored at -20 °C until further use. The MPO activity was assayed using a mouse MPO ELISA kit. Samples were diluted in phosphate citrate buffer (pH 5.0).

Histopathologic Evaluation

The lungs were fixed with 10% neutral formalin. For light microscopic examination, lung tissue was dehydrated with graded alcohol and then embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (HE). Pathological changes in the lung tissues were observed under a light microscope

Western Blot Analysis

Mice under diethyl ether anesthesia were killed 12 h after LPS administration. Tissues were harvested and frozen in liquid nitrogen immediately until homogenization. The tissue samples were homogenized in RIPA buffer and lysed for 30 min on ice. These protein extracts were used for western blot analysis, and protein concentrations were determined using a BCA protein assay kit. Equal amounts of protein were loaded into wells on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Subsequently,

proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (BIO-RAD) with a glycine transfer buffer. After blocking the nonspecific site with blocking solution (5% (wt/vol) nonfat dry milk), PVDF was incubated with specific antibodies against I κ B α , p-I κ B α antibodies in 5% (wt./vol) BSA dissolved in Tween 20/Tris-buffered saline (TBST). With the use of peroxidase-conjugated secondary antibody, bound antibodies were detected by ECL plus (GE Healthcare Buckinghamshire, UK).

Immunocytochemical Analysis

RAW 264.7 cells (4×10^5 cells/mL), cultured on glass coverslips, which were plated onto 24-well plates for 24 h, were pretreated with 1, 2, 4 mg/kg of ellagic acid 1 h prior to treatment with 1 μ g/mL LPS for 1 h in a 37 °C, 5% CO₂ incubator. Glass coverslips were washed with 0.01 M PBS and fixed in 4% formaldehyde for 30 min at room temperature; detergent extraction was performed with 3% TritonX-100 for 10 min at room temperature. Coverslips were saturated with PBS containing 5% bovine serum albumin for 30 min at room temperature and processed for immunofluorescence with rabbit anti-NF- κ B/p65 polyclonal antibodies followed by Cy3-conjugated sheep anti-rabbit IgG. Finally, coverslips were mounted on slides. Fluorescence signals were analyzed by Fluoview microscopy (OLYMPUS, Japan).

Statistical Analysis

All data were expressed as mean \pm SD. Data analysis used SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Survival rates were estimated using the Kaplan-Meier method and a log-rank test. Statistical significance was accepted when P<0.05 or <0.01.

RESULTS

Effect of Ellagic Acid on LPS-Induced Cytokine Production *In Vitro*

TNF- α , IL-1 β , IL-6 and IL-10 concentrations in the culture supernatant of RAW264.7 cells were measured by sandwich ELISA. Treatment of RAW 264.7 cells with LPS alone resulted in significant increases of cytokine production compared to control. However, TNF- α , IL-1 β and IL-6 levels in the cell supernatant treated with 1, 2 and 4 μ g/ml of ellagic acid significantly decreased compared to those of LPS group (P<0.05) in a dose-dependent manner. On the contrary, IL-10 level increased in a dose-dependent manner (**Figure 2**). To assess whether ellagic acid could also regulate the cytokines production of LPS-induced mRNA expression, we carried out RT-PCR. As shown in **Figure 3**, the results were consistent with ELISA assay.

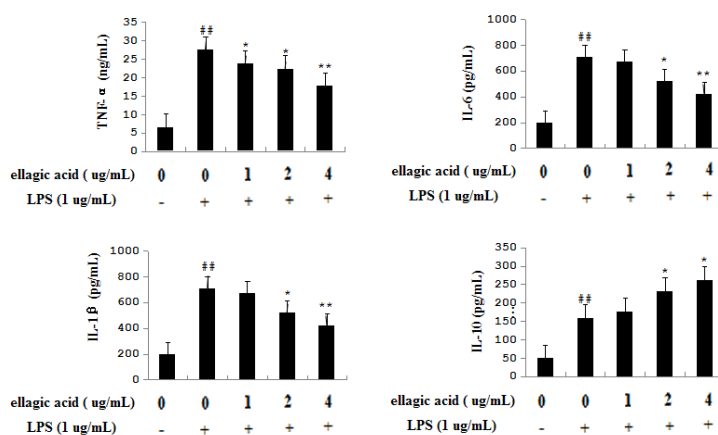


Figure 2. Effect of different concentrations of ellagic acid on TNF- α , IL-1 β , IL-6 and IL-10 expression at 24 h following LPS (1 μ g/mL) administration in RAW 264.7 cells. The values presented are the means \pm SD. ##p<0.01 vs control group; *p<0.05, **p<0.01 vs LPS group.

The cytotoxic effect of ellagic acid was evaluated using the MTT assay, and salidroside did not affect the cell viability of RAW 264.7 cells treated with concentrations ranging from 0 to 8 μ g/ml for 24 h (data not shown). These results assured us that the effects of ellagic acid on RAW 264.7 cells are due to its inhibition of TNF- α , IL-1 β , IL-6 and IL-10 productions directly or indirectly but not due to cell death.

Effect of Ellagic Acid on Production of TNF- α , IL-1 β , IL-10 and IL-6 in the BALF of LPS-Induced ALI Mice

Mice were given an intraperitoneally administration of ellagic acid (5 mg/kg) 1 h prior to an i.n. administration of LPS. BALF was collected at 12 h following LPS challenge to analyse the production of TNF- α , IL-1 β , IL-6, IL-10. As shown in **Figure 4**, the results were similar to that *in vitro*.

Effect of Ellagic Acid on Total Protein Concentration in the BALF

The total protein concentration significantly increased in the BALF of ALI mice at 12 h after LPS challenge compared to those of the control group (P<0.05 or P<0.01). Ellagic acid and DXM significantly decreased the total protein concentration (P<0.01) in the BALF compared to those in the LPS group (**Figure 5**).

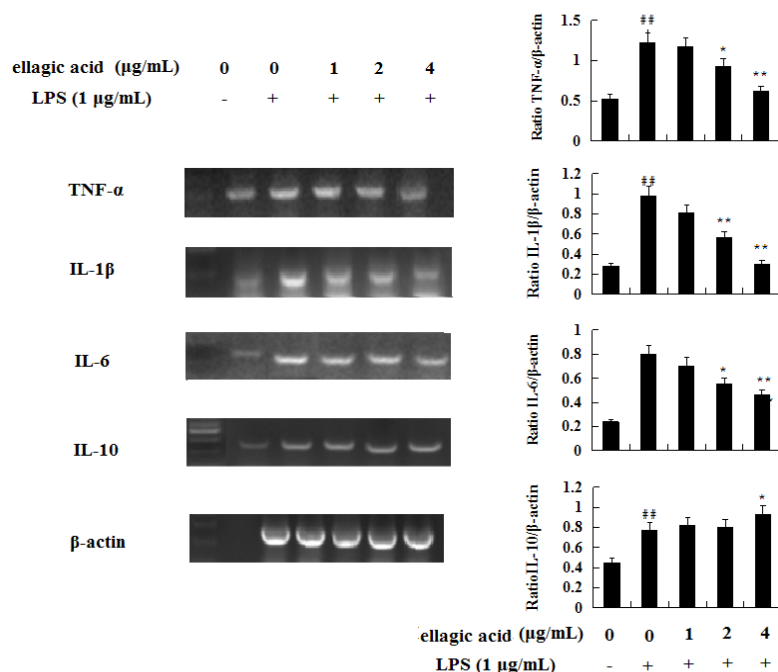


Figure 3. Effect of different concentrations of ellagic acid on mRNA expression of TNF- α , IL-1 β , IL-10 and IL-6 in LPS-induced RAW 264.7 cells. The cells were treated with 1 μ g/mL of LPS alone or LPS plus different concentrations (1, 2, 4 μ g/mL) of ellagic acid I for 24 h. Total RNA was isolated and mRNA expressions of the aforementioned cytokines were measured by RT-PCR. Shown in the down panel were from three independent experiments. A representative Western blot is shown in here. ## $p < 0.01$ vs control group; * $p < 0.05$, ** $p < 0.01$ vs LPS group.

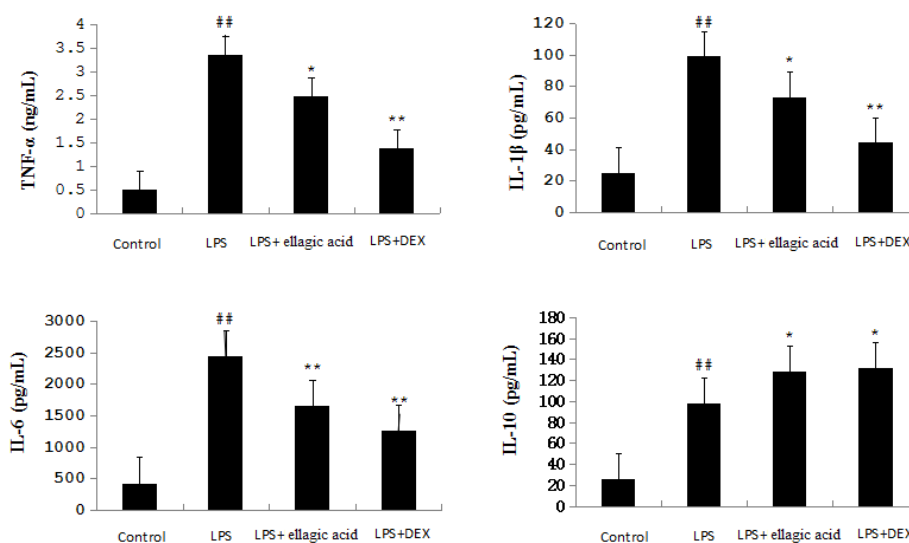


Figure 4. Effect of ellagic acid on production of TNF- α , IL-1 β , IL-10 and IL-6 in the BALF of LPS-induced ALI mice. Mice were given an intraperitoneally administration of ellagic acid (5 mg/kg) 1 h prior to an i.n. administration of LPS. BALF was collected at 12 h following LPS challenge to analyse the production of TNF- α , IL-6, IL-10 and IL-1 β . The values presented are the means \pm SD (n=6 in each group). ## $p < 0.01$ vs control group; * $P < 0.05$ or ** $P < 0.01$ vs LPS group.

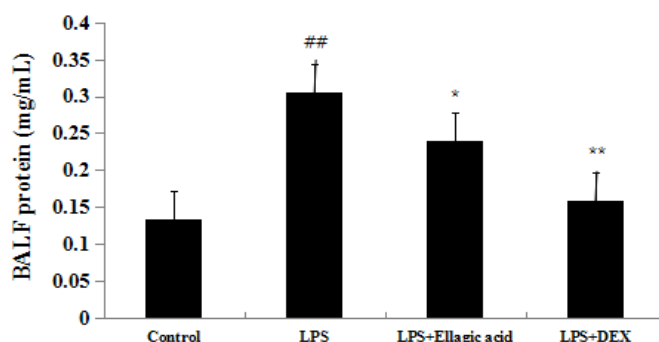


Figure 5. Effect of ellagic acid on total protein concentration in the BALF of LPS-induced ALI mice. Mice were given an intraperitoneally administration of ellagic acid (5 mg/kg) 1 h prior to an i.n. administration of LPS. Total protein concentration in the BALF was determined at 12 h after LPS challenge. The values presented are the means \pm SD (n=6 in each group). ## $p < 0.01$ vs control group; * $P < 0.05$ or ** $P < 0.01$ vs LPS group.

Effect of Ellagic Acid on MPO Activity

MPO activity is another measure of lung parenchymal phagocyte infiltration. As shown **Figure 6**, LPS challenge resulted in significant increases in lung MPO activity. Compared with the control group. Intraperitoneally, injection of a single dose of ellagic acid 1 h before LPS exposure significantly reduced MPO activity compared with the LPS group ($P<0.01$). Meanwhile, treatment with DXM significantly decreased LPS-induced increases in MPO activity in lung compared with the LPS group ($P<0.01$).

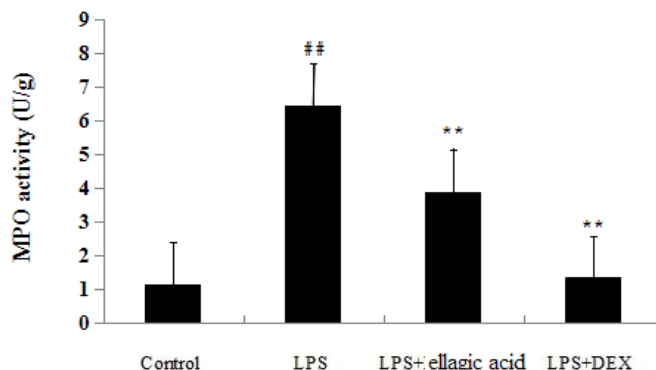


Figure 6. Effects of ellagic acid on MPO activity in lungs of LPS-instilled mice. 12 h after LPS instillation, lung homogenates were prepared for determination of MPO activity. MPO activity in the lungs was determined with an ELISA kit. Data are presented as the mean \pm SD ($n=6$ in each group). ## $p<0.01$ vs- control group; * $P<0.05$ or ** $P<0.01$ vs- LPS group.

Effect of Ellagic acid on Histopathological Changes in the Lung Tissue

To evaluate the histological changes after ceftiofur treatment in LPS-treated mice, lung sections were dyed with hematoxylin and eosin. Normal pulmonary histology was found in the control group (**Figure 7A**). In the LPS group (**Figure 7B**), mice exhibited marked increases in inflammatory cell infiltration, mostly including neutrophils, macrophages, etc. In addition, lung tissues from the experimental group administered LPS alone were significantly damaged, with interstitial edema, hemorrhage, and thickening of the alveolar wall. However, these histopathological changes were ameliorated in the ellagic acid and DEX group (**Figure 7C and 7D**).

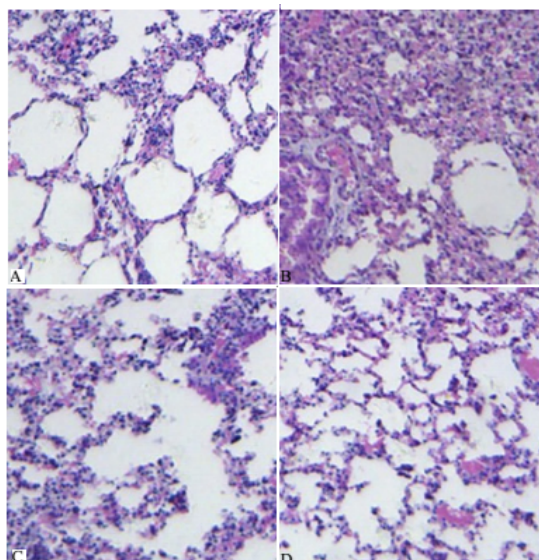


Figure 7. Effect of ellagic acid on histopathological changes in lung tissues in LPS-induced ALI mice. Mice were given a subcutaneous injection of ellagic acid 1 h prior to an i.n. administration of LPS. Lungs from each experimental group were processed for histological evaluation at 12 h after LPS challenge. Lungs were prepared for H&E staining. (A) Control group; (B) LPS group, (C) ellagic acid group; (D) DEX group. The panels show a $\times 100$ magnification.

Effects of Ellagic Acid on LPS-Induced P-I- κ B, I- κ B and NF- κ B Signaling Pathway Activation

First, we examined the effect of ellagic acid on the LPS induced P-I- κ B, I- κ B in the cytoplasm by Western blotting analysis using different phospho-specific antibodies (**Figure 8**). As shown in **Figure 8**, treatment with LPS resulted in up-regulation of I κ B. However, when we added Different concentrations ellagic acid, NF- κ B and the degradation of I κ B were significantly blocked by ellagic acid. To determine whether this I κ B degradation was related to I κ B phosphorylation, we examined the effect of ellagic acid on LPS-induced P-I κ B by western blot. The results showed that the phosphorylation of I κ B α in RAW264.7 cells was increased after LPS administration, but was significantly inhibited by ellagic acid treatment.

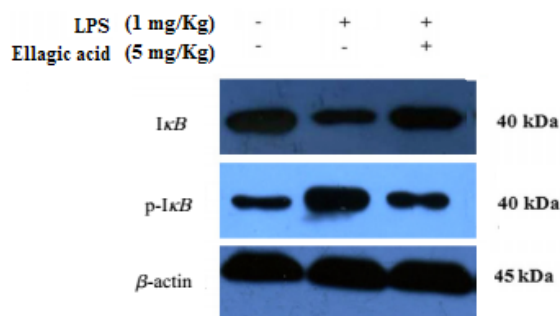


Figure 8. Effect of ellagic acid on LPS-induced phosphorylation of IκB. The cells were stimulated with or without LPS (1 μg/mL) for 30 min. Protein samples were analyzed by Western blot with phospho-specific antibodies. The total P-IκB and IκB levels were used as an internal control. Shown in the up panel were from three independent experiments. A representative Western blot is shown in the up panel.

Furthermore, we evaluated the effect of ellagic acid on LPS induction of the NF-κB pathway with Fluoview microscopy. As shown in **Figure 9**, treatment with LPS resulted in the accumulation of p65 in the nucleus. The majority of intracellular p65 translocated from the cytoplasm to the nucleus, demonstrated by strong p65 staining in the nucleus. However, nuclear translocation of p65 induced by LPS was dose-dependently inhibited with ellagic acid treatment by immunocytochemical staining.

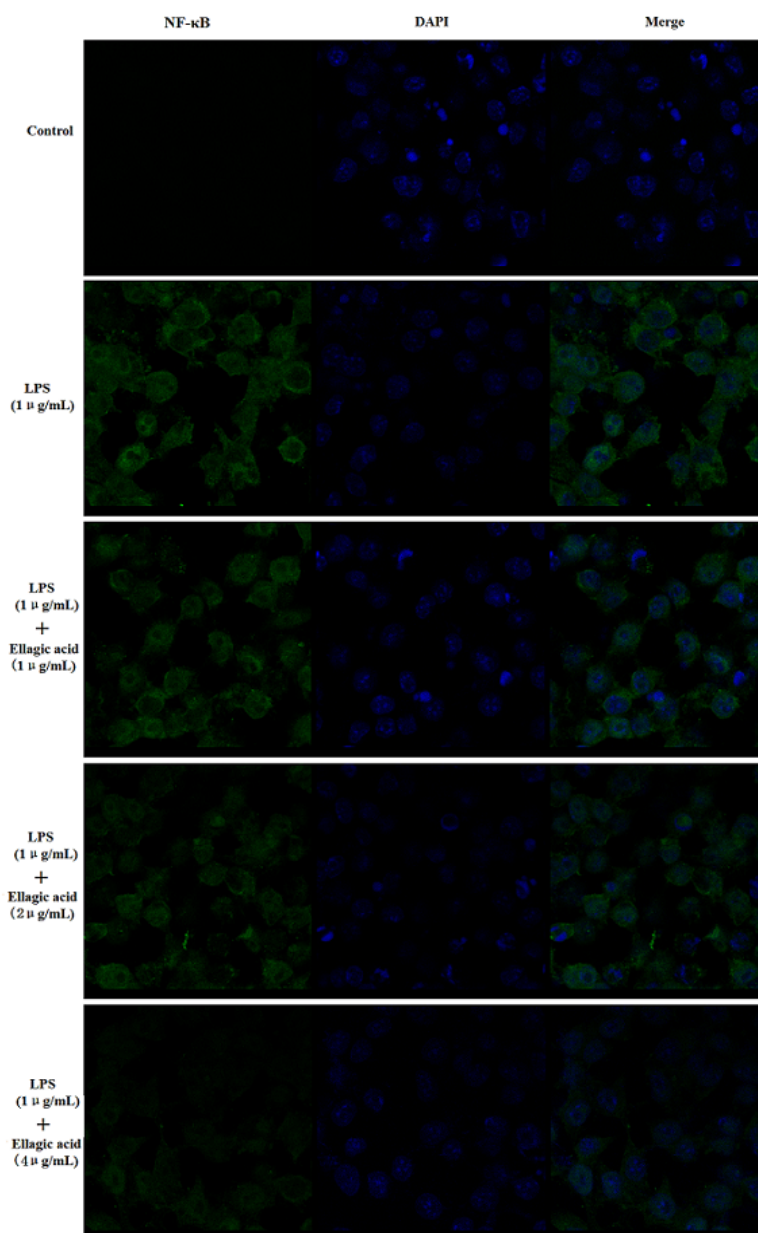


Figure 9. Effect of ellagic acid on LPS-induced NF-κB activation by immunocytochemistry. The cells were pretreated with different concentrations (1, 2, 4 μg/mL) of ellagic acid for 1 h prior to stimulation with 1 μg/mL of LPS for 1 h. They were then fixed, permeabilised, and incubated with rabbit anti-NF-κB/p65 polyclonal antibody followed by Cy3-conjugated anti-rabbit IgG. The nuclei of the corresponding cells were demonstrated by DAPI staining. Magnification for images was 600×.

DISCUSSION

In this study, we explored the ability of a natural polyphenolic compound from fruit and nut to modulate LPS response using macrophage-mediated inflammatory conditions and LPS-induced ALI. Dexamethasone (DEX) is the most frequently used anti-inflammatory drugs in the clinical treatment of ALI. So *in vivo* model, we used it as a positive control to evaluate effect of ellagic acid in LPS-induced ALI.

Increasing evidence has shown that macrophages play an essential role in the regulation of inflammation responses^[19,20]. Macrophages are important immune cells of innate immunity and represent the front line of host response to infection, invasion, and injury. LPS-mediated activation of macrophages leads to the production of various inflammation cytokines and mediators. The proper inflammatory response helps remove invading pathogens and subsides to restore immunologic homeostasis^[21,22]. However excessive production of these cytokines from durative or plentiful infection may result in systemic inflammatory response syndrome (SIRS), sepsis, and severe tissue damage, including ALI^[23]. Therefore, any approach that inhibits inflammation *in vitro* and *in vivo* may potentially have an effect on the prevention or treatment of ALI.

In the experiment, we first tested the effects of ellagic acid on LPS induced proinflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-10 *in vitro*, which are characterized cytokines in inflammatory process of ALI. The inhibition of inflammatory cytokines is an important target for anti-inflammatory therapies. But among the cytokines, IL-10 is a kind of important anti-inflammatory cytokine in controlling inflammatory responses^[24,25]. The outcomes showed that ellagic acid significantly down regulated the production of large amounts of TNF- α , IL-1 β , and IL-6, increased IL-10 *in vitro*. These results inspired us to further study the effects of ellagic acid *in vivo*. As we known, in patients with ALI and ARDS, elevated concentrations of IL-1 and TNF- α have been measured in BALF, and were related to poor outcome^[26]. Inhibiting the overproduction of pro-inflammatory cytokines showed the lessening of pulmonary injury in LPS induced ALI model^[27,28]. As expected, the data presented here demonstrates that ellagic acid may significantly inhibit the production of LPS-induced TNF- α , IL-6, IL-1, and increase IL-10 *in vivo*.

In ALI model, we also tested total protein concentration and myeloperoxidase (MPO) activity in the BALF. Total protein indicates epithelial permeability and pulmonary edema. MPO activity is a measure of lung parenchymal phagocyte infiltration and was considered as the marker of the activation and accumulation of neutrophils in inflammation. Treatment of ellagic acid and DEX before LPS exposure significantly reduced total protein and MPO activity compared with the LPS group. These findings indicated that the protective effect of ellagic on ALI induced by LPS depend on attenuation of inflammatory cell sequestration and migration into the lung tissue.

In addition, following treatment with ellagic acid, we used H&E staining to evaluate the histological changes in lung tissue from mice with acute lung injury. In the LPS group, the lung showed significant pathologic changes, such as alveolar wall thickening, alveolar hemorrhage, interstitial edema, inflammatory cells infiltration. However, treatment with ellagic acid and DEX significantly attenuated these changes. But the effect of ellagic acid was weaker than DEX.

To further characterize the nature of the inhibitory effect of ellagic acid on ALI, we examined the effects of ellagic acid on the activation of NF- κ B signaling pathways, which regulate the expression of many immune and inflammatory genes. NF- κ B is one of the most important transcription factors and is found in cell types that express cytokines, chemokine's, growth factors, cell adhesion molecules, and some acute phase proteins in healthy and disease states^[29,30]. The activation of NF- κ B involves the phosphorylation of I κ Bs at two critical serine residues (Ser32 and Ser36) via the I κ B kinase signal some complex. In unstimulated cells, NF- κ B is localized to the cytosol due to its binding with I κ B. However, when cells are activated by LPS, I κ B is phosphorylated by I κ B kinase and degraded, and then the released NF- κ B migrates into the nucleus and induces the transcription of pro-inflammatory mediators, e.g. iNOS, COX-2, TNF- α and IL-1 β , IL-6, and IL-8^[30]. Therefore, the activation of NF- κ B was assessed by measuring the degree of phosphorylation of I κ B protein. In the experiment, we evaluated the effect of ellagic acid on LPS induced NF- κ B pathway by western-blot and immunocytochemical analysis. As shown in **Figure 8**, we first determine whether this I κ B degradation was related to I κ B phosphorylation, The results showed that the phosphorylation of I κ B α in lung tissue were increased after LPS administration, but was significantly inhibited by ellagic acid treatment. I κ B α protein showed the opposite trend. In immunocytochemical analysis, treatment with LPS resulted in the accumulation of p65 in the nucleus. The majority of intracellular p65 translocated from the cytoplasm to the nucleus, demonstrated by strong p65 staining in the nucleus. However, nuclear translocation of p65 induced by LPS was dose-dependently inhibited with ellagic acid treatment by immunocytochemical staining. The data showed that ellagic acid efficiently blunted nuclear factor-kappa B (NF- κ B) activation by inhibiting the degradation and phosphorylation of I κ B α , as well as the translocation of p65 from cytoplasm to the nucleus.

CONCLUSION

In conclusion, pretreatment with ellagic acid results in a significant reduction in the amount of inflammatory cells, protein leakage in the BALF, and inflammatory cell infiltration into lung tissue. ELISA results indicated that ellagic acid can significantly suppress TNF- α , IL-6, IL-8 levels, increase IL-10 production *in vitro* and *in vivo*. The protective effect of ellagic acid on ALI induced by LPS may be through suppressing NF- κ B pathways activation.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the content of this article.

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