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ORIGINAL ARTICLE

Ameliorative Effects of Tannic Acid on Lipopolysaccharide-induced Sepsis and Acute Lung Injury in Mice

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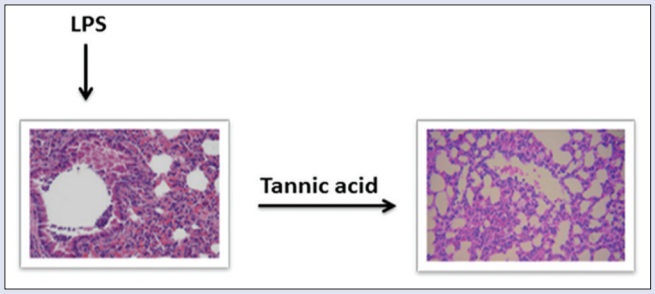
ABSTRACT

Background: Acute lung injury (ALI) caused by endotoxins is a severe complication causing lethal conditions. Prevention of the inflammatory response is necessary to overcome this condition. Tannic acid is a polyphenol known for its pharmacological properties such as antioxidant, antimicrobial, anti-inflammatory, anticancer, antitumor, and antimutagenic. This study aims to investigate the anti-inflammatory mechanism of tannic acid on lipopolysaccharide (LPS)-induced sepsis and ALI in mice. **Materials and Methods:** Male BALB/c mice were divided into five groups (n = 12) and induced with LPS (50 mg/kg body weight) and treated with tannic acid (25, 50, and 100 mg/kg) after 1 h. The bronchoalveolar lavage fluids were collected to determine the myeloperoxidase activity and levels of inflammatory cytokines (tumor necrosis factor-α, interleukin [IL]-6, and IL-1 β). Nuclear factor-kappa-β (NF-κβ) activities were examined through Western blot analysis, and hematoxylin and eosin staining was done for histopathological alterations of lung tissues. **Results:** Results of the study showed that tannic acid was able to prevent the infiltration of inflammatory cells and cytokines into the site of ALI which is also connected to the suppression of NF-κβ activation as shown in the Western blot analysis. The histopathological results further support these results. The safety of tannic acid was also proven on the survival of RAW264.7 cells. **Conclusion:** The anti-inflammatory mechanism of tannic acid on LPS-induced ALI and sepsis can be credited to the inhibition of inflammatory cytokines production mediated by NF-κβ pathway suppression.

Key words: Acute lung injury, anti-inflammatory, histopathology, lipopolysaccharide, nuclear factor kappa-β, tannic acid

SUMMARY

- Tannic acid is a polyphenol known for its pharmacological properties such as antioxidant, antimicrobial, anti-inflammatory, anticancer, antitumor, and anti-mutagenic
- The anti-inflammatory mechanism of tannic acid is attributed to the prevention of nuclear factor kappa-β activation along with the suppression of inflammatory cytokines and related mediator cells.



Abbreviations used: ALI: Acute lung injury; BALFs: Bronchoalveolar lavage fluids; NO: Nitric oxide; COX-2: Cyclooxygenase-2; iNOS: Inducible nitric oxide synthase; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1 β; IL-6: Interleukin-6.

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INTRODUCTION

A common tissue injury within the body induces numerous inflammatory responses in various pathways as a measure of protection to the site of injury. This phenomenon could lead to an increased infiltration of pro-inflammatory mediators, polymorphonuclear leukocytes, and other cells that could further damage the tissues surrounding the injured site.[1] Microbial infections and exposure to endotoxins cause similar inflammatory response in lungs. Such inflammatory response of enormous inflammatory cell accumulation, migration of polymorphonuclear leukocytes, and other complications in lungs lead to respiratory failure known as acute lung injury (ALI).[2] In patients with sepsis, ischemia-reperfusion, major trauma, and other clinical complaints, ALI is accompanied by a high risk of mortality. Sepsis is a condition known as the syndrome of systemic inflammation which is caused by hyperinflammatory response that injures the surrounding tissues and organs.[3] The actual inflammatory response is usually triggered by the intrusion of foreign organisms such as pathogens or microbes, but they do not directly cause sepsis. It is necessary to prevent or inhibit the initiation or progression of sepsis because this condition is generally fatal to patients.[4]

In order to study the inflammatory process and the underlying mechanism, lipopolysaccharide (LPS) is commonly used as an endotoxin to induce lung injury in animal models. LPS is a cell wall component of Gram-negative bacteria which can trigger the inflammatory response in humans and collapse the immune functions of multiple organs.[5] Transcription factors such as activator protein-1 and nuclear factor-κβ (NF-κβ) are triggered by LPS, which in turn releases the

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pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and other inflammatory mediators.[6] These inflammatory mediators and cytokines play vital roles in the development of ALI. Therefore, there is a need to find a potent inhibitor to prevent the activation of inflammatory response which can be studied using the LPS-induced ALI in animal model.

Plant-derived antioxidants have been studied and proven to have anti-inflammatory potential in various contexts.[7] Tannic acid is a naturally occurring polyphenol that could be found in a variety of plant species which includes various tea leaves, coffee, grapes, and in many more parts of plants such as root, bark, leaves, and fruit.[8] Tannic acid has been reported to possess strong pharmacological properties such as antioxidant, antimicrobial, anti-inflammatory, anticancer, antitumor, and antimutagenic.[9,10] It has also been reported that tannic acid was able to suppress the inflammatory mediators such as cyclooxygenase-2, inducible nitric oxide (NO) synthase, and several enzymes in an inflammatory mouse model.[11] However, there are no reports on the anti-inflammatory mechanism of tannic acid on LPS-induced sepsis and ALI. Therefore, we investigate the safety and effectiveness of tannic acid on RAW264.7 cells and LPS-induced ALI in mouse model.

MATERIALS AND METHODS

Materials

Tannic acid (purity >98%) and LPS were purchased from Sigma Aldrich Chemical Co., St. Louis, USA. All antibodies were purchased from Santa Cruz Biotechnology, USA. All enzyme-linked immunosorbent assay (ELISA) kits and Western blot analysis kits were purchased from Sigma Aldrich Chemical Co., St. Louis, USA. All the other chemicals obtained were of analytical grade.

Cell culture

The macrophage cell line (RAW264.7) was purchased from the American Type Culture Collection (Manassas, US). RPMI-1640 medium was used to culture the cells and supplemented with fetal bovine serum (10% v/v) and antibiotics (100 U/ml streptomycin and 100 U/ml penicillin) at 37°C in a humidified atmosphere (5% CO_2) until reaching 90% confluence. For experimental purpose, the cells were incubated in a 96-well plate with or without LPS and different concentrations of tannic acid (0–16 μM). The cells were initially treated with tannic acid for 1 h and then treated with LPS (1 μg/ml) for 24 h.

Cell viability assay

Cell survival analysis of RAW264.7 cells against different concentrations of tannic acid (0–16 μM) was conducted using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) following manufacturer's guidelines. The cells were seeded in a 96-well plate at a concentration of 1×10^5 cells/ml and treated with tannic acid. The cells were then replaced with fresh medium (100 μl) and MTT solution (10 μl) (5 mg/ml) for 4 h. Formazan was dissolved and the absorbance was read using a microplate reader (Tecan Austria GmbH, Austria) at 570 nm with a reference wavelength of 670 nm. The results were presented as percentage cell survival ± standard deviation (SD) of at least three independent assays.

Nitric oxide measurement in RAW246.7 cells

NO concentrations in cell culture supernatant were measured using Griess reaction. The murine macrophage cells RAW246.7 were seeded in the 96-well plate, pretreated with tannic acid (0–16 μM) for 1 h, and activated with LPS (1 μg/ml) for 24 h. The cell supernatant was mixed with an equal volume of Griess reagent following the manufacturer's protocol (Sigma Aldrich, St. Louis, USA). Sodium nitrite solution was used as a standard to measure the nitrite concentrations. The absorbance was read at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). The results presented (μM nitrite ± SD) were of at least three independent experiments.

Prostaglandin E_2 determination in RAW246.7 cells

The cells were seeded in the 96-well plate, pretreated with tannic acid (0–16 μM) for 1 h, and activated with LPS (1 μg/ml) for 24 h. The concentrations of inflammatory mediator prostaglandin E_2 (PGE_2) in RAW246.7 cells were determined using ELISA kit following the manufacturer's instructions. The results were presented as ng/ml concentration ± SD of at least three independent assays.

Animals and experimental design

Male BALB/c mice (18–22 g) were obtained from our institute's animal husbandry. The animals were acclimatized at a 12 h light/dark cycle in a temperature- and humidity-controlled room. The animals were treated with humane manner, and the experiment was conducted according to the specification of the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Briefly, sixty mice were randomly segregated into five groups (n = 12) which are the control group, LPS alone (50 mg/kg body weight)-treated group, and tannic acid (25, 50, and 100 mg/kg) + LPS-treated groups. ALI was induced in mice by intraperitoneal administration of LPS in sterile phosphate-buffered saline. One hundred microliters of tannic acid dissolved in DMSO at different concentrations (25, 50, and 100 mg/kg) was administered 1 h after the administration of LPS. The mice were sacrificed after 6 h of treatment, and the lungs were excised to collect the bronchoalveolar lavage fluids (BALFs). A portion of the lung from each mouse was fixed in 10% formalin for histopathological analysis.

Lung wet-to-dry weight ratio

The wet weight of the lung was obtained immediately upon excision, and then the lung was dried in oven (65°C) for 72 h to obtain the dry weight. The wet-to-dry weight (W/D) ratio was calculated to assess the tissue edema due to LPS and tannic acid treatment.

Inflammatory cell infiltration

The BALFs were collected from each group of animals and centrifuged for 10 min at 1000 g. The supernatant of BALF containing different inflammatory cells was stained with Wright–Giemsa for differential cell counts.

Myeloperoxidase activity assay

The myeloperoxidase (MPO) assay in lung tissue due to tannic acid and LPS treatment was assessed using a commercial MPO assay kit (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's protocol. The results were presented as U/g of MPO activity ± standard error of mean (SEM) in each treatment group.

Inflammatory cytokine analysis in bronchoalveolar lavage fluids

The concentrations of inflammatory cytokines (TNF-α, IL-1β, and IL-6) in BALF of each experimental group were determined using ELISA kits (Sigma Aldrich, St. Louis, USA) following the protocols of manufacturer. The results were presented as ng/ml concentration ± SEM of each experimental group.

Western blot analysis of nuclear factor kappa-β

Lung tissues were homogenized, and the proteins were extracted using a commercial protein extraction kit (Thermo) following the manufacturer's protocols. The supernatants of the protein were separated using a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked using skim milk (5%) for 2 h in Tris-buffered saline (TBS). The membranes were incubated with primary antibodies against p65, p-p65, Iκβ, p-Iκβ, and β-actin at 4°C overnight. After rinsing thrice with TBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The immunoblots were stained with enhanced chemiluminescence detection kit and subjected to detection by gel documentation system.

Histopathological analysis

The lung portions fixed in 10% formalin were dehydrated, embedded in paraffin wax, and sliced to 4-μm sections. The thin sections were stained with hematoxylin and eosin, mounted, and observed under a light microscope with photographic facility. The histopathological verifications were done by a pathologist who was not aware of the experimental groups.

Statistical analysis

All results were represented as the mean ± SEM of at least three independent experiments. The statistical analysis was done using one-way ANOVA followed by Tukey's *post hoc* analysis (SPSS 19.0 (IBM Corporation, Armonk, NY, USA)). The significance level was accepted at $P < 0.05$ or $P < 0.01$.

RESULTS

Effects of tannic acid on wet-to-dry weight ratio of lipopolysaccharide-induced lung

The protective effect of tannic acid on LPS-induced lung edema in mice was assessed through W/D weight ratio of lungs. The results are demonstrated in Figure 1, where LPS significantly increased the W/D ratio, but treatment with increasing dose of tannic acid prevented the elevation of W/D ratio significantly.

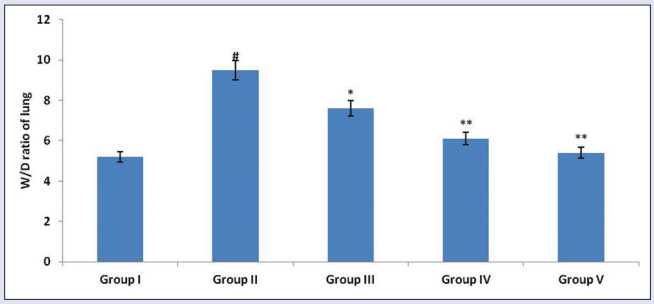

Figure 1: Effects of tannic acid on the lung wet-to-dry weight ratio of lipopolysaccharide-induced acute lung injury in mice. The values presented are the means ± standard error of the mean (n = 12 in each group) of three independent experiments. #$P < 0.01$ versus control group, *$P < 0.05$ and **$P < 0.01$ versus lipopolysaccharide group

Preventive effects of tannic acid on lipopolysaccharide-induced inflammatory cell infiltration

Inflammatory cell infiltration is common in LPS-induced ALI.[12] Therefore, the concentrations of neutrophils, macrophages, and total cells infiltrated in the BALF of LPS-induced mice were evaluated to measure the preventive effect of tannic acid. The results shown in Figure 2 indicate that LPS significantly increased the infiltration of macrophages, neutrophils, and total cells in the BALF of ALI mice. However, tannic acid dose dependently prevented the infiltration of neutrophils, macrophages, and total cells in the BALF of treated mice.

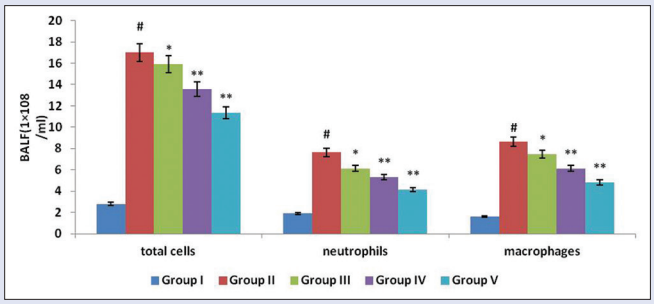

Figure 2: Effects of tannic acid on inflammatory cell infiltration in the bronchoalveolar lavage fluid. The values presented are mean ± standard error of the mean (n = 12 in each group) of three independent experiments. #$P < 0.01$ versus control group, *$P < 0.05$, **$P < 0.01$ versus lipopolysaccharide group

Protective effects of tannic acid on myeloperoxidase activity in lipopolysaccharide-induced acute lung injury

Infiltration of polymorphonuclear leukocytes plays a major role in inflammation and related tissue damage in LPS-induced ALI.[13] MPO is an important marker of polymorphonuclear leukocyte infiltration; therefore, the MPO activity was assessed in the BALF of LPS-induced ALI. Figure 3 shows the significant elevation of MPO activity in LPS-induced mice as compared to the control mice. However, tannic acid was able to reduce the MPO activity remarkably in increasing dose pattern.

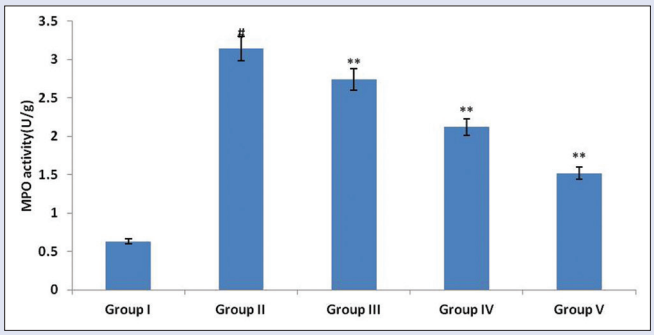

Figure 3: Effects of tannic acid on myeloperoxidase activity in lung tissues of lipopolysaccharide-induced acute lung injury. The values presented are the mean ± standard error of the mean (n = 12 in each group) of three independent experiments. #$P < 0.01$ versus control group, *$P < 0.05$, **$P < 0.01$ versus lipopolysaccharide group

Suppressive effect of tannic acid on lipopolysaccharide-induced inflammatory cytokine production

Inflammatory cytokines are normally produced during an inflammatory response to mediate the process.[14] The

anti-inflammatory effect of tannic acid was evaluated through the detection of inflammatory cytokine concentration (TNF-α, IL-1β, and IL-6) using ELISA kits in BALF of LPS-induced ALI mice. The results clearly indicate significant elevation of the levels of inflammatory cytokines in LPS-induced mice as shown in Figure 4. In converse, tannic acid administration was able to significantly suppress the levels of TNF-α, IL-1β, and IL-6.

Protective effect of tannic acid on lipopolysaccharide-induced nuclear factor kappa-β activation

The release of inflammatory cytokines and mediators is mainly controlled by the activation of NF-κβ through phosphorylation of Iκβ and translocation of p65 subunits into the nucleus.[15,16] Hence, the activation of NF-κβ in LPS-induced ALI was determined through Western blot analysis using β-actin as an internal control. The analysis showed that the NF-κβ subunits Iκβ and p65 were phosphorylated in LPS-induced ALI [Figure 5]. Tannic acid treatment was able to suppress the activation of NF-κβ in LPS-induced ALI, which can also be related to the results of suppression of inflammatory cytokine production.

Histopathological alterations due to tannic acid administration in lipopolysaccharide-induced acute lung injury

The overall protective effect of tannic acid was observed by histopathological evaluation of LPS-induced lung tissues. The results of all the treatment groups are collectively demonstrated in Figure 6. The control group exhibited normal lung cellular arrangement, whereas the LPS-induced lung of Group 2 showed extensive damage to the lung architecture such as alveolar hemorrhage, hyperemia, interstitial edema, infiltration of inflammatory cells, and interalveolar septal thickening. These changes were significantly altered in the dose-dependent pattern of tannic acid administration, thus protected the organ from ALI caused by LPS.

Cell viability, nitric oxide production, and prostaglandin E_2 expression in lipopolysaccharide-activated RAW264.7 cells

The cell viability test upon tannic acid treatment on macrophage RAW264.7 cells showed no signs of toxicity on the percentage of cell survival [Figure 7]. There were no statistical significance between each treatment group of tannic acid, which proves the safety and effectiveness of tannic acid.

The protective effect of tannic acid on the levels of NO and PGE_2 production was determined in LPS-activated RAW264.7 cells. The results exhibited that tannic acid remarkably prevented the production of NO and inflammatory mediator PGE_2 in LPS-activated RAW264.7 cells with increasing concentration manner as shown in Figure 7. These results further support the anti-inflammatory potential of tannic acid against LPS-induced damage.

DISCUSSION

The severity of ALI is a well-known condition that needs a real solution. The ALI condition caused by sepsis is one of the worst fatal diseases globally.[3] There are no proper treatment methods or drugs to cure ALI with sepsis till date. Plant-derived antioxidant compounds are widely being studied for their pharmacological significance which includes the therapeutic potential for ALI with sepsis.[17,18] Therefore, we studied the ameliorative potential of tannic acid on LPS-induced ALI in mice. In this study, tannic acid has been proven to possess anti-inflammatory properties against LPS-activated RAW264.7 cells and also in the LPS-induced lung injury in mice. The anti-inflammatory properties of tannic acid can be credited to its suppressive effect on NF-κβ activation.

Infiltration of neutrophils, macrophages, and other inflammatory mediator cells during an inflammatory response triggers an autonomous reactive oxygen species chain reaction and releases hydrolytic proteinase, thus causing damage to the surrounding cells.[19,20] LPS-induced lung injury in mice demonstrated high concentrations

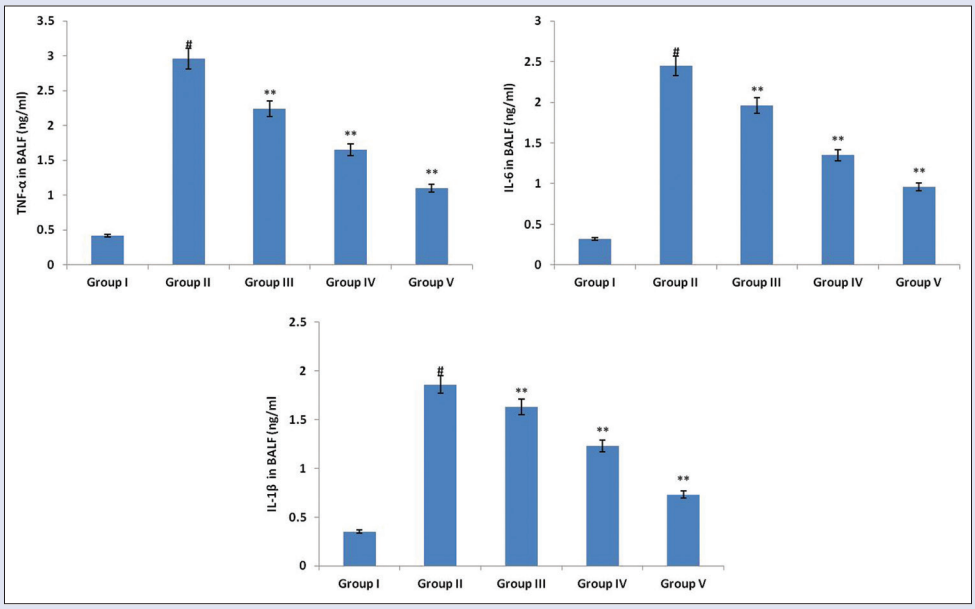

Figure 4: Effects of tannic acid on tumor necrosis factor-α, interleukin-1β, and interleukin-6 production in the bronchoalveolar lavage fluid of lipopolysaccharide-induced acute lung injury in mice. The values presented are mean ± standard error of the mean (n = 12 in each group) of three independent experiments. $^{\#}P < 0.01$ versus control group, $^{*}P < 0.05$, $^{**}P < 0.01$ versus lipopolysaccharide group

of cell infiltration, which was due to an initial lung injury and edema. Tannic acid significantly prevented the lung injury and spreading of the damage to the surrounding tissues through inhibiting the infiltration of inflammatory cells. This proves that preventing the infiltration of inflammatory cells could prevent the progress of ALI. Being a natural antioxidant, tannic acid might have also scavenged the reactive oxygen species triggered by neutrophil infiltration. The protective effect of tannic acid was exhibited in the histopathological results where the cellular arrangement was preserved; instead, LPS-induced lung tissues were extremely damaged with visible cell infiltrations. The MPO activity is a measure of neutrophil presence in the tissues. The MPO activity results were also supporting the protective effect of tannic acid because LPS-induced ALI exhibited highly elevated levels of MPO activity. Tannic acid administration successfully reversed the levels of MPO activity in a dose-dependent manner, indicating the prevention of neutrophil infiltration.

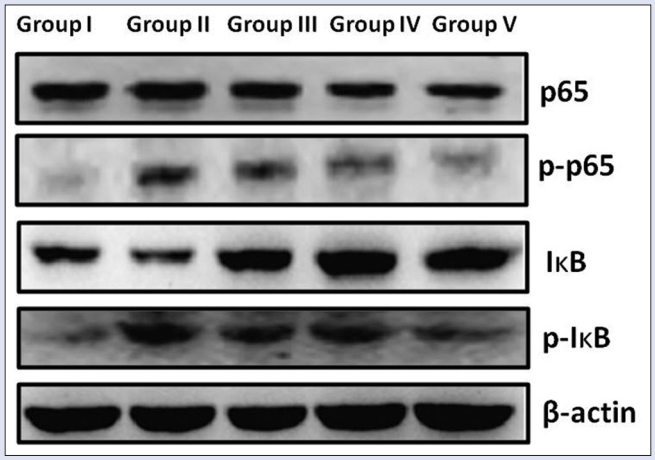

Figure 5: Tannic acid inhibits lipopolysaccharide-induced nuclear factor-kB activation

Inflammatory cytokines are activated through the infiltration of neutrophils and macrophages. The inflammatory cytokines and mediators such as TNF-α, IL-6, IL-1β, PGE_2, and NO are important enhancers of inflammatory reaction.[21,22] The production and regulation of inflammatory cytokines are mainly controlled by NF-κβ activation pathway and also other transcription factors.[23] LPS-induced ALI is mediated by these inflammatory cytokines which initiate a cascade reaction resulting in hyperinflammation, thus causing injury to the

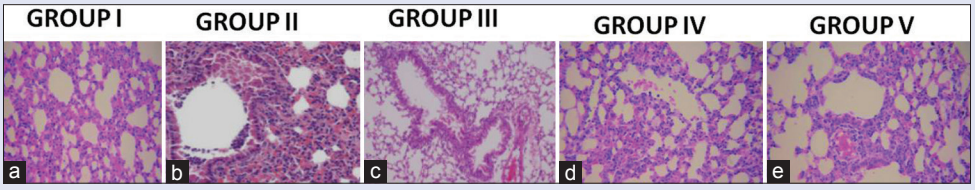

Figure 6: Effects of tannic acid on histopathological changes in lung tissues in lipopolysaccharide-induced acute lung injury in mice. Representative histological changes of lung obtained from mice of different groups. (a) Control group, (b) lipopolysaccharide group, (c) lipopolysaccharide + tannic acid (25 mg/kg) group, (d) lipopolysaccharide + tannic acid (50 mg/kg) group, and (e) lipopolysaccharide + tannic acid (100 mg/kg) group (H and E, ×200)

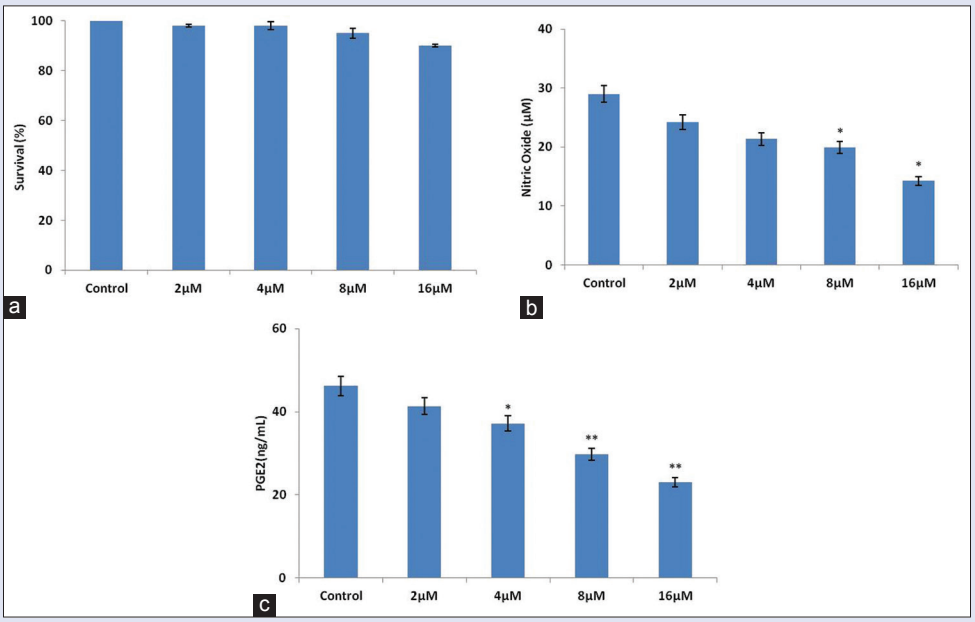

Figure 7: Effect of tannic acid on cell viability and prostaglandin E_2 and nitric oxide production by lipopolysaccharide-activated RAW264.7 cells. Cells were incubated with different concentrations of tannic acid for 1 h and then treated with lipopolysaccharide (1 μg/ml) for 24 h (a) Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, (b) nitric oxide concentrations in cell culture supernatant were measured by the Griess reaction, (c) prostaglandin E_2 concentrations in cell culture supernatant were measured by enzyme-linked immunosorbent assay. The data are presented as the mean ± standard deviation of three independent experiments

lungs.[24] This was shown in our study that LPS-induced lung injury was caused by elevated concentrations of inflammatory cytokines (TNF-α, IL-6, and IL-1β). Tannic acid was able to significantly control the levels of inflammatory cytokines and also prevent the phosphorylation of NF-κβ subunits Iκβ and p65, hence inhibiting the transcription of inflammatory mediators. This proves the anti-inflammatory potential of tannic acid in LPS-induced ALI. It can be said that preventing the translocation of NF-κβ subunits into nucleus and inhibition of inflammatory cytokine production could help in attenuating tissue injury progression in ALI.

CONCLUSION

We report for the first time the ameliorative and anti-inflammatory properties of tannic acid on LPS-induced ALI in mice. Our results have clearly shown the effective mechanism of tannic acid in protecting the lung against LPS-induced injury. The anti-inflammatory mechanism of tannic acid is attributed to the prevention of NF-κβ activation along with the suppression of inflammatory cytokines and related mediator cells. Tannic acid as a natural antioxidant might have scavenged the reactive oxygen species produced by LPS-induced lung injury, thus ameliorating the tissue injury progression as expressed by histopathological outcome. Taken together, tannic acid can be recommended as a potential drug candidate for the treatment of ALI as well as other inflammatory diseases.

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Conflicts of interest

There are no conflicts of interest.

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