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Diosmin downregulates the expression of T cell receptors, pro-inflammatory cytokines and NF-κB activation against LPS-induced acute lung injury in mice

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ABSTRACT

Diosmin, a natural flavonoid glycoside present abundantly in the pericarp of various citrus fruits. Because of its anti-inflammatory and antioxidant properties, it can be used in many diseases. In this study, we investigated the possible protective mechanisms of the diosmin on LPS-induced lung injury through inhibition of T cell receptors, pro-inflammatory cytokines and NF-κB activation. Animals were pretreated with diosmin (50 and 100 mg/kg, p.o.) for seven days prior to lipopolysaccharides (LPS) treatment. LPS administration increased neutrophils, monocytes, lymphocytes, total leukocyte count (TLC) and platelets which were decreased by diosmin. We observed that mice exposed to LPS showed increased malondialdehyde level and MPO activity whereas marked decrease in glutathione content. These changes were significantly reversed by treatment with diosmin in a dose dependent manner. Diosmin treatment showed a substantial reduction in T cell ($CD4^+$ and $CD8^+$) receptors and pro-inflammatory ($IL\text{-}2^+$ and $IL\text{-}17^+$) cytokines in whole blood. In addition, RT-PCR analysis revealed increased mRNA expression of IL-6, IL-17, TNF-α, and NF-κB in the LPS group, while reduced by treatment with diosmin. Western blot analysis confirmed the increased protein expression of IL-1β, TNF-α and NF-κB p65 in the LPS group and treatment of animals with diosmin reversed these effects. The levels of cytoplasmic p-IκB-α and p-NF-κB p65 expression also were mitigated by diosmin. The histological examinations revealed protective effect of diosmin while LPS group aggravated lung injury. These results support the potential for diosmin to be investigated as a potential agent for the treatment of lung injury and inflammatory diseases.

1. Introduction

Acute lung injury (ALI) is a major clinical complication with high rates of morbidity and mortality, characterized by alveolar-capillary membrane damage [1]. ALI, in more serious instances, can lead to acute respiratory distress syndrome [2], respiratory failure [3], and ultimately death. Severe hypoxemia, pulmonary endothelial injury, capillary leak, pulmonary edema, and neutrophil accumulation are the hallmark of ALI [4]. Furthermore, infiltration and activation of polymorphonuclear neutrophils (PMNs), results collateral damage to lungs tissue in response to environmental airborne toxicants such as ozone, particulate matter, and biogenic toxicants [5,6]. Mechanistically, in ALI, delay and abnormal secretion of neutrophil granulocytes and alter the release of cytokines are considered as the pathogenesis of apoptotic changes in the lungs tissue. Infiltrated neutrophils leads to production of reactive oxygen species (ROS) which play an important role in the pathogenesis of neutrophil-mediated airway injury and disease [7,8], resulted in an imbalance in oxidant/antioxidant factors [9,10]. Therefore, control of airways infiltration or activation of PMNs considered as therapeutic measures which may be useful in the mitigation of the morbidity in ALI [11]. Administration of LPS in mice increases oxidative stress and stimulates the release of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 by the airway epithelial cells and alveolar macrophages thus serving as a model of ALI

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[12,13]. IL-6 is one of the most common cytokines with strong influences on inflammatory responses and serves as a marker of acute phase reaction in the LPS-induced ALI model [14]. Nuclear factor-kappaB (NF-κB), a nuclear transcription factor, is required for maximal transcription and production of numerous cytokines including TNF-α and IL-6 that play a central role in inflammation and the pathogenesis of lung diseases [15]. Therefore, therapeutic approaches aimed at modulating NF-κB signalling may have potential therapeutic advantages for inflammatory diseases.

Diosmin (diosmetin 7-O-rutinoside), a natural flavone glycoside readily obtained by dehydrogenation of the corresponding flavanone glycoside hesperidin, is abundant in the pericarp of various citrus fruit [16,17]. Heusser and Osswald (1977) conducted several studies examining the sub chronic, chronic and teratogenic toxicity of diosmin. There were no systematic deviations in clinical, biochemical or hematological values, suggesting that there were no toxicological effects of diosmin [18]. However, there are no scientific reports available on the effect of diosmin against LPS-induced acute lung injury. Diosmin protects against cerebral ischemia/reperfusion injury through activating JAK2/STAT3 signal pathway in mice [19]. Pretreatment with diosmin improves cardiac function and suppresses oxidative stress [20]. Furthermore, diosmin treatment showed protection against ethanol-induced hepatic injury by inhibition of TNF-α and NF-κB activation [21].

Together, these investigations have provided the basis for potential biological applications of diosmin. The present study was designed to make an attempt to evaluate the preventive efficacy of diosmin against LPS-induced lung injury in mice. This study was conducted to assess the effect of diosmin on the levels of neutrophils, monocytes, lymphocytes, TLC, platelets, malondialdehyde and MPO activity. We, also investigated the effects of diosmin on T cell subsets ($CD4^+$ and $CD8^+$) and intracellular cytokines ($IL\text{-}2^+$ and $IL\text{-}17^+$) production and were analyzed by flow cytometry in heparinized whole blood. We furthermore assessed the effects of diosmin on the mRNA expression levels of IL-6, TNF-α, IL-17, and NF-κB p65 activation in lung tissue using reverse transcription real-time PCR (RT-PCR). IL-1β, TNF-α, NF-κB p65, p-IκB-α and p-NF-κB p65 was also demonstrated in lung tissue using Western blotting analysis. The effect of the diosmin treatment was further confirmed by histological investigation of lung tissue.

2. Materials and methods

2.1. Animals

In the present study male adult Balb/c mice, 6–7 weeks old and weighing 20–25 g was obtained from Experimental Animal Care Center, College of Pharmacy at King Saud University, Riyadh, KSA. They were housed under ideal laboratory conditions (12 h light/12 h darkness cycle, 45–55% relative humidity and temperature 23–25 °C), maintained on standard pellet diet and water ad libitum throughout the experimental period. All experiments were carried out according to the guidelines of the animal care and use committee at King Saud University.

2.2. Chemicals

Diosmin, Heparin and LPS were obtained from Sigma–Aldrich (St Louis, USA); Fluoroisothiocyanate (FITC), labeled CD4 and IL-17, Phycoerythrin (PE), labeled CD8 and IL-2 anti-mouse monoclonal antibodies; FcR blocking reagent fixation/Permeabilization and Permeabilization solutions were obtained from Miltenyi Biotech (Bergisch Gladbach, Germany). The primers used in the current study for gene expression were purchased from Applied Biosystems (Paisley, UK) and Genscript (Piscataway, USA). High Capacity cDNA Reverse Transcription kit and SYBR® Green PCR Master Mix were purchased from Applied Biosystems (Paisley, UK). TRIzol were purchased from Life Technologies (Grand Island, USA) Primary and secondary antibodies used for Western blotting were obtained from Santa Cruz (Dallas, USA). Nitrocellulose membrane was purchased from Bio-Rad Laboratories (Hercules, USA). Chemiluminescence Western blot detection kits were obtained from GE Healthcare Life Sciences (Piscataway, USA).

2.3. Experimental design

Mice were randomly divided into four groups: Group 1, normal control (NC) group, received 50 μL PBS only, intranasal (i.n.), under light ether anesthesia. Group 2, toxic group (LPS), received LPS 20 μg/mice in 50 μL of PBS, i.n., to induce lung injury on day 7. Group 3, treatment group, received LPS similar to group 2, and also diosmin (50 mg/kg, p.o.) for 7 days. Group 4 received LPS similar to group 2, and also diosmin (100 mg/kg, p.o.) for 7 days. Dose of diosmin were selected on the basis of previous studies [20,21].

All the mice were sacrificed at the end of the study by decapitation under ether anesthesia, as per the protocol. Whole blood was collected for hematology and flow-cytometery analysis and serum separation was done for biochemical analysis at 3000xg for 10 min. The mice lungs were isolated and washed in ice cold PBS and used for assessment of oxidative stress, histopathology, mRNA and protein expression. Samples were kept at −70 °C until analysis.

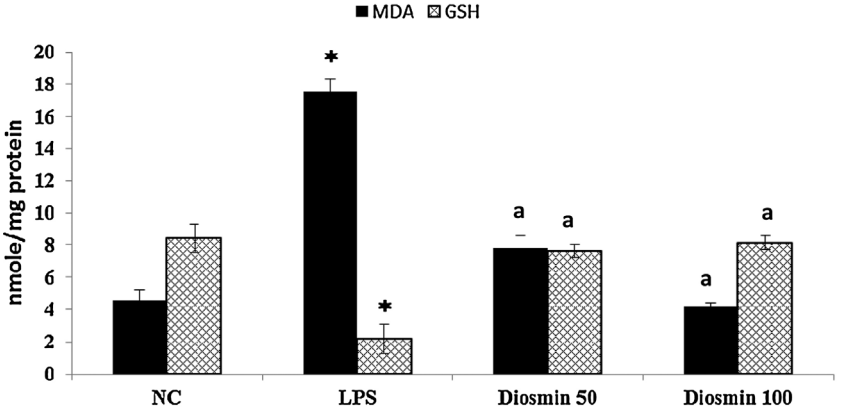

Fig. 1. Effects of Diosmin on MDA and GSH levels assessed in lung tissue. Each value indicates the mean ± S.E.M of six animals per group. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer post-test. $^*p < 0.05$ compared with control group; $^ap < 0.05$ compared with the LPS group.

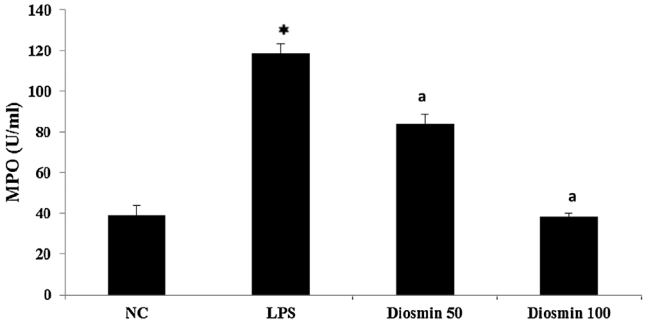

Fig. 2. Effects of Diosmin on MPO activity assessed in lung tissue. Each value indicates the mean ± S.E.M of six animals per group. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer post-test. $^{*}p < 0.05$ compared with control group; $^{a}p < 0.05$ compared with the LPS group.

2.4. Preparation of tissue homogenates

The lung tissues were removed quickly, perfused immediately with ice cold normal saline. A portion of lung sample were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing potassium chloride (1.17% w/v), using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 700 × *g* for 10 min at 4 °C in a refrigerated centrifuge to separate the nuclear debris. The supernatant so obtained was centrifuged at 9000 × *g* for 20 min at 4 °C to get the post mitochondrial supernatant (PMS).

2.5. Determination of lipid peroxides, measured as malondialdehyde (MDA)

Level of MDA, a product of membrane lipids peroxidation, was estimated in lungs tissue by the method of Okhawa [22], using the standard calibration curve prepared with tetraethoxy propane. MDA was expressed as nmoles of MDA per milligram of protein. Protein was estimated by the method of Lowry [23].

2.6. Determination of reduced glutathione (GSH)

GSH content was estimated in lung tissue by the method of Sedlak and Lindsay [24]. The absorbance of reaction mixture as read within 5 min of addition of dithiobis-2-nitrobenzoic acid at 412 nm against a reagent blank.

2.7. Determination of MPO Activity

MPO activity, which reflects the infiltration of neutrophils into the lung, was measured according to the modified method of Suzuki et al. utilizing 3,3′,5,5′ tetramethyl benzidine (TMB) in 96-well microtitre plates [25]. The reaction mixture was assayed for MPO activity by measuring the OD at 460 nm.

2.8. Determination of the number of $CD4^+$ and $CD8^+$ T cells in whole blood

Whole blood was collected in heparinized tubes from the retro-orbital plexus after the administration of light ether as an anesthetic for the assessment of the CD4 and CD8 surface marker. FITC labeled CD4 and PE labeled CD8 monoclonal antibodies were used to determine the percentage of $CD4^+$ and $CD8^+$ T cells. These antibodies were added directly to 100 μL of whole blood, protected from light, and red cells were lysed using a whole blood lysing reagent. Approximately 20 μL of the corresponding fluorescently labeled monoclonal antibodies were then added to the suspension of mononuclear leucocytes (Miltenyi Biotech, Germany), followed by incubation at room temperature for 20 min while protected from light, and the cells were then washed twice with 2 mL of wash buffer [26]. After one final washing, the stained cells were analysed immediately by flow cytometry. Analyses of the acquired data were accomplished using the Beckman Coulter CXP software application.

2.9. Flow cytometric analysis of $IL\text{-}2^+$ and $IL\text{-}17^+$ cytokines in heparinized whole blood

Heparinized whole blood was collected from the retro-orbital from all groups after the administration of light ether as an anesthetic. FITC labeled IL-17 and PE labeled IL-2 monoclonal antibodies were used to determine the percentage of $IL\text{-}2^+$ and $IL\text{-}17^+$ cytokines. Approximately, 100 μL of blood was pipetted directly into a 12 × 75 mm fluorescence-activated cell sorting (FACS) tube for the assessment of IL-2 and IL-17 in the presence of GolgiStop (BD Biosciences, USA) for intracellular staining. The RBCs were lysed for 8 min using 3 mL of 1x lysing solution (Miltenyi Biotech, Bergisch Gladbach, Germany). After centrifugation at 300 × *g* for 5 min, the supernatant was aspirated and 1 x fixation/permeabilizing solution (500 μL, Miltenyi Biotech) was added into the pellet and incubated for 10 min at room temperature in the dark. The cells were then centrifuged at 300 × *g* for 5 min; the supernatant was aspirated and 1 x permeabilizing solution (500 μL) and FcR blocking reagent (10 μL), were added into the pellet and incubated for 10 min at room temperature in the dark. After washing with 3 mL of wash buffer, cytokine-specific antibodies (20 μL) against IL-2, and IL-17 were added to the cells and incubated for 30 min at room temperature in the dark. Measurements were performed on a flow cytometer (Beckman Coulter, USA). The analysis of the acquired data was accomplished using CXP software application [27].

2.10. RNA Extraction and cDNA Synthesis

All extraction procedures were performed on ice using ice-cold reagents. The total cellular RNA from mouse lung tissue was isolated from homogenates using TRIzol reagent (Invitrogen®, Carslbad, California) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. RNA quality was determined by measuring the 260/280 ratio (>2.0). High-Capacity cDNA reverse transcription kit (Applied Biosystems®) was utilized to synthesize first strand cDNA, according to the manufacturer's instructions. Briefly, 1.5 μg of total RNA from each sample was added to a mixture of 2.0 μL of 10x reverse transcriptase buffer, 0.8 μL of 25x dNTP mix (100 mM), 2.0 μL of 10x reverse transcriptase random primers, 1.0 μL of Multi-scribe reverse transcriptase, and 3.2 μL of nuclease-free water. The final reaction mixture was kept at 25 °C for 10 min, and then heated to 37 °C for 120 min, followed by 85 °C for 5 min, and finally cooled to 4 °C.

2.11. Quantification of mRNA Expression in lung tissue via RT-PCR

Quantitative analysis of specific gene mRNA expression was performed via RT-PCR by subjecting the resulting cDNA obtained from the above preparation methods to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Bio systems®). The 25-μL reaction mixture contained 0.1 μL of 10 μM forward primer and 0.1 μL of 10 μM reverse primer (40 μM final concentration of each primer), 12.5 μL of SYBR Green Universal Master mix, 11.05 μL of nuclease-free water and 1.25 μL of cDNA sample. The primers used in these assays were purchased from Integrated DNA technologies (IDT, Coralville, IA), selected from PubMed and other databases, and are listed in Table 1. The fold change in the level of target mRNA between control and treated animals were corrected by the level of β-actin. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. The RT-PCR data were ana-

Table 1
Primers sequence. IL, Interleukin; Interferon gamma IFN-γ; TNF-α, Tumor necrosis factor-alpha; NF-kB, Nuclear Factor-KappaB; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

Targeted gene	Direction and sequence
IL-6	F: 5′-CCGGAGAGGAGACTTCACAG-3′ R: 5′-GGAAATTGGGGTAGGAAGGA-3′
IL-17	F: 5′-ATCCCTCAAAGCTCAGCGTGTC-3′ R: 5′-GGGTCTTCATTGCGGTGGAGAG-3′
TNF-α	F: 5′-GCGGAGTCCGGGCAGGTCTA-3′ R: 5′-GGGGGCTGGCTCTGTGAGGA-3′
NF-kB p65	F: 5′- GGACAGCACCACCTACGATG-3′ R: 5′-CTGGATCACTTCAATGGCCTC-3′
GAPDH	F: 5′-CCCAGCAAGGACACTGAGCAAG-3′ R: 5′-GGTCTGGGATGGAAATTGTGAGGG-3′

Table 2
Effects of diosmin on neutrophils, Monocytes, Lymphocytes, TLC and Platelets count in heparinized whole blood. D-50 = 50 mg/kg diosmin, D-100 = 100 mg/kg diosmin, WBC = White Blood Cells, LPS = Lipopolysaccharides, SEM = Standard Error of Mean. Each value indicates the mean ± S.E.M of six animals per group. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer post-test.

Groups	Neutrophils (%) Mean ± SEM	Monocytes (%) Mean ± SEM	Lymphocytes (%) Mean ± SEM	TLC ($10^3/\mu L$) Mean ± SEM	Platelets ($10^3/\mu L$) Mean ± SEM
Control	29.21 ± 1.572	2.18 ± 0.338	45.80 ± 2.192	8.20 ± 0.231	235.50 ± 10.012
LPS	63.75 ± 1.196*	10.63 ± 0.336*	81.50 ± 1.276*	13.76 ± 0.141*	675.75 ± 12.379*
Diosmin 50	44.63 ± 1.528[a]	5.60 ± 0.125[a]	72.29 ± 1.549[a]	11.56 ± 0.218[a]	420.50 ± 8.342[a]
Diosmin 100	32.00 ± 1.503[a]	3.86 ± 0.405[a]	62.45 ± 1.311[a]	10.09 ± 0.172[a]	259.25 ± 8.189[a]

[a] $p < 0.05$ compared with the LPS group.

lyzed using the relative gene expression (i.e., ΔD CT) method as described in Applied Biosystems User Bulletin No. 2. Briefly, the data are presented as the fold change in gene expression normalized to an endogenous reference gene (β-actin) and relative to a calibrator [28].

2.12. Western blot analysis

Protein was extracted from lung tissue as previously described [29]. Briefly, lung was washed in ice cold PBS, cut into small pieces, and homogenized separately in cold protein lysis buffer and protease inhibitor cocktail [30]. Total cellular proteins were obtained by incubating the cell lysates on ice for 1 h, with intermittent vortex mixing every 10 min, followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. Total protein was measured by the Lowry method [23]. Western blot analysis was performed using a previously described method [30]. Briefly, 25–50 μg of protein from each group was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred to nitrocellulose membranes (Bio-Rad, USA). Protein blots were blocked overnight at 4 °C, followed by incubation with primary antibodies against IL-1β, TNF-α, NF-κB, p-IκB-α and p-NF-κB p65 (Santa Cruz, USA) and peroxidase-conjugated secondary antibodies at room temperature. Bands were visualized using the enhanced chemiluminescence method (GE Health Care, Mississauga, Canada) and quantified relative to β-actin bands using the ImageJ® image processing program (National Institutes of Health, Bethesda, USA). Images were taken using a C-Digit chemiluminescent Western blot scanner (LI-COR, Lincoln, USA) (Table 2).

2.13. Histopathology assessment of lung tissue

The lungs tissue were harvested on day 7 following after 4 h of i.n. LPS administration, fixed for 4 days in 10% buffer formosaline, decalcified in decal solution (EDTA) in 5% formic acid embedded in paraffin and sectioned (3–4 μm). Tissue sections were stained with haematoxylin and eosin (H&E) for histopathological examination under light microscopy. Alveolar destruction, vascular proliferation, and inflammatory cell infiltration were assessed.

2.14. Statistical analysis

All data are presented as the mean ± S.E.M, and six animals are included in each group. Statistical analysis was performed using a one-way ANOVA followed by the Tukey–Kramer post-test. Each value indicates the mean ± S.E.M. of six animals. $p < 0.05$ is accepted as the level of significance; *$p < 0.05$ compared to the normal control (NC) group; [a]$p < 0.05$ compared to the LPS group. Statistical analysis was carried out using Graph pad prism 3.0 (La Jolla, CA).

3. Results

3.1. Effects of LPS and diosmin on neutrophils, monocytes, lymphocytes, TLC and platelets count

LPS is reported to cause alteration in hematological parameters [31]. Neutrophils are the most abundant white blood cell, constituting 60–70% of the circulating leukocytes. Administration of LPS to mice significantly ($p < 0.05$) decreased in neutrophils and monocyte count. Treatment with diosmin (50 and 100 mg/kg) reversed LPS-induced decreased in neutrophils and monocyte count. However lymphocyte, number of total white blood cells per microliter of blood and platelets count were increased significantly ($p < 0.05$) by LPS dose dependently (Table 1). Treatment with diosmin (50 and 100 mg/kg) ameliorated LPS-induced increase in lymphocytes, TLC and platelets count (Table 1).

3.2. Effects of LPS and diosmin on lipid peroxidation & GSH

In general, it is reported that oxidative stress induced membrane damage attenuated by supplementation of an antioxidant. MDA, a product of lipid peroxidation, is the biomarker for estimating the status of oxidative stress [32]. In this study, administration of LPS to mouse caused a significant ($p < 0.05$) increase in lungs MDA contents approximately by 4 fold. Treatment with diosmin (50 and 100 mg/kg) showed a dose dependent reversal in LPS-induced increase in MDA levels (Fig. 1). Consequently, administration of LPS to mouse caused significant ($p < 0.05$) decrease in GSH level approximately by 4 fold. Treatment with diosmin 50 mg/kg and 100 mg/kg showed a dose dependent reversal in LPS-induced decrease in GSH level (Fig. 1).

3.3. Effects of diosmin on MPO activity in LPS-induced acute lung injury

MPO activity in lungs was determined to assess the effects of diosmin on neutrophil accumulation. LPS challenge caused significant ($p < 0.05$) increase in lung MPO activity. Dose dependent

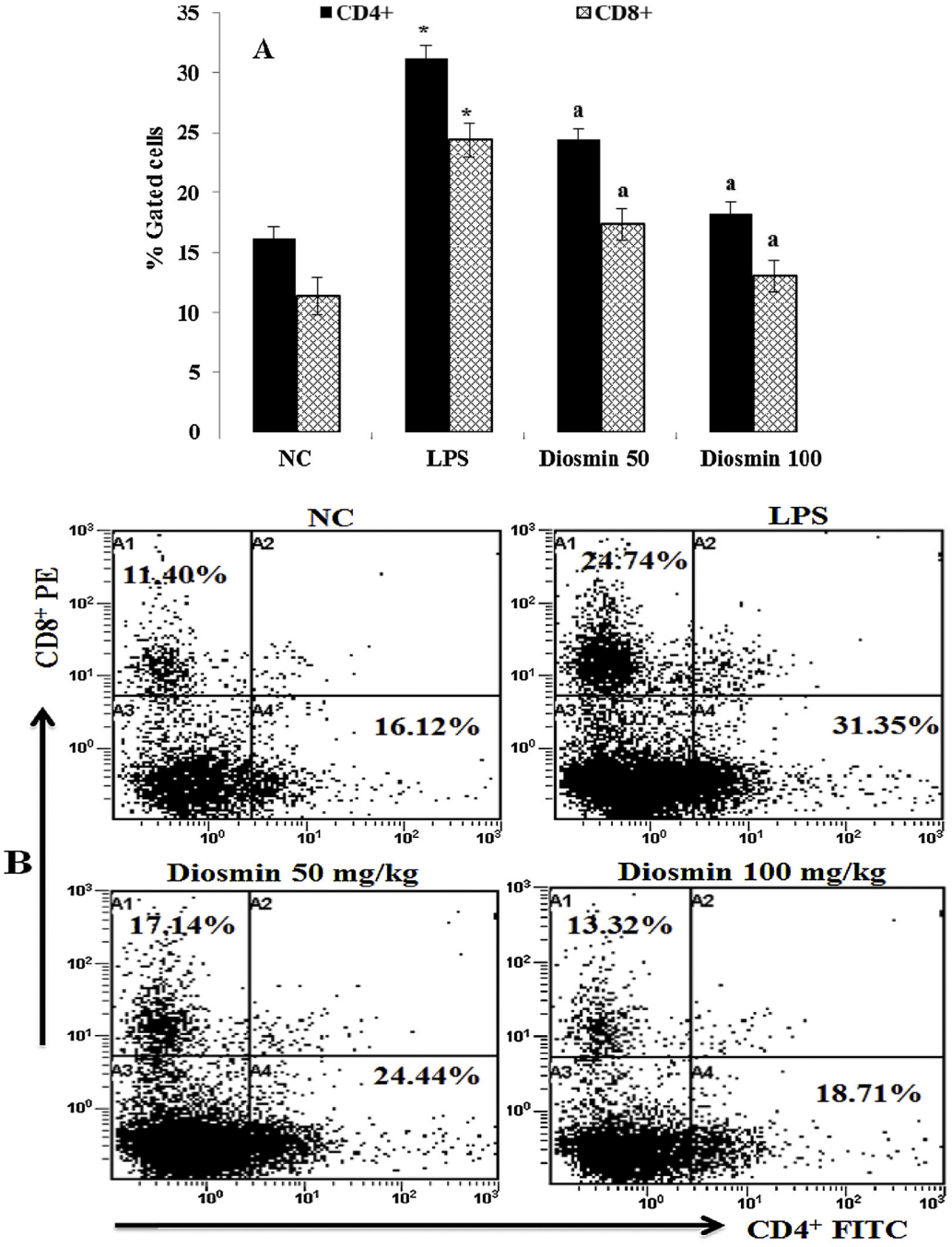

Fig. 3. (A) Effect of diosmin on $CD4^+$ and $CD8^+$ T cell numbers as assessed by flow cytometric analysis of heparinized whole blood. (B) Each dot plots represents one representative mouse from the normal control (NC), LPS, LPS with Diosmin 50 mg/kg, and Diosmin 100 mg/kg treated groups. Each value indicates the mean ± S.E.M of six animals per group. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer post-test. $^*p < 0.05$ compared with the normal control (NC) group; $^a p < 0.05$ compared with the LPS group.

reversal in MPO activity was seen with diosmin treatment (Fig. 2). This infers that diosmin treatment reversed LPS-induced increase neutrophils accumulation reflected by MPO activity in lungs.

3.4. Effect of diosmin on the number of T cell subsets in the whole blood

The number of $CD4^+$ T cells exhibited a substantial increase in the LPS group compared with the normal control (NC) group (Fig. 3). As shown in Fig, the groups of animals treated with diosmin at 50 and 100 mg/kg exhibited a significant inhibition of $CD4^+$ T cells relative to the LPS group. Fig. 3 demonstrates that LPS induced a significant increase in the number of $CD8^+$ T cells compared with the NC group. Treatment with diosmin produced a substantial reduce in $CD8^+$ T cells compared with the LPS group. The extreme effect was observed using diosmin at 100 mg/kg (Fig. 3).

3.5. Effect of diosmin on the intracellular IL-2^+ and IL-17^+ cytokines production

We next observed the effects of diosmin treatment on the intracellular IL-2^+ and IL-17^+ cytokines production. On day 7 after

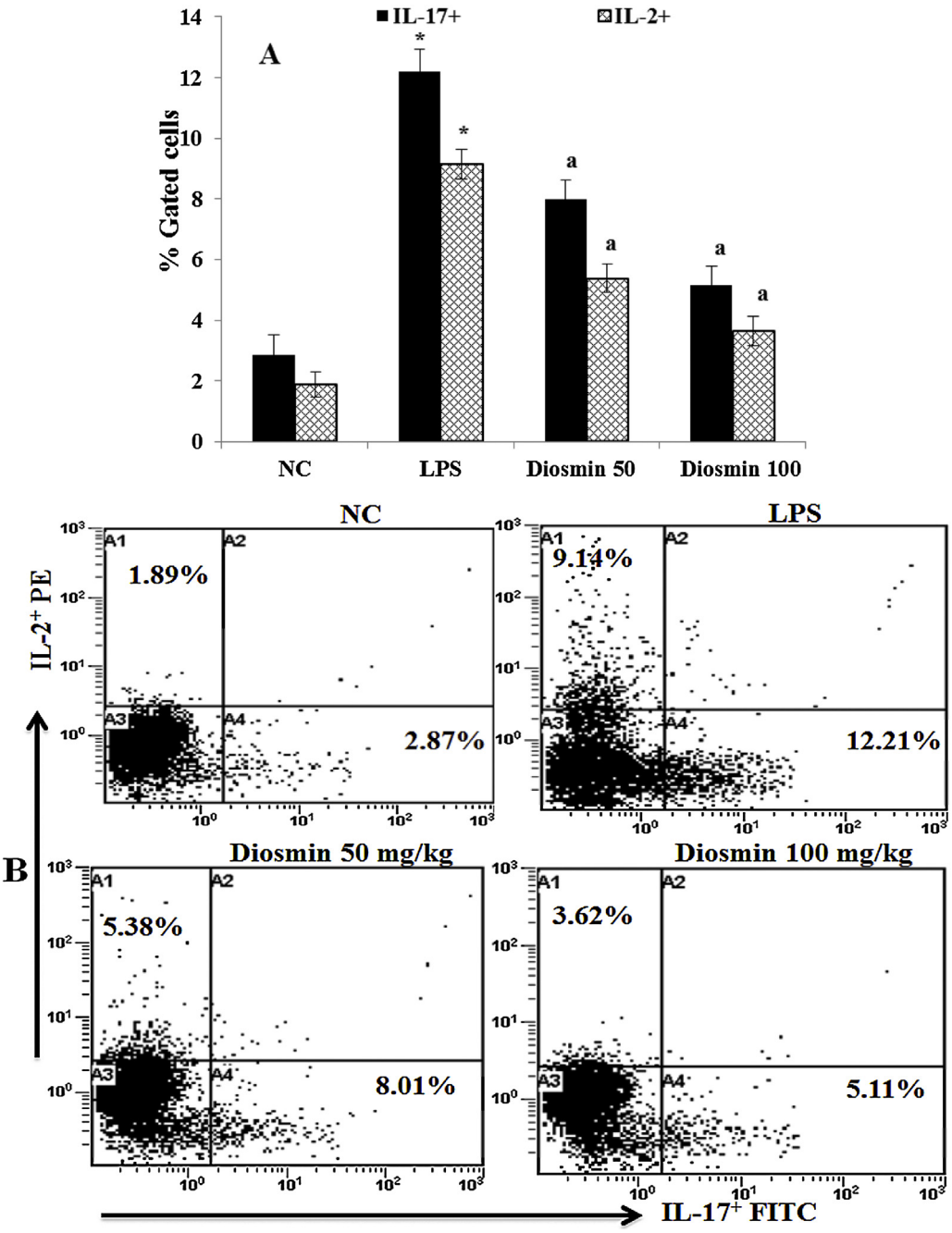

Fig. 4. (A) Flow cytometric analysis of the effect of diosmin on total intracellular IL-2$^+$ and IL-17$^+$ cytokines production in heparinized whole blood. (B) Dot plots represent one representative mouse from the normal control (NC), LPS, LPS with Diosmin 50 mg/kg, and Diosmin 100 mg/kg treated groups. Each value indicates the mean ± S.E.M of six animals per group. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer post-test. $^*p < 0.05$ compared with the normal control (NC) group; $^a p < 0.05$ compared with the LPS group.

treatment, whole blood was analyzed for intracellular IL-2$^+$ and IL-17$^+$ cytokines production by flow cytometry. While there were an increase in IL-2$^+$ and IL-17$^+$ cytokines production in the LPS group, treatment with diosmin at 50 and 100 mg/kg significantly inhibited these effects (Fig. 4).

3.6. *Effect of diosmin on IL-6, TNF-α, IL-17 and NF-κB p65 mRNA expression*

We further evaluated the effect of diosmin in LPS-induced lung injury by measuring mRNA levels of pro-inflammatory IL-6, TNF-α and IL-17 cytokines in the lung tissue. Our results showed that induction of lung injury by LPS significantly increased the levels of IL-6, TNF-α and IL-17 mRNA as compared to mice in the normal control (NC) group (Fig. 5). In contrast, as compared to mice treated with LPS only, pre-treatment of LPS injected mice with diosmin at 50 and 100 mg/kg resulted in a marked decrease in the mRNA expression levels of pro-inflammatory IL-6, TNF-α and IL-17 cytokines (Fig. 5). As illustrated in Fig. 5, there was significant difference in transcription factors of NF-κB p65 mRNA expression level between NC and LPS groups. LPS group markedly increased NF-κB p65 as compared with NC group. In the group treated with diosmin at 50 and 100 mg/kg the expression level of NF-κB p65 triggering were inhibited as compared with LPS group (Fig. 5). This inhibition was more prominent in the diosmin at 100 mg/kg treated group compared with the LPS group (Fig. 5).

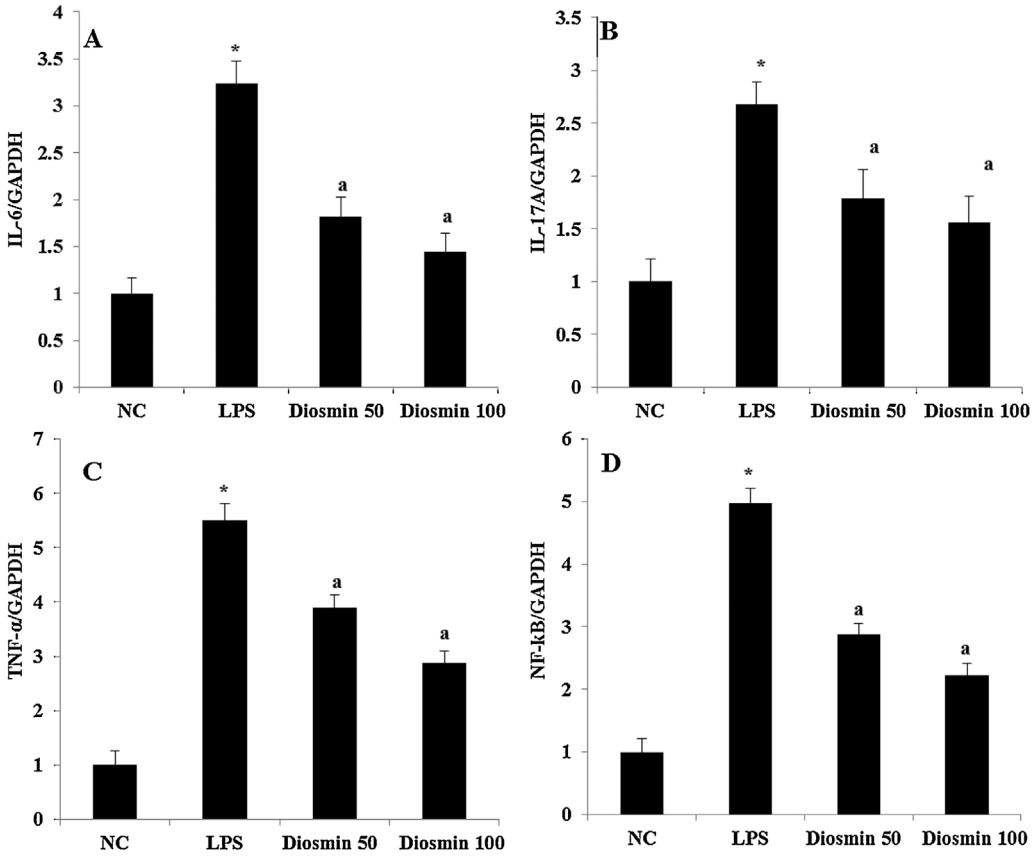

Fig. 5. Effect of diosmin on expressions of IL-6 (A), IL-17A (B), TNF-α (C) and NF-κB (D) mRNA. mRNA expressions were measured by quantitative RT-PCR analysis. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer post-test. $^{*}p < 0.05$ compared with the normal control (NC) group; $^{a}p < 0.05$ compared with the LPS group.

3.7. Effect of diosmin on IL-1β, TNF-α and NF-κB p65 protein expression

We studied the effects of diosmin on protein expression of IL-1β, TNF-α and NF-κB p65 pro-inflammatory mediators. Western blotting analyses of lysates generated from lung tissue isolated from untreated control mice, LPS-injected mice, and LPS-injected mice pre-treated with diosmin revealed that the LPS-injected group had significantly increased IL-1β protein expression as compared to the NC group. Importantly, treatment of mice with diosmin at 50 and 100 mg/kg significantly prevented the stimulation of IL-1β (Fig. 6). Similarly, TNF-α protein expression was significantly elevated after LPS administration and was markedly reduced by diosmin treatment as compared to LPS-injected group (Fig. 6). As expected, expression of NF-κB p65 protein in the lung tissue was significantly augmented in LPS alone group compared to the untreated normal control (NC) group, and treatment with diosmin significantly diminished the LPS-induced activation of NF-κB p65 (Fig. 6).

3.8. Effect of diosmin on phosphorylated IκBα and NF-κB p65 levels

To elucidate the molecular mechanisms involved in the attenuated inflammatory responses by diosmin, we examined the effects of diosmin on IκB/NF-κB p65 pathway. Diosmin inhibited LPS-induced phosphorylated IκB-α (p-IκB-α) and p65, a subunit of NF-κB, nuclear translocation were measured by Western blot analysis in mice lung tissue (Fig. 7). These results suggest that diosmin inhibits LPS-induced activation of NF-κB signaling by inhibiting IκB phosphorylation (Fig. 7).

3.9. Effects of diosmin on histopathological changes in LPS-induced acute lung injury

Normal morphological structures of lungs tissue were observed in the control group. However, treatment with LPS showed marked inflammatory response characterized by presence of interstitial edema, hemorrhage, infiltration of inflammatory PMNs, leucocytes and destruction of lung parenchyma. These changes were reversed in a dose dependent manner by the treatment with diosmin (Fig. 8).

4. Discussion

It has been reported that diosmin has broad-spectrum beneficial health effects including anti-oxidant, anti-inflammatory and anti-tumor effects and therapeutically used to improve the symptoms of venous and lymphatic vessel insufficiency [16,33–36]. It has also been reported that pre-treatment with diosmin, showed a gradual decrease in the expression of TNF-α [37]. Diosmin has been investigated in a number of animal models and human cancer cell lines, and has been found to be chemopreventive and antiproliferative [38,39].

Lung injury induced by LPS in rodents represents a frequently used ALI model, mimicking many features of ALI in humans [40]. Despite the fact that effective therapies for ALI have been developed, ALI still has a high mortality rate (35–45%), which has been unchanged since 1994 [41]. Several clinical studies have indicated that acute lung injury (ALI) is provoked by an excess of pro-inflammatory cytokines, produced by the active neutrophils, that accumulate in the lung, which can directly damage the pulmonary capillary endothelial cells and induce the release of other inflamma-

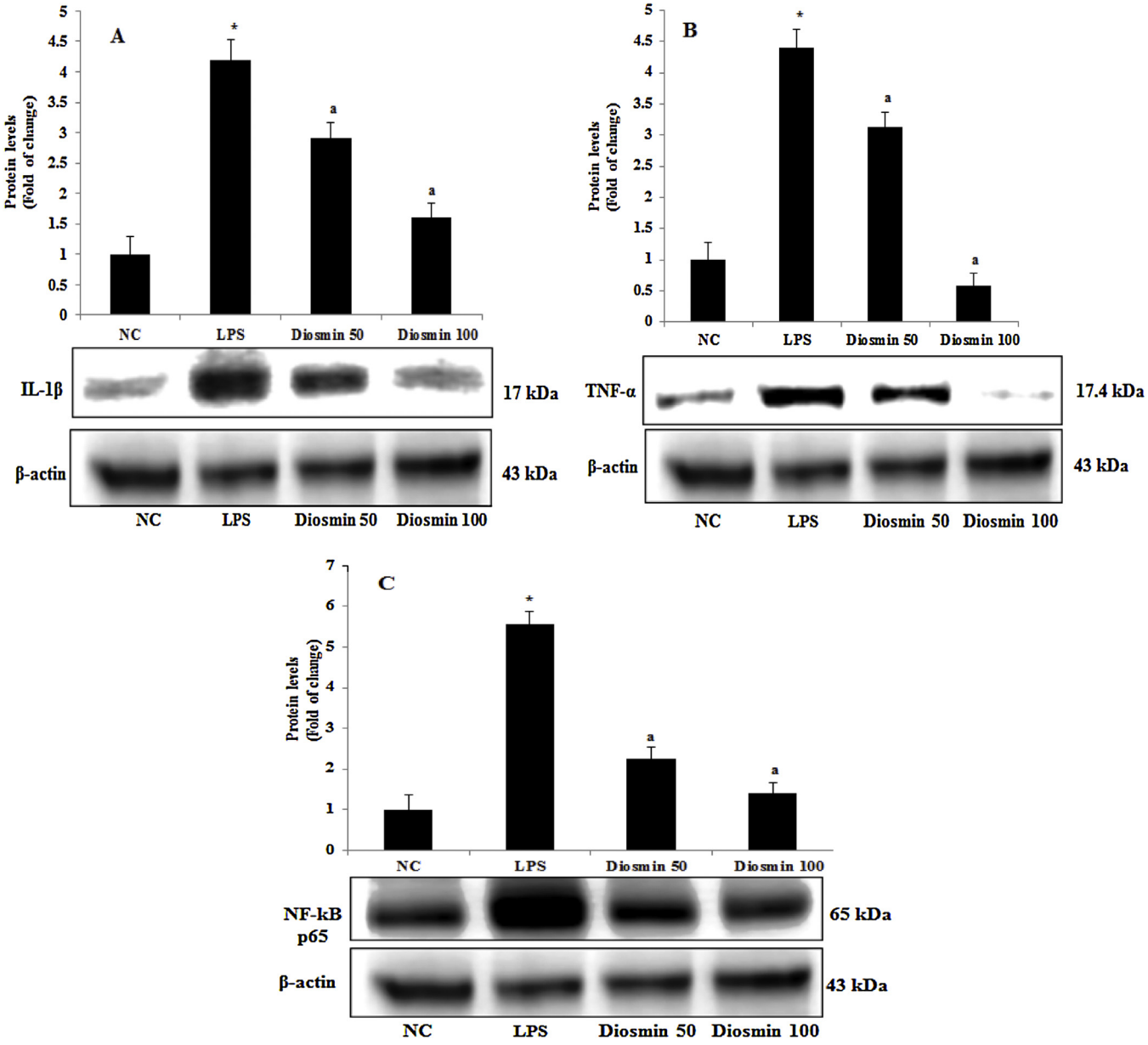

Fig. 6. Effect of Diosmin on the expressions of IL-1β, TNF-α and NF-κB p65 protein. Protein expressions were measured by Western blot analysis of lung tissue. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer post-test. $^{*}p < 0.05$ compared with the normal control (NC) group; $^{a}p < 0.05$ compared with the LPS group.

tory mediators [42]. LPS is one of the most potent cytotoxic inducers of inflammation, and of a cascade of intracellular events involved in cell death [43].

Neutrophils arrive quickly at sites of infection and form the first line of defense against invading micro-organisms. Neutrophil is the main inflammatory cell types in ALI [44,45]. In LPS-induced inflammation, neutrophils were activated by multiple inflammatory mediators [46]. Neutrophils significantly increased in LPS-induced ALI animal model [47]. MPO activity in pulmonary parenchyma, reflecting the activation of neutrophils, was largely up-regulated in ALI condition [47]. Administration of LPS has been reported to generate lipid peroxidation of membrane phospholipids and culminates in the formation of MDA [48]. The significant recruitment of neutrophils in LPS-induced ALI was diminished by diosmin in the current study. Accordingly, MPO activity was also notably suppressed by diosmin. These findings might partially explain that the reduction in neutrophils and down-regulation of MPO activation could prevent the animals from developing ALI and implied that the occurrence of ALI was due to the continuous and cumulative damages from the activation of neutrophils. Diosmin reduced MDA induced lipid peroxidation levels in LPS-injected lung tissue. Granules of neutrophils mainly contain MPO enzyme and thus adhesion and margination of neutrophils in the lungs parenchyma predicted by increased MPO activity [49]. In the present study, administration of LPS decreased GSH contents, while treatment with diosmin reversed LPS-induced decrease in lungs GSH contents in a dose-dependent manner. Increased intracellular GSH content might be due to up-regulation of enzymatic/nonenzymatic antioxidants or decreased ROS production in diosmin treated group.

LPS induces activation of innate immune cells such as monocytes and dendritic cells resulting in increased production of pro-inflammatory cytokines [50]. Thus, it is well established that LPS can act as a powerful adjuvant for T cell responses [51]. Tincati et al., reported that LPS-induces T-cells stimulation which signif-

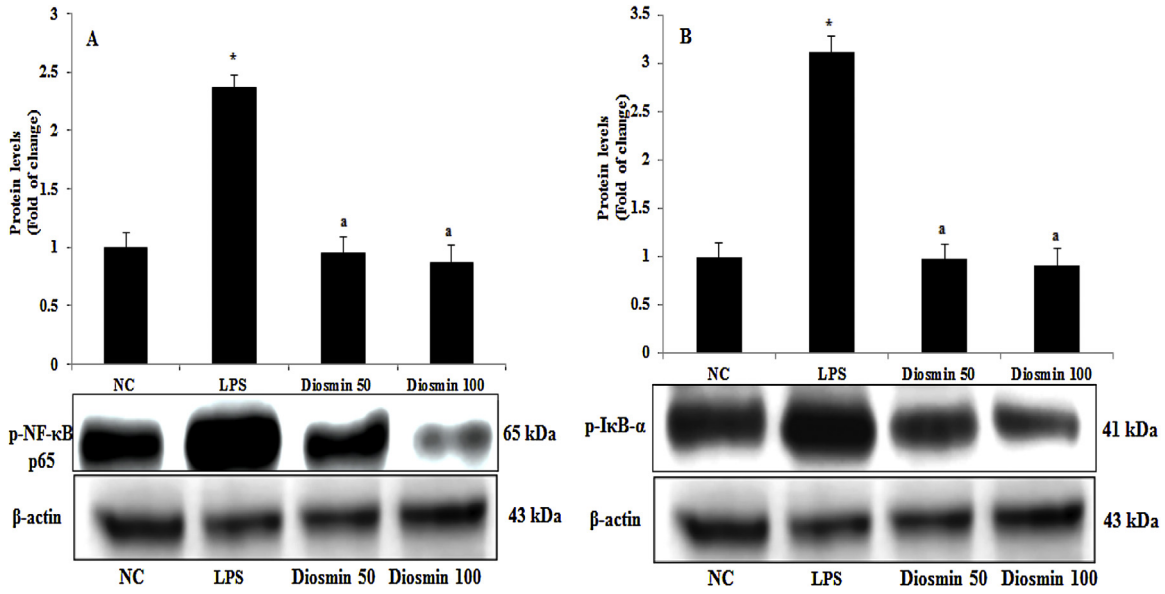

Fig. 7. Effect of diosmin on the expressions of p-NF-κB p65 and p-IκB-α protein. Protein expressions were measured by Western blot analysis of lung tissue. Statistical analysis was performed using one-way ANOVA followed by the Tukey–Kramer post-test. $^{*}p < 0.05$ compared with the normal control (NC) group; $^{a}p < 0.05$ compared with the LPS group.

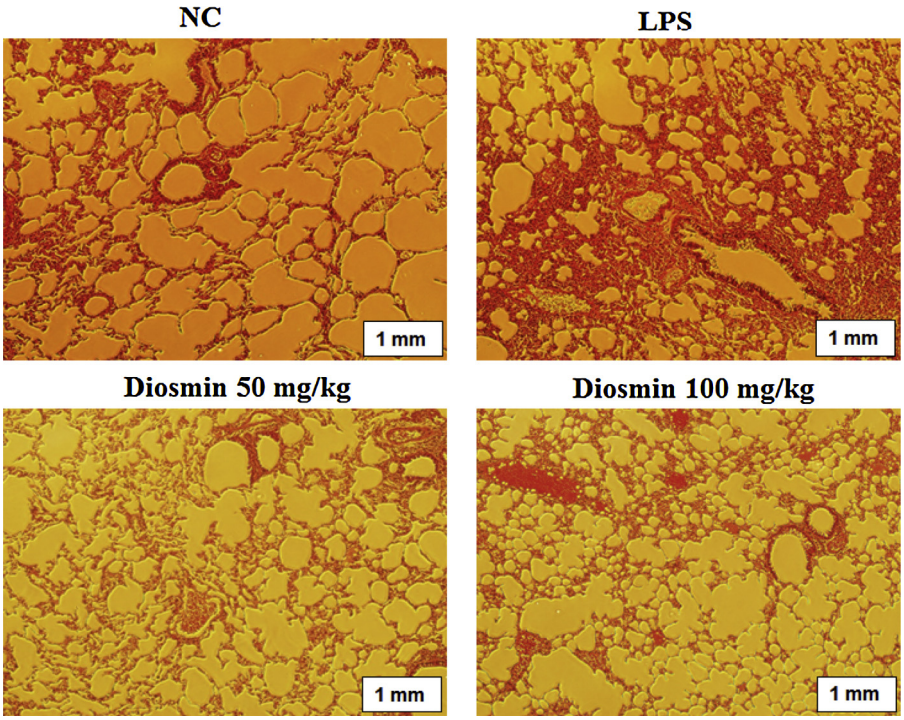

Fig. 8. Histopathological examination of lung tissue using light microscopy of sections stained with haematoxylin and eosin. Diosmin (50 and 100 mg/kg) was administered p.o for seven days.

icantly increases $CD4^+$ and $CD8^+$ cells [52]. It has been also found that, LPS-induced inflammation triggers mononuclear recruitment, along with an increased population of $CD4^+$ T lymphocytes [53]. The present results demonstrate that, inflammation caused by the LPS administration resulted, substantial increase in the production of $CD4^+$ and $CD8^+$ T cells. Considering the importance of $CD4^+$ and $CD8^+$ cells, we analysed the effect of diosmin on these parameters and found that diosmin significantly inhibited the production of $CD4^+$ and $CD8^+$ T cell receptors. The present study shows that the anti-inflammatory action of diosmin in the mouse model of ALI can be explained by the reduction of $CD4^+$ and $CD8^+$ T cell receptors at the site of inflammation.

Studies showed that inflammatory cytokines played critical roles in LPS-induced inflammatory response [54]. Intensive studies have been carried out on the potential pro-inflammatory properties of IL-17, this seem to be important in the neutrophil infiltration and activation at lung or airway inflammation [55,56]. In addition, IL-17 deficient mice have an impaired neutrophilic response to

allergen [56]. In the present study, we evaluated the protective role of diosmin in LPS-induced lung injury. The data presented here demonstrate that diosmin significantly inhibit the production of IL-2 and IL-17 cytokines. IL-2 and IL-17 clearly increased in whole blood after LPS exposure, while diosmin pre-treatment significantly decreased these cytokines compared to the LPS group. Consequently, diosmin confers protection to LPS-induced ALI mice model through the inhibition of IL-2 and IL-17 cytokines production.

Pro-inflammatory cytokines, notably TNF-α and IL-6, participate in the early development of inflammation and have been shown to play a crucial role in ALI [42]. These cytokines play a critical role in the process of acute lung injury and contribute to the severity of lung injury [57]. TNF-α is the primary cytokine responsible for initiating an acute inflammatory response [42]. IL-6 is one of the first and the key inflammatory cytokines that are upregulated in various inflammatory conditions [58]. Inhibition of TNF-α by its pharmacological inhibitor or mRNA transcription inhibitor exhibits protective effects in preclinical models of ALI [59]. We found that the mRNA expression levels of IL-6, IL-17 and TNF-α in the lung tissue were dramatically increased after LPS induction. Pretreatment with diosmin significantly lowered the mRNA levels of the LPS-induced pro-inflammatory cytokines, and the cytokine expression levels were similar to those seen in the NC group. In tissue inflammatory and immune response, NF-κB signaling plays a central role via transcriptionally regulating gene expressions [60]. In experimental animal models of ALI, NF-κB activation is increased [61]. Pharmacological inhibition of NF-κB pathway shows decreased production of pro-inflammatory mediators and protective effects against endotoxin-induced ALI in animals [61]. In the present study, we found LPS challenge induced the activation of NF-κB and the activation of NF-κB signaling was inhibited by diosmin treatment, suggesting that inhibition of NF-κB signaling plays a role in the protective effects of diosmin on ALI.

To further clarify the mechanism of the action of diosmin, we examined protein expressions of IL-1β, TNF-α and NF-κB in the lung tissue by Western blot analysis. Pro-inflammatory cytokines, such as IL-1β and TNF-α, are key mediators and play an important role in the pathogenesis of acute lung injury [62]. Therefore, we next examined whether diosmin could therapeutically attenuate LPS-induced inflammatory responses in the lungs. The expression of the pro-inflammatory cytokines IL-1β and TNF-α were significantly increased in the presence of LPS, whereas treatment with diosmin significantly decreased protein expressions of IL-1β and TNF-α as compared to LPS group. These results indicated that diosmin may reduce immune response in mice with LPS-induced ALI. To further characterize the mechanism of diosmin on ALI in LPS-challenged mice, we investigated the protein level of NF-κB p65. NF-κB is a critical transcription factor which plays an essential role in regulating expression of many inflammatory cytokines genes, including TNF-α and IL-6 [63]. Administration of LPS leads to increased activation of NF-κB p65 which was reversed by diosmin. Accumulating evidence indicated that diosmin had anti-inflammatory ability by inhibiting the expression of pro-inflammatory cytokines through blocking the activation of NF-κB pathways. Activation of the NF-κB pathway is central to the expression of several pro-inflammatory cytokines. In this study we focused to determine whether diosmin inhibits NF-κB activation by measuring p-IκB-α. Our data showed that pre-treatment of animals with diosmin markedly inhibited LPS-induced phosphorylation of IκBα and nuclear translocation of NF-κB p65 in mice lung. These results demonstrated that diosmin inhibited NF-κB signaling pathway activation.

In conclusion, our results showed that pretreatment with diosmin decreases inflammatory cell infiltration into the lung tissue, MPO activity and MDA content. Animals treated with diosmin not only attenuated T cell subsets, but also the production of pro-inflammatory cytokines. Histological investigation also showed that diosmin has substantial anti-inflammatory effects against LPS-induced ALI. Furthermore, the protective effects of diosmin are associated with attenuation of inflammatory reactions, and the compound's mechanism of action may involve blocking the NF-κB signaling pathway activation. Therefore we suggest that diosmin may be used as protective agent for preventing and treating LPS-induced ALI.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.09.001.

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