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ORIGINAL ARTICLE

p-Coumaric Acid Attenuates Lipopolysaccharide-Induced Lung Inflammation in Rats by Scavenging ROS Production: an *In Vivo* and *In Vitro* Study

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Abstract— Lipopolysaccharide (LPS), known as lipoglycans and endotoxins found in the cell wall of some type of Gram-negative bacteria, causes acute lung inflammation (ALI). p-Coumaric acid (p-CA) possesses anti-inflammatory and anti-oxidative activities. The main purpose of our research was to explore the effect of p-CA on LPS-induced inflammation. In part I, 32 rats were divided into four groups: Control, LPS (5 mg/kg), p-CA (100 mg/kg), and LPS + p-CA to investigate acute lung inflammation caused by LPS. In part II, the effect of LPS-stimulated inflammatory response on A549 cells was investigated. The dosage of LPS and p-CA which used in this part was 1 μg/ml and 20 mM, respectively. ALI rats showed an elevation in antioxidant activity, TNF-alpha, IL-6, MDA, inflammatory parameters, and Nrf2 gene expression. Although pre-treatment with p-CA could return these changes approximately to normal condition in all two-part studies (*in vivo* and *in vitro*). The results of *in vivo* and *in vitro* study showed that LPS induced lung inflammation. Pre-treatment with p-CA causes modulating of oxidative stress in inflammatory condition in lung injury and A549 cell.

KEY WORDS: LPS; p-coumaric acid; ALI; inflammation; Nrf2; A549 cells.

INTRODUCTION

A major ligand for Toll-like receptor 4 (TLR4) is lipopolysaccharide (LPS), which causes acute lung injury (ALI), leads to serious inflammation in airway, and can result in oxidative stress. Long-term exposure to inflammation causes changes in the structure of alveolar walls and airway components [1, 2]. ALI is related to acute respiratory infections with high rate of fatality [3]. Intratrachealy injection of LPS promotes inflammation and stimulates inflammatory cytokines including TNF-alpha and interleukin-6 which play an important role for expression of adhesion molecules [4].

Nuclear factor erythroid-derived 2-like 2 (Nrf2), as an important transcription factor, in pathological condition maintained cellular antioxidant capacity, resulting in disable or prevent NF- B signaling pathway. It was shown that in Nrf2-deficient mouse, LPS leads to stimulation of TNF-alpha and NF- B pathway in embryonic fibroblasts [5]. In another study performed on Nrf2 knockout mice, cigarette smoke and elastase increased the susceptibility to emphysema [6]. In inflammatory diseases, accumulation of free

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radicals causes activation of inflammatory cytokines and many other processes such as dilatation of local vasculature, leukocyte migration, increased permeability, and blood flow [7].

Epidemiological studies have shown that dietary factors, which are rich in herbs, fruits, and vegetable, have low or no side effect for use in preventing human diseases and reduce the risk of cardiovascular diseases [8]. p-Coumaric acid (p-CA) as a flavonoid agent, is associated with protection effects against several pathological conditions such as carcinogenesis [9], oxidative cardiac damage, atherosclerosis [10], neuronal injury [11], and anti-inflammatory activities [12].

In the current study, the role of oxidative stress activation during TLR signaling in lung and cell culture was investigated. Since anti-oxidant therapy can inhibit oxidative stress, we investigated the anti-inflammatory effect of p-coumaric acid as an antioxidant agent, against LPS-induced lung injury involved in this pathway.

MATERIAL AND METHODS

Chemicals

LPS (E.coli, 055:B5), p-coumaric acid, xylazine, and ketamine HCl were obtained from Sigma-Aldrich Co., USA, and Alfasan Co., Netherlands, respectively. Antioxidant assay and cytokine ELISA kits were obtained from ZELLBIO and IBL (Germany). The material used in part II: polyclonal anti-Nrf2 (Abcam, Cambridge, MA); Nrf2 primers for RT-PCR (Bioneer, South Korea); FBS (HyClone Laboratories, Logan, UT); penicillin/streptomycin and RPMI-1640 (Invitrogen, Burlington, Canada);

Animals' Treatments

Thirty-two Sprague-Dawley rats (male, 200–250 g) were purchased from the Animal House in Ahvaz Jundishapur University. The rats were divided into 4 groups ($n = 8$): control (intrapretunaly injection of normal saline for 10 days + intratracheal instillation of normal saline in the 8th day), p-CA (intrapretunaly injection of p-CA100 mg/kg for 10 days + intratracheal instillation of normal saline in the 8th day), LPS (intrapretunaly injection of normal saline for 10 days + intratracheal instillation of LPS 5 mg/kg in the 8th day), p-CA + LPS (intrapretunaly injection of p-CA 100 mg/kg for 10 days + intratracheal instillation of LPS in the 8th day). To produce the effective dose of p-CA, concentration-effect study (25, 50, and 100 mg/kg, ip) was done. In BALF, p-CA 25 mg/kg cannot decrease inflammatory cell counts, also p-CA 50 and 100 decrease inflammatory cell count level significantly ($P < 0.05$) and ($P < 0.001$) respectively, p-CA at 100 mg/kg effectively decreased inflammatory cell counts. Therefore, it was used as an effective dose (Fig. 1). The experiments were carried out in accordance with the ethical guidelines, and the protocol was approved by the Ethics Committee for Animals at Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (No: IR.AJUMS.REC.1396.275).

LPS Instillation

After anesthetizing the animals by xylazine and ketamine (ip), the normal saline (0.2 ml) containing 5 mg/kg of *E. coli* lipopolysaccharide was instilled into the airways. Control animals received saline by the same route.

Bronchoalveolar Lavage Procedure

In the end of treatments, the rats were placed on their dorsal side, then their thoracic cavities were opened, and their trachea was cannulated. One milliliter of PBS was flushed into the lung (3 times) and then collected for examination. The procedure was made in triplicate for every sample. The BALF was centrifuged (4 °C, 4000 rpm, 10 min), and the supernatant was kept at − 80 °C for other examination [13]. Total cell count was done in Neubauer chamber, and differential count was done on air-dried slide stained by Wright-Giemsa. Under × 400 magnification, the total and differential leukocyte counts were determined.

Determination of Cytokines in BALF

Interleukin-6 and TNF-α produced in BAL fluid supernatant were determined by using ELISA kits (IBL, Germany) as introduced by the manufacturer.

Histopathology of Lung Tissue

In all groups, the lung tissue from each rat was fixed in 10% buffered formalin (overnight), then proceeded to paraffin, tissues cut into 4 μm sections, and stained with H&E, then by using a light microscope, pathological changes in the lung tissues were determined [14].

Levels of Antioxidant Enzymes and MDA in Lung Tissue and A549 Cell

One hundred milligrams of lung tissue was homogenized in PBS and then centrifuged (1000*g*, 10 min). Cell

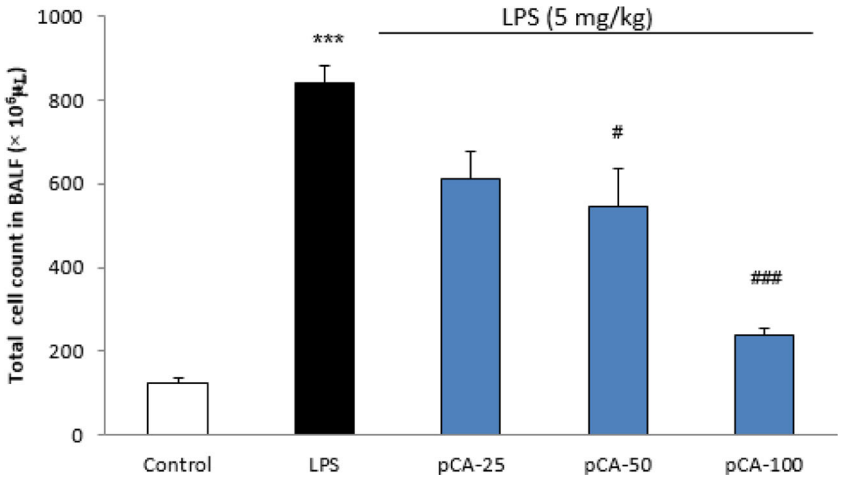

Fig. 1. Concentration-effect study (25, 50, and 100 mg/kg of p-CA, ip) in BALF: Data are expressed as the mean ± SEM ($n = 8$ for each group). ***$P < 0.001$ *vs.* control group, ###$P < 0.001$ and #$P < 0.05$ *vs.* LPS group. p-CA, p-coumaric acid.

samples (in part II) and supernatant of lung tissue were used for measurement of antioxidant enzyme activities and MDA by using ZellBio kits (Germany).

Expression of Nrf2 Gene in Lung Tissue and A549 Cell

The total RNA was extracted from lung tissue homogenates and cells (in part II) using RNeasy plus mini kit (Qiagen Co., Netherlands). By spectrophotometry (BioPhotometer Plus; Eppendorf, Germany) at 260 and 280 nm, total RNA concentration and OD value was calculated. Then 1 μg of the total RNA was used for synthesis of the complementary DNA (cDNA synthesis kit, Qiagen, USA). For determining levels of the Nrf2 mRNA and the housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH) in the lung tissues and A549 cell (in part II), reverse transcription polymerase chain reaction (RT-PCR) was used [15]. The primer sequences were used for PCR analysis: Nrf2 (forward: GGTTTCTTCGGCTA CGTTTC; reverse: CCTCCCAAACTTGCTCAATG) and GAPDH (forward: GTATTG GGC GCC TGG TCA CC; reverse: CGCTCCTGGAAGATGGTGATGG). In A549 cells: GAPDH (forward: GCTCACTGTTCTCT CCCTC; reverse: GAGGTCAATGAAGGGGTCAT) and Nrf2 (forward: GGTTTCTTCGGCTACGTTTC; reverse: CCTCCCAAACTTGCTCAATG).

Part II

Cell Culture and Treatments. The human alveolar epithelial cell line (A549) was purchased from Pasteur Institute at National Cell Bank of Iran (NCBI), and maintained in culture medium supplemented with 1% FBS. The cells were divided into 5 groups: control (treated only with DMEM), p-CA (treated with p-CA 20 mM for 2 h), LPS (treated with LPS 1 μg/ml for 24 h), LPS + p-CA (treated for 24 h with LPS 1 μg/ml + p-CA 20 mM for 2 h), H_2O_2 (100 μM) a famous oxidant, as positive control in selective experiments [16]. Concentration effect of p-CA was down through MTT assay (20, 40, 80, 160 mM).

MTT Reduction Assay. By using the MTT assay, cell viability was determined. A549 cells were cultured with density of 1–10^5 cells/ml, and after various treatments with p-CA (20, 40, 80, 160 mM), the medium was removed. Subsequently, the cells were incubated with MTT, and then by using a microplate reader (Bio-Rad, CA, USA), the optical density was read (OD) at 570 nm. We used the following formula: Optical density test/optical density control×100. p-CA at 20 mM showed a larger OD value.

Flow cytometry for Reactive Oxygen Species. To determine the percentage of the reactive oxygen species in all groups, we used an oxidant-sensitive fluorescent probe, known as 5-(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA). After various treatment of cells, and removing the culture medium, the cells were washed with PBS and incubated with 10M H2DCFDA at 37 °C for 30 min. Flow cytometric analysis was performed using a flow cytometer (ELITE, Coulter, Hialeah, FL) after trypsinizing the cells, washing, and resuspending in PBS (≥ 10,000 events acquired per sample) [16].

Nrf2 Immunocytochemistry. Immunocytochemistry was performed for Nrf2 localization in response to LPS in A549 cells. A549 cells were cultured in 24-well plates, and after various treatments, washing with PBS, and fixing for 30 min with 4% paraformaldehyde, the cells were washed with PBS. Then, permeabilizing and washing was done using Triton X-100 for 10 min, blocking with PBS containing 2% bovine serum albumin for 30 min. Then the cells were incubated overnight with rabbit polyclonal anti-Nrf2 antibody (Abcam, Cambridge, MA). The cells washed with PBS and incubated with FITC-labeled anti-rabbit IgG for 1 h at room temperature in the dark. Finally, after washing with PBS, to demonstrate cell nucleus (blue stains), the cells were stained with DAPI (Roche) for 30 min then observed under UV light microscopy [16].

Statistical Analysis. Statistical comparisons were made by the one-way ANOVA and followed by *post hoc* Tukey's test. Data are expressed as means ± SEM; if p values were lower than 0.05, data were considered statistically significant.

RESULTS

Levels of Inflammatory Cell Count in BALF

In BAL Fluid, LPS treatment significantly increased the total cell count ($P < 0.001$), percentage of macrophages ($P < 0.05$), and polymorphonuclear leukocytes in LPS group compared with control rat ($P < 0.001$), which is presented in Table 1. Pre-treatment of rats with p-CA (100 mg/kg) significantly reduced LPS-enhanced total cell numbers ($P < 0.001$) and percentage of macrophages ($P < 0.05$), lymphocyte, and neutrophil cell ($P < 0.001$).

Cytokine Activity in BALF

TNF-alpha and IL-6 are important cytokines for index of lung inflammation. Levels of IL-6 and TNF-alpha in BAL fluid were analyzed to evaluate the inflammatory changes in all groups as presented in (Fig. 2); there was a significant increase in IL-6 ($P < 0.001$) and TNF-alpha ($P < 0.001$) in the LPS group. However, IL-6 and TNF-α activity significantly decreased by pre-treatment of p-CA in the LPS group compared with LPS alone.

Histology of Lung Tissue in ALI

To investigate the effect of p-CA on histological changes after treatment with LPS-induced inflammation in rats, significant pathologic changes were observed in the lungs of LPS-treated rats when the lung sections were stained with hematoxylin and eosin. These changes included inflammatory cell infiltration and rupture of air space. Pre-treatment with p-CA (100 mg/kg) reduced these histopathological changes compared with the control group (Fig. 3).

Levels of Antioxidant Enzymes and Lipid Peroxidation in ALI

As shown in Fig. 4, MDA significantly increased in the LPS-treated rats in comparison with the control group ($P < 0.001$). In groups receiving p-CA, the MDA levels significantly decreased in lung tissue in comparison with LPS alone ($P < 0.001$). The activity of GSH, SOD, and GPx in lung tissue of the LPS group was significantly higher in comparison with controls ($P < 0.01$). Pre-treatment with p-CA in the LPS group leads to a significant decrease in activity of antioxidant enzymes ($P < 0.05$ and $P < 0.01$).

RT-PCR for Nrf2 Gene Expression in Lung Tissue

As presented in Fig. 5, the expression of Nrf2 gene significantly increased after 72 h in the LPS group compared with the controls ($P < 0.01$). In group receiving p-CA, Nrf2 gene expression significantly decreased compared with LPS alone ($P < 0.01$).

Part II

Toxicity of p-CA to A549 Cells. Cell viability was assayed to evaluate the toxicity of p-CA to human alveolar epithelial A549 cells. Treatment of A549 cells with 20, 40, 80, and 160 mM p-CA did not show any cytotoxic effect on the viability in A549 cells in 20 and 40 mM concentrations (Fig. 6).

ROS Production in A549. To investigate the effect of p-CA on the ROS scavenging in A549 cells, the cells were treated with LPS (1 μg/ml) in the presence and absence of p-CA (2 h, 20 mM). By flow cytometry which used the fluorescent probe H2DCFDA, ROS levels were determined in A549 cells. LPS leads to a significant production of ROS ($P < 0.001$). Pre-treatment with p-CA attenuated LPS-induced ROS production ($P < 0.001$), suggesting the ability of p-CA to ROS scavenging (Fig. 7).

Nuclear Translocation of Nrf2 in A549 Cells. Nrf2 Nuclear translocation was localized in cytoplasm of the control

Table 1. Inflammation cell count in the BALF

Groups	Total cell ($\times 10^6$ µL)	Macrophages (%)	Lymphocytes (%)	Polymorphonuclear leukocytes (%)
Control	125 ± 9.74	53 ± 1.41	28.6 ± 1.36	18.2 ± 1.8
p-CA	107.8 ± 4	52.8 ± 0.91	24.6 ± 2.37	18.4 ± 2.75
LPS	840 ± 43.01***	59.4 ± 1.69*	10 ± 1.34***	30.8 ± 1.15***
LPS + p-CA	239.6 ± 16.79###	52.2 ± 1.49#	27.8 ± 1.93###	19.6 ± 1.36###

Airway inflammation was induced by intratracheal injection (i.t) of LPS 5 mg/kg on the 8th day. *$P < 0.05$ and ***$P < 0.001$ *vs.* control group (# $P < 0.05$, ### $P < 0.001$ *vs.* LPS group. Data are expressed as the mean ± SEM ($n = 8$)
p-CA p-coumaric acid

group. After treatment with LPS for 24 h and p-CA (2 h) alone or with LPS and in H_2O_2 (as a positive control) increased Nrf2 protein translocation in the nucleus compared with the control group (Fig. 8).

Nrf2 Expression A549 Cells. The levels of Nrf2 mRNA expression were examined by RT-PCR analyses. LPS treatment can increase expression of Nrf2 ($P < 0.001$), but the pre-treatment of p-CA (20 mM) for 2 h resulted in decreased Nrf2 mRNA expression ($P < 0.01$) (Fig. 9).

Antioxidant Enzyme Activity and Lipid Peroxidation in Cell Culture Supernatant. At the end of the experiment, MDA level in the LPS group was significantly higher in comparison with the control group ($P < 0.001$). In groups receiving p-CA, there was a significant decrease in the MDA levels in cells compared with LPS alone ($P < 0.05$). GSH, SOD, and GPx activity in the LPS group significantly

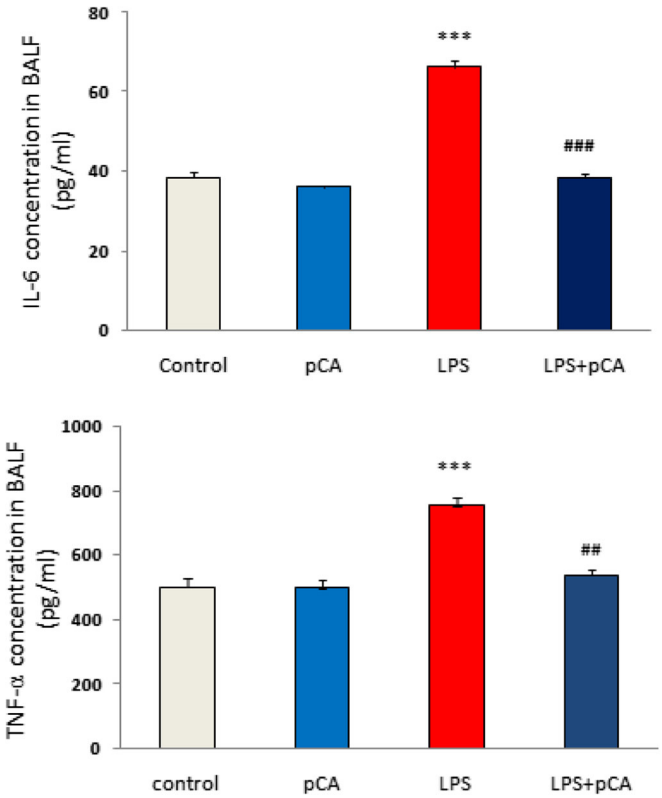

Fig. 2. Effect of p-CA (100 mg/kg) on TNF-α and IL-6 in tissue of LPS-treated rat: ***$P < 0.001$ *vs.* control group, ###$P < 0.001$ *vs.* LPS group. Data are expressed as the mean ± SEM of ($n = 8$).

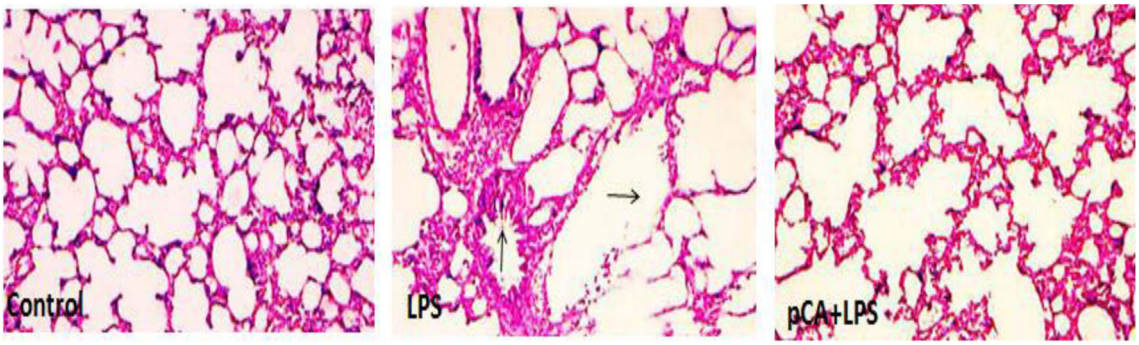

Fig. 3. Effect of p-CA (100 mg/kg) on histopathological examination in lung tissues in LPS-induced ALI. Lung sections were stained with hematoxylin and eosin (H&E). Control: no inflammation, LPS treatment (5 mg/kg); infiltration of inflammatory cells (), LPS + p-CA (100 mg/kg); showed very low inflammation (magnification × 200). Scale bars, 100 μm.

increased compared with controls ($P < 0.01$). These antioxidant enzymes decreased significantly ($P < 0.01$) by pretreatment of p-CA in the LPS group, as shown in Fig. 10.

DISCUSSION

According to the results of this study, anti-inflammatory effect of p-CA on acute lung injury (ALI) was shown as in *in vivo* and *in vitro*. We showed that injection of LPS intratrachealy induced ALI in rats and numbers of total cells and differential cell count including macrophages and polymorphonuclear leukocytes also increased the level of TNF-alpha and IL-6 which are pro-inflammatory mediators, in the BALF of LPS rats compared with controls. These findings are consistent with other reports [17]. In the pathological inflammatory process, TNF-alpha is produced by several cell types in the airways, including mast cells, macrophages, and lymphocytes, and it induces airway smooth muscle cell

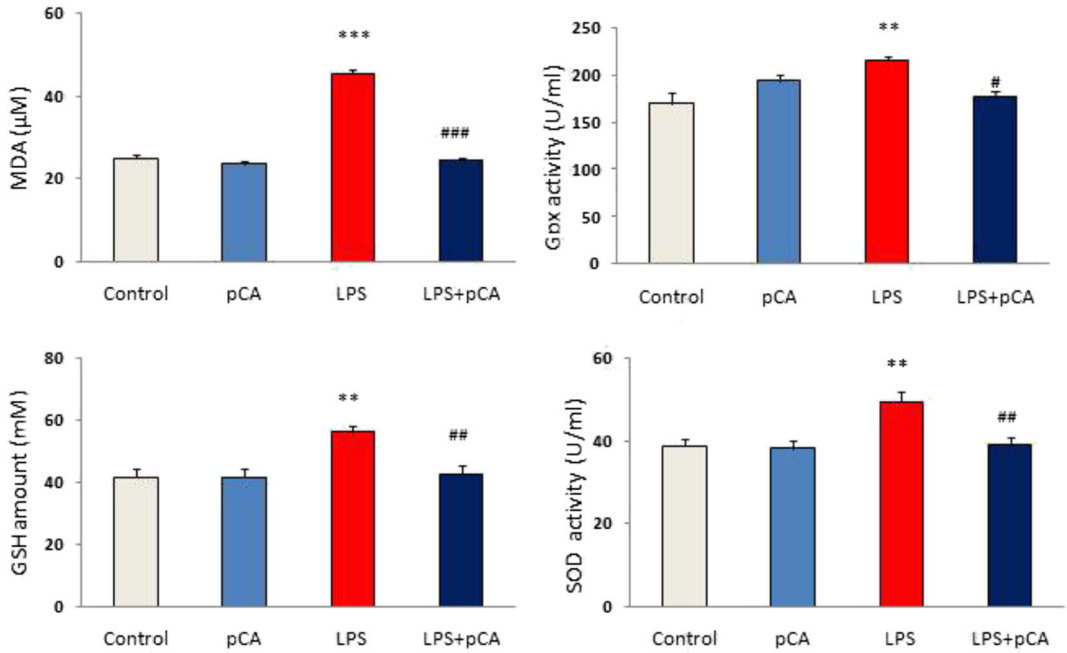

Fig. 4. Effect of p-CA (100 mg/kg) on antioxidant enzymes and MDA levels in lung tissue of LPS-treated rat. $^{***}P < 0.001$ and $^{**}P < 0.05$ *vs.* control group, $^{###}P < 0.001$, $^{##}P < 0.01$ and $^{#}P < 0.05$ *vs.* LPS group. Data are expressed as the mean ± SEM ($n = 8$).

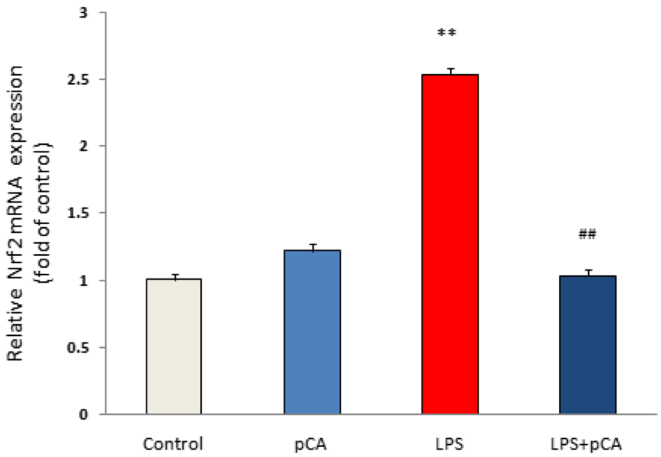

Fig. 5. Effect of p-CA (100 mg/kg) on Nrf2 mRNA expression in lung tissue of LPS-treated rat: $^{**}P < 0.01$ *vs.* control group, $^{\#\#}P < 0.01$ *vs.* LPS group. Data are expressed as the mean ± SEM ($n = 8$).

contractility. IL-6 has been shown to be elevated in several inflammatory lung diseases, resulting in the generation of reactive oxygen species (ROS) which causes damage to the macromolecules such as DNA [18–20]. To assess the anti-inflammatory effect of p-CA, we examined the p-CA effect in the model of the LPS-induced inflammation in rats. In group receiving pre-treatment with p-CA, the number of pro-inflammatory macrophages and polymorphonuclear leukocytes (Table 1) was reduced, and in the BALF of LPS-treated rats, there was a decline in TNF-alpha, and IL-6 level was seen (Fig. 2). Nowadays, the herbal medicine exhibits various anti-inflammatory activity [21]. p-CA, a natural phenolic compound, has an important role in reducing lung inflammation caused by LPS. Histopathological results of this study showed a significant difference between the LPS group and the group pre-treated with p-CA, with a significant decrease in inflammatory cell and increase in alveolar air space. Oxidative damage and induction of the matrix metalloproteinase are induced by inflammatory agents such as leukocytes. According to the findings of this study, the increase and activation of inflammatory cells and pro-inflammatory factors is a reason for

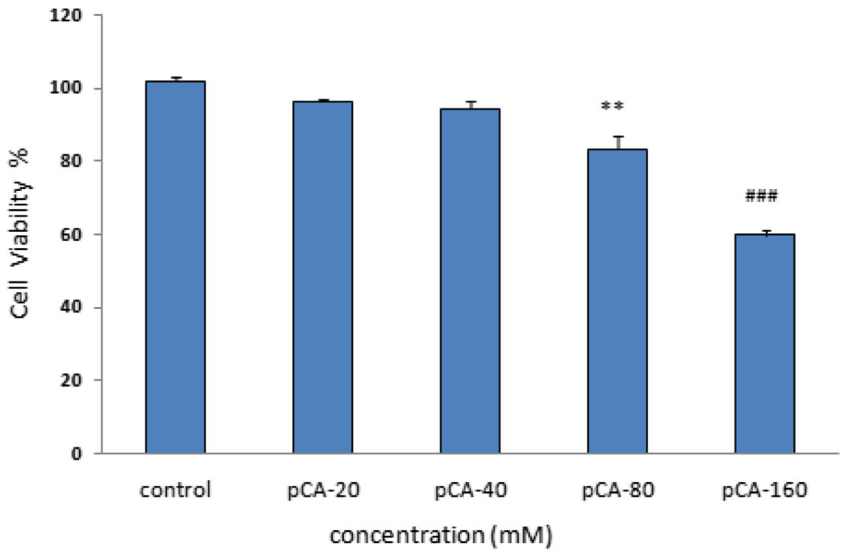

Fig. 6. Effect of p-CA (20, 40, 80, and 160 mM) on cell viability. Cytotoxic effect of p-CA on A549 cells was measured by MTT assay. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control group. Data are expressed as the mean ± SEM.

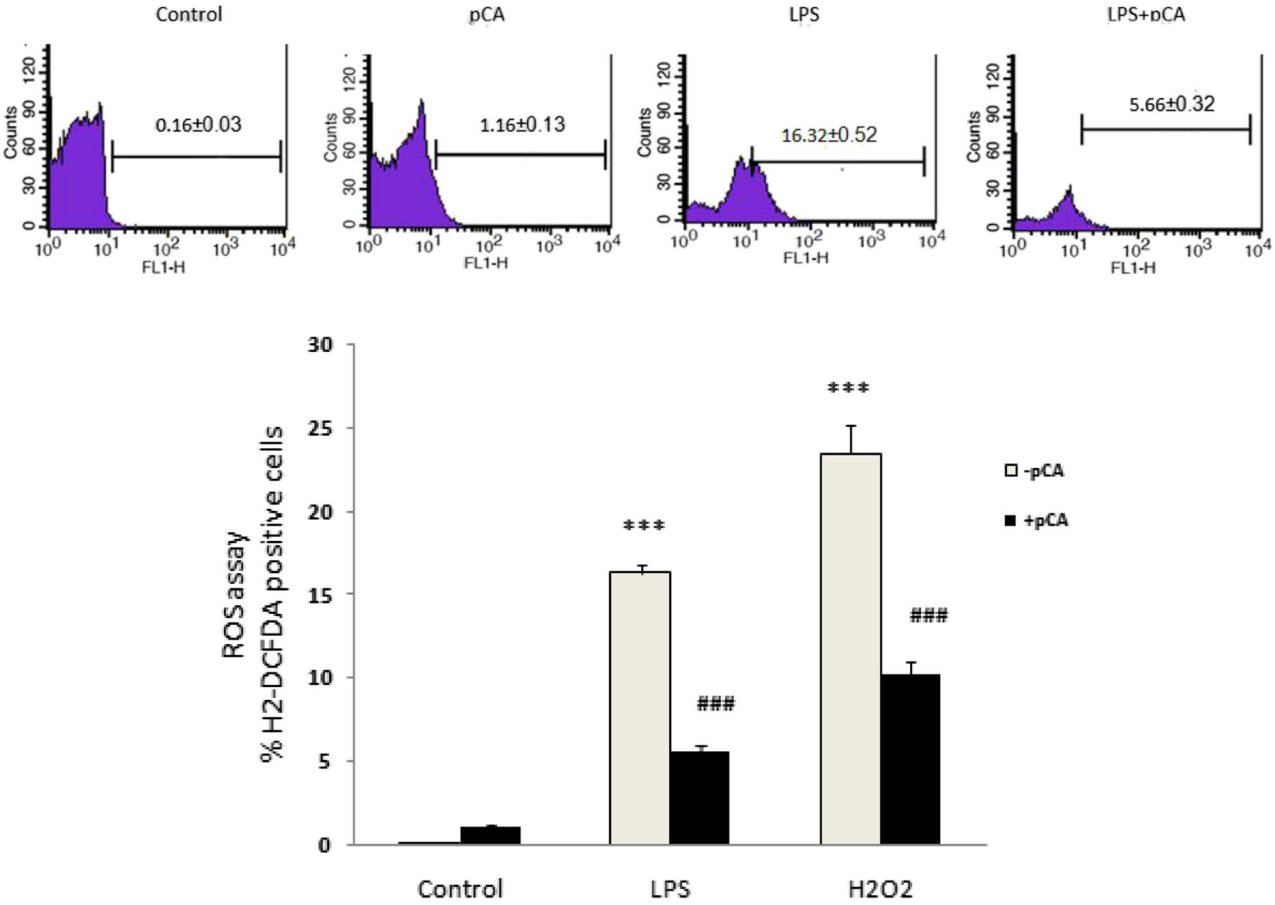

Fig. 7. Effect of p-CA (20 mM) on LPS-induced reactive oxygen species (ROS) in cell sample (A549): $^{***}P < 0.001$ *vs.* control group, $^{###}P < 0.001$ *vs.* LPS group. Data are expressed as the mean ± SEM.

the inflammatory and degenerative processes in the lung tissue.

We also demonstrated the ability of p-CA to reduce the MDA level in lung tissue. Our results showed that LPS treatment leads to increased oxidative stress *via* lipid peroxidation (MDA) after 72 h. p-CA significantly decreased LPS-induced MDA production, compared with treatment with LPS alone (Fig. 4). These results confirmed that p-CA has a potential to reduce lipid peroxidation. Important antioxidant enzymes such as SOD, CAT, and GPx protect the lung from oxidative stress by decreasing hydrogen peroxide [22]. Unexpectedly, increased SOD, GSH, and GPx activity was observed in the LPS group, which reflected an imbalance between oxidants and antioxidants, as confirmed by Rahman et al. The increase of oxidants (ROS) is proportional and parallel to an increase in its substrate such as SOD, GSH, and GPx (Fig. 11). Pretreatment with p-CA leads to changes in levels of SOD, GSH, and GPx which was similar to the controls, and this alteration indicates the balance kept between antioxidants and oxidants and another possibility is the direct effect of p-CA in quenching and reducing free radicals.

Antioxidant capacity and lipid peroxidation are important indexes of the oxidative damage induced by free radicals [23, 24]. Moura et al. (2012) showed the level of SOD, CAT, and GPx increased in the cs group compared with control rats (in acute inflammation caused by cigarette smoke) and treatment with *Euterpe oleracea* Mart. Extract leads to decrease antioxidant activity similar to control group. Ben-Shaul et al. found that treatment with LPS (ip) alone causes an approximately 20% increase in SOD and catalase activity in heart compared with the control group and apocynin decreased SOD and CAT level in heart tissue after 24 h post LPS [25]. However, in another study, different results have been reported. Shi et al., for example, found bovine mammary epithelial cells stimulated with 1

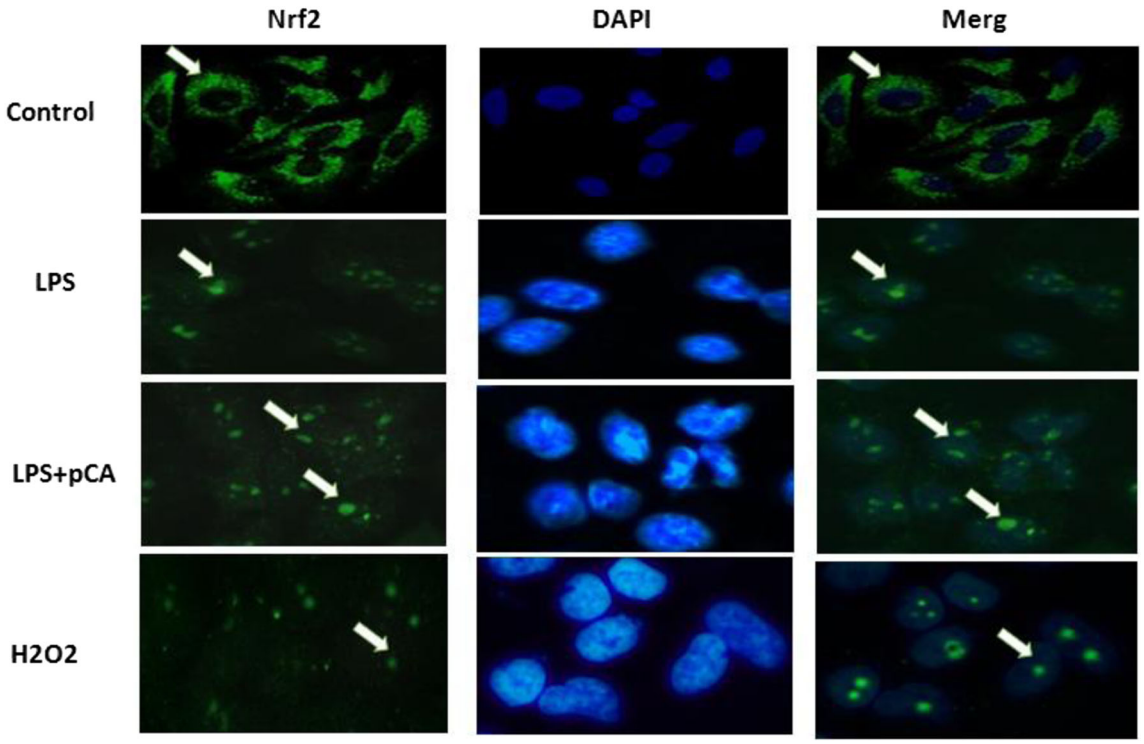

Fig. 8. Immunocytochemistry of A549 cells showing localization of Nrf2 in response to LPS treatment. H_2O_2 (100 μM) was used as a positive control. After treatment, cells were incubated with Nrf2 antibody. Cells were counter stained with DAPI to show nuclear morphology and visualized under a fluorescent microscope.

and 100 μg/ml LPS showed a significantly lower antioxidant enzyme activity compared with the control group. Chu et al. investigated the effect of the rosmarinic acid on LPS-induced ALI and showed SOD activity was lower in ALI mice. Hung-Te Hsu et al. showed propofol reduce oxidative stress induced by LPS through the Nrf2 pathway, which increased the total GSH and reduced the level of cellular ROS in A549 cells compared with the LPS-treated

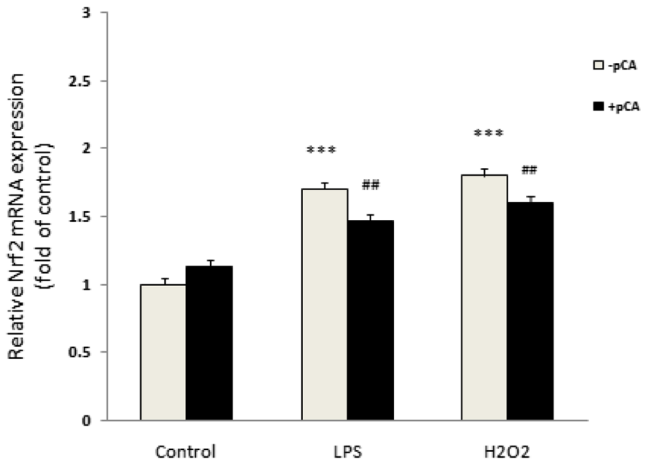

Fig. 9. Effect of p-CA (20 mM) on Nrf2 mRNA expression in cell sample (A549): inflammation was induced by 1 μg/ml LPS for 24 h. $^{***}P < 0.001$ *vs.* control group, $^{\#\#\#}P < 0.001$ *vs.* LPS group. Data are expressed as the mean ± SEM.

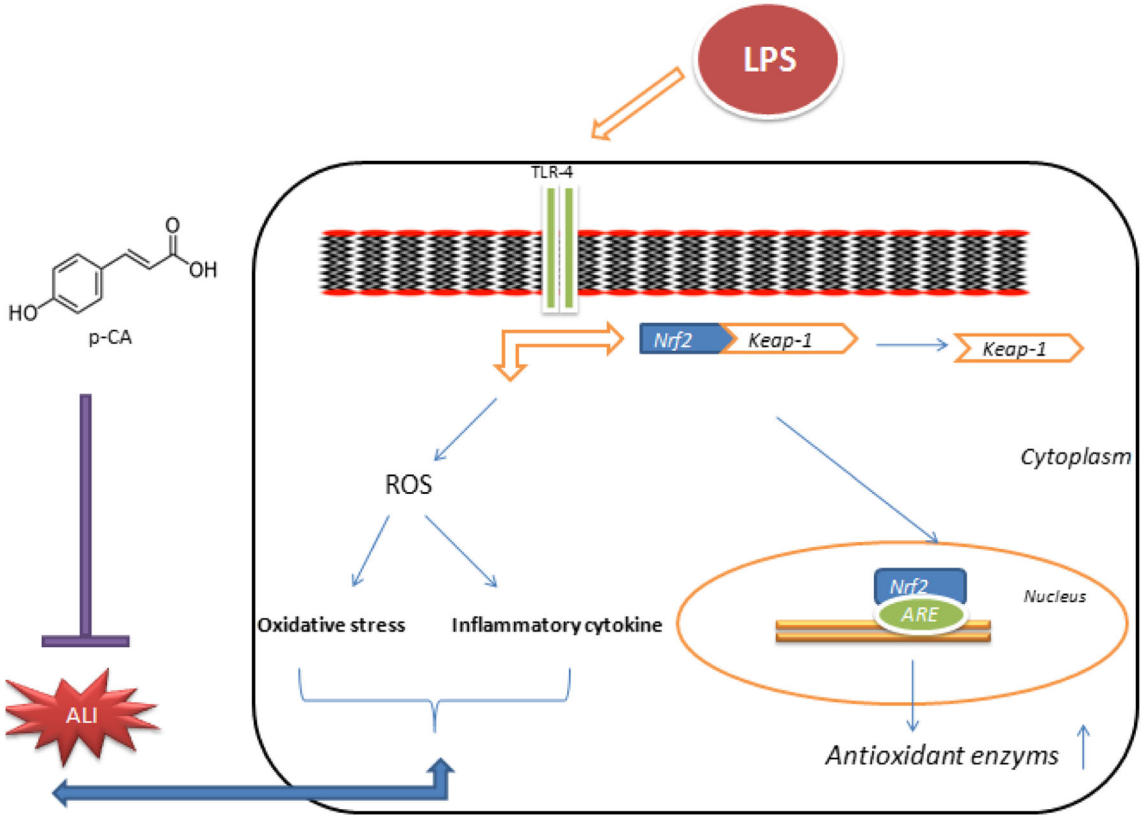

Fig. 11. Schematic pathway of LPS-induced ALI *via* oxidative stress and protective role of p-coumaric acid (p-CA).

A549 cells. These different reports may be due to differences in the type of animal, cells, dosage, or test model.

In our study, LPS upregulated the Nrf2 expression in the ALI group, and in rats that pre-treated with p-CA, Nrf2 expression was similar to controls *in vivo* (Fig. 5). Oxidative damage is a marker of lung injury during respiratory diseases. Under acute pathological conditions such as ALI, the balance between oxidant and antioxidant systems changes. Thus, anti-oxidant therapy is one of the strategies for the treatment of ALI, and p-CA may be a potential therapeutic candidate as an anti-inflammatory agent.

We tested our hypothesis in human airway epithelial cells. LPS exposure led to ROS and MDA production, in lung epithelial cells. Our results are consistent with a previous study [16].

Alveolar epithelial cells (AEC) are considered as a key defense against environmental pathogens because of the location of these cells during the onset of a pathogen attack, which forms the inner layer of the pulmonary airways and alveoli. Therefore, AEC played a key role in inflammatory condition [26]. The injury of the pulmonary blood-air barrier is the main pathophysiologic characteristic of ALI/ARDS [27]. ROS production, MDA level, and antioxidant enzyme activity in group treated with p-CA significantly decreased compared with LPS alone.

When chemopreventive compounds or oxidative stress affected the cells, Nrf2, the critical factor that regulated cellular defense response, translocates into the nucleus and leads to expression of endogenous antioxidants and phase II detoxifying enzymes [28].

We showed that in the absence of LPS, Nrf2 was detected in the cytoplasm of A549 cells, but was detected in the nucleus of A549 cells after exposure to LPS, H_2O_2 (which is an oxidant) or p-CA (Fig. 7). An increase in Nrf2 levels is associated with nuclear translocation of Nrf2 in A549 cells. Treatment with p-CA for 2 h after LPS resulted in translocation of Nrf2 to the nucleus. It seems that LPS and H_2O_2 elicit conformational changes in Nrf2/Keap1 complex, which leads to separation of Nrf2 from Keap1, then Nrf2 translocates to the nucleus similar to what gets done under conditions like oxidative stress and exposure to

electrophilic compounds, which have the ability to modify thiol groups of Keap1 or Nrf2 [29]. In addition, we reported nuclear accumulation of Nrf2 in A549 cells treated with p-CA plus LPS. One possible justification can be the ability of p-CA to augmentation of Keap1 or upstream kinases of this pathway such as mitogen-activated kinases, phosphatidyl inositol 3-kinase, and protein Kinase-C may be involved in this process [30].

In conclusion, exposure to LPS increased Nrf2 expression, increased antioxidant capacity, and increased lipid peroxidation in *in vivo* and in *in vitro*. This indicates that the imbalance of oxidants and anti-oxidants has caused injury. Treatment of A549 cells and rats with p-CA significantly restores this change. It seems that p-CA prevents LPS-induced ALI by reducing oxidative stress, inflammatory factors, and scavenging of ROS.

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COMPLIANCE WITH ETHICAL STANDARDS

The experiments were carried out in accordance with the ethical guidelines, and the protocol was approved by the Ethics Committee for Animals at Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (No: IR.AJUMS.REC.1396.275).

Conflict of Interest. The authors declare that they have no conflict of interest.

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