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Original Research Article

Myricetin attenuates lung inflammation and provides protection against lipopolysaccharide-induced acute lung injury by inhibition of NF-κB pathway in rats

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Abstract

Purpose: *To investigate whether myricetin ameliorates lipopolysaccharide (LPS)-induced acute lung inflammation (ALI) in a rat model, and to elucidate the probable molecular mechanism of action involved.*
Methods: *Myricetin (10, 20 and 40 mg/kg) was administered to rats 30 min after intratracheally administering LPS (5 mg/kg). BALF protein concentration, lung wet-to-dry weight ratio, myeloperoxidase (MPO) activity, cytokine production and migration of inflammatory cells were evaluated.*
Results: *Myricetin significantly ($p \leq 0.05$) attenuated lung inflammation as evident from the decreased wet-to-dry weight ratio of lungs, concentration of protein in the BALF, activity of MPO, cytokine production, and inflammatory cell migration. A decrease was also seen in TLR4, MyD88 and NF-κB expression. Additionally, an elevated antioxidant enzyme activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) was observed in all the treatment groups.*
Conclusion: *Myricetin provides protection against LPS-induced ALI. The underlying mechanisms of its anti-inflammatory action may include inhibition of NF-κB-mediated inflammatory responses.*

Keywords: *Acute lung injury, Anti-inflammatory, Myricetin, Inflammation, Cytokine, BALF, Flavonoid*

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INTRODUCTION

Acute lung injury (ALI) causes extensive disruption of blood-air barrier, leading to the development of acute respiratory failure and capillary edema, and hence making it a life-threatening clinical disorder. Together, these effects result in high mortality in patients [1]. Its pathology includes sustained and severe pulmonary inflammation leading to an increase in permeability of pulmonary vessels, pulmonary edema and neutrophil infiltration inside the alveolar cavities, leading to impairment of respiratory functions [2].

ALI is primarily caused due to Bacterial infection. The pathogenesis of ALI follows a multistep developmental process involving several inflammatory cells and mediators. Gram-negative bacteria have been recognized as the main cause of this infection. Lipopolysaccharide (LPS), present in the bacterial cell wall is an important virulence factor and is a primary factor known to cause the pulmonary inflammation [3,4]. LPS has been reported to cause ALI through several inflammatory mechanisms [5,6].

The transmembrane protein, Toll-like receptor 4 (TLR4) acts as a transduction molecule during the pathogenesis of ALI [7]. TLR4 activates a

signaling pathway that plays an important function in the innate immunity against pathogens [8]. TLR4 also acts as a primary sensor of LPS and activates a downstream signal cascade. LPS binds to TLR4 which induces a pro-inflammatory MyD-88 dependent pathway and causes the expression of cytokines which is induced by the NF-κB activation [9]. TLR4 mediated NF-κB activation also induces cytokine generation. These cytokines include TNF-α and IL-6. Such cytokines are known to further activate more NF-κB molecules in a feedback loop, thus amplifying the pro-inflammatory signal and playing a fundamental role in LPS-induced ALI [1].

Flavonoids are a group of natural substances known for their several anti-inflammatory and anti-oxidative properties [10]. Some flavonoids have previously been indicated for their role in ALI for example, Quercetin [11]. The natural flavonoid, Myricetin is abundantly found in plants, for example, berries, tea leaves and several medicinal plants [12]. Myricetin is known to possess anti-inflammatory properties [13]. Although anti-inflammatory properties of myricetin have been shown in previous studies, no study has yet shown its function in acute lung injury that was induced by LPS. In the present study, we explored, whether myricetin can potentially protect against LPS-induced ALI. Additionally, we also elucidated its potential molecular mechanism of action.

EXPERIMENTAL

Study animals and experimental design

Sprague–Dawley rats (adult male, n = 120, weighing 250 to 300 g) were provided by the animal house of Tongren Hospital (Shanghai, China). The animals were kept in a 12 by 12 hour light and dark cycle and controlled temperature (22 - 24 °C) and humidity (40 - 60 %). Moreover, the rats were fed *ad libitum,* with the standard rat diet and water. The animal study was carried out after receiving approval from the institutional ethical committee of Shanghai Jiao Tong University School of Medicine (approval no. EME6231-F), and followed the guidelines of Principles of Laboratory Animal Care [14].

Myricetin and *Escherichia coli* LPS were procured from Sigma (USA). The animals were assigned to six groups at random *viz.* 1) Control (phosphate buffered saline, negative control-treated); 2) Myricetin (40 mg/kg) group; 3) LPS group (5 mg/kg, administered through intratracheal instillation); (4) LPS + Myricetin (10 mg/kg) group; (5) LPS+ Myricetin (20 mg/kg) group; (6) LPS + Myricetin (40 mg/kg) group. Saline and Myricetin were intra-peritoneally (i.p.) administered 30 minutes after LPS challenge. The rats were euthanized at 7 hours post-LPS administration. The euthanasia was followed by the collection of lung samples for subsequent experimental analysis.

Estimation of 'wet-to-dry' weight ratio

After euthanasia, the extent of pulmonary edema in the experimental groups was assessed by measuring the 'wet-to-dry' weight of the lungs**.** For this, the left lung was removed and immediately rinsed in phosphate buffer saline (PBS) and then and weighed to determine the lung 'wet' weight. Same lung was subsequently desiccated for 72 hours at 80 °C temperature in an oven. The lung was again weighted to estimate its 'dry' weight. To quantify pulmonary edema, the ratio of two values was calculated.

Determination of the broncho-alveolar lavage (BAL) protein concentration and cell counts

Broncho-alveolar lavage (BAL) was done by lavaging with 5 ml of PBS and a gentle aspiration. Approximately 90 % of the BALF was recovered. Centrifugation of the BALF was carried out at 1000 g for 30 min at 4 °C temperature. The estimation of total protein in the supernatants was performed by the Bradford's method and using the Protein Assay Dye (Bio-Rad, USA). Protein estimation was performed by generating a standard curve of bovine serum albumin protein (BSA). Moreover, estimation of total cells count in the BALF was carried out post re-suspension of the cell pellet in 50 µl PBS.

Evaluation of pulmonary myeloperoxidase (MPO) activity

ALI is characterized by increased neutrophil accumulation in lungs. This elevated neutrophil accumulation can be detected by the MPO activity. For this, equal weights of the lung tissues were homogenized and centrifuged at 15000 g for 30 min at 4 °C temperature. The supernatant thus separated was then evaluated for MPO activity using the MPO ELISA kit (Hycult Biotech, Netherlands) as per the manufacturer's protocol and absorbance was measured at 450 nm. The absorbance of the samples was measured at 450 nm wavelength. All the evaluations were performed in replicates and the units were depicted in per gram.

Measurement of the pulmonary oxidative stress

Excised lungs were homogenized at 10 % (w/v) and subsequently centrifuged at 15000 g and 4 °C temperature for 30 minutes and the supernatant thus obtained was used for oxidative stress evaluations using colorimetric methods (spectrophotometer MARCEL S350 PRO).

The activity of superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GPx) was estimated using Bioxytech-MDA-586 (OxisResearch, USA) and Ransod and RanseL kits (Randox Laboratories, Crumlin, UK), respectively. The activity of catalase (CAT) was determined by the breakdown of hydrogen peroxide at 37 °C.

Examination of pulmonary histopathology

The upper lobes of the right lungs were excised from all the six experimental groups. The pulmonary tissues were fixed in 8 % paraformaldehyde for 24 hours, dehydrated in ethanol and sliced into 4 µm sections after embedding them in paraffin. Post sectioning, the tissues were deparaffinized and subsequently stained using the hemotoxylin-eosin (H&E) stain. Histopathological variations in lungs were analyzed under a light microscope.

Cytokine measurements

The concentrations of cytokines (IL-6 and TNF-α) in the BALF were estimated using ELISA kit (MN, USA), following the manufacturer's protocol.

Western blot analysis

Protein extracts (15 µg), separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were washed with 0.2 % Tween 20 and blocked with 2 % non-fat dry milk. Binding of primary antibodies onto the membranes was performed by incubating it with primary antibody solution in TBST and 2 % NFDM for 8 hours at 4 °C temperature.

The blots were probed with antibodies TLR4, NF-κB p65, p-NF-κB p65 and MyD88 (Santa Cruz Biotec. Inc. USA). The antibodies for IκB-α and p-IκB-α antibodies were procured from the Cell Signaling Tech., Inc. (USA). Finally, blots were incubated with horse radish peroxidase (HRP) conjugated secondary antibodies. The blots were developed using ECL system. Western blot densitometry was performed using ImageJ (NIH, USA) software.

Statistical evaluation

The quantitative results were depicted as means ± SEM. All the quantitative results were statistically analyzed by one-way analysis of variance (ANOVA). The analysis for multiple tests correction was performed by Bonferroni's method. All the statistical calculations were performed in Statistica 8 software program. The maximum threshold for statistically significant difference was set at $p < 0.05$.

RESULTS

Effect of myricetin on pulmonary hyperpermeability and edema

Exposure to LPS caused 2.16-fold increase in the wet-to-dry weight ratio of the lungs ($p < 0.01$, Figure 1A) when compared to the negative control-treated animals. Additionally, the BALF protein concentration also showed a significant increase of 5.4-fold ($p < 0.01$, Figure 1B) in comparison to the negative control-treated animals. These two parameters were significantly reduced by myricetin treatment ($p < 0.01$), whereas, no statistically significant difference was observed in the total concentration of BALF protein and the wet-to-dry weight ratio between the negative control and the myricetin treated animal groups.

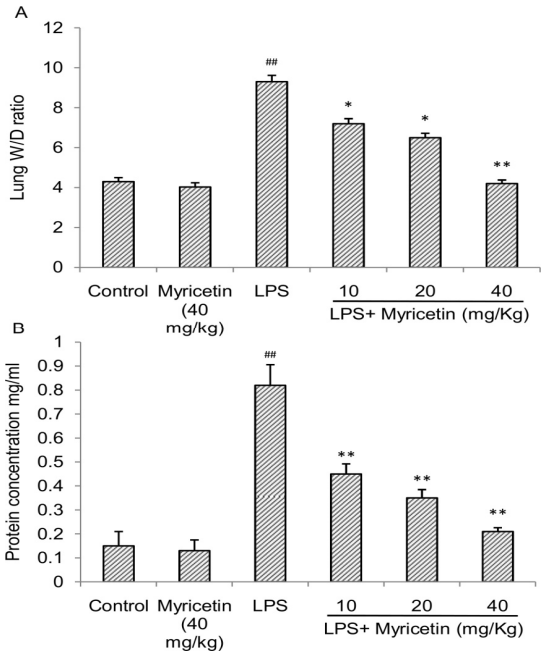

Figure 1: Effect of myricetin on (A) lung wet/dry weight ratio (B) total protein concentration in BALF in the LPS-induced ALI rats. The error bars indicate ± SEM (n = 20); # compared with control group; * compared with LPS group; # or * denotes $p \leq 0.05$; **##** or ** denotes $p \leq 0.01$

Effect of myricetin treatment on MPO activity in lung tissues

In comparison to animals of the negative control-treated group, the MPO activity was found to be significantly increased (5.46-fold) in LPS-induced animals (Figure 2). However, treatment with myricetin caused 1.82 to 3.90-fold decrease ($p < 0.01$) in the activity of MPO in animals that were previously challenged with LPS.

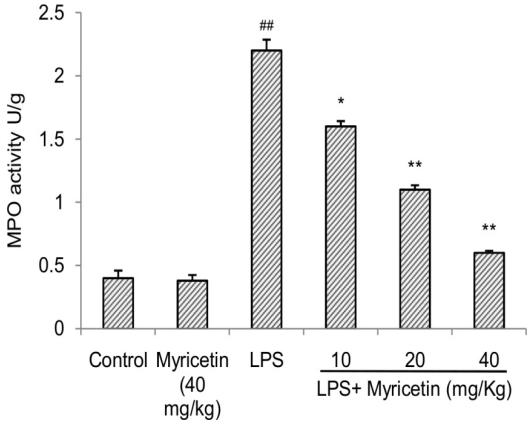

Figure 2: Effect of myricetin on the MPO activity. The error bars indicate ± SEM (n = 20); # compared with control group; * compared with LPS group; # or * denotes $p \leq 0.05$; ## or ** denotes $p \leq 0.01$

Effect of myricetin on the inflammatory cells count in the BALF

LPS-induction 10.5-fold increase ($p < 0.01$) in the total BALF cell count. The cell count of neutrophils and macrophages also followed a similar trend (Figure 3). Upon administration of myricetin, a significant and large decrease in the BALF cell count was observed.

Effect of myricetin on LPS-induced oxidative stress in lungs

The levels of MDA were increased significantly ($p < 0.05$) in animals induced with LPS in comparison to that of the negative control-treated group (Table 1). Myricetin treatment reduced the MDA levels by 29, 60.8 and 72.4 % for 10, 20, and 40 mg myricetin treatment groups, respectively. The activity of three antioxidant enzymes *viz.* SOD, CAT, and GPx was significantly reduced upon LPS treatment in relation to negative control-treated animals. However, their activity was significantly restored ($p < 0.05$) upon treatment of myricetin. In all the three antioxidant enzymes the highest activity was observed in the case of 40 mg/Kg treatment group.

Effect of myricetin on the histopathologic variations

Histological examination of the lung tissue was performed 7 hours post myricetin treatment. As depicted in the Figure 4A, the lung tissues of the negative control-treated animal group had a normal appearance and were devoid of any histopathological changes. It was observed that exposure to LPS treatment exhibited extensive damage to the lung tissue. The large scale intrusion of the inflammatory cells insides the pulmonary interstitium along with migration into the alveolar cavities (Figure 4C).

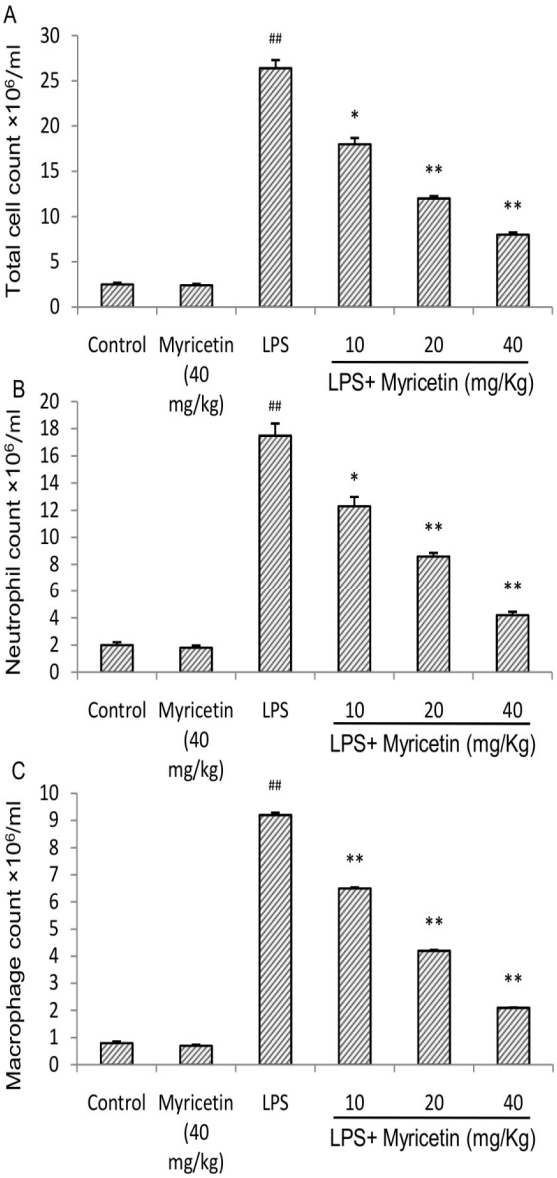

Figure 3: Effect of myricetin on the numbers of total cells (A), neutrophils (B) and macrophages (C) in BALF of LPS-induced ALI rats. The error bars indicate ± SEM (n = 20); # compared with control group; * compared with LPS group; # or * denotes $p \leq 0.05$; ## or ** denotes $p \leq 0.01$

Table 1: Effect of myricetin on oxidative stress biomarkers in the lung; mean ± SEM (n = 20); # compared with control group; * compared with LPS group; # or * denotes $p \leq 0.05$; ## or ** denotes $p \leq 0.01$

Treatment group	Malondialdehyde	Superoxide dismutase	Glutathione peroxidase	Catalase
Control	6.92 ± 0.94	274.56 ± 21.3	205.65 ± 15.15	21.84 ± 2.11
LPS	26.06 ± 1.51##	141.63 ± 16.32 ##	121.62 ± 19.21 ##	11.65 ± 1.54 ##
LPS+Myricetin (10 mg/kg)	18.5 ± 1.06*	156.23 ± 17.11*	165.47 ± 21.29*	13.91 ± 1.09*
LPS+Myricetin (20 mg/kg)	10.21 ± 0.97**	211.11 ± 11.21**	181.24 ±11.84**	16.52 ± 1.91*

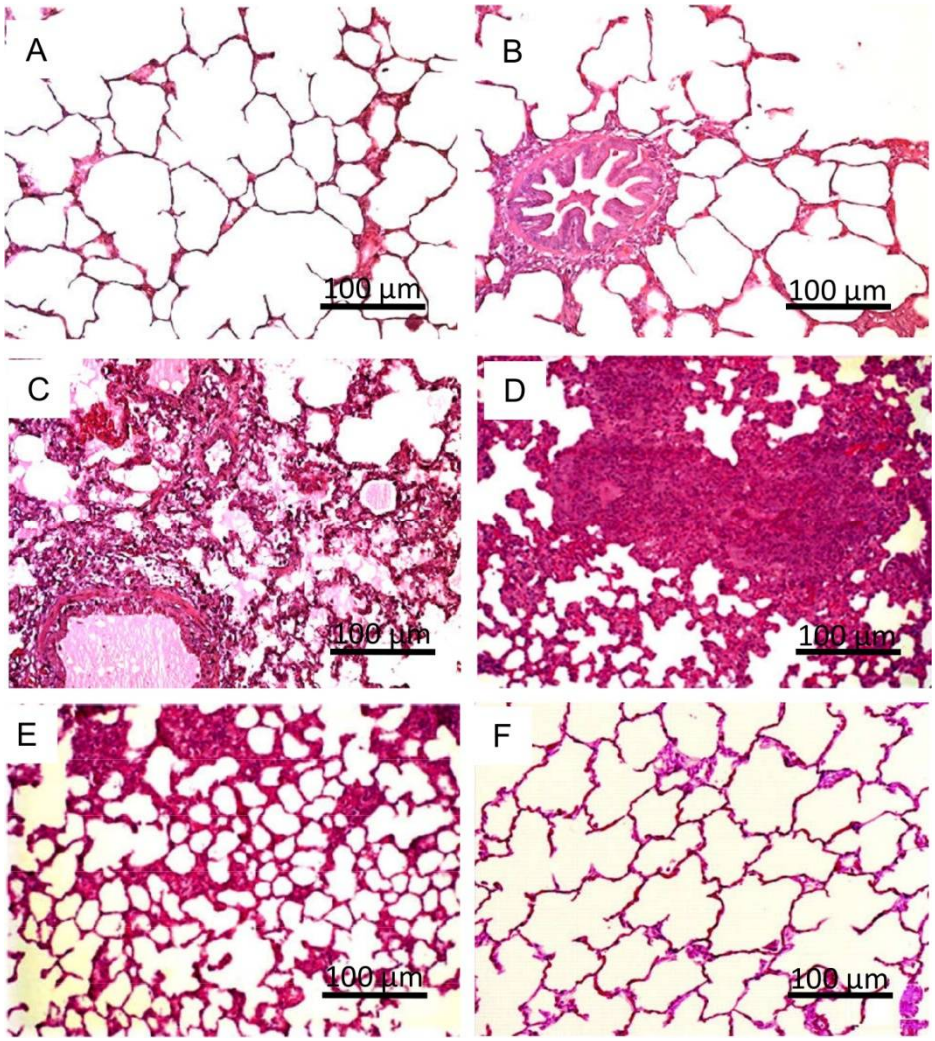

Figure 4: Histological estimation for the effects of myricetin on LPS-induced ALI. (A) Control, (B) myricetin (40 mg/kg) and (C) LPS, (D) LPS + myricetin (10 mg/kg), (E) LPS + myricetin (20 mg/kg), (F) LPS + myricetin (40 mg/kg)

Moreover, the LPS treatment also resulted into pulmonary edema, hemorrhagia in the stroma, alveolar collapse, and alveolar wall thickening. The histology of myricetin treated animal group had close similarities with that of negative control-treated group (Figure 4B). The LPS group that was treated with myricetin showed significant attenuation of the histopathological changes especially in the group of animals that were treated with 20 mg and 40 mg/kg of myricetin (Figure 4D, Figure 4E and Figure 4F).

Effect of myricetin on the BALF cytokine (TNF-α and IL-6) concentration

The anti-inflammatory action of myricetin was further assessed by evaluating the concentration of the pro-inflammatory cytokines i.e. TNF-α and IL-6 in the BALF. Cytokine assay was performed

by ELISA at 7 hours post-LPS administration. The BALF concentrations of IL-6 and TNF-α was significantly increased (Figure 5A and Figure 5B) in the LPS-induced group. However, myricetin treatment caused 27.5- and 29.6-fold ($p < 0.01$) increase in the concentration of IL-6 and TNF-α, respectively. Western blotting was used to detect the expression of TLR4 and MyD88. The levels of TLR4 and MyD88 proteins showed a significant up-regulation in the LPS-induced group (Figure 6). However, this increase was significantly down-regulated by myricetin treatment.

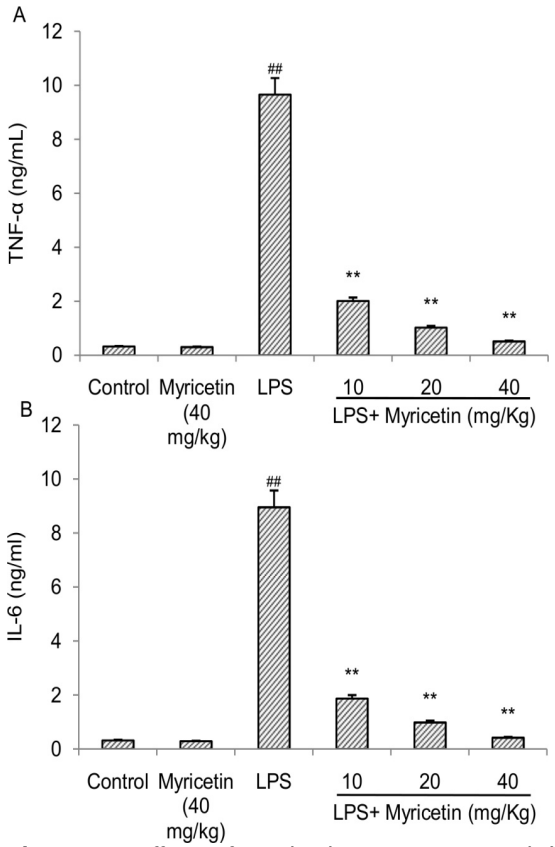

Figure 5: Effect of myricetin on TNF-α and IL-6 concentrations in the BALF. TNF-α (A) and IL-6 and (B). The error bars indicate ± SEM (n = 20); # compared with control group; * compared with LPS group; # or * denotes $p \leq 0.05$; ## or ** denotes $p \leq 0.01$

Effect of myricetin on NF-κB activation in lung tissues

IκB-α and NF-κB p65 phosphorylation was estimated using immunological (western blot) analysis. These phosphorylations reflected the NF-κB activation in pulmonary tissues. As illustrated in Figure 7, administration of LPS induction resulted in 17- and 11-fold increase ($p < 0.01$) in the NF-κB p65 and IκB-α phosphorylation in comparison to the negative control-treated group. However, a significantly large down-regulation in the phosphorylation of NF-κB p65 and IκB-α was observed when treated with myricetin.

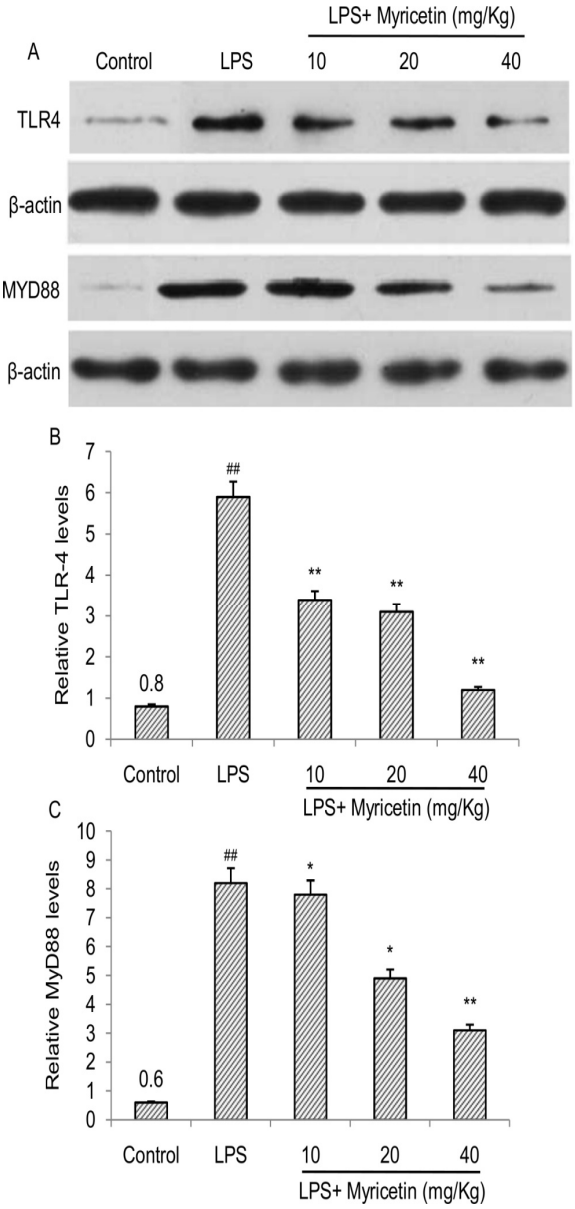

Figure 6: Effect of myricetin on the expression of TLR4 and MyD88 in pulmonary tissues. (A) Western blot analysis of TLR4 and MyD88. Data for TLR4 (B) and MyD88 (C) levels, compared with control. The error bars indicate ± SEM (n = 20); # compared with control group; * compared with LPS group; # or * denotes $p \leq 0.05$; ## or ** denotes $p \leq 0.01$

DISCUSSION

The anti-inflammatory and anti-oxidative effects of myricetin on the LPS-induced ALI in rats were identified. This study also attempts to determine the potential mechanism of action of myricetin on pulmonary inflammation. This is the first such study on myricetin, a natural flavonoid found in several plants.

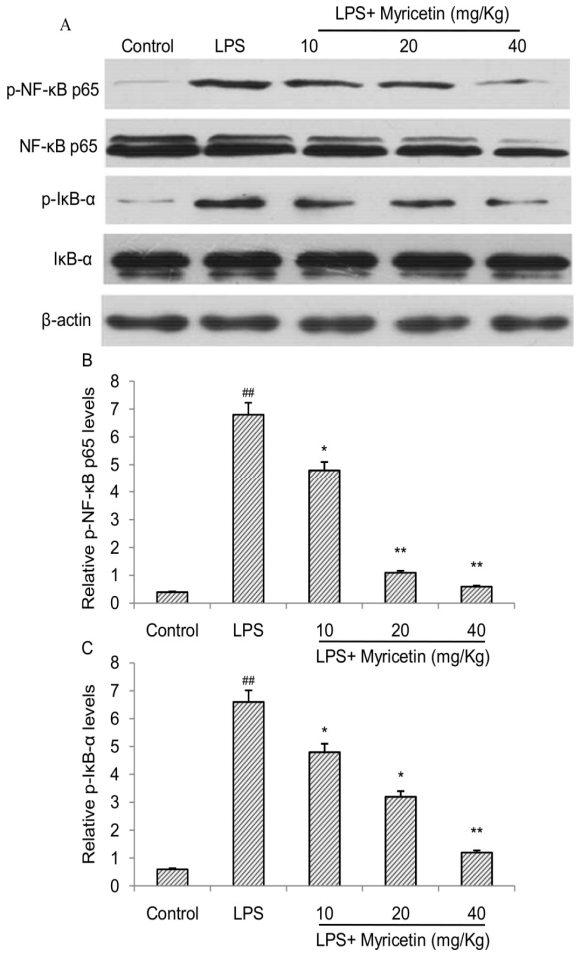

Figure 7: Effect of myricetin on phosphorylation of NF-κB p65 and IκB-α in lung tissues. (A) Western blot of p-NF-κB p65, NF-κB p65, p-IκB-α and IκB-α where β-actin was used as control, the phosphorylation of NF-κB p65 and (B) IκB-α (C) were detected by western blot densitometry. The error bars indicate ± SEM (n = 20); # compared with control group; * compared with LPS group; # or * denotes $p \leq 0.05$; ## or ** denotes $p \leq 0.01$

Results showed a significant attenuation of lung inflammation which was evident from parameters such as decreased wet-to-dry weight ratio of the lungs, the concentration of BALF protein, the activity of MPO, cytokine production and migration of inflammatory cells. Moreover, the expression of TLR4, NF-κB and MyD88 were also found to be significantly decreased. Moreover, in all the treatment groups a significantly large increase in the antioxidant enzyme (SOD, GPx and CAT) activity was observed.

Pulmonary inflammation and edema are typical signs of ALI [15]. Increase in the wet-to-dry weight ratio of the lungs and an elevated BALF protein concentration due to protein extravasation are strong indicators of pulmonary inflammation and edema [16]. Both of these parameters were significantly increased upon LPS-induction and there was a subsequent significant decrease after myricetin treatment. These results indicated attenuation of lung edema and leakage of vesicles in LPS-challenged rats after myricetin treatment.

Pulmonary edema indicated by the wet-to-dry ratio of lungs as well as the BALF protein concentration was further confirmed by the observations in lung histology. We observed several inflammatory changes in our rat ALI-model for example, pulmonary edema, thickening of pulmonary epithelium and migration of the inflammatory cells into the pulmonary interstitial spaces. A marked improvement in the histopathology of lungs was noticed when the LPS-induced rats were treated with myricetin. This observation provided a direct proof of the effectiveness of myricetin in the LPS-induced ALI, apart from the various measured parameters.

Infiltration of neutrophils into pulmonary cavities is one of the hallmarks of ALI [15]. After LPS-induction, neutrophils migrate across the pulmonary endothelium and epithelium and reach the alveolar spaces. Here, the activated neutrophils cause elevated production of ROS, the release of MPO, cytotoxic and pro-inflammatory mediators and increase in microvascular permeability [17]. Therefore, the persistent presence of neutrophils in the pulmonary cavity is a strong indicator of ALI. In this study, upon myricetin treatment, the total cell count in BALF and also neutrophil and macrophage count were significantly decreased. Neutrophils and alveolar macrophages are known to release MPO during acute inflammation of the lungs [18]. Increased activity of MPO is a strong indicator of neutrophil migration into the pulmonary parenchyma and the alveolar cavities [19]. MPO concentration has been used for estimation of LPS-induced ALI in many studies for example, D Rittirsch *et al* and CL Tsai *et al* [20,21]. In the myricetin treated rats, as compared to LPS treated mice, a marked reduction in the activity of MPO was found. This further indicated a reduced neutrophil infiltration and diminished inflammation of lungs.

ROS are produced by activated cytokines [22]. ROS can cause several damages to lung tissue such as DNA damage, protein oxidation, and lipid peroxidation. Heightened levels of MDS and reduced levels of pulmonary anti-oxidants (GPx, SOD, CAT) in the LPS-induced ALI were observed. These levels were significantly normalized upon myricetin treatment. In addition to being an anti-inflammatory agent, myricetin

was also known to possess antioxidant properties [23].

The release of cytokines is known to have an important function in the pathogenesis of ALI, for instance, the hyper permeability of the capillary-alveolar barrier [24]. The cytokines like IL-6 and TNF-α have also been reported to be involved in the development of LPS-induced acute lung injury [25]. In our results, the BALF levels of IL-6 and TNF-α showed marked increase upon induction with LPS. However, after administration of myricetin these levels decreased significantly and came down to almost normal levels (compared to negative control-treated).

Several studies in past have shown the regulatory function of NF-κB in the inflammatory and immunological responses in ALI [26]. TLR4-MyD88-NF-κB signaling pathway is widely reported to have an important function in the inflammatory signaling of ALI [9]. The concentration of these mediators was determined by western blot densitometry which indicated the role of this pathway in case of anti-inflammatory action by myricetin. In our results, the TLR4-MyD88-NF-κB pathway was found to be significantly upregulated in LPS-induced animals. This was also corroborated by the increased phosphorylation of NF-κB and IκB-α, observed in the western-blot. After treating LPS-induced ALI rats with myricetin, an effective inhibition of the TLR4-MyD88-NF-κB pathway was observed.

CONCLUSION

The decreased expression of cytokines and TLR4-MyD88-NF-κB pathway upon treatment with the plant flavonoid, myricetin, clearly indicated its anti-inflammatory effect on NF-κB signaling. Thus, myrecitin is a promising therapeutic agent for the treatment of ALI. However, further studies are required to elucidate the molecular mechanism responsible for the regulation of NF-κB signalling pathway by myrecitin.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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