Effects of Total Flavonoids of Allium mongolicum on Inflammatory Mediators in Lipopolysaccharide-Induced Mouse Peritoneal Macrophages (Post-print)

Authors: Wang Teregle, Wang Cuifang, Danni, Saruli, Du Hongxi, Guo Chunli, Has-Erdene, Cao Qina, Ao Changjin

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Abstract

This study employed lipopolysaccharide (LPS)-induced mouse peritoneal macrophages as an inflammatory model to investigate the anti-inflammatory effects of total flavonoids from Allium mongolicum. The CCK-8 assay was used to screen concentrations of total flavonoids from Allium mongolicum that promote the viability of mouse peritoneal macrophages. Based on these results, the following groups were established: control group, LPS group (stress model, 1 g/mL LPS), low-dose total flavonoids group (25 g/mL total flavonoids + 1 g/mL LPS), medium-dose total flavonoids group (50 g/mL total flavonoids + 1 g/mL LPS), and high-dose total flavonoids group (100 g/mL total flavonoids + 1 g/mL LPS). The Griess method was employed to determine nitric oxide (NO) content in cell culture supernatant; enzyme-linked immunosorbent assay (ELISA) was utilized to measure tumor necrosis factor- (TNF-), interleukin (IL)-6, IL-1 , and IL-10 levels in cell culture supernatant; reverse transcription polymerase chain reaction (RT-PCR) was applied to determine mRNA expression levels of TNF- , IL-6, IL-1 , IL-10, and inducible nitric oxide synthase (iNOS) in cells. The results demonstrated: 1) Compared with the control group, supplementation with total flavonoids from Allium mongolicum at concentrations of 25.0~100.0 g/mL significantly enhanced cell proliferation rate ($P<0.05$); 2) Compared with the control group, the LPS group significantly increased NO content in cell culture supernatant ($P<0.05$); compared with the LPS group, various concentrations of total flavonoids significantly inhibited NO production ($P<0.05$); 3) Compared with the control group, the LPS group significantly elevated the levels of TNF- , IL-6, IL-1 , and IL-10 in cell culture supernatant as well as gene expression levels in cells ($P<0.05$); compared with the LPS group, with the exception of IL-1 in

the low-dose group, 25, 50, and 100 g/mL concentrations of total flavonoids significantly reduced TNF- , IL-6, and IL-1 levels in cell culture supernatant (P<0.05), and with the exception of IL-10 in the low-dose group, significantly increased IL-10 content (P<0.05); with the exception of iNOS in the low-dose group, 25, 50, and 100 g/mL concentrations of total flavonoids significantly suppressed mRNA expression of TNF- , IL-6, IL-1 , and iNOS in cells (P<0.05), while exhibiting a trend toward promoting IL-10 mRNA expression, though this difference was not statistically significant (P>0.05). In summary, total flavonoids from Allium mongolicum exert significant anti-inflammatory effects on LPS-induced mouse peritoneal macrophages.

Full Text

Effects of Total Flavonoids from *Allium mongolicum* Regel on Inflammatory Mediators in Lipopolysaccharide-Induced Mouse Peritoneal Macrophages

WANG Terigele, WANG Cuifang, DAN Ni, SA Ruli, DU Hongxi, GUO Chunli, Khas-Erdene, CAO Qina, AO Changjin*

(College of Animal Science, Inner Mongolia Agricultural University, Hohhot 010018, China)

Abstract

This study employed lipopolysaccharide (LPS)-induced mouse peritoneal macrophages as an inflammatory model to investigate the anti-inflammatory effects of total flavonoids from *Allium mongolicum* Regel. The CCK-8 assay was used to screen concentrations of the flavonoids that promoted macrophage viability. Based on these results, five treatment groups were established: control, LPS group (stress model, 1 g/mL LPS), low-dose flavonoid group (25 g/mL flavonoids + 1 g/mL LPS), medium-dose flavonoid group (50 g/mL flavonoids + 1 g/mL LPS), and high-dose flavonoid group (100 g/mL flavonoids + 1 g/mL LPS). The Griess method was used to measure nitric oxide (NO) content in cell supernatants, while ELISA was employed to determine levels of tumor necrosis factor- (TNF-), interleukin (IL)-6, IL-1 , and IL-10. RT-PCR was utilized to quantify mRNA expression of TNF- , IL-6, IL-1 , IL-10, and inducible nitric oxide synthase (iNOS). The results demonstrated: (1) Compared with the control group, flavonoid concentrations of 25.0–100.0 g/mL significantly enhanced cell proliferation rates (P<0.05); (2) The LPS group showed significantly elevated NO content in supernatants compared to the control (P<0.05), while all flavonoid treatment groups significantly inhibited NO production relative to the LPS group (P<0.05); (3) The LPS group exhibited significantly increased supernatant levels and mRNA expression of TNF- , IL-6, IL-1 , and IL-10 compared to controls (P<0.05). Relative to the LPS group, flavonoid treatments at 25, 50, and 100 g/mL significantly reduced

TNF- , IL-6, and IL-1 supernatant concentrations (except IL-1 in the low-dose group) (P<0.05) and significantly increased IL-10 levels (except in the low-dose group) (P<0.05). Additionally, these flavonoid concentrations significantly suppressed mRNA expression of TNF- , IL-6, IL-1 , and iNOS (except iNOS in the low-dose group) (P<0.05), while showing a trend toward increased IL-10 mRNA expression that did not reach statistical significance (P>0.05). In conclusion, total flavonoids from *Allium mongolicum* Regel exert significant anti-inflammatory effects on LPS-induced mouse peritoneal macrophages.

Keywords: *Allium mongolicum* Regel; total flavonoids; lipopolysaccharide; mouse; peritoneal macrophages; stress model

Introduction

Inflammation is a common pathological process in humans and animals, characterized by classic symptoms including redness, swelling, heat, pain, and itching. The progression and outcome of inflammation are determined by the balance between tissue damage caused by inflammatory factors and the body' s counteractive defense mechanisms. While conventional anti-inflammatory drugs are effective, their substantial side effects have prompted the search for safer alternatives. During pathogen-induced inflammation, mononuclear macrophages undergo varying degrees of proliferation. Macrophages are primary inflammatory cells that release a cascade of inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor- (TNF-) upon antigenic stimulation, thereby promoting inflammatory responses and tissue damage. Local inflammatory reactions can also affect the entire organism, producing varying degrees of systemic responses, though the body' s intrinsic condition can constrain the development of local inflammation. Consequently, macrophages play crucial roles in maintaining internal homeostasis and regulating immunity and anti-inflammatory processes.

Allium mongolicum Regel, belonging to the Liliaceae family and *Allium* genus, possesses medicinal properties and therapeutic efficacy. Extracts from this plant contain various bioactive substances including ketones, aldehydes, and sugars, with flavonoids representing important constituents. Flavonoid compounds are a significant class of oxygen-containing heterocyclic natural organic compounds that exhibit antioxidant, antibacterial, anti-inflammatory, antiviral, and anticancer activities. Therefore, this study utilized LPS-induced mouse peritoneal macrophages as an inflammatory model to investigate the anti-inflammatory effects of total flavonoids from *Allium mongolicum* Regel, providing a scientific basis for further development and utilization of these compounds.

Materials and Methods

1.1 Experimental Animals

Male C57BL/6J mice aged 6–8 weeks were purchased from the Experimental Animal Center of Inner Mongolia Medical University.

1.2 Drugs and Reagents

Total flavonoids from *Allium mongolicum* Regel (extracted and prepared in our laboratory with an extraction rate of (12.85±0.03) mg/g), thioglycollate medium (7017946, BD), LPS (L2880, Sigma), fetal bovine serum (FBS, FND500, ExCell Bio), RPMI-1640 medium (C11875500BT, Gibco), CCK-8 (ck04, Dojindo), dimethyl sulfoxide (DMSO, D8371, Solarbio), nitric oxide assay kit (G2930, Promega), RNA extraction kit (Axygen), reverse transcription kit (RR047A, TaKaRa), PCR kit (RR820A, TaKaRa), TNF- ELISA kit (430904, Biolegend), IL-6 ELISA kit (431304, Biolegend), IL-10 ELISA kit (88-7105, Invitrogen), and IL-1 ELISA kit (432604, Biolegend).

1.3 Experimental Instruments

SynergyHT microplate reader (BioTek, USA), IX51 inverted microscope (Olympus), Roche480 PCR system (Roche), CO incubator (Thermo Fisher), and low-temperature centrifuge (Shanghai Puze Trading Co., Ltd.).

1.4 Culture of Mouse Peritoneal Macrophages

Male C57BL/6J mice aged 6–8 weeks received intraperitoneal injection of 2 mL 3% thioglycollate to stimulate macrophage recruitment. After 72 hours, mice were euthanized by cervical dislocation, immersed in 75% ethanol for 2–3 minutes, and dissected in a sterile laminar flow hood. The peritoneal cavity was exposed and lavaged three times with 5 mL ice-cold phosphate-buffered saline (PBS) each time. The lavage fluid was collected and centrifuged at 5,000 rpm for 3 minutes at 4°C. After discarding the supernatant, cells were resuspended in RPMI-1640 medium containing 10% FBS and seeded into culture plates for incubation at 37°C with 5% CO .

1.5 Experimental Procedures

1.5.1 CCK-8 Assay for Cell Proliferation Cells were seeded at 4×10 cells/well in 96-well plates. After 2 hours of adherence, the control group received complete medium containing 0.1% DMSO, while flavonoid treatment groups were treated with various concentrations (12.5, 25.0, 50.0, 100.0, and 200.0 g/mL) for 24 hours. Subsequently, 100 L of complete medium containing 10% CCK-8 solution was added to each well. Following 2 hours of additional incubation, optical density (OD) values were measured at 450 nm.

1.5.2 Griess Assay for NO Content in Cell Supernatants Cells were seeded at 2.5×10 cells/well in 24-well plates. After 2 hours of adherence and two washes with ice-cold PBS, cells were divided into five groups: control, LPS group (stress model, 1 g/mL LPS), low-dose flavonoid group (25 g/mL flavonoids + 1 g/mL LPS), medium-dose flavonoid group (50 g/mL flavonoids + 1 g/mL LPS), and high-dose flavonoid group (100 g/mL flavonoids + 1 g/mL LPS). The control group received complete medium with 0.1% DMSO, the LPS group received 1 g/mL LPS, and flavonoid groups were pretreated with respective flavonoid concentrations for 1 hour before addition of 1 g/mL LPS for 24 hours. Cell supernatants were collected for NO content determination using the Griess method.

1.5.3 RT-PCR Analysis of TNF- , IL-10, IL-1 , IL-6, and iNOS mRNA Expression Cells were seeded at 2.5×10 cells/well in 6-well plates. Following 2 hours of adherence and two PBS washes, cells were treated as described above. Flavonoid groups received 1-hour pretreatment before LPS addition, with total treatment durations of 4 and 12 hours. After incubation, supernatants were removed, cells were washed with ice-cold PBS, lysed, and collected in 1.5 mL EP tubes for total RNA extraction. cDNA was synthesized according to the reverse transcription kit instructions. Specific primers were used to amplify TNF- , IL-10, IL-1 , IL-6, iNOS, and the internal reference -actin. Primer sequences are listed in .

The RT-PCR reaction system (20 L) consisted of 6.4 L nuclease-free water, 10 L SYBR Green dye, 0.8 L each of forward and reverse primers, and 2 L reverse transcription product.

1.5.4 ELISA for Cytokine Levels in Cell Supernatants Cells were seeded at 2.5×10 cells/well in 6-well plates. After adherence and washing, cells were pretreated with flavonoids for 1 hour before addition of 1 g/mL LPS for 24 hours. Cell supernatants were collected and analyzed according to ELISA kit instructions, with OD values measured at 450 nm.

1.6 Statistical Analysis

Data are expressed as mean ± standard error. Statistical analysis was performed using SAS 9.0 software. Inter-group comparisons were conducted using one-way ANOVA, with $P<0.05$ considered statistically significant and $P>0.05$ considered non-significant.

Results

3.1 Effects of Total Flavonoids on Macrophage Proliferation

As shown in [Figure 1: see original paper], flavonoid concentrations of 12.5-200.0 g/mL significantly enhanced the proliferation rate of mouse peritoneal macrophages compared to the control (0 g/mL) ($P<0.05$). Proliferation rates

increased with concentration from 12.5 to 100.0 g/mL, but declined at 200.0 g/mL. Therefore, concentrations of 25.0–100.0 g/mL were selected for subsequent experiments. Data bars with different letters indicate significant differences (P<0.05). The same applies to [Figure 2: see original paper] through [Figure 7: see original paper].

3.2 Effects of Total Flavonoids on NO Production

As depicted in [Figure 2: see original paper], LPS stimulation significantly increased NO content in macrophage supernatants compared to the control group (P<0.05). All flavonoid treatment groups significantly inhibited NO production in a dose-dependent manner relative to the LPS group (P<0.05).

3.3 Effects of Total Flavonoids on mRNA Expression of Inflammatory Mediators

As illustrated in [Figure 3: see original paper] through [Figure 7: see original paper], control cells exhibited low mRNA expression of TNF- , IL-6, IL-1 , and iNOS, which increased markedly following LPS stimulation. Compared to the LPS group, all flavonoid concentrations significantly suppressed mRNA expression of TNF- , IL-6, IL-1 , and iNOS (except iNOS in the low-dose group) (P<0.05) in a dose-dependent manner. For IL-10, the highest mRNA expression was observed at 100 g/mL flavonoids, which was significantly higher than the control (P<0.05) but not significantly different from the LPS group (P>0.05).

3.4 Effects of Total Flavonoids on Cytokine Secretion

As presented in , LPS stimulation significantly elevated levels of pro-inflammatory cytokines (TNF- , IL-6, IL-1) and the anti-inflammatory cytokine IL-10 compared to controls (P<0.05). Flavonoid treatments at all concentrations significantly reduced TNF- , IL-6, and IL-1 levels (except IL-1 in the low-dose group) relative to the LPS group (P<0.05). Conversely, flavonoid treatments significantly increased IL-10 content compared to both control and LPS groups (P<0.05). Values with different letter superscripts indicate significant differences (P<0.05).

Discussion

Under normal conditions, physiological and biochemical functions are maintained when pro-inflammatory and anti-inflammatory factors remain balanced. However, excessive production of cytokines and inflammatory factors beyond the body' s protective capacity leads to inflammatory pathology. Inflammation represents a defensive response against disease, increasing oxygen and nutrient delivery to cells, but can become detrimental under certain conditions. Inflammation is classified as acute or chronic, with sustained high concentrations of inflammatory factors during acute inflammation potentially leading to chronic

inflammation, which is closely associated with diseases such as coronary heart disease, hypertension, and cancer.

Macrophage activation plays a crucial role in inflammatory responses. Resting macrophages exhibit minimal immune function, but upon stimulation by LPS and other factors, activated macrophages release numerous cytokines including TNF- , IL-1 , IL-6, and IL-10, which interact to regulate inflammation. Therefore, this study selected representative pro-inflammatory cytokines (IL-1 , IL-6, TNF-) and the anti-inflammatory cytokine IL-10 as key indicators. IL-1 exists in two forms: IL-1 is cell-bound and requires cell-cell interaction for activity, whereas IL-1 exhibits strong biological activity, enhancing immune cell cytotoxicity and promoting inflammatory and immune responses. IL-6 has broad physiological roles in immune responses and cell growth, while also stimulating production of IL-1 receptor antagonists and soluble TNF- receptors to inhibit early synthesis of pro-inflammatory factors. IL-10 is a dual-function cytokine with both immunosuppressive and immunostimulatory properties that inhibits release of immune mediators and pro-inflammatory cytokines from mononuclear macrophages. TNF- is an early inflammatory mediator produced by activated macrophages that plays multiple roles in physiological responses.

4.1 Effects on Macrophage Proliferation

Cell viability is a critical indicator of proliferation and relative growth rate. Macrophages are essential immune cells with diverse biological functions, including recognition and phagocytosis of pathogens and harmful substances, thereby triggering immune responses. Previous studies have demonstrated the importance of macrophage function in the resolution of inflammatory diseases such as rheumatoid arthritis, fibrotic pneumonia, and ulcerative colitis. Inflammatory cytokines and LPS can activate macrophages, with LPS (endotoxin) being particularly potent for mononuclear macrophage activation. This study revealed that total flavonoids significantly enhanced mouse peritoneal macrophage proliferation, with maximal effects at 100.0 g/mL and decreased efficacy at 200.0 g/mL, indicating that both insufficient and excessive flavonoid concentrations can affect cell viability.

4.2 Effects on NO Production and Inflammatory Factor Expression

LPS stimulation enhances macrophage activity, leading to synthesis and release of substantial amounts of NO, TNF- , IL-1 , IL-10, IL-12, and IL-6. The interaction of various inflammatory cells and cytokines maintains normal physiological processes. Studies have shown that flavonoids, flavonols, and chalcones can inhibit LPS-induced NF- B target gene expression and IL-1 production both in vitro and in vivo. In animal models of acute lung injury, flavonoid and flavonol interventions reduced inflammatory factor protein content in bronchoalveolar lavage fluid and decreased neutrophil infiltration, alleviating inflammation. Tanshinone IIA from *Salvia miltiorrhiza* exhibits anti-inflammatory activity by suppressing IL-10, TNF- , and platelet expression. Isoliquiritigenin

from licorice flavonoids inhibits IL-1 and IL-6 gene expression and cytokine release in RAW264.7 cells. Similarly, total flavonoids from *Abutilon* leaves significantly reduced inflammatory factor content while increasing IL-10 levels in macrophages. Studies on LPS-induced RAW264.7 cells have demonstrated that certain flavonoids significantly decrease TNF- , NO, and IL-6 production. Flavonoids also inhibit LPS-induced downstream NF- B target gene expression, TAK1 protein kinase activation, and inflammatory factor release, underscoring their important role in anti-inflammatory responses. Our results showed that while LPS stimulation dramatically increased NO, TNF- , IL-1 , and IL-6 levels, flavonoid treatment significantly reduced these inflammatory mediators while increasing IL-10 content. Thus, total flavonoids from *Allium mongolicum* Regel can decrease mRNA expression of TNF- , IL-1 , IL-6, and iNOS while enhancing IL-10 production in LPS-induced inflammatory models.

Conclusion

Total flavonoids from *Allium mongolicum* Regel enhance mouse peritoneal macrophage proliferation, reduce supernatant levels of NO, TNF- , IL-1 , and IL-6, and suppress mRNA expression of TNF- , IL-1 , IL-6, and iNOS, while simultaneously increasing IL-10 content and mRNA expression. These findings demonstrate that total flavonoids from *Allium mongolicum* Regel exert significant anti-inflammatory effects on LPS-induced mouse peritoneal macrophages.

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Note: Figure translations are in progress. See original paper for figures.