

Potential Effect of Astragaloside IV on the Lipopolysaccharide Induced Inflammation *via* the Inactivation of NF- κ B Signaling Pathway

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ABSTRACT

Background: The host treats lipopolysaccharides (LPS) as a sign of microbial invasion by pathogenic Gram-negative bacteria. In lungs, LPS activates a cascade of inflammatory reactions leading to inflammation. Previous investigation suggests that astragaloside IV (AG) has an anti-inflammatory and antioxidant effect, but the potential mechanism of lung inflammation is still unknown. In this experimental study, we aimed to investigate the anti-inflammatory potential of AG against LPS-induced lung inflammation.

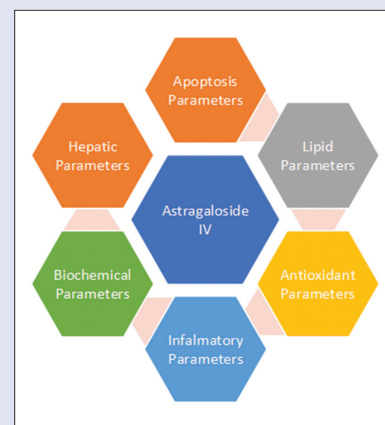
Materials and Methods: In this study, we used Sprague Dawley rats for the experimental protocol. Animals were injected with LPS (10 mg/kg, b. w.) for the induction of lung inflammation and were subsequently administered with AG (1.25, 2.5, and 5 mg/kg). At the end of the experiment, acute phase response and lipid parameters were estimated. Pro-inflammatory cytokines and inflammatory mediators were also estimated. Furthermore, quantitative real-time polymerase chain reaction was used to estimate the inflammatory markers and expression of mRNA of apoptosis markers.

Results: AG treatment significantly increased the survival rate as compared to LPS control. AG significantly ($P < 0.001$) altered the renal, hepatic, lipid, and antioxidant parameter at dose dependent manner. AG significantly ($P < 0.001$) decreased the level of malondialdehyde and reduced glutathione and increased the activity of superoxide dismutase. AG significantly ($P < 0.001$) decreased the level of nuclear factor kappa B (NF- κ B). In addition, AG significantly ($P < 0.001$) down-regulated the expression of inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-1 β , tumor necrosis factor- α , IL-18 and upregulated the expression of IL-4 and IL-10. **Conclusion:** In summary, these findings indicate that AG effectively suppressed the LPS-induced inflammation through inactivation of NF- κ B signaling pathway.

Key words: Antioxidant, apoptosis, astragaloside IV, lipopolysaccharide, lung inflammation

SUMMARY

- Astragaloside IV showed the lung protective effect against the LPS induced lung toxicity. AG considerably reduced the oxidative stress via altering the antioxidant enzymes level. AG significantly suppress the inflammatory reaction via decreased the level of pro-inflammatory cytokines and inflammatory mediators. On the basis of result we can say that Astragaloside IV could be potential drug for the treatment of lung toxicity



Abbreviations used: LPS=Lipopolysaccharides, AG=Astragaloside IV, IL-1=Interleukin-1, IL-6=Interleukin-6, IL-1 β = Interleukin-1 β , TNF- α =Tumor necrosis factor- α , IL-18= Interleukin-18, IL-4= Interleukin-4, IL-10=Interleukin-10, ALI=Acute lung injury, ARDS=Acute respiratory distress syndrome, IFN- γ =Interferon, NO=Nitric oxide, CINC-1=Cytokine-induced neutrophil chemoattractant, IL-8=Interleukin-8, PMNs=Polymorphonuclear neutrophils, iNOS=Inducible nitric oxide synthase, ERK=Extracellular signal-regulated kinase, MAPKs=Mitogen-activated protein kinases, STAT=Signal transducer and transcription activator, ROS=Reactive oxygen species, MDA=Malondialdehyde, GSH=Glutathione, SOD=Superoxide dismutase, CMC=Carboxymethyl cellulose, ALT=Alanine aminotransferase, ALP=Alkaline phosphatase, AST=Aspartate aminotransaminase, TC=Total cholesterol, HDL=High-density lipoprotein, TG=Triglycerides, SEM=Standard error of mean, ANOVA=One-way analysis of variance, MPO=Myeloperoxidase, CRP=C-reactive protein

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INTRODUCTION

Environmental pollution has led to acute inflammation of the parenchyma in the lung tissue which in turn leads to lung diseases such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS) ensuing in elevated mortality.^[1,2] These ailments might arise due to contact with numerous pathogenic micro-organisms releasing harmful molecule such as LPS present in the Gram-negative bacteria (outer-membrane portion), which can stimulate receptors for host pattern recognition 2.^[3,4] Researchers suggest that the acute lung inflammation that extravasations of plasma contains plentiful polymorphonuclear neutrophils (PMNs) and the existence of products of polymorphonuclear leukocytes in patient bronchoalveolar lavage fluid is associated with pathological manifestations inside the pulmonary tract.^[3-5]

During inflammation in the lungs, immune-related cells get activated and stimulate the production of various pro-inflammatory cytokines such as interferon- γ , tumor necrosis factor (TNF)- α , and interleukin (IL)-1 β that migrate to the inflamed area. The epithelium in the respiratory passage is the main cause of nitric oxide (NO) (inflammatory mediators), cytokine-induced neutrophil chemoattractant-1, and chemokines (IL-8) is also involved in lung injury.^[6,7] The relocation and recruitment of activated PMNs and other immune cells in the affected lung area induce respiratory illness. It has been clinically proven that the amelioration of lung inflammation occurs through the reduction in the number of PMNs or through blockade of recruitment of PMNs in the affected area.^[8-10] It has been revealed that inducible NO synthase is controlled through various signaling pathways, such as extracellular signal-regulated kinase, mitogen-activated protein kinases p38, and signal transducer and transcription activator pathways in various cell types.^[8,9,11,12]

Bacterial lipopolysaccharide (LPS), an endotoxin, is extracted from the Gram-negative bacterial cell walls which possess pro-inflammatory properties. It is also present in air, smoke generated from cigarette, and dusts of organic products.^[9,10,13] It is known to cause ARDS in people. LPS activates the human innate immune system and causes tissue damage, organ failure, and over production of NO.^[14,15] It stimulates the immune system in a manner similar to extreme sepsis by inducing systemic inflammation through the development of reactive oxygen species, which induces lung tissue damage through oxidative effect of the microvasculature in the lung. Injury in lung cause inflammatory cell infiltration and trauma in tissue leads to fibrosis. Previous studies have shown that long-term exposure to LPS reduces pulmonary function by

triggering a strong inflammatory response in the lungs.^[3,4] However, the extent of these patients' inflammatory processes in the lung pathology is still uncertain. Nuclear factor kappa B (NF- κ B), which is a transcription factor regulates various genes responsible for causing inflammation. It activates TNF- α and IL-6 gene promoter region which initiates inflammatory reaction during infection.^[3-5]

The principal active compound of *Astragalus membranaceus* is astragaloside IV (AG).^[16] The chemical structure of AG

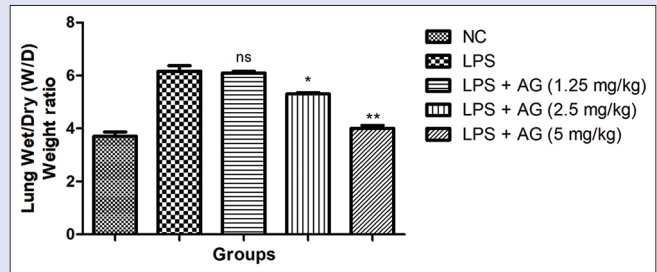


Figure 1: Showed the effect of astragaloside IV on the lung weight ratio of LPS induced lung inflammation in standard deviation rats. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

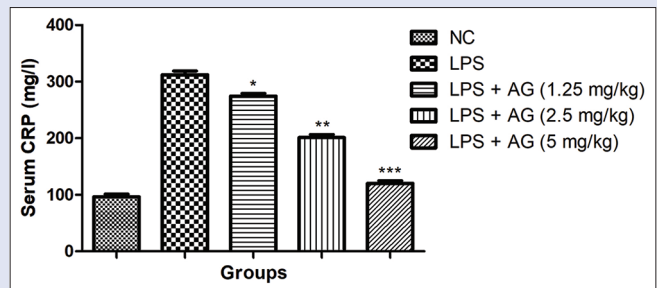


Figure 2: Showed the effect of astragaloside IV on the C-reactive protein of LPS induced lung inflammation in standard deviation rats. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

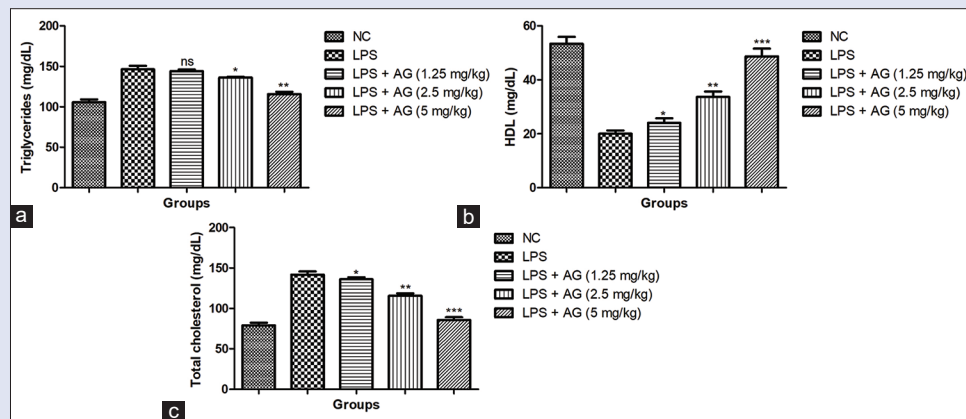


Figure 3: Showed the effect of astragaloside IV on the lipid profile of LPS induced lung inflammation in standard deviation rats. (a) Triglyceride, (b) high-density lipoprotein and (c) Total cholesterol. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

is shown in Figure 1. Follow-up studies showed that AG (3-*O*- β -D-xylopyranosyl-6-*O*- β -D-glucopyranosylcycloastragenol) has strong anti-inflammatory, antidiabetic, antioxidant properties, and other therapeutic properties.^[16-20] So far, *A. membranaceus* has been studied with respect to immune-regulatory functions; however, there are no reports on the pathophysiological mechanisms of AG in LPS-induced lung inflammation. Therefore, in this study, we explored how AG suppresses inflammation of the lungs by inhibiting the pathway of inflammatory cytokines.

MATERIALS AND METHODS

Chemicals

LPS was purchased from Sigma Company (*Escherichia coli* serotype O111:B4, St. Louis, MO, USA). AG (98%) was purchased from Sigma Company (St. Louis, MO, USA). Thiobarbituric acid; 1,1,3,4-tetramethoxypropan; vanadium III chloride (VC12); N-(1-naphthyl)-ethylenediamine dihydrochloride; pyrogallol; reduced glutathione (GSH); 5,5-dithiobis (2-nitrobenzoic acid); and pyrogallol were purchased from Sigma-Aldrich Company (Germany). Triglycerides (TGs), total protein, cholesterol, urea, creatinine, alanine aminotransferase (ALT), Aspartate aminotransferase (AST), malondialdehyde (MDA), GSH, and superoxide dismutase (SOD) kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

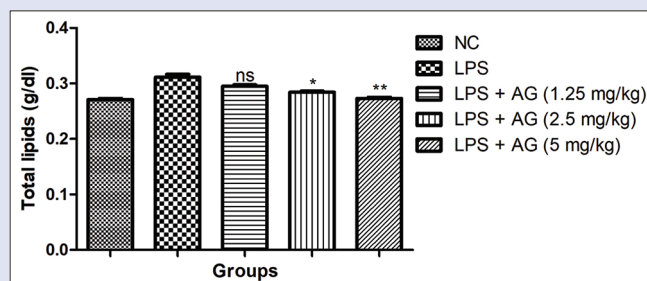


Figure 4: Showed the effect of astragaloside IV on the total lipid of LPS induced lung inflammation in standard deviation rats. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

Animals

In this study, male Sprague-Dawley rats weighing around 180–230 g were used. The animals were kept in the polyethylene cage under standard experimental conditions ($20^{\circ}\text{C} \pm 3^{\circ}\text{C}$; 60% relative humidity; 12/12 h light and dark cycle) and were provided with standard diet (rat chew, obtained from Nanjing, China) and water *ad libitum*. The entire experimental protocol for the ethical use of laboratory animals was carried out in accordance with the institutional and international guidelines.

Preparation of drugs

LPS was dissolved in sterile saline and was freshly prepared before use in the animal experiments.^[3] AG was prepared by dissolving it in the carboxymethyl cellulose (CMC).

Experimental design

Before the experiment, all the animals were acclimatized for 2 weeks. Then, the animals were divided into five groups containing 12 rats each. The rats were divided as follows:

- Group A: Normal control (received 0.5% CMC)
- Group B: LPS (single dose 10 mg/kg)
- Group C: LPS + AG (1.25 mg/kg)
- Group D: LPS + AG (2.5 mg/kg)
- Group E: LPS + AG (5 mg/kg), respectively.

After 12 h of LPS treatment, the animals were anesthetized and the blood sample was collecting by puncturing the retro-orbital plexus. The samples were centrifuged at 1000 g for 20 min at 4°C to separate the plasma and serum. The serum and plasma fractions were stored at -80°C for further biochemical analysis. The animals were scarified through cervical dislocation, and the lung tissue was removed and washed using phosphate-buffered saline, and finally stored at -80°C for the biochemical analysis.

Acute phase response

Acute phase response parameters including C-reactive protein (CRP) and NO were estimated in the plasma using the commercially available assay kits following the manufacture instruction (Nanjing Jiancheng Bioengineering Institute, China).

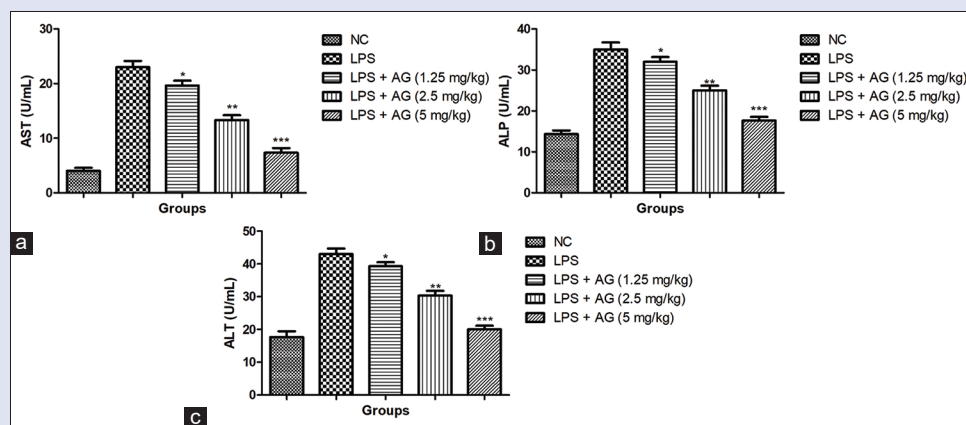


Figure 5: Showed the effect of astragaloside IV on the hepatic parameters of LPS induced lung inflammation in standard deviation rats. (a) AST, (b) ALP and (c) alanine aminotransferase. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

Hepatic parameters

Hepatic parameters such as ALT, alkaline phosphatase (ALP) and aspartate aminotransaminase (AST) were scrutinized using the commercially available assay kits following the manufacture protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Lipid parameters

Lipid parameters, including total cholesterol (TC), high-density lipoprotein (HDL), and TGs were determined based on the manufacture's protocol of assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Pro-inflammatory cytokines

Levels of IL-1 β , IL-1, IL-4, TNF- α , IL-10, IL-6, and IL-18 were estimated in serum and tissue samples based on the manufacturer's instructions provided in the enzyme immunosorbent assay kits (San Jose, CA, USA).

Quantitative real-time polymerase chain reaction

After the experimental protocol, quantitative real-time polymerase chain reaction (qRT-PCR) was performed to estimate the mRNA expression of different genes involved in the apoptosis and inflammation process. TRIzol reagent was used to isolate the total

RNA using the manufacture's protocol. A spectrophotometer was used to estimation the level of RNA at 260 nm, and purity of RNA was estimated by estimating the ratio of absorbance at A260/A280. The RNA with a ratio A260/280 between the 1.8 and 2.0 was further used for the experimental study. All primers were procured from Sigma. Table 1 shows the details of the primers.

Statistical analysis

The result was shown as mean $\pm$ standard error of mean (SEM). We used GraphPad prism software to analyze the data. One-way analysis of variance followed by the Dennett method was examined for a major difference among the group. A $P < 0.05$ was considered as the statistically significant.

RESULTS

W/D ratio

The ratio of W/D in lungs was calculated to examine the changes in the vascular permeability of pulmonary tissue to water caused by LPS. Our results demonstrated a higher level of lung W/D ratio in LPS-induced animals, whereas AG significantly decreased the W/D ratio in a dose-dependent manner when compared to the control group [Figure 1].

Determination of C-reactive protein

AG significantly decreased the levels of serum CRP levels, which demonstrated its anti-inflammatory effects. Increased levels of CRP suggested an acute inflammatory situation observed in the LPS-treated animals as represented in Figure 2.

Lipid profile

LPS treatment significantly increased the levels of serum TG. When compared to control group, AG significantly reduced the levels of serum TG [Figure 3]. Furthermore, LPS significantly increased the level of total lipids (serum) in control animals, whereas in AG-treated group, the level of total lipids decreased [Figure 4].

Effect of astragaloside on hepatic biomarkers

Treatment with LPS outfalls in significant elevations in the level of serum ALT, ALP, and AST and significant reduction in the albumin level contrast to control. Amelioration by different doses of AG resulted in

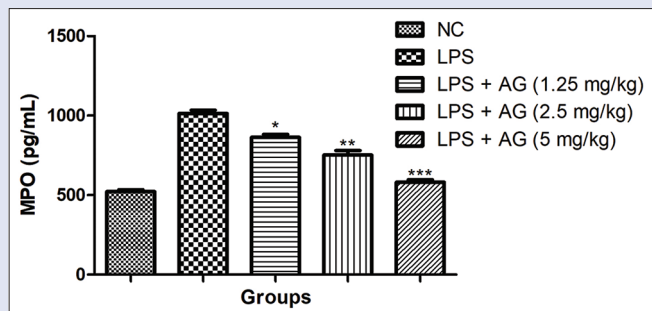


Figure 6: Showed the effect of astragaloside IV on the Myeloperoxidase activity of LPS induced lung inflammation in standard deviation rats. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

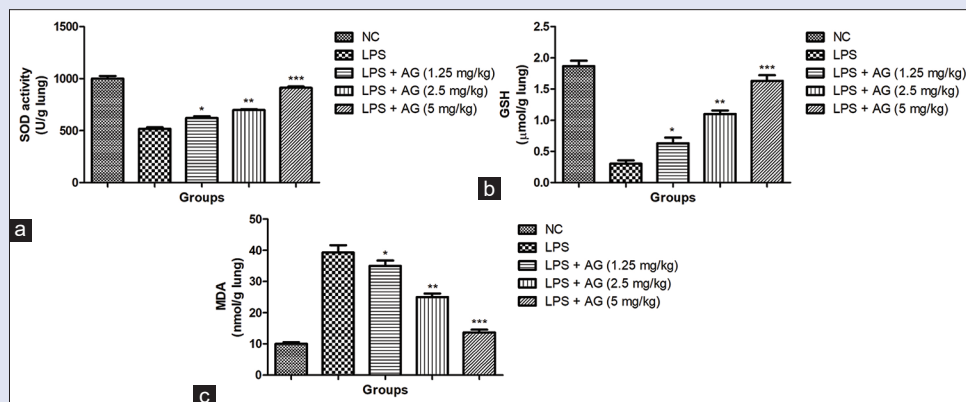


Figure 7: Showed the effect of astragaloside IV on the antioxidant parameters of LPS induced lung inflammation in standard deviation rats. (a) Superoxide dismutase, (b) GSH and (c) malondialdehyde. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

the increase in the levels of serum ALT, ALP, and AST and decrease in the level of albumin significantly which revealed down-regulation in LPS-induced lung inflammation [Figure 5].

Myeloperoxidase activity

Myeloperoxidase (MPO) is a biomarker used to determine the infiltration of neutrophils and macrophages in the lung tissue. According to our results, there was a significant upregulation in the activity of MPO in LPS-treated animals when compared to the control rats. AG reduced the activity of MPO significantly in a dose-dependent manner [Figure 6].

Impact of antioxidant parameters

Oxidative stress contributes to the disease progression. In this study, LPS-induced standard deviation (SD) rats exhibited higher levels of MDA and GSH and reduced activity of SOD. AG significantly decreased the levels of MDA in a dose-dependent manner ($P < 0.001$) and increased the activity of SOD and levels of GSH [Figure 7].

LPS-induced production of cytokines

In this study, we investigated the production of various inflammatory cytokines such as IL-1 β , IL-18, IL-1, IL-6, IL-4, IL-10, and TNF- α in

Table 1: List of primers

S. No	Gene	Primers	
		Forwarded	Reverse
1	TNF- α	ACGGCATGGATCTCAAAGAC	GTGGGTGAGGAGCACGTAGT
2	IL-1	CATTGTGGCTGTGGAGAAG	ATCATCCCACGAGTCACAGA
3	IL-1 β	CAGGCAGGCAGTATCACTCA	TGTCCTCATCCTGGAAGGTC
4	IL-4	CTGTAGGGCTTCCAAGGTGC	CTCTCATTGTGCCAGGTCAC
5	IL-6	TCTACCCCAACTTCCAATGCTC	TTGGATGGTCTTGGTCCTTAGCC
6	IL-10	GCCAGTTAGAAAGCCACCAC	GGTTCAGCCTGTTTCCCAAC
7	IL-18	GGAAGACCAGAGACATCCACTG	ACACTAGACCAAAGGGCTTG
8	β -actin	CGTTGACATCCGTAAGAC	TGGAAGGTGGACAGTGAG

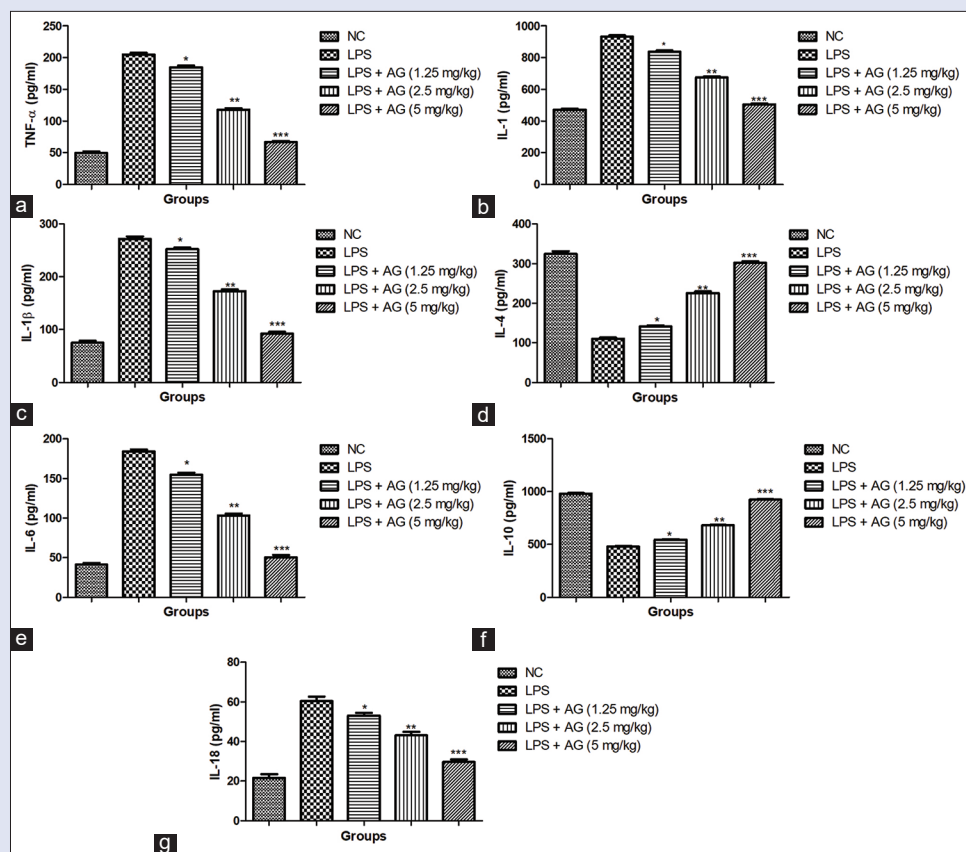


Figure 8: Showed the effect of astragaloside IV on the pro-inflammatory parameters of LPS induced lung inflammation in serum of standard deviation rats. (a) tumor necrosis factor- α , (b) interleukin-1, (c) interleukin-1 β , (d) interleukin-4, (e) interleukin-6, (f) interleukin-10 and (g) interleukin-18. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

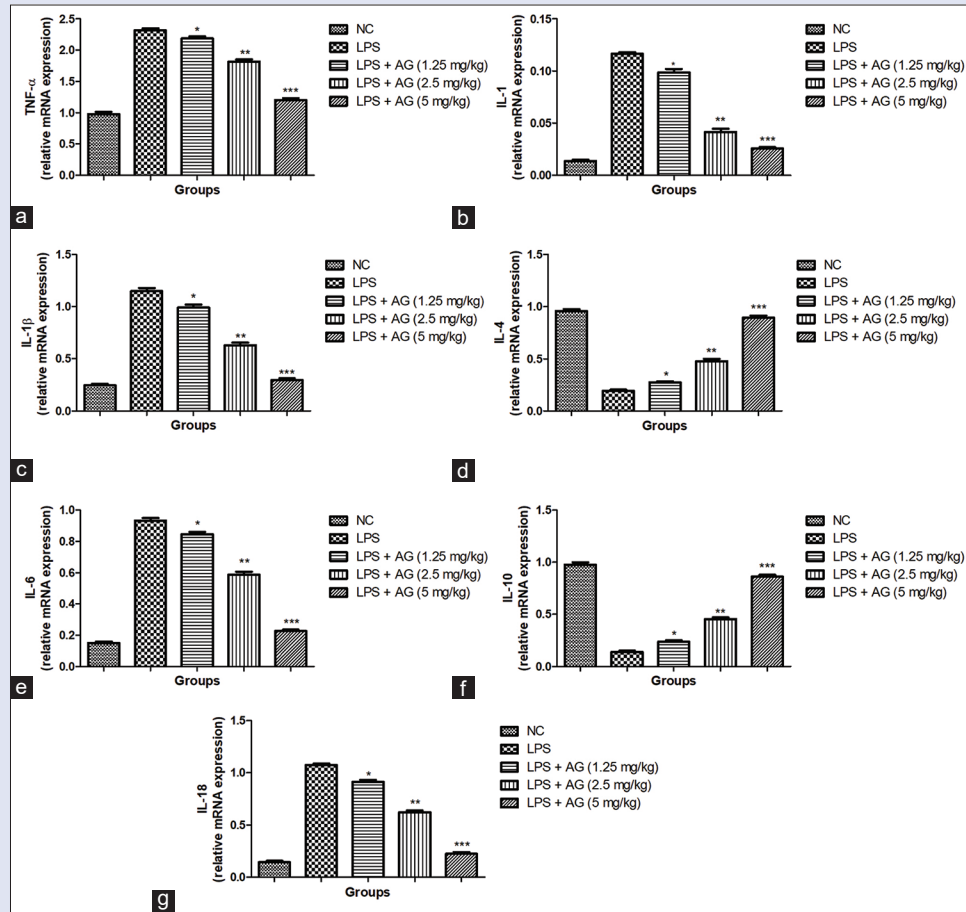


Figure 9: Showed the effect of astragaloside IV on the mRNA expression of pro-inflammatory parameters of LPS induced lung inflammation of standard deviation rats. (a) Tumor necrosis factor- α , (b) interleukin-1, (c) interleukin-1 β , (d) interleukin-4, (e) interleukin-6, (f) interleukin-10 and (g) interleukin-18. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

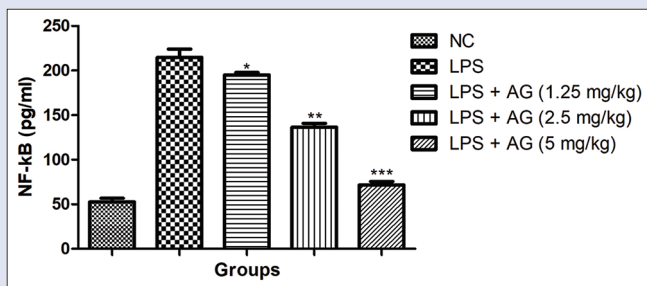


Figure 10: Showed the effect of astragaloside IV on the nuclear factor kappa B level of LPS induced lung inflammation in standard deviation rats. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

BALF to assess the anti-inflammatory activity of AG. Figure 8 shows significant up-regulation in levels of IL-1, IL-18, TNF- α , IL-6, and IL-1 β and lower levels of IL-4 and IL-10 in the LPS-induced animals than that of the control group. AG significantly down-regulated the levels of inflammatory cytokines namely IL-6, IL-1, IL-18, TNF- α , and IL-1 β and up-regulated the levels of IL-4 and IL-10 in a dose-dependent manner [Figure 8].

Effect of astragaloside on the level of gene and protein expression of the cytokines:

In this study, qRT-PCR was used to assess the gene expression of cytokines in the tissues of the lungs. The levels of cytokines in BALF agreed with the levels of expression of pro-inflammatory namely, IL-18, IL-6, IL-1 β , IL-4, and IL-10. A similar result was obtained in the mRNA expression of cytokines [Figure 9].

Effect of astragaloside on nuclear factor kappa B level

LPS-induced rats showed a reduction in the levels of NF- κ B compared to normal control. AG substantially increased in a dose-dependent manner [Figure 10].

Effect of astragaloside on expression of caspase-1 expression and NLRP3

In this study, we tested NLRP3 inflammasome activation to explore the underlying molecular mechanism of the anti-inflammatory properties of AG. Figure 11 shows up-regulated expression of caspase-1 and NLRP3 in the LPS-induced group compared with the control group. AG significantly down-regulated the protein expression [Figure 11].

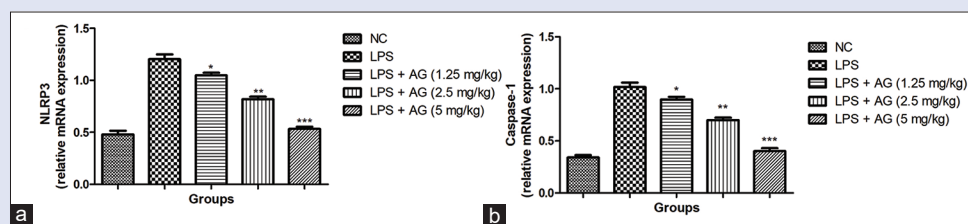


Figure 11: Showed the effect of astragaloside IV on the mRNA expression of NLRP3 and Caspase-1 of LPS induced lung inflammation in standard deviation rats. (a) NLRP3 and (b) Caspase-1. All the data presented as $\pm$ standard error of mean. Where * $P < 0.05$ consider as significant, ** $P < 0.01$ consider as more significant and *** $P < 0.001$ consider as extreme significant

DISCUSSION

According to the literature, ALI is responsible for the increased rate of patient death with no appropriate treatment available so far.^[21] Hepatic injury is significantly linked with lung inflammation;^[22] therefore, a patient with lung injury requires a proper monitoring of liver function.^[22] In this study, our results indicate that AG treatment significantly reduced the level of ALT and AST. AG shows hepatoprotective activity and therefore, in this study, we used AG to determine hepatic function along with lung function.

In acute and chronic inflammatory conditions, CRP is an acute phase protein which is released by hepatocytes in association with IL-6, which are commonly observed in the circulating blood and not alter to IL-1 β and TNF- α .^[23] IL-6 activates the replication of mRNA of CRP in hepatocytes which is increased by TNF- α and IL-1 β .^[24,25] In this study, we found that AG potentially inhibited the effect of TNF- α and IL-6.

Data presented in Figure 3 shows about the changes in lipid metabolism corresponding to the increase in the amount of total lipids, TC and TG in LPS-induced animals in contrast to the control group. LPS reduced the amount of HDL-c in contrast to the control group. Our results agree with those of Van Oosten *et al.*,^[26] which explains that increased levels of serum lipoprotein might be due to the physiological resistance mechanism.^[27] This study also shows the important property of serum lipoproteins, which bind with LPS to reduce its toxic effects and prevent inflammation. AG reduced the effects of LPS-induced inflammation, which proves that it also acts as a hypocholesterolemic agent.

LPS up-regulated the mRNA and protein expression of inflammatory cytokines in the lung tissue.^[3,4] Literature has reported the anti-inflammatory properties of AG in particular, the release of IL-1 β and IL-6 under *in vitro* and *in vivo* conditions.^[17,28] In addition, AG regulates the immune response by elevating the levels of anti-inflammatory cytokine such as IL-4 and IL-10. Our results suggest that AG can be used as a therapeutic agent to treat LPS-induced lung inflammation.

The results of this study demonstrate that AG reduced LPS-induced inflammation in a dose-dependent manner in rats. We also found that AG down-regulated the production of inflammatory cytokines in bronchoalveolar lavage fluid. These findings show the beneficial effects of AG on LPS-induced lung inflammation. Neutrophils are the most primitive immune cells recruited to the injury site in lung inflammation that produces cytotoxic products.^[8]

A previous study shows that neutrophil removal could alleviate ALI severity. The activity of MPO, a neutrophil marker, in tissues is a measure used to quantify tissue aggregation of neutrophils.^[8-10] In this study, AG significantly reduced the activity of MPO. The primary characteristic of hepatic injury is pulmonary edema. The ratio of the lung W/D was calculated to determine the severity of pulmonary edema.^[4]

AG considerably reduced the ratio of lung W/D, which suggests that significant reduced the edema in the liver tissue.

The inflammatory response in LPS-induced lung inflammation is mediated by a complex cytokine network, involving IL-1 β , IL-6, and IL-18. Such cytokines are produced mainly by LPS-activated monocytes and macrophages, and they recruit neutrophils into the lung tissue, which leads to the production of lung inflammation.^[8] In addition, the AG could be reduced the level of IL-4 and IL-10, which are showed the potential anti-inflammatory cytokines effect.

The production and release of IL-1 β and IL-18 is driven by NF- κ B and NLRP3 pathways. NF- κ B is a significant transcription factor which plays an essential part in the management of inflammatory mediators.^[29] The expression of pro-IL-18 and pro-IL-1 β could be regulated by activated NF- κ B when exposed to LPS. In the innate immune system, inflammasome NLRP3 is an important factor which regulates the production of IL-1 β and IL-18.^[30] Triggering inflammasome NLRP3 activate the caspase-1 that provokes the IL-1 β and IL-18 growth and oozing.^[31-33]

Moreover, previous data suggest that the amelioration of LPS-induced lung inflammation might be due to the suppression of signaling pathway (NLRP3).^[31,32] To scrutinize the possible underlying mechanism of the anti-inflammatory properties of AG, we studied the activity of AG in a dose-dependent manner on the NF- κ B expression and NLRP3 inflammasome activation. AG remarkably reduced the expression of inflammatory cytokines, down-regulated the expression of NF- κ B, and reduced the activation of the NLRP3 inflammasome. In contrast, AG up-regulated the expression of anti-inflammatory cytokines, such as IL-10 and IL-4 in a dose-dependent manner.

CONCLUSION

In summary, the results of this study confirmed that AG shows anti-inflammatory properties by blocking the inflammatory response to LPS-induced inflammation in experimental models using animals. The underlying mechanism suggests the blockade of signaling pathway (NLRP3/NF- κ B). Overall study outcomes demonstrate the potential activity of AG against lung inflammation which can be used as a novel therapeutic agent in the management of lung injury.

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Conflicts of interest

There are no conflicts of interest.

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