Original Research

Protective effect of quercetin on lipopolysaccharide-induced acute lung injury in mice by inhibiting inflammatory cell influx

Lian Wang[1], Jinming Chen[2], Bo Wang[3], Dingqian Wu[2], Hao Li[4], Huchen Lu[5], Haiwei Wu[4] and Ying Chai[1]

[1]Department of Thoracic Surgery, Second Affiliated Hospital, Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou 310009, China; [2]Department of Emergency Medicine, Second Affiliated Hospital, Zhejiang University School of Medicine; [3]Department of Cardiothoracic Surgery, Nanjing Chest Hospital, 215 Guangzhou Road, Nanjing 210029, China; [4]Department of Cardiothoracic Surgery, Jinling Hospital, Clinical Medicine School of Nanjing University, 305 East Zhongshan Road, Nanjing 210009, China; [5]Department of Neurological Surgery, Nanjing Brain Hospital, 264 Guangzhou Road, Nanjing 210029, China
Corresponding author: Ying Chai. Email: z2ychai@126.com

Abstract

Sepsis may result in lung injury through a complex cascade of events including interstitium infiltration of inflammatory cells. Quercetin, the most abundant dietary flavonoid found in various plants and food products, possesses potent anti-inflammatory and antioxidative properties. The purpose of this study was to investigate whether preventive administration of quercetin could exert beneficial effects on experimental septic acute lung injury induced by lipopolysaccharide (LPS). C57/BL6 mice were challenged with LPS and survival time was monitored from 0–96 h after LPS treatment. Quercetin markedly rescued lethality, improved survival time, and inhibited serum necrosis factor α, interleukin 1β, and interleukin 6, and nitric oxide (NO), and increased IL-10 secretion. Moreover, quercetin decreased lung pathological changes, myeloperoxidase activity, and malondialdehyde levels. Quercetin also reduced the lung permeability changes and neutrophil and macrophage recruitment to the bronchoalveolar lavage fluid compared to the vehicle. Additionally, quercetin significantly reduced COX-2, HMGB1, iNOS expression, and NF-κB p65 phosphorylation. These results suggest that treatment with quercetin in septic mice improved survival time and lung injury. Quercetin may be a promising potential therapeutic reagent for LPS-induced acute lung injury.

Keywords: Quercetin, acute lung injury, lipopolysaccharide, high-mobility group box 1, cyclooxygenase-2

***Experimental Biology and Medicine* 2014; 239: 1653–1662. DOI: 10.1177/1535370214537743**

Introduction

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are severe forms of diffuse lung disease that impose a substantial health burden in both developing and developed countries. ALI/ARDS is caused by an increase in permeability of the alveolar-capillary barrier and subsequent impairment of arterial oxygenation in response to direct or indirect insults, most commonly sepsis, pneumonia, and trauma.[1] Despite the improvement in supportive care has been great, current mortality and morbidity remain high, while therapies targeting the underlying lung injury are limited.[2]

Multiple organ dysfunction syndrome is the most devastating complication of sepsis, and one of the first organs to fail is the lung. Indeed, almost half of all patients with severe sepsis will go on to develop ALI/ARDS.[3] Although the pathophysiology is still not completely understood, sepsis may result in ALI through a complex cascade of events. In sepsis, the initiating stimuli of systemic inflammation are often bacterial components such as lipopolysaccharide (LPS), which induce the secretion of pro-inflammatory cytokines, nitric oxide (NO) and reactive oxygen species (ROS) predominantly from leukocytes.[4] Toll-like receptor 4, which plays an important role in LPS recognition, triggers activation of an intracellular signaling pathway involving nuclear factor-κB (NF-κB), leading to the upregulation of many genes responsible for the secretion of inflammatory mediators including tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6).[5] Excessive inflammatory cytokines in circulation injure vascular endothelium and induce the infiltration of inflammatory cells as well as the movement of fluid into the interstitial space. The subsequent local activation of neutrophils and macrophages leads to tissue damage via the release of cytotoxic and immune cell-activating agents such as cytokines, cationic polypeptides, free radicals, and

proteinases.[6] High-mobility group box 1 (HMGB1) is a ubiquitous nuclear protein that functions as a late-acting mediator of endotoxin and sepsis lethality.[7] When released into the extracellular milieu, HMGB1 can stimulate the expression of TNF-α, IL-1, and IL-6 from macrophages.[8] Many studies have shown that HMGB1 is not only involved in sepsis, but also participates in the process of LPS-induced ALI.[9] Administration of anti-HMGB1 antibodies significantly attenuated endotoxin-induced lung injury in mice. Thus, strategies that target HMGB1 with specific antagonists may have the potential to treat lethal systemic inflammatory responses including ALI.[10]

Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone), which represents the most abundant dietary flavonoid found in various plants and food products, possesses potent anti-inflammatory[11] and antioxidative[12] properties that contribute to the beneficial effects on multiple cell types, both in animal and human. Several *in vitro* studies using different cell lines have shown that the flavonoids are capable of inhibiting LPS-induced cytokine production. For instance, quercetin inhibits TNF-α production in LPS-stimulated macrophages,[13,14] IL-6 production in neutrophils,[15] and IL-8 in pulmonary epithelial cells.[16] In addition, flavonoids are nature-derived NF-κB inhibitors,[17] and quercetin inhibits NF-κB activation both *in vivo*[18,19] and *in vitro*.[20,21] Moreover, it was demonstrated that this polyphenol compound significantly inhibited HMGB1 release from macrophages and prevented the accumulation of serum HMGB1 levels in mice with lethal endotoxemia.[22] Numerous reports have demonstrated the inhibitory effects of naturally occurring flavonoids such as quercetin on cyclooxygenase-2 (COX-2) expression,[23] which is a critical inducible enzyme in multiple pathophysiological processes including ALI.[24] Furthermore, quercetin is the most potent neutralizer of free radicals within the flavonoid family.[25] With the increased knowledge about the pharmacokinetics and the molecular mechanisms of action, this unique bioflavonoid has been proposed as a treatment for acute and chronic inflammatory processes.[23] Nevertheless, little is known about the effect of quercetin on LPS-induced ALI. Therefore, we hypothesized that quercetin may inhibit inflammatory response and reverse oxidative stress, particularly through decreasing the expression of HMGB1 and COX-2, thereby preventing the progression of sepsis and resultant ALI. To test this hypothesis, we investigated whether preventive administration of quercetin could exert beneficial effects on experimental septic ALI induced by LPS in mice.

Methods

Animals and reagents

C57/BL6 mice (male, 7–8 weeks of age, 22–23 g) were obtained from Nanjing Medical University Laboratory Animal Center (Certificate No. SCXK-JIANGSU-2008-0004). Animal care was in compliance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care published by the National Academy Press (Washington, DC, 1996). Mice were randomly assigned into four groups ($n = 20$ in each group). The experimental protocol was approved by the Animal Care and Use Committee, Nanjing University. Group 1 (Control group), orally administered daily with vehicle for one week and received an intraperitoneal injection of PBS at day 8; group 2 (Quer group), orally administered daily with quercetin for one week and received PBS injection at day 8; group 3 (LPS group), orally administered daily with vehicle for 1 week and received an intraperitoneal LPS injection at day 8; and group 4 (Quer + LPS group), orally administered daily with quercetin and then received an intraperitoneal injection of LPS at day 8.

Escherichia coli LPS (026:B6) and quercetin were purchased from Sigma-Aldrich (Shanghai, China). The purity of quercetin is ≥98%. Carboxymethyl cellulose sodium (CMC) was from Sinopharm Chemical Reagent (Shanghai, China). Quercetin was suspended in 0.5% CMC to achieve a final working concentration of 18 mg/mL. The dosage of quercetin (60 mg/kg) was based on previous studies demonstrating beneficial effects of the drug.[18,19]

Antibodies for COX-2 and HMGB1 were purchased from Abcam (Cambridge, MA US). Anti-inducible nitric oxide synthase (iNOS) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-NF-κB p65, anti-phospho-p65, and anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Survival time study (Experiment 1)

For the survival study, the dose of LPS in mice ($n = 10$ in each group) was 20 mg/kg. The mortality of mice was recorded every 12 h during 96 h after PBS/LPS injection. No serum or tissue sample was collected in this first experiment.

Experiment 2

ALI was induced by intraperitoneal injection of 10 mg/kg LPS ($n = 10$ in each group).[26] After 24 h, mice (no death observed) were sacrificed with an overdose of pentobarbital (60–90 mg/kg) injected intraperitonealy. The chest space was opened by the median sternotomy. Both lungs were excised from the thoracic space by dissecting the trachea. The left lung was used to obtain bronchoalveolar lavage fluid (BALF) as described previously.[27] Serum and lung tissue samples were collected for subsequent analysis. The superior lobe of right lung was excised for histopathologic examination. The middle lobe of right lung was excised for analysis of lung wet/dry weight ratio. The lower lobe of right lung was rapidly removed and cut into two parts of similar size. A part of the lower lobe was homogenized for oxidative stress parameters measurements, and the other part was used to extract proteins for Western blot.

Determination of BALF protein and leukocyte counts

The BALF was immediately centrifuged at 1400 *g* for 10 min at 4°C to pellet the cells. The cell-free supernatant was stored at −80°C for subsequent analysis of protein levels. The cell pellets were resuspended in PBS for total cell counts by a hemacytometer (Qiujing Inc., Shanghai, China). For differential cell counts, slides were prepared

with a cytospin centrifuge (PrepStain, Becton Dickinson, Franklin Lakes, USA) and stained with Wright-Giemsa. These preparations were analyzed by a pathologist blind to group assignments. Protein concentrations in the supernatant of the BALF were measured by a Coomassie brilliant blue protein assay reagent kit (Jiancheng Bioengineering, Nanjing, China). A standard curve was performed with an albumin (bovine serum) standard saline solution.

Assay for TNF-α, IL-1β, IL-6, IL-10, and NO

Serum samples were collected for TNF-α, IL-1β, IL-6, IL-10, and NO detection. According to the manufacturer's instructions, the concentrations of all cytokines, including TNF-α, IL-1β, IL-6, and IL-10 were detected by ELISA kits (eBioscience Inc, San Diego, CA, USA) as described previously.[28] NO production in the mouse serum was detected by a commercial kit (R&D System, Minneapolis, MN, USA). Absorbance was measured at 540 nm with a T-max microplate reader (Molecular Device Corp., Sunnyvale, CA, USA).

Histopathological examination

The superior lobe of right lung was fixed in 10% neutral buffered formalin for 24 h. The tissues were embedded in paraffin and cut into 5 μm sections. Hematoxylin-eosin stains were performed using standard protocol. The pathological changes were assessed by a pathologist blinded to the animal groups. Lung injury was scored based on the findings in 10 randomly selected high-power fields (400×) for each tissue slide, as described earlier.[29] For immunohistochemistry, lung sections were incubated with COX-2 antibody followed by incubation with horseradish peroxidase-conjugated goat antibody against rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were then developed with diaminobenzidine and counterstained with hematoxylin. Quantification of images was done by scanning densitometry with Image J 1.44 (National Institute of Health, Bethesda, MD, USA).

Assessment of lung water content

The middle lobe of right lung was removed and the wet weight was determined. The lung tissue was placed in an oven at 60°C for 24 h to obtain the dry weight. The lung water content (reflecting pulmonary edema) was derived using the formula: $W/D\% = [(\text{wet weight} - \text{dry weight})/\text{wet weight}] \times 100$.

Measurements of myeloperoxidase activity and malondialdehyde in lung tissues

The snap-frozen lung tissue was crushed in a prechilled mortar and resuspended at a concentration of 50 mg/mL in PBS. The tissues were centrifuged for 10 min at 10,000*g* at 4°C. The supernatants were collected for myeloperoxidase (MPO) and malondialdehyde (MDA) detection.

MPO activity (as a marker of neutrophil infiltration) was assessed according to the methods previously described[29] using a commercial kit (Jiancheng Bioengineering, Nanjing, China). MPO activity was expressed in units per gram weight of wet tissue.

The lipid peroxidation product MDA was assessed by using the thiobarbituric acid reactive substances (TBARS) assay kit (Jiancheng Bioengineering). Briefly, 100 μL of supernatant was added to 100 μL sodium dodecyl sulphate (SDS) lysis solution and mixed thoroughly. After 250 μL of thiobarbituric acid reagent addition, samples were incubated at 95°C for 1 h and centrifuged at 3,000 rpm at room temperature for 15 min. The supernatant was transferred to a microplate for determination of the absorbance at 532 nm. Values of TBARS are expressed as nmol equivalents of MDA per milligram protein.

Western blotting analysis of HMGB1, iNOS, NF-κB p65, and phospho-p65 expression

Total proteins were obtained from the supernatants of tissue homogenate with T-PER tissue protein extraction reagent (Pierce, Rockford, IL, US). Equal amounts (30 μg) of total protein were separated on 10% polyacrylamide gels, using an electrophoresis system (#164-5051, Bio-Rad, Hercules, CA, USA), and transferred to nitrocellulose membranes (#77010, Pierce) using a semidry transfer cell (#221BR13870, Bio-Rad) at 10 V for 40 min. The membranes were blocked for 60 min with 5% nonfat milk in Tris-buffered saline Tween (TBST) and washed subsequently. Primary antibodies for HMGB1, iNOS, NF-κB p65, and p-p65 were added and incubated overnight at 4°C. Additionally, all blots were incubated with anti-β-actin antibody to confirm protein loading levels. Membranes were washed with TBST, incubated with horseradish peroxidase-conjugated species-appropriate secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature, and developed by an enhanced chemiluminescence (ECL) kit (MB154410B, Pierce). Quantification of images was done by scanning densitometry with Image J 1.44.

Statistical analysis

The data were reported as the means ± SD. Statistical analyses were performed with SPSS for Windows version17.0 (SPSS Inc., Chicago, IL, USA). Survival data were presented by the Kaplan–Meier method and comparisons were made by the log rank test. The means from different treatments were compared by one-way ANOVA followed by Dunnett's test. Values of $P < 0.05$ were considered statistically significant.

Results

The survival time

As shown in Figure 1, LPS (20 mg/kg) administration dramatically resulted in mouse death. At 96 h after LPS injection, the survival rate of mice in LPS group was 10%. The accumulative mortalities during four days were 40% in quercetin pretreatment ALI mice, which were significantly lower than that in LPS group (90%, $P < 0.05$). These data indicated that pretreatment with quercetin could significantly protect mice with endotoxemia from death.

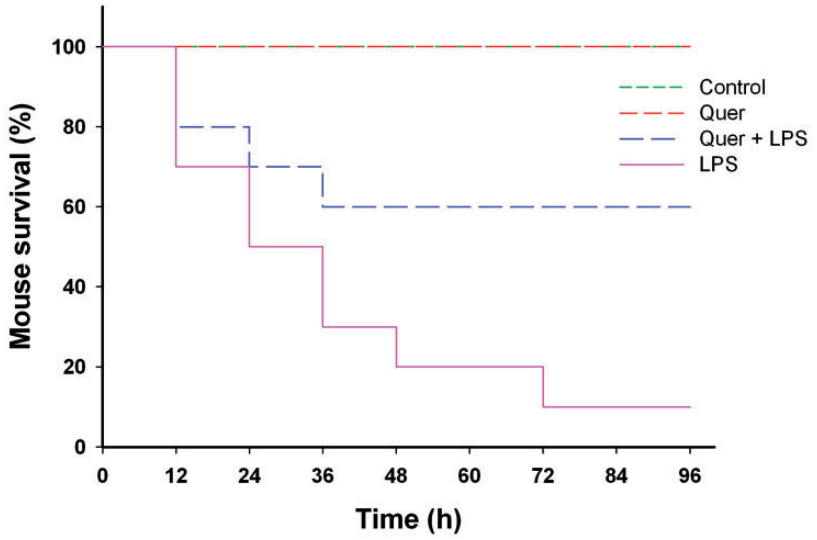

Figure 1 Effect of quercetin on the survival of septic mice. Mice were orally administered daily with vehicle/quercetin for one week and received an i.p. injection of PBS/LPS (20 mg/kg) at day 8. The mortality of mice was recorded every 12 h during 96 h. The survival rates were constructed using the Kaplan–Meier curve, and differences in mortality were compared using the log-rank. (A color version of this figure is available in the online journal.)

Table 1 Quercetin pretreatment attenuates serum TNF-α, IL-1β, IL-6, and NO levels, and elevates anti-inflammatory cytokine IL-10 concentration ($n = 5$ in each group)

	Control	Quer	LPS	Quer + LPS
TNF-α (pg/mL)	45.6 ± 10.1	38.8 ± 14.2	138.6 ± 24.7*	87.3 ± 17.2#
IL-1β (pg/mL)	30.1 ± 7.7	34.0 ± 9.6	98.0 ± 14.6*	67.6 ± 10.3#
IL-6 (pg/mL)	35.7 ± 13.8	43.0 ± 7.8	1368.7 ± 134.7*	987.6 ± 97.3#
IL-10 (pg/mL)	160.4 ± 57.9	148.1 ± 49.4	2380.3 ± 350.5*	3370.2 ± 410.8#
NO (μmol/L)	31.7 ± 7.5	33.7 ± 9.2	224.5 ± 24.9*	117.7 ± 30.0#

Note: Circulating cytokine levels were measured by ELISA of sera prepared at 24 h after PBS/LPS injection.
*$P < 0.05$ vs control.
#$P < 0.05$ vs LPS.

Serum levels of TNF-α, IL-1β, IL-6, NO and IL-10

After 10 mg/kg of LPS treatment, TNF-α, IL-1β, IL-6, and NO contents in serum were remarkably elevated at 24 h (Table 1). However, quercetin markedly decreased the serum levels of these cytokines. Of note, quercetin significantly increased IL-10 expression compared with that in the LPS group ($P < 0.05$; Table 1).

Histopathological changes

To evaluate histopathological characteristics of the lungs, the tissue sections were stained with hematoxylin and eosin. In the Control and Quer groups, lung tissues showed a normal structure and clear pulmonary alveoli (Figure 2a). On the other hand, the lungs from LPS group had extensive alveolar wall thickness, alveolar and interstitial hemorrhage, and obvious inflammatory cells infiltration (Figure 2a). When the mice were pretreated with quercetin, the pulmonary edema and infiltration were improved significantly (Figure 2a). Generally, Figure 2(b) shows that the lung injury score in Quer + LPS group was significantly lower ($P < 0.05$) compared with that in LPS group, although the injury was not completely prevented.

Lung water content, protein concentration, and inflammatory cell count in BALF

To investigate the effect of quercetin on LPS-induced lung edema, we evaluated the lung water content and protein concentration in BALF 24 h after PBS/LPS treatment. Compared with the LPS group, administration of quercetin prior to LPS significantly reduced the increase in the lung water content and protein concentration ($P < 0.05$; Figure 3(a) and (b)), suggesting that quercetin prevents the LPS-induced increase in lung vascular permeability. Moreover, quercetin treatment led to a significant lowering of the number of total cells, neutrophils, and macrophages in the BALF, as compared to LPS group ($P < 0.05$; Figure 3(c) to (e)).

MPO activity and MDA in lung tissues

To assess the neutrophil infiltration within pulmonary tissues, MPO activity was detected. The results revealed that

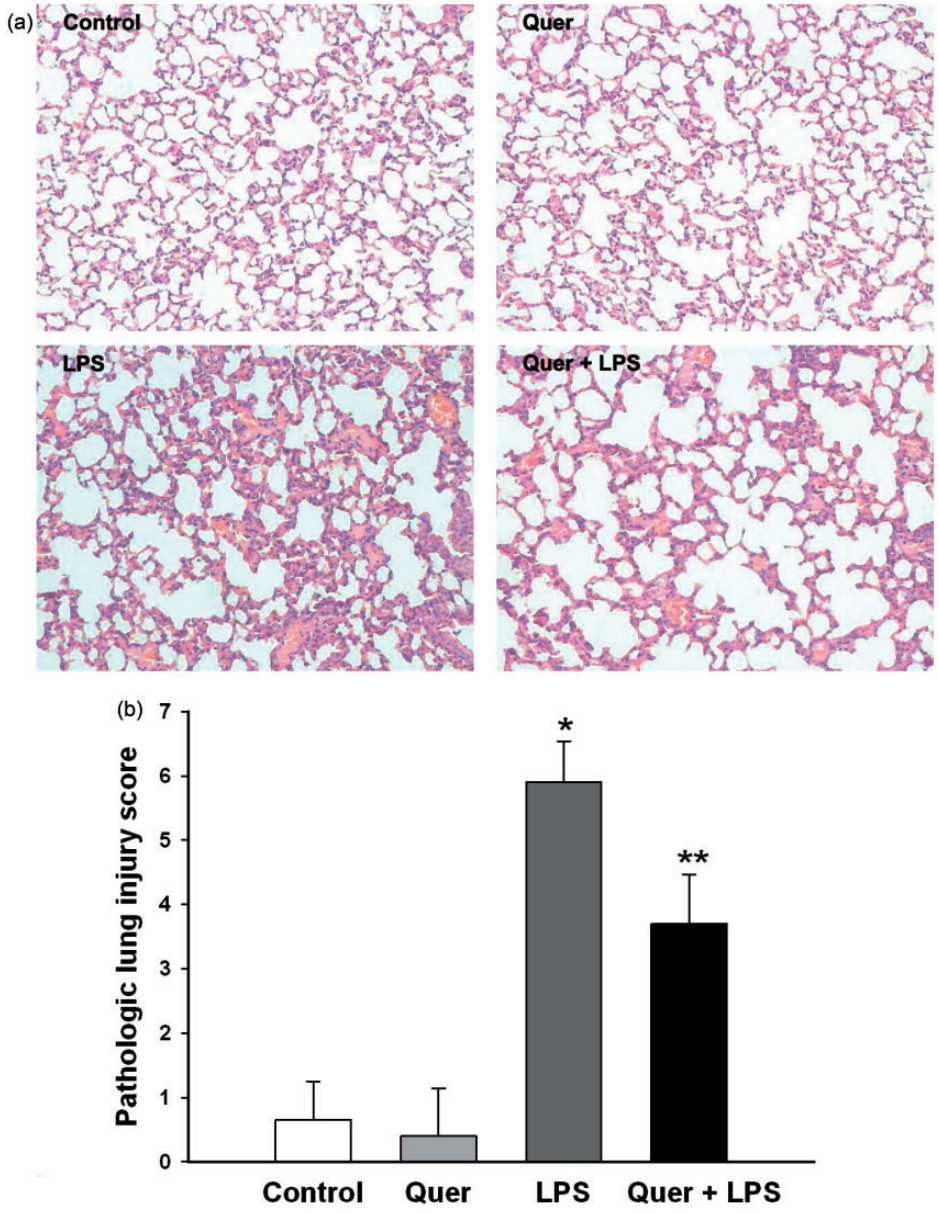

Figure 2 Quercetin attenuated LPS-induced ALI. Mice were orally administered daily with vehicle/quercetin for one week and received an i.p. injection of PBS/LPS (10 mg/kg) at day 8. (a). The superior lobe of right lung was stained with hematoxylin and eosin (magnification 100×). (b). Histopathological scores. Lung injury was scored based on the findings in 10 randomly selected high-power fields for each tissue slide ($n = 5$ in each group). *$P < 0.05$ vs control, **$P < 0.05$ vs LPS. (A color version of this figure is available in the online journal.)

MPO activity was markedly attenuated in Quer + LPS group ($P < 0.05$; Table 2). The level of MDA, an oxidative stress biological marker, was also increased in LPS group ($P < 0.05$; Table 2), which could be partially reversed by quercetin.

Levels of COX-2, HMGB1, iNOS expression, and NF-κB p65 phosphorylation

We next analyzed expression of COX-2 implicated in LPS-induced lung injury using immunohistochemistry. COX-2 protein distribution was upregulated after 24 h following LPS administration; this was predominant in alveolar macrophages and neutrophils, as well as alveolar epithelial type II cells. Nevertheless, pretreatment with quercetin efficiently prevented the increase of COX-2 (Figure 4).

Results of Western blot show the levels of HMGB1 and iNOS expression in lung tissue are significantly increased at 24 h after LPS challenge, which could be reversed by quercetin pretreatment (Figure 5(a) and (b)). We simultaneously determined the effects of quercetin on NF-κB. As shown in Figure 5(a) and (b), NF-κB p65 phosphorylation was increased with total p65 unchanged after LPS stimulation, and drug treatment also suppressed NF-κB p65 activation.

Discussion

In the present study, it was shown that quercetin treatment increased the survival rate and attenuated LPS-induced ALI in a mouse model of sepsis (Figure 6). Quercetin reduced

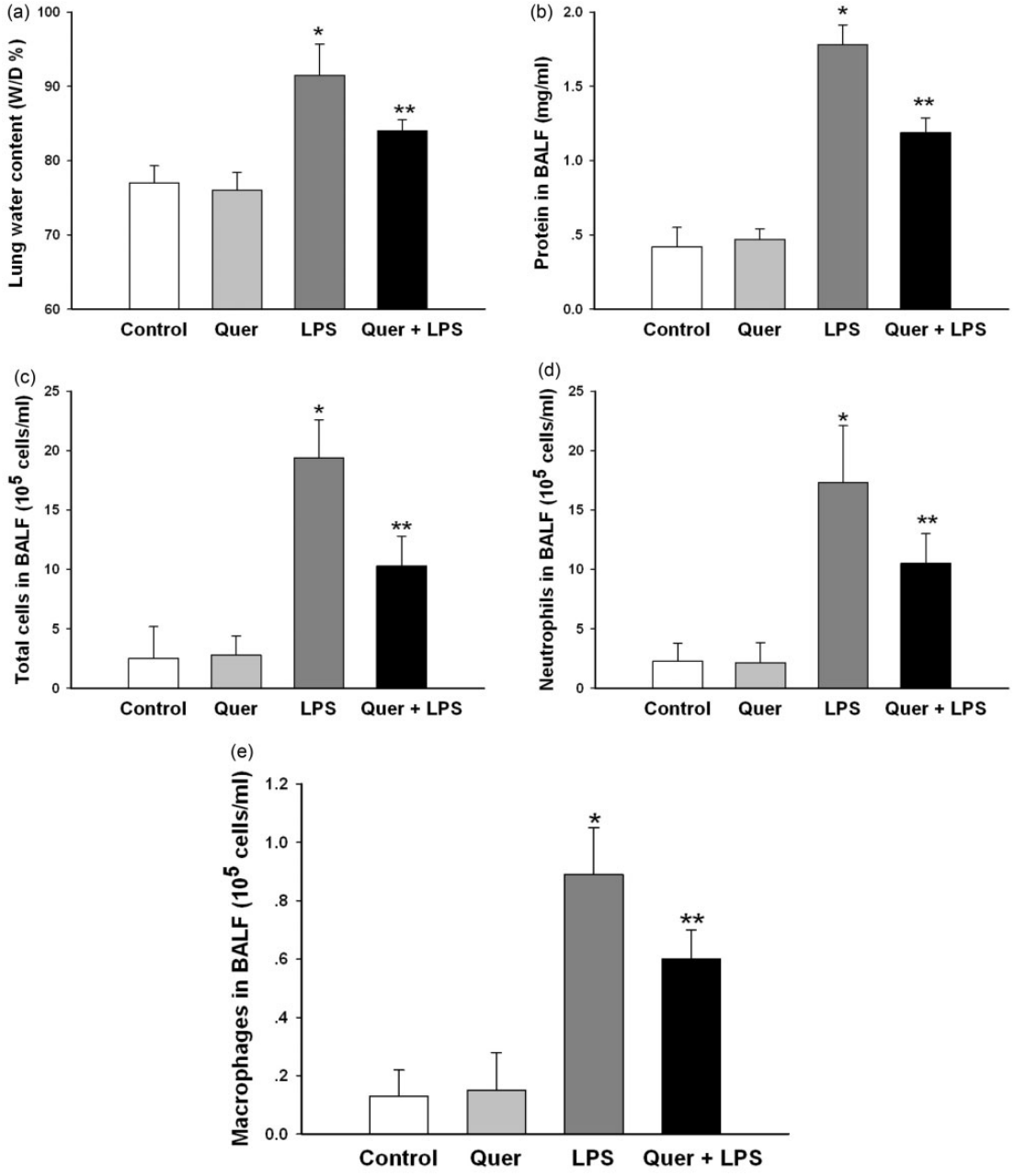

Figure 3 Effect of quercetin on lung edema, protein concentration and inflammatory cell counts in BALF. Mice received vehicle/quercetin gavage once daily for one week, and PBS/LPS (10 mg/kg) was injected intraperitoneally at day 8 ($n = 10$ each group). The mice were killed after 24 h, and the BALF and lung tissue was then collected. Lung water content (a), protein concentration (b), the numbers of total cells (c), neutrophils (d), and macrophages (e) in the BALF were estimated as described in the Methods section. *$P < 0.05$ vs control group, **$P < 0.05$ vs LPS group

Table 2 Effect of quercetin on MPO activities and MDA in lung tissues

	MPO activities (U/mg)	TBARS (nm MDA/mg)
Control	0.40 ± 0.13	0.610 ± 0.130
Quer	0.35 ± 0.18	0.553 ± 0.077
LPS	1.50 ± 0.24*	1.680 ± 0.134*
Quer + LPS	$1.15 \pm 0.17^{\#}$	$0.978 \pm 0.097^{\#}$

Note: Lung homogenates were prepared for determination of MPO activities and MDA according to protocols 24 h after LPS/PBS challenge ($n = 10$).
*$P < 0.05$ vs control.
$^{\#}P < 0.05$ vs LPS.

the secretion of cytokines including TNF-a, IL-6, and IL-1β in serum, and elevated the expression of IL-10, an important anti-inflammatory and immunoregulatory cytokine. More importantly, this was followed by an improvement in several inflammatory parameters, including the vascular permeability alterations and inflammatory cells recruitment to the lung and BALF. Meanwhile, it reduced levels of oxidative stress markers such as MPO activity and MDA in lung tissue. Together, these findings showed that quercetin has potential therapeutic effects for ALI.

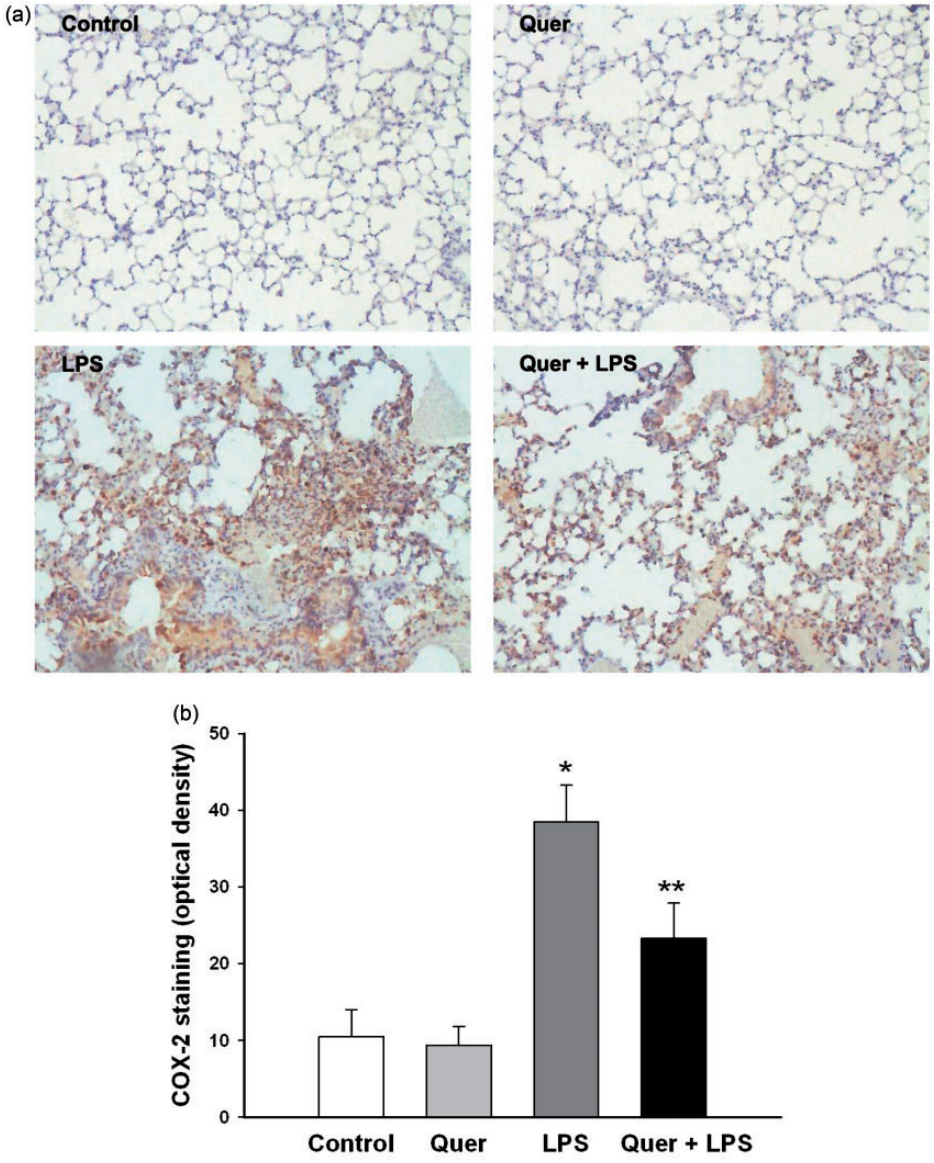

Figure 4 Immunohistochemistry analysis of the effect of quercetin pretreatment on COX-2 protein expression in lung tissue. Representative images from three separate experiments are shown (magnification 100×). The mean intensity of COX-2 staining was determined from image analysis and represented as arbitrary units ($n = 5$ each group). *$P < 0.05$ vs control group, **$P < 0.05$ vs LPS group. (A color version of this figure is available in the online journal.)

It was shown here that administration of quercetin substantially elevated the survival rate of septic mice and inhibited systemic inflammatory response to endotoxin-induced sepsis in mice, as evidenced by decreased serum levels of TNF-α, IL-1β, and IL-6. These inflammatory cytokines are perhaps the most extensively studied group of mediators involved in sepsis[30] and ALI.[31] Traditionally, sepsis was viewed as an immunoparalysis state characterized by impaired innate and adaptive immune responses to invasive microbial pathogens. Cytokines are a group of endogenous inflammatory and immunomodulating proteins, which have a crucial role in the complex pathophysiology underlying sepsis. The imbalance between inflammatory and anti-inflammatory cytokines may lead to endothelial dysfunction and leakage syndrome, which is associated with hypotension, tissue hypoxemia, edema, and organ dysfunction.[32] Furthermore, IL-10, a cytokine with anti-inflammatory properties, was significantly increased after quercetin treatment in LPS-induced mice. The role of IL-10 in sepsis was demonstrated by numerous reports, both in experimental animal models of septic shock and humans with sepsis.[32] A single injection of recombinant IL-10 protein protected BALB/c mice from a lethal intraperitoneal injection of endotoxin. In contrast, the immunoneutralization of IL-10 reversed the ability of IL-10 to protect mice from lethal endotoxemia and led to elevated levels of circulating TNF-α in mice.[33] Therefore, the decreased levels of proinflammatory cytokines (including TNF-α, IL-1β, and IL-6) and increased levels of anti-inflammatory cytokines including IL-10, may partly underlie the protective effect of quercetin against LPS-induced lethal endotoxemia and ALI.

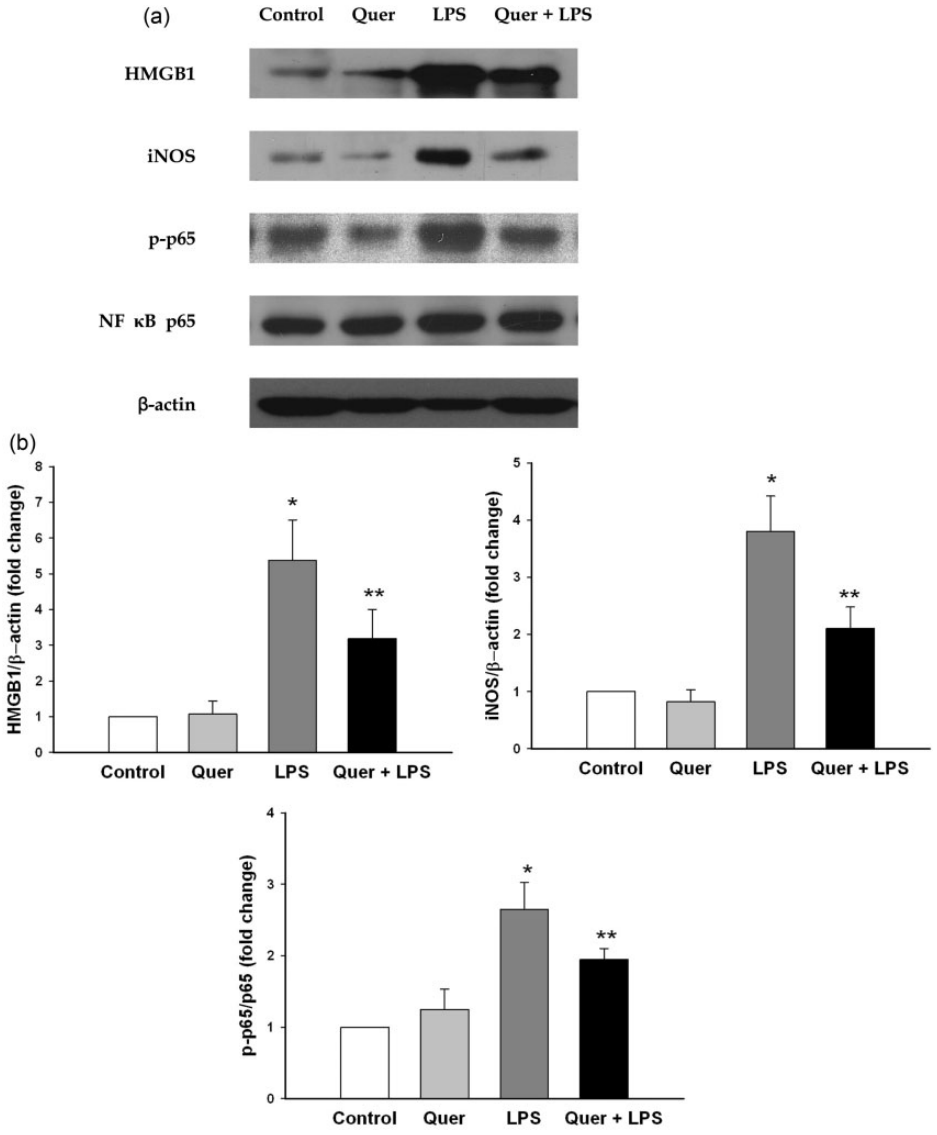

Figure 5 Western blot analysis of HMGB1 and iNOS expression and NF-κB p65 phosphorylation in the lung. (a) Representative blots are shown. (b) Densitometric quantification data are expressed as the intensity ratio of target proteins to *internal* standard ($n = 5$ in each group). *$P < 0.05$ vs control, **$P < 0.05$ vs LPS

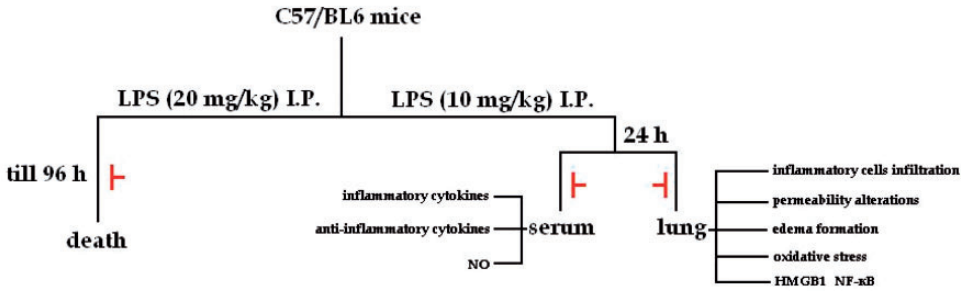

Figure 6 Quercetin pretreatment (red bars) improved survival time and lung injury in LPS-induced septic mice. The scheme shows some of the mechanisms whereby quercetin exerts important beneficial effects in ALI. (A color version of this figure is available in the online journal.)

Intraperitoneal injection of bacterial LPS, a known complement activator, represents a frequently used sepsis-associated model of ALI.[26] Apoptosis of endothelial cells develops rapidly following administration of LPS and causes modulation of epithelial permeability and disruption of the alveolar epithelial barrier. In addition, LPS induces changes in neutrophil deformability and the entrapment of neutrophils in the pulmonary capillaries,

followed by permeability alterations and edema formation. Pathologically, it is characterized by influx of neutrophils into the interstitium and broncheoalveolar space, disruption of endothelial and epithelial integrity, interstitial edema, and leakage of protein into the alveolar space.[6] In the current study, LPS exposure led to elevated lung W/D ratio, increased protein concentration and a large influx of white cells, which might be related with the increased epithelial and endothelial permeability. However, pretreatment with quercetin significantly inhibited the LPS-induced elevation of W/D ratio, protein leakage, white cells migration, and reduced the inflammatory histological changes in the lung tissue. These results indicated that quercetin had an anti-inflammatory activity during ALI, which were associated with its attenuation of the sequestration and migration of inflammatory cell into the lung tissue.

Our results also implicated a decreased oxidative stress in the lung of quercetin-pretreated mice. It is generally recognized that oxidative stress plays an important role in the development and manifestation of inflammatory response, including sepsis and ALI.[34] MPO, a heme protein abundantly expressed in neutrophils, is released during neutrophil activation. The major role of MPO is to aid in microbial killing, but under various conditions, MPO produces excess oxidant leading to tissue damages. Accordingly, the MPO activity reflects the function and activated state of PMNs in the pulmonary parenchyma.[35] In the present study, we found that quercetin significantly decreased the activity of MPO and lipid peroxidation products (MDA). The antioxidative capacities of quercetin are attributed to the phenolic hydroxyl groups within the molecule that have the optimal configuration for free radical scavenging.[36] Therefore, the protective effect of quercetin on LPS-induced ALI was partly explained by its anti-oxidative properties.

We simultaneously analyzed several key proteins involved in the development of ALI. HMGB1 has recently been proposed as one of the proinflammatory molecular and late mediators of endotoxin or sepsis lethality.[7] This ubiquitous DNA-binding nuclear protein is released by activated macrophages. It amplifies and extends the inflammatory response by inducing cytokine release through its functional proinflammatory cytokine domain resides within the primary amino sequence.[8] It was reported that quercetin significantly reduced circulating levels of HMGB1 in mice with established endotoxemia.[22] Moreover, HMGB1 may play a key role in the pathogenesis of clinical and experimental ALI. Expression of HMGB1 was apparent in epithelial lining fluid and alveolar macrophages of patients with ALI and mice instilled with LPS.[37] In addition, HMGB1 modulates the inflammatory cascade in LPS-activated macrophages by inducing proinflammatory cytokines such as TNF-α and IL-1β, while suppressing anti-inflammatory responses.[38] HMGB1 given intratracheally produced acute inflammatory injury to the lungs *in vivo*, including neutrophil accumulation, the development of lung edema, and increased pulmonary production of cytokines. Administration of anti-HMGB1 antibodies decreased the migration of neutrophils to the lungs as well as lung edema in LPS-induced ALI.[10] Targeting HMGB1 has proven to be a successful strategy in experimental models of diverse infectious and inflammatory diseases,[39] and here we observed that quercetin significantly inhibited HMGB1 expression in sepsis-induced ALI.

NF-κB is a protein transcription factor and typically a heterodimer of p50 and p65 subunit. Stimulation of cells with endotoxin triggers a series of signaling events that result in NF-κB translocation, followed by synthesis of multiple molecules involved in inflammatory responses, including iNOS and COX-2.[40] A variety of agents and strategies has been proven to successfully suppress experimental ALI development through an NF-κB mechanism.[41] In our study, Western blot analysis demonstrated that phospho-NF-κB p65 protein was significantly increased after LPS administration, accompanied by marked increases in iNOS and COX-2, and quercetin pretreatment prevented these manifestations. This finding is consistent with previous reports which suggested the inhibitory effect of quercetin on NF-κB in different animal models.[18,19,42]

Although many of the effects still need to be confirmed by human intervention trials, quercetin is a most promising compound for disease prevention and therapy. In this study, quercetin exerted potent anti-inflammatory and anti-oxidant effects, and decreased the exacerbation of the inflammation process during LPS-induced ALI in a mouse model. Collectively, these results suggest the potential of quercetin as a candidate for the treatment of sepsis-associated ALI.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data. LW, BW, and H-Li conducted the experiments; LW, DW, and H-Lu wrote the manuscript; and JC, HW, and YC carried out the review of the manuscript.

ACKNOWLEDGEMENTS

This study was funded by the National Natural Science Foundation of China (grant no. 81272594 to LW and 30972969 to HW) and the Natural Science Foundation of Zhejiang province (grant no. LY13H160016 to YC). We thank Dr Fengchun Lin for the pathology analysis.

REFERENCES

1. Ware LB. Pathophysiology of acute lung injury and the acute respiratory distress syndrome. *Semin Respir Crit Care Med* 2006;**27**:337–49
2. Tsushima K, King LS, Aggarwal NR, De Gorordo A, D'Alessio FR, Kubo K. Acute lung injury review. *Intern Med* 2009;**48**:621–30
3. Sevransky JE, Levy MM, Marini JJ. Mechanical ventilation in sepsis-induced acute lung injury/acute respiratory distress syndrome: an evidence-based review. *Crit Care Med* 2004;**32**:S548–53
4. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF. Neutrophils in development of multiple organ failure in sepsis. *Lancet* 2006;**368**:157–69
5. Raetz CRH, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev of Biochem* 2002;**71**:635–700
6. Grommes J, Soehnlein O. Contribution of neutrophils to acute lung injury. *Mol Med* 2011;**17**:293–307
7. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005;**5**:331–42
8. Li J, Kokkola R, Tabibzadeh S, Yang R, Ochani M, Qiang X, Harris HE, Czura CJ, Wang H, Ulloa L, Warren HS, Moldawer LL, Fink MP,

Andersson U, Tracey KJ, Yang H. Structural basis for the proinflammatory cytokine activity of high mobility group box 1. *Mol Med* 2003;**9**:37–45
9. Lutz WSJ. High mobility group box 1 protein as a late-acting mediator of acute lung inflammation. *Int J Occup Med Environ Health* 2004;**17**:245–54
10. Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ. Cutting edge: HMG-1 as a mediator of acute lung inflammation. *J Immunol* 2000;**165**:2950–54
11. Chirumbolo S. The role of quercetin, flavonols and flavones in modulating inflammatory cell function. *Inflamm Allergy Drug Targets* 2010;**9**:263–85
12. Boots AW, Haenen GRMM, Bast A. Health effects of quercetin: From antioxidant to nutraceutical. *Eur J Pharmacol* 2008;**585**:325–37
13. Manjeet KR, Ghosh B. Quercetin inhibits LPS-induced nitric oxide and tumor necrosis factor-α production in murine macrophages. *Int J Immunopharmacol* 1999;**21**:435–43
14. Kumazawa Y, Kawaguchi K, Takimoto H. Immunomodulating effects of flavonoids on acute and chronic inflammatory responses caused by tumor necrosis factor alpha. *Curr Pharm Des* 2006;**12**:4271–79
15. Liu J, Li X, Yue Y, Li J, He T, He Y. The inhibitory effect of quercetin on IL-6 production by LPS-stimulated neutrophils. *Cell Mol Immunol* 2005;**2**:455–60
16. Geraets L, Moonen HJJ, Brauers K, Wouters EFM, Bast A, Hageman GJ. Dietary flavones and flavonoles are inhibitors of poly(ADP-ribose)polymerase-1 in pulmonary epithelial cells. *J Nutr* 2007;**137**:2190–95
17. Nam NH. Naturally occurring NF-kappaB inhibitors. *Mini Rev Med Chem* 2006;**6**:945–51
18. Moreira AJ, Fraga C, Alonso M, Collado PS, Zetller C, Marroni C, Marroni N, González-Gallego J. Quercetin prevents oxidative stress and NF-kappaB activation in gastric mucosa of portal hypertensive rats. *Biochem Pharmacol* 2004;**68**:1939–46
19. Dias AS, Porawski M, Alonso M, Marroni N, Collado PS, González-Gallego J. Quercetin decreases oxidative stress, NF-kappaB activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats. *J Nutr* 2005;**135**:2299–4
20. Ying B, Yang T, Song X, Hu X, Fan H, Lu X, Chen L, Cheng D, Wang T, Liu D, Xu D, Wei Y, Wen F. Quercetin inhibits IL-1 beta-induced ICAM-1 expression in pulmonary epithelial cell line A549 through the MAPK pathways. *Mol Biol Rep* 2009;**36**:1825–32
21. Vidya Priyadarsini R, Senthil Murugan R, Maitreyi S, Ramalingam K, Karunagaran D, Nagini S. The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF-kappB inhibition. *Eur J Pharmacol* 2010;**649**:84–91
22. Tang D, Kang R, Xiao W, Zhang H, Lotze MT, Wang H, Xiao X. Quercetin prevents LPS-induced high-mobility group box 1 release and proinflammatory function. *Am J Respir Cell Mol Biol* 2009;**41**:651–60
23. Tunon MJ, Garcia-Mediavilla MV, Sanchez-Campos S, Gonzalez-Gallego J. Potential of flavonoids as anti-inflammatory agents: modulation of pro- inflammatory gene expression and signal transduction pathways. *Curr Drug Metab* 2009;**10**:256–71
24. Fukunaga K, Kohli P, Bonnans C, Fredenburgh LE, Levy BD. Cyclooxygenase 2 plays a pivotal role in the resolution of acute lung injury. *J Immunol* 2005;**174**:5033–39
25. Hanasaki Y, Ogawa S, Fukui S. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radic Biol Med* 1994;**16**:845–50
26. Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. *Am J Physiol-Lung Cell Mol Physiol* 2008;**295**:L379–99
27. Li H, Qiang Y, Wang L, Wang G, Yi J, Jing H, Wu H. Repair of lipopolysaccharide-induced acute lung injury in mice by endothelial progenitor cells, alone and in combination with simvastatin. *Chest* 2013;**144**:876–86
28. Wang L, Wang B, Li H, Lu H, Qiu F, Xiong L, Xu Y, Wang G, Liu X, Wu H, Jing H. Quercetin, a flavonoid with anti-inflammatory activity, suppresses the development of abdominal aortic aneurysms in mice. *Eur J Pharmacol* 2012;**690**:133–41
29. Liu L, Qiu H-B, Yang Y, Wang L, Ding H-M, Li H-P. Losartan, an antagonist of AT1 receptor for angiotensin II, attenuates lipopolysaccharide-induced acute lung injury in rat. *Arch Biochem Biophys* 2009;**481**:131–36
30. de Jong HK, van der Poll T, Wiersinga WJ. The systemic pro-inflammatory response in sepsis. *J Innate Immun* 2010;**2**:422–30
31. Goodman RB, Pugin J, Lee JS, Matthay MA. Cytokine-mediated inflammation in acute lung injury. *Cytokine Growth Factor Rev* 2003;**14**:523–35
32. Schulte W, Bernhagen J, Bucala R. Cytokines in sepsis: potent immunoregulators and potential therapeutic targets-an updated view. *Mediators Inflamm* 2013;**2013**:165974
33. Howard M, Muchamuel T, Andrade S, Menon S. Interleukin 10 protects mice from lethal endotoxemia. *J Exp Med* 1993;**177**:1205–08
34. Guo RF, Ward PA. Role of oxidants in lung injury during sepsis. *Antioxid Redox Signal* 2007;**9**:1991–2002
35. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol* 2005;**77**:598–625
36. Heijnen CGM, Haenen G, Oostveen RM, Stalpers EM, Bast A. Protection of flavonoids against lipid peroxidation: the structure activity relationship revisited. *Free Radic Res* 2002;**36**:575–81
37. Ueno H, Matsuda T, Hashimoto S, Amaya F, Kitamura Y, Tanaka M, Kobayashi A, Maruyama I, Yamada S, Hasegawa N, Soejima J, Koh H, Ishizaka A. Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am J Respir Crit Care Med* 2004;**170**:1310–16
38. El Gazzar M. HMGB1 modulates inflammatory responses in LPS-activated macrophages. *Inflamm Res* 2007;**56**:162–67
39. Yang H, Wang HC, Czura CJ, Tracey KJ. HMGB1 as a cytokine and therapeutic target. *J Endoxtin Res* 2002;**8**:469–72
40. Tsatsanis C, Androulidaki A, Venihaki M, Margioris AN. Signalling networks regulating cyclooxygenase-2. *Int J Biochem Cell Biol* 2006;**38**:1654–61
41. Fan J, Ye RD, Malik AB. Transcriptional mechanisms of acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2001;**281**:L1037–50
42. Comalada M, Camuesco D, Sierra S, Ballester I, Xaus J, Gálvez J, Zarzuelo A. In vivo quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-κB pathway. *Eur J Immunol* 2005;**35**:584–92

(Received January 1, 2014, Accepted April 3, 2014)