Issues in Biological Sciences and Pharmaceutical Research
Vol.12(1),pp.1-8, March 2024
Available online at https://www.journalissues.org/IBSPR/
https://doi.org/10.15739/ibspr.24.001
ISSN 2350-1588 (Online) ISSN:2814-3809(Print)

Original Research Article

Protective effect of Monflu on lipopolysaccharide-induced acute lung injury in Wistar rats

Received 4 December,2023 | Revised 14 January, 2024 | Accepted 19 January, 2024 | Published 4 March, 2024

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Monflu is a granule that develops from Lider-7 traditional prescription, and used for the treatment of respiratory contagious diseases. This study aimed to evaluate quality control, total biological activity and to analyze the activity of this substance in Wistar rat with acute lung injury (ALI). Seventy Wistar rats were randomized into four groups as follows: control group, Lipopolysaccharide (LPS) group, and LPS+Monflu groups (242 mg/kg and 484 mg/kg Monflu before LPS injection). Results showed that, Monflu contained 6g granule in a packet, alkaloids were 95±16.6 µg/g, phenols were 3.36±0.45 mg/g, flavonoids were 1.18±0.1 mg/g. The rats pretreated with Monflu high and low doses had significantly decreased levels of interleukin (IL)-1β (p=0.001 at 3-12 h), IL-6 (p=0.001 at 9 and 12 h) and tumor necrosis factor (TNF)-α (p=0.05 at 9 and 12 h). The rats pretreated with Monflu groups significantly increased levels of prostaglandin E2 (PGE2) at 3, 6, and 9 h (p<0.05), but it had reduced at 12 hours (p=0.001). Monflu groups showed significantly decreased inflammation and change of pulmonary structure. This study showed that Monflu exhibited a protective effect against LPS-induced ALI which was revealed to be mediated by suppressing the release of pro-inflammatory cytokines.

Keywords: Traditional medicine, herbal granule, total alkaloid, acute lung injury, lipopolysaccharide, monflu, inflammatory cytokines.

INTRODUCTION

Inflammation is a mechanism to defend against exogenous pathogens and is involved in various physiological and pathological processes. Various progressive diseases, including ALI, are associated with inflammation (Medzhitov, 2008; Ding et al., 2018) The acute lung injury model is a commonly used experimental model in rats to study acute lung injury and acute respiratory distress syndrome (ARDS). This model is designed to mimic the pathological changes observed in human ARDS, which is characterized by widespread inflammation and tissue damage. In the acute lung model, rats are typically subjected to various injurious stimuli such as direct lung injury, systematic inflammation, or mechanical ventilation with high tidal volumes. These stimuli can induce lung inflammation, increased permeability of the lung blood vessels, and impaired gas exchange, resembling the clinical features of ARDS. Researchers utilize the acute lung to investigate the underlying mechanisms of lung injury, identify potential therapeutic targets, and evaluate the efficacy of novel treatments for ARDS. (Matute-Bello et al., 2008, Domscheit et al., 2020).

Acute lung disease, among the most common respiratory disorders occurring worldwide, continues to escalate in magnitude and significance due to changing air pollution such as increased infection virus and bacterium increased pneumonia mainly resulting from air pollution and

urbanization. Mongolia is high, cold, and windy It has an extreme continental climate with long, cold winters and short summers, during which most of its annual precipitation falls. It is a harsh climate and nomadic lifestyle make the population vulnerable to acute respiratory infection and inflammation.(Darmaa et al., 2021).

Monflu granule which is made from prepared Lider-7 tang or seven herbal medicines, Radix Sophoroe alopecuroides, Radix Inulae helenium, Fructus Gardeniae, Fructus Terminaliae billericae, Fructus Terminaliae chebulae, Herba Gentianae barbatae and Herba Lagotis integrifoliae. It is useful for treat cold and flu symptoms such as nasal congestion, headache, body ache, fever, sore throat pain, and cough (Baavgai and Boldsaikhan 1990).

The advantages of the Monflu granule are easy to use for patients, the traditional use of the medicine has not been lost, the solid precipitated pharmaceutical form, quality assurance is provided, and the activity of the medicine has been conducted. In our previous investigation, lider-7 tang a Mongolian medicine was found to exert a protective effect on lung damage and significantly reduced levels of TNFα, IL-6 cytokines lipopolysaccharide-induced acute model in rats and Lider-7 tang contains phenolic, flavonoid, alkaloid and iridoid, etc biological component (Erdenechimeg et al., 2017). In the world, many researchers and pharmaceutical companies study together to create herbal-based formulas many different hospitals create their own formulas and they also try to formulate them into granules, tablets, pills, capsules, and injections instead of traditional drugs. The objective of the present study carried out standardization of Monflu granule and evaluated the effects of Monflu on levels of inflammatory cytokines by observing histopathological changes associated with LPS-induced ALI.

MATERIALS AND METHODS

Materials

Monflu granules manufactured at the “Tavin-Us pharma” drug factory (Ulaanbaatar, Mongolia) has been used. Their patch numbers are 040522 and 061122.

Ethics statement

Experimental procedures performed in this study were in accordance with the Guide for the Care and Use of Laboratory Animals, proposed by the Institute of Traditional Medicine and Technology. The study protocol was approved by the Medical ethics subcommittee of the Institute of Traditional Medicine and Technology, Mongolia

Experimental animals

In this study, we used adult 42 male and 28 female total 70 Wistar rats (200-240 g). All experimental animals were acquired from the experiment animal house, Institute of traditional medicine and Technology. The rats were housed in cages maintained at room temperature with a 12-h light/dark cycle. Rats were feed a standard pellet diet and tap water.

Reagents

Standards of rutin, oxymatrine, and gallic acid were obtained from Sigma-Aldrich. The Folin Ciocalteu’s phenol reagent and aluminum chloride were purchased from Sangon, China. Other solvents and chemicals were of analytical grade. Escherichia coli 055:B5 endotoxin was purchased from Sigma-Aldrich (USA). The cytokine immunoassay kits were purchased from Shanghai MLBIO Biotechnology Co.Ltd (China).

Chemical analysis

Estimation of total flavonoid contents

The method was based complex with aluminum nitrate, and slightly made modification (Quettier DC, 2000 and Erdenechimeg et al., 2021). 3 g of the sample were accurately weighed to a flask, 50 ml 70% ethanol were added, heated under reflux at 70^0C for an hour, cooled and filtered to a 100 ml volumetric flask, then, diluted up to volume with 70% ethanol (A solution). 3 ml of A solution were transferred to a 25 ml volumetric flask, 6 ml distilled water and 1 ml 5% sodium nitrite were added, shaked for 6 minutes, and 1 ml 10% aluminum nitrate was added. After 6 minutes, 10 ml 4% sodium hydroxide were added and diluted up to volume with distilled water. After 15 minutes, the absorbance was measured at 500 nm of the test solution as directed under Ultraviolet-visible Spectrophotometry. Total flavonoid content was reported as rutin equivalent and calculated as mg/g.

Determination of total phenolic contents

The content of total phenolics was estimated according to the Folin-Ciocalteu method (Singleton VL, 1999). 3 g of the dried and powdered sample were accurately weighed to a flask, 50 ml 70% ethanol were added , heated under reflux at 70^0C for an hour, cooled and filtered to a 100 ml volumetric flask, diluted up to volume with 70% ethanol. 0.5 ml of the extract were transferred to a 25 ml volumetric flask, 1ml Folin-Ciocalteu reagent (diluted 1:10 in water) was added, and diluted up to volume with aqueous Na_2CO_3 (10.75%). The mixture was left for 30 min, then absorbance was measured at 760 nm. Gallic acid was used to establish the calibration curve, and total polyphenolic content was expressed as mg/g.

Determination total alkaloids

The content of total alkaloids was estimated using the spectrophotometric method based on the reaction with

bromocresol green (BCG) (Shamsa et al., 2008), then measured the absorbance at 420 nm.

6 g of the granule were accurately weighed to a paper filter, 50 ml methanol were added, heated under Soxhlet apparat for 24 hours, and filtered. The extract was evaporated and dried under vacuum evaporator at 45°C. The dry residue was dissolved with a 5 ml 2 mol/l hydrochloric acid, filter (A solution). 1 ml of A solution was transferred to a separatory funnel and washed with 10 ml chloroform (3 times). The pH of A solution was adjusted to neutral with 0.1 mol/l sodium hydroxide (pH-7-8), then 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture and extract were shaked with 1, 2, 3, and 4 ml chloroform. The extracts were collected in a 10 ml volumetric flask and diluted up to volume with chloroform. The absorbances were measured at 470 nm of the test solution as directed under Ultraviolet-visible Spectrophotometry. Total alkaloids content was reported as oxymatrine equivalent as μg/g and the calibration curve was established.

Moisture

1g of the granule was accurately weighed to porcelain bowls that was pre-dried, placed in a closed bag with a constant weight, and placed in a drying cabinet at a temperature of 80°C for 3-4 hours. Drying was carried out until constant weight. The moisture content of the granule was be calculated using by percentages (Mongolian national pharmacopoeia, 2011).

Average weight

10 pieces of the granule were taken, each one was accurately weighed, and the variance was calculated compared to the average weight (Mongolian national pharmacopoeia, 2011).

Experimental protocols

Rats were randomized into four groups: LPS group (n=20), in which LPS (7.5 mg/kg, dissolved in 0.5 mL sterile saline) was administered by an intravenous (iv) injection via the tail vein; LPS+Monflu high (n=20), and low (n=20) doses group in which Monflu (242 and 484 mg/kg, orally) were administered 30 min before injection of LPS (7.5 mg/kg, dissolved in 0.5 mL sterile saline, iv); and a control group (n=20), in which the rats were administered saline at a volume equivalent to that in the other groups. Rats were sacrificed with an overdose of sodium pentobarbital (100 mg/kg, ip). Then lung tissue and blood samples were taken 3, 6, 9, and 12 hours later for analysis (Li et al., 2016).

Histological investigation

12 hours after the LPS injection, rats were euthanized (n=5, the control, LPS, and LPS+Monflu (242 mg/kg), LPS+Monflu(484 mg/kg) groups. The obtained lung tissue specimens were fixed with 10% formalin, embedded in paraffin, cut into 5 mm-thick sections. The sections were then stained with hematoxylin and eosin (H&E) as per the standard staining method. Histologic changes were graded by a pathologist blinded to the clinical status of the rats. The lung tissue samples were then scored for the degree of intra-alveolar edema, intra-alveolar hemorrhage, and neutrophil infiltration using grades 0 to 4 (0, absent; 1, mild; 2, moderate; 3, severe; 4, overwhelming) with a maximum score of 12, as described previously (Chen et al., 2012).

Serum levels of cytokines (TNF-α, IL-6, IL-1β, PGE2)

Blood samples were collected via cardiac puncture at 3, 6, 9, and 12 h after the administration of LPS. All rats were euthanized with phenobarbital sodium before blood collection. The collected blood samples were centrifuged at 377.3g for 10 min at 4°C, and the serum supernatant retrieved was stored at −20°C for further analysis. The serum levels of TNF-α, IL-6, IL-1β and PGE2 were detected using solid-phase sandwich enzyme-linked immune sorbent assay (ELISA, Shanghai MLBIO Biotechnology Co.Ltd) kits specific for the detection of these factors, and the absorbance was measured at 450 nm by a plate reader (Chromate 4300 microplate) (Erdenechicmeg, 2017).

Statistical analysis

The experimental data were reported as the mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance followed by one-way analysis of variance (ANOVA), Tukey's multiple comparison test. The differences were considered to be significant at p= or <0.05.

RESULTS

The total content of alkaloid, flavonoid, and phenolic

The calibration curves by rutin, oxymatrine, and gallic acid were established. The flavonoid content of the extract of rutin equivalent and the standard curve equation was y =11.815x – 0.0092 r2 = 1.000 and concentration was between 4.0 to 40.0 μg/ml (Table1). The flavonoid content in the Monflu granule was 1.18±0.1 mg/g. Also, Table 1 shows the contents of total phenols that were measured at 760 nm. The standard curve of gallic acid was y =110.77 x - 0.0736, r2 = 0.995 and concentrations were between 0.72 to 2.1 μg/ml. The total phenol varied from 3.36±0.45 mg/g in the Monflu granule. The content of alkaloids was measured by Bromocresol green reagent in term of oxymatrine equivalent (the standard curve equation: y =5.5435 x + 0.0613, r2 = 0.957) and concentration was between 4.0 to 50.0μg oxymatrine per ml. The total alkaloids were determined 95±16.6 μg/g in Monflu granule (Table1 and Table 2).

Table 1. Total phenolics, flavonoids, and alkaloids in extracts of the Monflu granule (n=3)

№	Chemical compounds	Amount
1	Total flavonoid	1.18±0.1 mg/g
2	Total phenolics	3.36±0.45 mg/g
3	Total alkaloids	95±16.6 μg/g

Table 2. Quality control parameters of Monflu granule

№	Parameters	Result
1	Color	Orange
2	Odor	Light herb
3	Taste	Bitter and sour
4	Moisture	2.8±0.2 %
5	Average weight	6.0±0.3 g

Protective effect of Monflu in acute lung injury

Monflu pre-treatment reduced LPS-induced ALI in lung tissue

The control group showed no histological alterations. The group of LPS rats showed raised alveolar wall thickness, less air space, swelling, and infiltration of inflammatory cells 12 hours after LPS injection, indicating ALI. Rats pre-treated with Monflu (LPS+Monflu 242 and 484 mg/kg) groups showed significantly decreased inflammation and pulmonary structure alteration, together with normal alveolar majority air space, as compared to non-treated group (Figure 1A-C; H&E staining, x400 magnification). The total scores of the histological changes in the groups indicated that the degree of pulmonary injury or acute pneumonia in the LPS+Monflu 242 mg/ group was significantly less than in the LPS group (p=0.05, Figure 1)
The LPS group had a significantly higher histological score than the healthy group, indicating the presence of pulmonary inflammation and damage (p=0.05). However, the damage of histological score in the LPS+Monflu 242 mg/kg group was significantly decreased than that in the LPS group, indicating that Monflu reduced the degree of pulmonary edema induced by LPS (p=0.01 Figure 2).

Effect of Monflu pretreatment on inflammatory cytokines in ALI model

In the LPS group, the levels IL-1β significantly increased after LPS injection and peak levels were at 9 hours. However, the levels of IL-1β significantly decreased in Monflu groups 242 mg/kg, 484 mg/kg doses in all studied hours.
In addition, the rats pretreated with Monflu 242 mg/kg showed significantly decreased levels of IL-6 (LPS+Monflu 242 mg/kg group vs LPS group: p=0.001 at 9 and 12 hour). LPS+Monflu 242 mg/kg group had significantly decreased levels of TNF-α (LPS+Monflu 242 mg/kg group: p=0.05 at 9 and 12 hour) at the valid time points (Figure 3).

The rats pretreated with Monflu 242mg/kg, 484 mg/kg showed significantly increased levels of PGE2 (LPS+Monflu 242, 484 mg/kg group vs LPS group: p=0.05 at 3, 6 and 9 hour). In contract, the rats pretreated with Monflu 242, 484 mg/kg showed significantly decreased levels of PGE2 (LPS+Monflu 242, 484 mg/kg group vs LPS group: p=0.001 at 12 h).

DISCUSSION

In the present study, a rat model of ALI was successfully induced by the intravenous injection of LPS. We found that LPS exposure caused a dramatic increase in the proinflammatory cytokines level and pulmonary histological score, reflecting the occurrence of neutrophil infiltration. Furthermore moreover, histological analysis revealed the increase in thickness alveoli integrity and loss air space. Taken together, these reconstructions confirmed the model of LPS-induced ALI. Pretreatment with Monflu high and low doses also decreased the extent of histopathological changes, neutrophil infiltration, and secretion of proinflammatory cytokines in rat blood serum.

Lipopolysaccharide (LPS), the major component of the gram-negative bacterial cell wall, can induce the production of pro-inflammatory cytokines and activate various types of cells, including macrophages, epithelial cells, and endothelial cells (Ding et al., 2018). LPS is the principal component of the outer membrane of gram-negative bacteria and is a potent stimulator of rapid pro-inflammatory cytokine production. The elevated expression of TNF-α, IL-1β, and IL-6 is an important step in the pathogenesis of ALI and acute respiratory distress syndrome (Li et al., 2016) .

In response to pathogen infection, pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) and anti-inflammatory cytokine (IL-10) increased in patients with sepsis. Importantly, a decrease in IL-6 was associated with a better prognosis and overproduction of IL-10 was found to be the main predictor of severity and

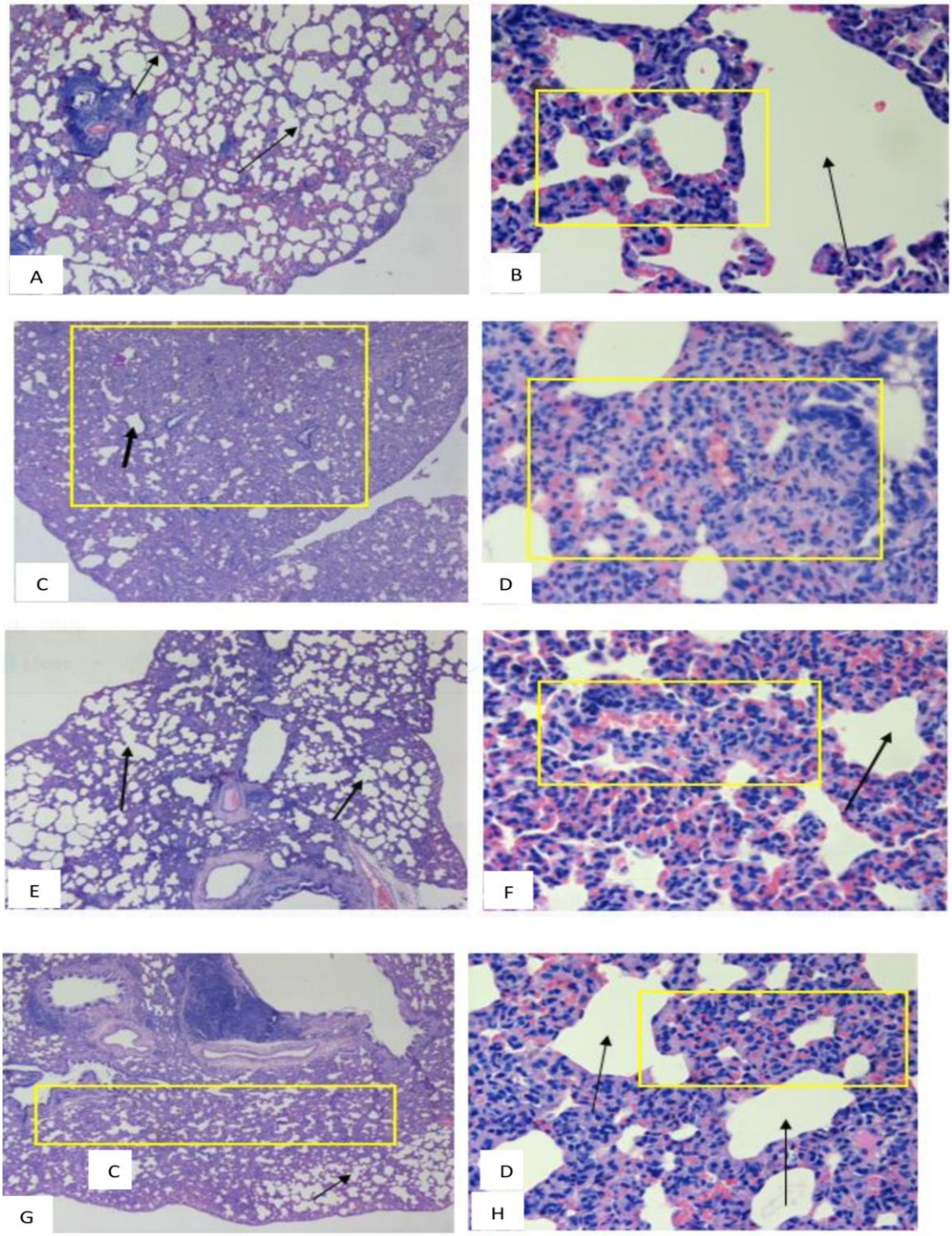

Figure 1 A-H: Histopathological alterations in lung section samples of the four groups. Hematoxylin and eosin (x40, x400 magnification). A,B Control group with normal lung structure. C,D, Lipopolysaccharide (LPS) group with grown up alveolar wall thickness, less air space infiltration of inflammatory cells. E, F LPS+Monflu 242 and G,H LPS+Monflu 484 mg/kg group showed less structure destruction and inflammatory infiltration. (Arrow -alveoli), (box-wall of alveoli)

fatal outcome (Chaudhry et al., 2013).

Also, in the present study we observed an increase in TNF-α and IL-6, IL-1β levels. In our results showed pretreated with Monflu high and low doses had significantly decreased levels of interleukin IL-1β , IL-6 and TNF α cytokine. This effect may be due to the biologically

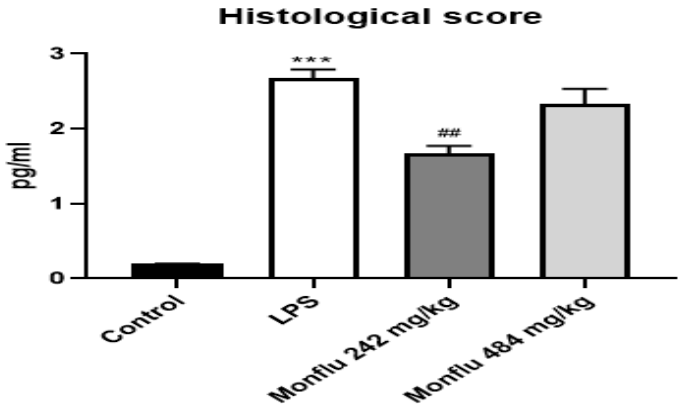

Figure 2: Comparison of the pulmonary histological scores of the four groups. Data are reported as means±SD. ##P=0.001, LPS+Monflu 242 group compared to control group; ***P=0.001, LPS group compared to control group.

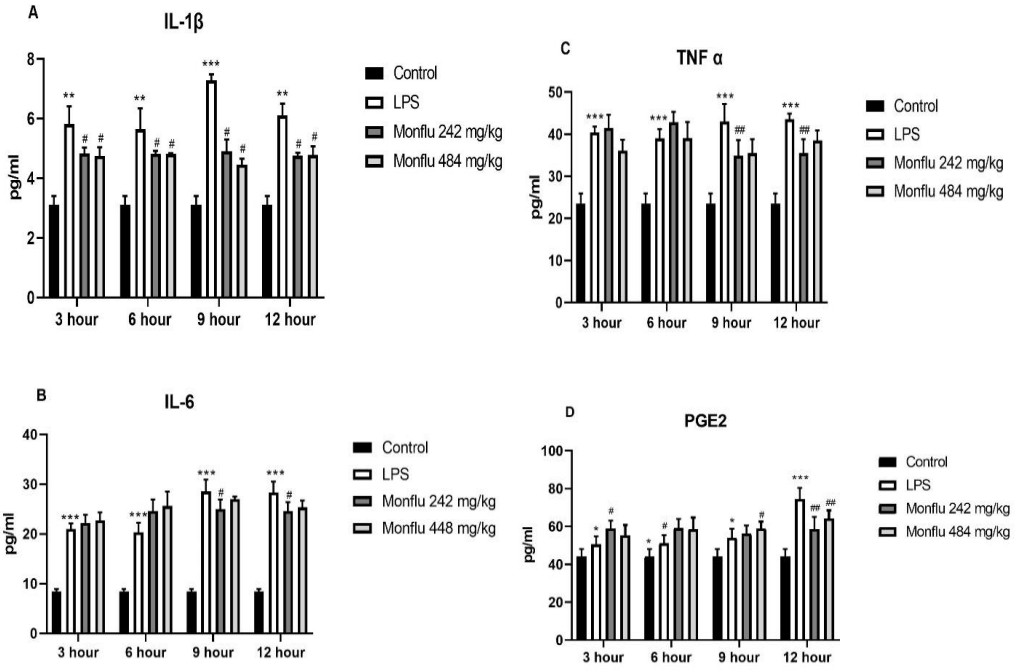

Figure 3: (A-C) IL-1β, IL-6, TNF α , PGE2 level LPS induced ALI model. Data was expressed as mean±SD (n = 5), *p = 0.05, **p=0.01, ***p=0.001 compared with the control group; #p=0.05, ##p=0.01 compared with the model or LPS group.

active substances contained in Monflu. Because Monflu is a composed Sophoroe alopecuroides, Radix Inulae helenium, Fructus Gardeniae, Fructus Terminaliae billericae, Fructus Terminaliae chebulae, Herba Gentianae barbatae and Herba Lagotis integrifoliae. Chemical compound is a consisted phenolic compound, alkaloids, iridoid etc (Erdenechimeg et al., 2017).

Flavonoids are widely present in natural plants and have a variety of biological activities, such as antioxidant, anti-inflammatory, anticancer, antibacterial, and antiviral activities. Their anti-inflammatory and antioxidant activities render them useful preventive and therapeutic candidates for inflammatory lung diseases. (Yu, 2021).

Our findings indicated that Monflu pretreatment increased PGE2 amount early times 3.6.9 hours in the serum, after that at the 12 hour may thus mitigate the inflammatory response and lung injury symptoms through its inhibitory effect on PGE2 amount in rats. A reduction of

the PGE2 may be related to apigenin active ingredient. Apigenin is contained in Montflu and has been studied for ALI model anti-inflammatory effects. Apigenin inhibits the activation of COX-2 and NF-*κ*B (Wang, 2014).

Besides that matrine is the main active ingredient of *Sophora flavescens* (Liou, 2016) found that it can prevent ALI in LPS-induced mice by decreasing the expression levels of COX-2, intercellular cell adhesion molecule-1 (ICAM-1), TNF-*α*, and IL-6. Therefore, several publications revealed that a herbal medicine had beneficial effects in animal models of acute lung injury.

Isoalantolactone is a sesquiterpene lactone extracted from roots of *Inula helenium L* and has shown anti-inflammatory effects. Isoalantolactone has an effect inhibit the production of inflammatory mediators in LPS-stimulated BMDMs. The inhibitory effect of isoalantolactone was partly attributed to the downregulation of non-degradable K63-linked ubiquitination of TRAF6, suppressing the activation of the NF-κB pathway. This biological substance has exerted an anti-inflammatory effect against responses in the LPS-induced ALI mice model (Ding et al., 2018).

Geniposide, a main iridoid glucoside component of gardenia fruit, has been shown to possess anti-inflammatory activity. The geniposide protective effects against LPS-induced ALI by mitigating inflammatory responses and that the compound's mechanism of action may involve blocking nuclear factor-kappaB (NF- κB) and mitogen-activated protein kinases (MAPK) signalling pathway activation (Xiaofeng, 2012). These anti-inflammatory effects of medicinal plant and biological active substance related to had significantly stimulatory influence on exhibites a protective effect against LPS-induced ALI and which was revealed to be mediated by suppressing the release of pro-inflammatory cytokines it was similar to our result.

Conclusions

This study showed that Monflu exhibites a protective effect against LPS-induced ALI, which was revealed to be mediated by suppressing the release of pro-inflammatory cytokines.

Funding

This project was funded by the Mongolian Foundation for Science and Technology and the contract number of the research project was ShUA-2022/03.

Authors' contributions

Dejidmaa Buyantogtokh and Erderenchimeg Chuluunbaatar wrote the original draft, also Chimedragchaa Chimedtseren provided a consultant on methodology. The research team included Dejidmaa Buyantogtokh, Erderenchimeg Chuluunbaatar and Narangerel Ganbaatar. All authors read and approved the last version of the manuscript.

Conflict of interest

All authors have none to declare.

Data availability

The article's research did not utilize any data.

Acknowledgment

We would like to express our special thanks to the Mongolian Academy of Science and Mongolian Foundation for Science and Technology. Also, we want to thank the research team at the research center of the Institute of Traditional Medicine and Technology for their help during this study.

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