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ORIGINAL ARTICLE

Resveratrol Exerts Anti-Inflammatory Effect in Lipopolysaccharide-Induced Lung Inflammation via Downregulation of Antioxidant and Inflammatory Mediators

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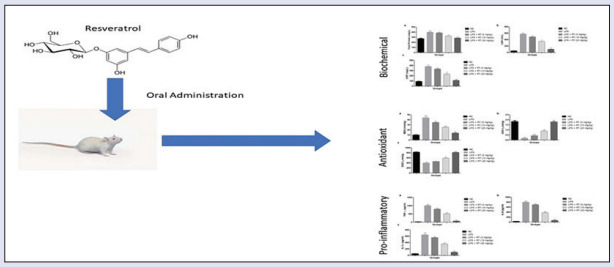
ABSTRACT

Background: Acute lung inflammation (ALI) is a serious health condition that causes severe pulmonary distress, tissue loss, and ultimately leads to death of the patient. Previously, theantagonisticrole of flavonoids has been extensively studied with respect to inflammation in cancer. Inflammatory reaction targeted during the lung cancer therapy. **Aims and Objectives:** In this study, the chemoprotective effect of resveratrol and its of mechanism of action against the Lipopolysaccharide (LPS)-induced lung inflammation was investigated. **Materials and Methods:** In this in vitro study, we performed experiments using RAW 264.7 cells. LPS was used to induce inflammation in the lungs of Swiss Wistar rats that were randomly divided into different groups. The rats were treated with resveratrol in a dose-dependent manner. The lung tissue and dry/wet weight of lung tissue was estimated. The antioxidant activity and anti-inflammatory parameters were also estimated. **Results:** According to results, resveratrol significantly reduced the secretion of pro-inflammatory cytokines including interleukin (IL)-1β, IL-4, IL-6, tumor necrosis factor-α (TNF-α), IL-18, IL-10, and antioxidant parameters viz., glutathione (GSH), malondialdehyde (MDA), and superoxide dismutase (SOD), respectively. Resveratrol significantly ($P<0.001$) reduced the weight of lung tissue and dry/wet of lung tissue at dose-dependent manner. **Conclusion:** In summary, resveratrol plays the role of a chemo-protective agent against the LPS-induced lung inflammation via anti-inflammatory and antioxidant mechanism.

Key words: Antioxidant parameter, inflammatory mediators, pro-inflammatory cytokines, resveratrol

SUMMARY

- In this experimental study, resveratrol significantly altered the cell viability, nitric oxide production and pro-inflammatory cytokines such as TNF-α, Il-1β, IL-4, IL-10, IL-18 and NF-κB. Resveratrol significantly reduced the lung index and lung wet/dry ratio. Resveratrol significantly reduced the total cell count in BALF. Resveratrol significantly reduced the level of total protein, LDH and CRP at dose dependent manner. Resveratrol also decreased the total cells, neutrophils and macrophiles at dose dependent manner. Resveratrol significantly reduced the level of MDA and improved the level of GSH and SOD and suggesting the antioxidant effects. Resveratrol significantly reduced the level of TNF-α, Il-1β and IL-6. On the basis of result, we can conclude that resveratrol having dual effect against the lung inflammation.



Abbreviations used:ALI=Acutelunginflammation,LPS=Lipopolysaccharide, Il-1β=Interleukin-1β, IL-4= Interleukin-4, IL-6=Interleukin-6, TNF-α=Tumor necrosis factor-α, IL-18= Interleukin-18, IL-10=Interleukin-10, GSH=Glutathione, MDA=Malondialdehyde, SOD=Superoxide dismutase, ARDS=Acute respiratory distress syndrome, BALF=Bronchoalveolar lavage fluid, TLR-4=Toll-like receptor 4, NF-κB=Nuclear factor kappa B cells, MAPKs=Mitogen-activated protein kinase, NLRP3=NOD-, LRR- and pyrin domain-containing protein 3, iNOS=Inducible nitric oxide synthase, COX-2=Cyclooxygenase-2, DMEM=Dulbecco'smodified Eagle medium, CCK=Cell Counting Kit – 8, ROS=Reactive oxygen species, CAT=Catalase, LPO=Lipid peroxide, GPx=Glutathione peroxidase, GR=Glutathione reductase, GGT=γ-glutamyltranspeptidase, LDH=Lactate dehydrogenase, ADA=Adenosine deaminase, 5-HT=5′-nucleotidase, QRT-PCR=Quantitative real-time polymerase chain reaction, DCFH-DA=2′,7′-Dichlorofluorescin Diacetate

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INTRODUCTION

Acute Lung Inflammation (ALI) is a serious health condition that causes severe respiratory dysfunction and tissue damage and ultimately leads to death. Clinically, the term ALI is commonly replaced with the term Acute Respiratory Distress Syndrome (ARDS), which is distinct based on the various diagnostic principles.[1] Patients suffering from ARDS have high mortality (around 30%–50%) and need advanced treatment.

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[2] However, in the case of ALI, inflammation of the lung tissue plays a significant role in the development of inflammation and dissemination of lung disorders.[1] The bacterial cell wall of Gram-negative bacteria contains lipopolysaccharide (LPS), which is responsible for sepsis and endotoxemia leading to the gradual breakdown of multiple organs such as the skin, kidney, liver, and heart.[3,4] LPS is commonly used to induce inflammation. Its features are similar to that of lung tissue injury caused due to ALI/ARDS such as lung edema, leukocyte infiltration, abnormal gas exchange, and mortality. LPS caused ALI/ARDS by undermining the cohesion of the alveolar-capillary membrane and allowing uncontrolled penetration of neutrophils into the alveolar spaces.[5,6] Inflammation of the lungs plays a major role in generating multiple inflammatory responses related to pro-inflammatory cytokines and reactive oxygen species (ROS). Macrophages play a crucial role in controlling the host's immune response during ALI.[2,4,7] It is well documented that macrophages can induce infiltration of neutrophils and trigger inflammatory process. In addition, improved evidence indicates the relationship between oxidative stress and inflammation, which plays an important role in the pathophysiology of ALI. During the oxidative stress, a large number of ROS is generated and the level of endogenous antioxidant enzymes might be decreased.[2,4] During the LPS-induced lung inflammation, alveolar and endothelial cells get damaged which results in the activation of macrophages and the secretion of inflammatory cells, especially the neutrophils.[8] LPS induced ALI similar to the human lung inflammation. It increased the level of cytokine content and neutrophil count in bronchoalveolar lavage fluid (BALF) during the creation of the ALI model in rodents.[9] Neutrophils in the lung tissue not only secrete the pro-inflammatory cytokines but also induce oxidative stress and ROS production. Previous studies indicate that Toll-like receptor 4 (TLR-4) is a transmembrane receptor and is an essential protein that arbitrates LPS and cell contact.[9,10] LPS induced the inflammation reaction, which further increased the level of TLR-4 and finally activation of mitogen-activated protein kinases (MAPKs), NLRP3, and nuclear factor-kappa B (NF-κB) during the inflammatory pathway.[11] NF-κB is a pleiotropic pathway that is involved in various processes such as immune response, inflammatory pathway, cell survival, and cell expansion.[9] A similar effect causes ALI induced by MAPK during LPS activation. The modulation of MAPK and NF-κB pathways activates various inflammatory mediators, such as inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6, IL-10, and IL-18.[9,11]

Synthetic drugs have a lot of limitations and side effects; therefore, natural plant-based drugs have attracted greater attention from various researchers. Natural products made from plants, minerals, and animals have been used to treat various human diseases.[12,13] Practically, the history of medicines dates back to the existence of human civilization. The recently accepted therapy is allopathy, and it is more popular due to short time of action. However, allopathy has limitations as it can cause various side effects.[14,15] Plant-based therapy is better and has less or no side effects; therefore, in this study, we focused our research on resveratrol, which is a plant-based therapeutic agent. In addition, we examined the anti-inflammatory and antioxidant role of resveratrol in LPS-mediated RAW 264.7 macrophages and rat model of ALI induced by LPS.

MATERIALS AND METHODS

Chemical

Resveratrol and LPS were purchased from Sigma-Aldrich (USA). Reagent chemicals were bought from the Proteintech firm. Cell counting kit (CCK)-8 and 3-methyladenine were purchased from the Selleck (USA). The rest of the chemicals and reagent used in the current experimental analysis was bought from the licensed vendor

Cell culture

RAW 264.7 mouse macrophage cells were procured from the Chinese Academy of Sciences Center for Cell Resources, Shanghai, China. Briefly, Dulbecco's modified Eagle's medium (DMEM) was used for routine cell culture. The medium was supplemented with penicillin (100 units/mL), fetal bovine serum (10%), and streptomycin (100 units/mL). The cells were incubated in a humidified incubator at 37°C (5% CO_2).[9]

Cell viability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cell viability. Briefly, RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in 96-well plates and incubated at 37°C for 24 h in a CO_2 incubator (5%). Then, various concentrations of resveratrol were added to the cells and incubated for 18 h. Then, H_2O_2 was added to the reaction mixture and incubated for 1 h. Then, MTT (5 mg/mL) was added to each well and incubated again for the next 4 h. The formazan crystals formed were dissolved by adding dimethyl sulfoxide in each well, and the absorbance of the color developed was read at 570 nm.[9]

Cell counting kit-8 assay

RAW 264.7 cells were seeded at a density of 1×10^4 cells/well in 96-well plates for CCK-8 assay. The cells were incubated for 24 h at 37°C in a CO_2 incubator (5%). Then, CCK-8 (10 μL) reagent was added to each well and the mixture was incubated at 37°C for the next 2 h. EXL-800 Multiscan range was used to measure the absorbance at 450 nm.

Intracellular reactive oxygen species estimation

To determine the intracellular ROS production, the cells (RAW 264.7) were seeded at a density of 1×10^4 cells/well in 96-well plates and incubated for 24 h. Then, the cells were treated with resveratrol (0–10 μg/ml) in a serum-free DMEM and incubated for the next 18 h. Then, the cells were incubated with 50 μM 2′,7′-Dichlorofluorescin Diacetate (DCFH-DA) for half an hour and 300 μM H_2O_2 was added and incubated for 10 min to generate ROS. In addition, RAW 264.7 cells were seeded in 12-well plates and incubated for 24 h. Then, the cells were treated with the various concentrations of resveratrol (0–10 μg/ml) for the next 1 h, and finally, the cells were exposed to 300 μM H_2O_2 and incubated for the next 18 h. Finally, DCFH-DA dye (50 μM) was added to each well and incubated for 30 min. Finally, the emission and excitation were read at 535 and 485 nm, respectively, using a flow cytometry.[9]

Animals

In this study, Swiss Wistar rats (150–180 g, both males and females) were used in the experimental analysis. The rats were procured from the institutional animal house and under standard laboratory condition (20°C ± 5°C; 60%–70% relative humidity and 12/12 h day/night cycle). The rats were fed with standard diet and water *ad libitum*.

Acute lung inflammation model

Previously reported method was used for the ALI model. The mice were divided into five groups as follows:

- Group I: Normal control
- Group II: LPS group
- Group III–V: LPS + resveratrol (10, 20, and 40 mg/kg), respectively.

For the induction of ALI, the mice were anesthetized using anesthesia (diethyl ether) and LPS (0.5 mg/kg) was intranasally administered to cause lung injury. After the administration of LPS (12 h), the mice were euthanized, and their lung tissue was harvested for the estimation of various biochemical parameters.[16]

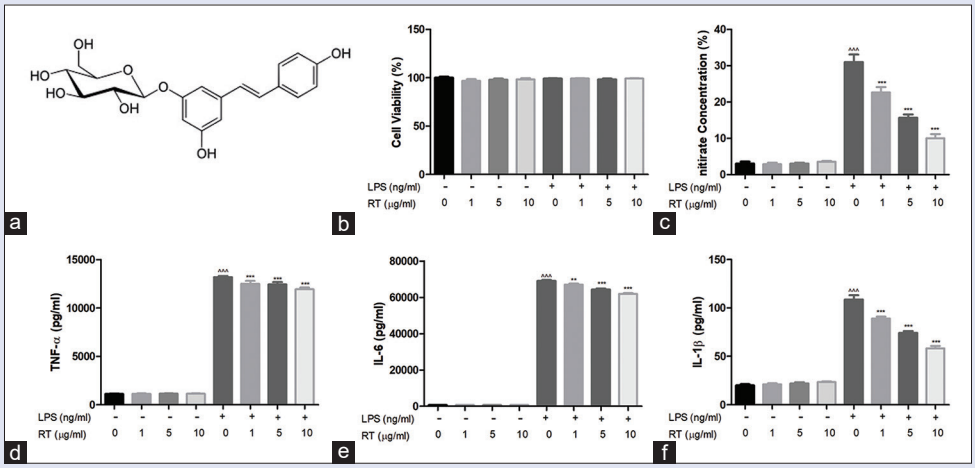

Figure 1: The effect of resveratrol on the inflammatory mediators in lipopolysaccharide-induced RAW 264.7 cells. (a) Structure of resveratrol, (b) Cell viability of resveratrol via using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, (c) nitric oxide, (d) tumor necrosis factor-α, (e) interleukin-6, and (f) interleukin-1β in culture supernatant and estimated via using the ELISA. All the values present as ± standard deviation of three or more replicates. ^^^$P < 0.001$ compared with control; **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

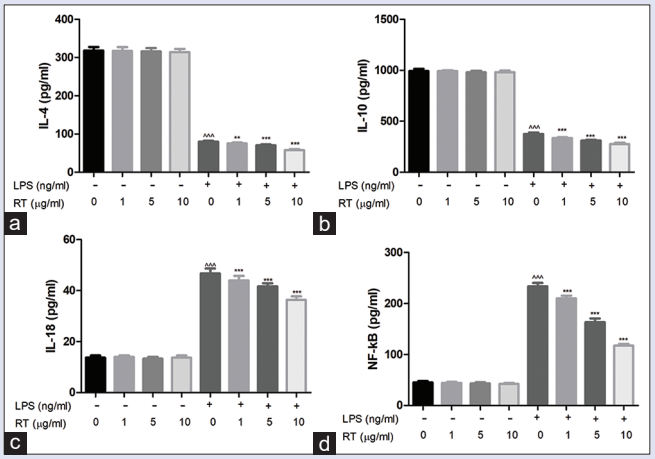

Figure 2: The effect of resveratrol on the pro-inflammatory cytokines and inflammatory mediators in lipopolysaccharide-induced RAW 264.7 cells. (a) Interleukin-4, (b) interleukin-10, (c) interleukin-18, and (d) nuclear factor-kappa B in culture supernatant and estimated via using the ELISA. All the values present as ± standard deviation of three or more replicates. ^^^$P < 0.001$ compared with control; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

Biochemical analysis

Biochemical analysis was performed using the liver and lung homogenate. Catalase (CAT), glutathione (GSH), lipid peroxide, superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and vitamins including C and E were estimated via using the kits and by following the manufacturer's instructions (Abcam technology).

For the determination of aryl hydrocarbon hydroxylase, previously reported method was used with minor modifications. The activity of γ-glutamyl transpeptidase, lactate dehydrogenase (LDH), adenosine deaminase, polyamines, and 5′-nucleotidase was estimated in the lung and liver for the microsomal fraction. The tissue homogenate was centrifuged at 105,000 g for 60 min and further used in the estimation of cytochrome b5 and cytochrome P450, uridine diphosphate-glucuronyl transferase, quinone reductase, and GSH S-transferase. The sample was further used to estimate the level of putrescine, histamine, spermine, and spermidine by using the carboxymethyl (CM)-cellulose column chromatography. Briefly, the lung tissue samples were homogenized (100 mg) in ice-cold $HClO_4$ containing ethylenediaminetetraacetic acid (2 mM) and further centrifuged for 5 min at 3000 rpm, and the supernatant was used for CM-cellulose column chromatography. Finally, the reaction was conducted for 10 min at 50°C and terminated by cooling the reaction mixture in water, and finally, the absorbance was read at 420 nm.

Table 1: Showed the effect of resveratrol on the body weight and lung weight.

S. No	Groups	Weight (gm)	
		Body weight	Lungs Weight
1	NC	234.4±5.45	8.45±1.03
2	LPS	194.2±4.83^^^	12.34±1.84^^^
3	LPS + RT (5 mg/kg)	203.8±5.26*	11.2±1.45*
4	LPS + RT (10 mg/kg)	213.2±6.34**	10.6±1.23**
5	LPS + RT (15 mg/kg)	222.5±5.32***	9.02±1.01***

All the values present as ± standard deviation of three or more replicates. ^^^$P < 0.001$ compared with control; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

Statistical analysis

In this study, we performed one-way analysis of variance using the Dunnett's comparative model. All data are reported as mean ± standard error of mean. $P < 0.05$ was found to be statistically significant.

RESULTS

Inflammatory mediators in RAW 264.7 cells

ELISA techniques were used to determine the levels of cytokines in the cell culture medium. Figure 1a shows the chemical structure of resveratrol. Figure 1b exhibits the effect of resveratrol on the cell viability. Figure 1c-f shows the effect of resveratrol on the formation of LPS-induced pro-inflammatory cytokines, and it shows the decrease in the amount of pro-inflammatory cytokines, namely IL-6, IL-1β, and TNF-α, in a dose-dependent resveratrol treatment.

Figure 2a shows the decreased levels of IL-4 and increased levels of IL-10 [Figure 2b] and IL-18 [Figure 2c] levels, and resveratrol reduced the levels of pro-inflammatory cytokines in a dose-dependent manner. Figure 2d shows the increased levels of NF-κB in the LPS-induced RAW cells, and resveratrol significantly reduced its level.

Effect of resveratrol on body weight and lung tissue on lipopolysaccharide-induced lung inflammation

During the lung inflammation, the weight of lung tissue was found to be increased, whereas the body weight decreased. Table 1 shows that the LPS-induced rats exhibited decreased body weight when compared to normal rats.

Figure 3a shows the effect of LPS and resveratrol on lung weight. LPS-induced control group rats showed increased lung weight compared to other controlled group rats. Resveratrol significantly ($P < 0.001$) decreased the lung weight. LPS-induced rats showed the enhanced lung dry/wet and resveratrol significantly ($P < 0.001$) reduced the dry/wet lung tissue weight [Figure 3b].

Effect of resveratrol on total cell count and nitrite/nitrate

Figure 4a shows the total cell count in BALF. According to our results, LPS-induced rats demonstrated a considerably increased total cell count, whereas resveratrol-treated rats demonstrated reduced total cell count.

A similar finding has been reported for the level of nitrite or nitrate in LPS-treated rats. Resveratrol treatment significantly ($P < 0.001$) downregulated the level of nitrite/nitrate [Figure 4b].

Biochemical parameters

During the lung inflammation, the biochemical parameters including total protein, C-reactive protein (CRP), and LDH increased. LPS-induced rats exhibited increased levels of CRP, total protein, and LDH, and resveratrol significantly ($P < 0.001$) downregulated the level of total protein, LDH, and CRP [Figure 5a-c, respectively].

Differential lymphocytes

In LPS-induced rats, the differential lymphocyte parameter increased throughout the process of inflammation. The LPS-induced community rats exhibited elevated levels of total cells, neutrophils, and macrophages [Figure 6a-c, respectively] compared with standard controls. Resveratrol significantly ($P < 0.001$) decreased the level of total cells, neutrophils, and macrophages [Figure 6a-c, respectively] as compared to LPS-induced rats.

Antioxidant parameters

Figure 7 shows the effect of resveratrol on antioxidant parameters of LPS-induced lung inflammation. LPS-induced control rats displayed the increased levels of malondialdehyde (MDA) and decreased levels of SOD, GSH and resveratrol treatment significantly ($P<0.001$) decreased the MDA level, and increased the GSH, SOD levels [Figure 7a-c, respectively].

Pro-inflammatory cytokines

Resveratrol effect on pro-inflammatory cytokines of LPS-induced lung inflammation rats is shown in Figure 8. LPS-induced group rats treated with resveratrol significantly ($P < 0.001$) downregulated the level of TNF-α, IL-6, and IL-1β [Figure 8a-c, respectively].

DISCUSSION

Previous studies suggest that the intraperitoneal, intravenous, and intratracheal administration of LPS in rodents induces ALI, which is similar to that of human ALI/ARDS.[8,16,17] Therefore, in this study, we used ALI model in rats induced by LPS.[8,18] According to our results, there were similar characteristics such as enhancing the lung wet/

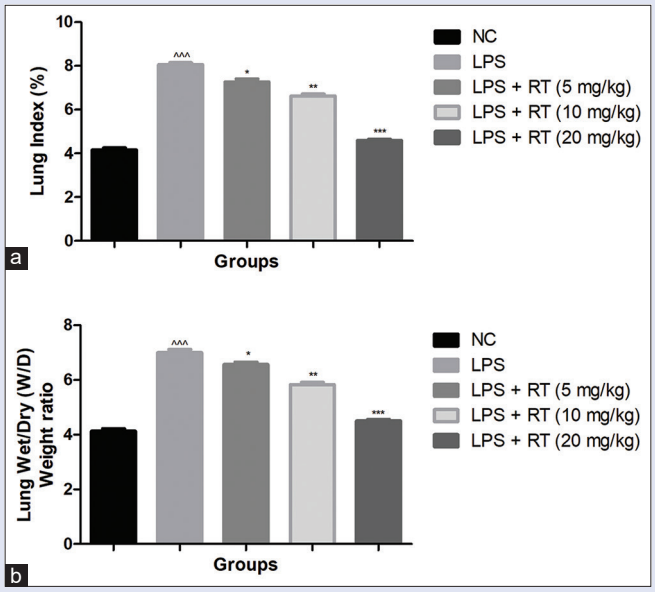

Figure 3: The effect of resveratrol on the lung index in lipopolysaccharide-induced lung inflammation. (a) Lung index and (b) lung dry/wet tissue ratio. ^^^$P < 0.001$ compared with control; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively. ^^^ $P < 0.001$ compared with control; *<0.05, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

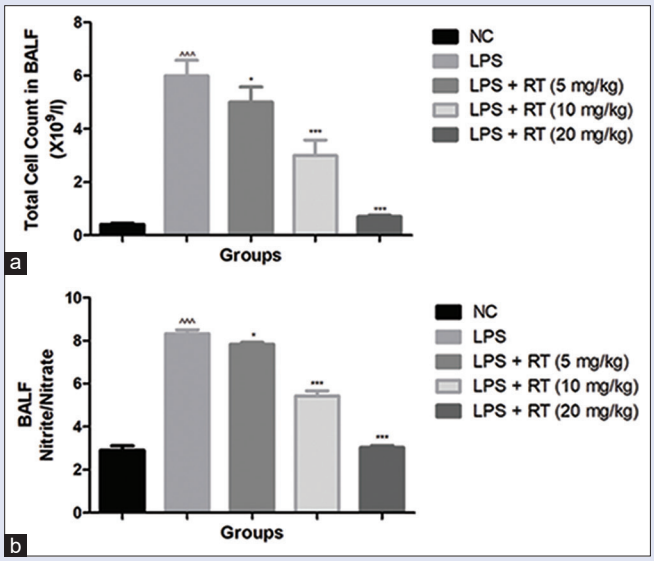

Figure 4: The effect of resveratrol on the bronchoalveolar lavage fluid count in lipopolysaccharide-induced lung inflammation. (a) Total cell count in bronchoalveolar lavage fluid and (b) bronchoalveolar lavage fluid nitrite/nitrate. ^^^$P < 0.001$ compared with control; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

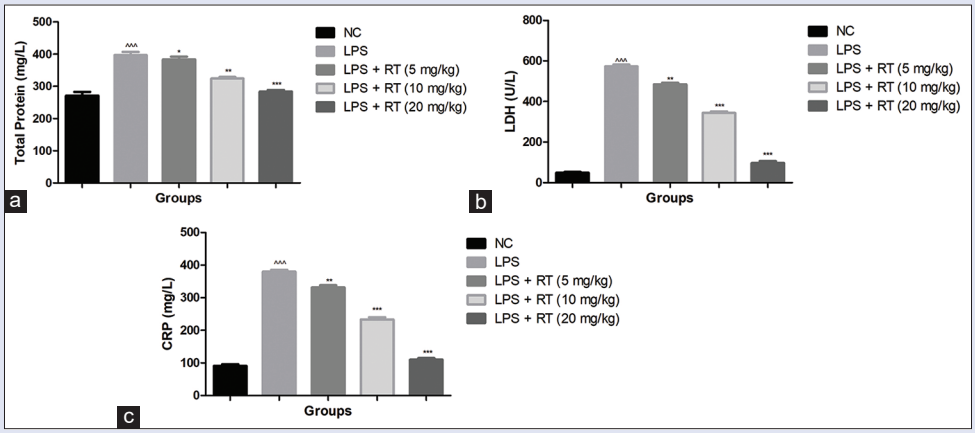

Figure 5: The effect of resveratrol on the biochemical parameters in lipopolysaccharide-induced lung inflammation. (a) Total protein, (b) lactate dehydrogenase, and (c) C-reactive protein. ^^^$P < 0.001$ compared with control; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

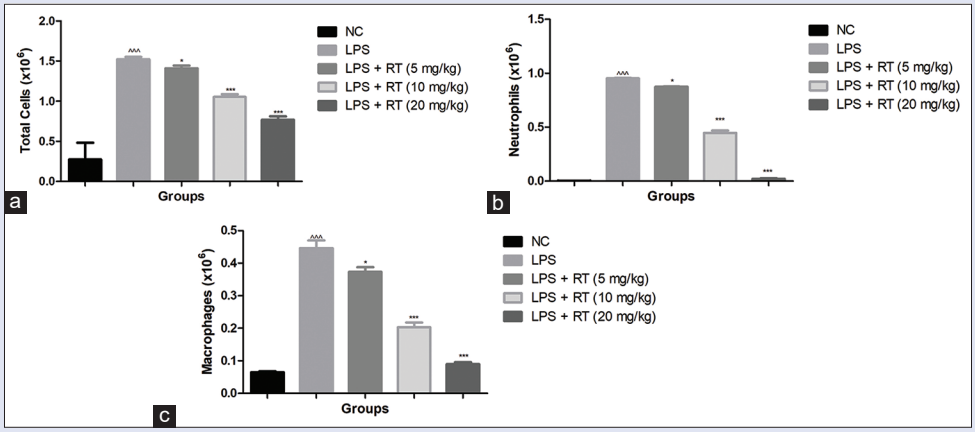

Figure 6: The effect of resveratrol on the differential leukocyte count in lipopolysaccharide-induced lung inflammation. (a) Total cells, (b) neutrophils, and (c) macrophage. ^^^$P < 0.001$ compared with control; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

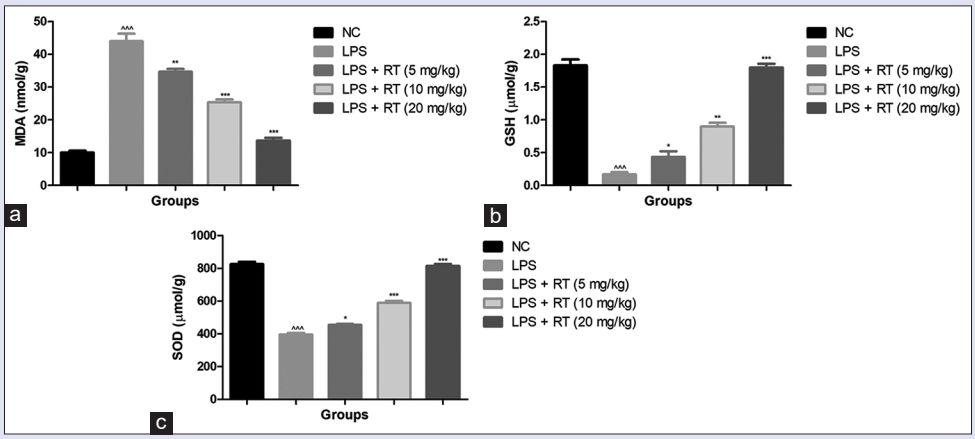

Figure 7: The effect of resveratrol on the antioxidant parameters in lipopolysaccharide-induced lung inflammation. (a) Malondialdehyde, (b) glutathione, and (c) superoxide dismutase. ^^^$P < 0.001$ compared with control; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

dry ratio and increase in the level of BALF. Along with the increase in total protein, there was a change in the levels of LDH (showing the barrier's enhanced alveolar-capillary membrane permeability) and the functioning of the lung tissue.[19,20]

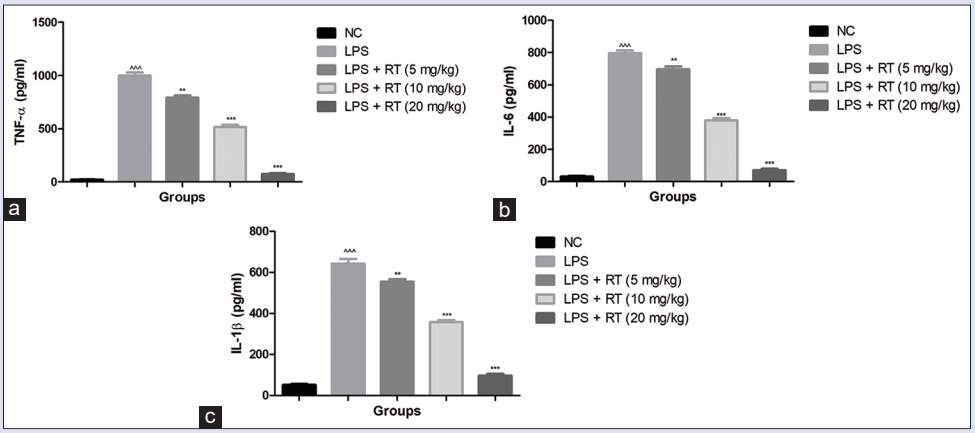

Figure 8: The effect of resveratrol on the pro-inflammatory cytokines in lipopolysaccharide-induced lung inflammation. (a) Tumor necrosis factor-α, (b) interleukin-6, and (c) interleukin-1β. ^^^$P < 0.001$ compared with control; *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the lipopolysaccharide treated with alone, respectively

Oxidative stress is a state of imbalance between protection against antioxidants and development of oxidants. Specific physiological pathways produce ROS, which include superoxide radical, hydrogen peroxide, hydroxyl, and hydroperoxyl radicals.[19-21] Each cell develops an array of antioxidative enzymatic and non-enzymatic pathways to detoxify the ROS. Oxidative stress is well characterized as xenobiotic bioactivation which creates an imbalance between detoxification and ROS production. The ROS formed can damage the cellular components such as DNA, lipids, and proteins.[22,23] It is well documented that ROS/reactive nitrogen species play a significant role in the expansion of various diseases. In the LPS-induced ALI method, there was an increased amount of ROS formed, especially after the activation of neutrophils.[20,24,25] In addition, if ROS is continuously produced, then the endogenous antioxidant protection mechanism cannot eliminate the free radical formed, ultimately leading to the inflammation of the tissue.[1,26] In this study, LPS-induced rats showed decreased levels of endogenous antioxidants and resveratrol significantly ($P < 0.001$) increased their levels.

It is well demonstrated that the lungs are highly susceptible to the environmental pollutants and are commonly directly or indirectly exposed to various harmful elements such as infectious micro-organisms.[27] ALI is a clinical condition in which there is involvement of the chronic inflammation in the lung tissue and is categorized with lung edema, diffuse alveolar injury, surfactant dysfunction, neutrophil, infiltration, and so on. It is well documented that lung inflammation is commonly induced by some of the Gram-negative bacteria, which are commonly found during serious lung infection. LPS is commonly present in the cell wall of Gram-negative bacteria, and it is an effective stimulator of macrophages.[28-30] LPS binds with TLR-4, which further activates macrophages and monocytes, which results in the secretion of pro-inflammatory cytokines. These molecules activate various signaling pathways in LPS-induced macrophages and consequently generate the pro-inflammatory mediators to neutralize the infectious agents.[19,20] LPS-induced lung inflammation is characterized by alveolar hemorrhagic edema and also increased levels of protein, cytokine, and neutrophil count in the BALF. Previous studies have suggested that the cytokines and inflammatory proteins are closely related to the expansion of inflammatory processes.[20] In this study, we found that the resveratrol effectively reduced the LPS-induced pulmonary edema and lesions in the lungs and decreased the production of LDH in LPS-induced ALI rats. This effect might be due to anti-inflammatory effects of resveratrol. Furthermore, lung tissue injury boosted the activity of macrophages and neutrophils in pulmonary tissue and also triggered the secretion of huge amount of pro-inflammatory cytokines as well as free radicals that can be continued the inflammatory reaction, leading to the more chronic tissue inflammation and apoptosis.[31,32] In this study, we observed increased levels of pro-inflammatory cytokines in the BALF after induction with LPS and resveratrol significantly reduced the level of cytokines. Pro-inflammatory cytokines, namely IL-18, one of the significant markers, take an important part in the pathogenesis of the numerous inflammatory processes. Previous studies suggest that epithelial cells, neutrophils, and macrophages increase the production of various pro-inflammatory cytokines such as IL-6, IL-8, G-CSF, GM-CSF, metalloproteases, and chemokines by the activation of pro-inflammatory cytokines, especially IL-18.[32-34] The model developed in this study is good to study chronic inflammatory conditions, autoimmune diseases, allergies, and various types of tumors.

CONCLUSION

This study shows the protective effect of resveratrol against the LPS-induced lung inflammation by altering the inflammatory cytokines and scavenging the free radicals formed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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