IJTMGH International Journal of Travel Medicine and Global Health

Int J Travel Med Glob Health. 2023 Mar;11(1):220-226
http://ijtmgh.com

doi 10.30491/IJTMGH.2023.381813.1339

Original Article Open Access

The effects of oral administration of peiminine on the functions of mouse peritoneal macrophages

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Received 2022-12-16; **Accepted** 2023-02-21; **Online Published** 2023-03-01

Abstract

Introduction: Peiminine is an alkaloid extracted from the bulbs of *Fritillaria thunbergii* with profound pharmacological effects. However, there is no or limited information about the possible effect of the peiminine on the function of immune cells. This study was done to investigated the impact of oral administration of peiminine on the functions of peritoneal macrophages isolated from BALB/c mice.

Methods: The male BALB/c mice were randomly allocated to four equal groups (n=10) and treated orally with doses of 0, 1, 3, and 6 mg/kg of peiminine (dissolved in PBS) for one month. After 30 days, the peritoneal macrophages of the mice were isolated, and their function was evaluated ex vivo.

Results: Data analysis indicated an increase in phagocytosis in macrophages obtained from mice treated with peiminine in a non-dose-dependent manner. NR uptake did not show any change between the vitality of macrophages isolated from different studying groups. Also, receiving peiminine by mice in a dose-dependently has reduced the production of oxygen and nitrogen free radicals by macrophages. Ex vivo secretion of IL-12 by LPS-stimulated macrophage significantly down-regulated in a dose-dependent manner compared to macrophages isolated from mice without treatment. Also, the production of IL-10 by LPS-stimulated macrophages isolated from mice received low to moderate doses of peiminine significantly increased compared to macrophages alone.

Conclusion: These findings proposed that the macrophage isolated from mice that orally received peiminine had an anti-inflammatory phenotype.

Keywords: Peiminine, Macrophage, Immunomodulation, BALB/c mice.

Citation: Mansouri Motlagh B, Abtahi Froushani SM, Delirezh N. The effects of oral administration of peiminine on the functions of mouse peritoneal macrophages. 2023;11(1):220-226. doi: 10.30491/IJTMGH.2023.381813.1339

Introduction

Macrophages, among the most critical cells, play a role in the initiation and expansion of innate immune responses, as well as shaping the function of the acquired immune system.[1] Unlike neutrophils, macrophages have not passed the final stages of differentiation and can still perform cell division at the site of inflammation. Macrophages act almost at the same speed as neutrophils respond to microbes, but their life span is much longer than neutrophils in places of inflammation. Therefore, macrophages are the primary executive cells in the final stages of The innate immune response (a few days after the beginning of the infection).[2, 3] Macrophages play an essential role in maintaining homeostasis and tissue repair by removing cellular debris and clearing apoptotic cells.[1]

Fritillaria thunbergii is a plant from the Liliaceae family. It is native to China, Turkey, and Iran. In Iran, it grows in the Zagros mountain range at an altitude of 2000 meters.[4] It is a perennial, bulbous plant, and has great potential for use in ornamental plants.[5] Due to the lack of protection and excessive livestock grazing, in There is a danger of destruction and extinction, and now a species is strictly protected.[4] In traditional medicine, the bulb of this plant is primarily used for cancer treatment.[5] Peiminine is an alkaloid extracted from the bulbs of *Fritillaria thunbergii*.[6, 7] It is documented that peiminine suppresses colon cancer.[7, 8] It also inhibits Glioblastoma in vitro and

in vivo by induction of autophagic cell death.[9] Scientific literature indicated that peiminine has anti-inflammatory, antitussive, and sound expectorant effects.[7] Peiminine is effective in reducing bleomycin-induced acute lung injury in rats. Peiminine could suppress RANKL-induced osteoclastogenesis by Inhibiting the NF-κB, NFATc1, and ERK signaling pathways.[7] In another study, results indicated peiminine regressed injuries of myocardial infarction via regulating MAP kinase pathway.[7, 10] Peiminine also protects dopaminergic neurons from neuroinflammation by inhibiting the NF-κB pathways and extracellular-regulated protein kinase (ERK1/2).[7] However, there is no or limited information about the possible effect of the peiminine on the functions of immune cells. This study was done to investigate the impact of oral administration of peiminine on the functions of peritoneal macrophages isolated from BALB/c mice.

Materials and Methods

Reagents:

In this experimental study, fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Gibco/Life Technologies, Inc. (Gaithersburg, MD). Lipopolysaccharide (LPS), natural red (NR), nitroblue tetrazolium (NBT), dioxin, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phosphate-buffered saline (PBS), and tetradecanoylphorbol acetate (TPA) were purchased Sigma-Aldrich (St. Louis, MO).

Grouping and treatment of animals

Male BALB/c mice are aged 6-8 weeks were purchased from the Pasteur Institute of IR. Iran. The BALB/c mice were maintained at a constant temperature (22±2°C) with a 12-hour light/dark cycle and had free access to water and food. After adaptation, the animal was randomly allocated to four equal groups (n=10). Mice were treated orally with doses of 0, 1, 3, and 6 mg/kg of peiminine (dissolved in PBS) for one month.

It is necessary to mention, that animal procedures were done by the Ethics Committee of the Faculty of Veterinary Medicine of Urmia University, Urmia, Iran (Ethics Code: IR-UU-6045).

Isolation of macrophage cells from the peritoneum of mice:

After one month of Peiminine treatment, the animals were euthanized by cervical dislocation. To remove peritoneal resident macrophages, 5 ml of ice-cold PBS was injected into the peritoneal cavity of each mouse. The peritoneal fluid was aspirated and centrifuged at 600 g for 10 minutes at 4°C. The pellets were rinsed three times in PBS and re-suspended in DMEM that contained 10% heat-inactivated FBS. The isolated cells were counted in the Neubauer chamber. Their viability was assessed the trypan blue dye exclusion method. Then, 100 µl of the alive cell (2×10^6 cells/ml) was pre-incubated in 96-well microplates for 60 minutes at 37°C in a moist atmosphere of 5% CO2. According to the ability of macrophage cells to adhere to the bottom of culture flasks, the non-adherent cells were discarded by vigorously washing three times with ice-cold PBS. The cell viability monitored by trypan blue exclusion was never below 95%.[11]

Neutral red uptake

Macrophages were incubated with Neutral Red Solution (0.33%) for 1 hour. To eliminate the neutral red that was not phagocytized by macrophages, the supernatants were discarded, and the cells were washed twice with PBS. Then, the cells were lysed by lysing buffer (ethanol and 0.01% acetic acid at a 1:1 ratio, 200 µl/well). The optical density (OD) at 490 nm was measured by a microplate reader (Dynatech, Denkendorf, Germany).

Macrophage phagocytosis

Macrophages were cultured with neutral-red-stained, heat-stabilized, zymosan suspension for 30 minutes at a 1:10 ratio as previously described. The supernatant was deleted, and phagocytosis was inhibited by the addition of Baker's formol calcium solution. The macrophages were washed 2 times by centrifugation in PBS. Neutral red was solubilized by acidified alcohol, and the absorbance was recorded spectrophotometrically at 550 nm.

Assessment of respiratory burst:

Five hundred microliters of 10% NBT solution along with N-formylmethionine leucyl-phenylalanine (f-MLP, Sigma) was added to each well at a final concentration of 1 µM. The plates were incubated for half an hour at 37°C. After the incubation time, the cells were washed three times and fixed with methanol. Then, the fixed formazan crystals were dissolved by adding 1400 µl of DMSO and 1200 µl of 2M KOH. Two hundred microliters of the solution were removed and transferred to a 96 plate under the bed and read at 620 nm wavelength by an ELISA reader.[12]

Measurement of nitric oxide production:

Five hundred microliters of opsonized yeast suspension (2×10^7 cells per milliliter) were poured into the wells of

24-well plates. In another group of wells, N-formylmethionine leucyl-phenylalanine (fMLP) was added to each well at a final concentration of 1 Mμ. These cells were incubated for 8 minutes at 37°C and 5% carbon dioxide. The amount of nitric oxide production was determined by the Griess colorimetric method and using the sodium nitrite standard curve. Briefly, 100 μL of neutrophil cell culture supernatant was poured into the wells of a 96-well plate at the bottom of the bed in pairs. Then 100 μL of 1% sulfanilamide solution (Sigma - USA) was added to the wells. The plate was incubated for 10 minutes. It was kept in the dark and at room temperature. Then, 100 μL of 1% N-1-1-naphthylethylenediamine dihydrochloride solution (Sigma Company) was added to all the wells and kept again for 10 minutes in the dark at room temperature. Finally, the light absorption of the sample during The wave of 530 nm was read by an ELISA device. At the same time, using different concentrations of sodium nitrite, a standard curve was drawn, and through regression and linear equation, the concentration of nitrite in the samples was determined.[13]

Cytokine assay

The peritoneal macrophages were pulsed with LPS (10 pg/mL) for 24 hours. The cell-free supernatant was collected and used to monitor the levels of IL-10 and IL-12 by an ELISA kit (Bender MedSystems, Austria) according to the manufacturer's guidelines.

Statistical analysis

The Kolmogorov-Smirnov test was used to confirm the normal distribution of data. Afterward, the findings were analyzed using one-way ANOVA plus Dunnett's post hoc test. Data were expressed as means ± SD. $P<0.05$ were presumed statistically significant.

Results

The ability of endocytosis and phagocytosis are two essential characteristics of macrophage cells .The present study indicated no significant change in the potential of endocytosis of neutral red between macrophages isolated from untreated or treated rats with different doses of peiminine (Figure 1.A). Macrophages obtained from mice treated with peiminine showed more tremendous potential in phagocytosis of opsonized zymosan particles in a dose-independent manner (Figure 1.B). The phagocytic potential of opsonized zymosan particles by macrophages obtained from mice receiving peiminine at a dose of 3 and 6 mg/kg was greater than that of macrophages obtained from mice receiving 1 mg/kg of peiminine. Nevertheless, there was no significant difference between the phagocytic power of macrophages obtained from mice receiving peiminine with a dose of 3 mg/kg or receiving a dose of 6 mg/kg (Figure 1.B).

The respiratory burst ability of macrophages stimulated with tetradecanoylphorbol acetate (TPA) in the groups receiving peiminine increased in a dose-dependent manner, so that the macrophages isolated from the mice receiving the highest dose of peiminine (6 mg/kg) had the lowest respiratory burst rate (Figure 2.A). A similar pattern with respiratory burst changes was observed regarding nitric oxide production in peritoneal macrophages. So that by increasing the dose of peiminine, the ability to produce nitric oxide by the macrophages isolated from them increased significantly (Figure 2.B).

Oral intake of peiminine for one month led to a dose-independent decrease in the production of IL-12 by macrophages isolated from the peritoneum of mice after stimulation with LPS (Figure 3.A). The IL-12 production by macrophages from mice receiving peiminine at a dose of 3 and 6 mg/kg was lower than that of macrophages obtained from mice receiving 1 mg/kg of peiminine. Nevertheless, there was no significant difference in IL-12 production between the macrophages obtained from mice receiving peiminine with a dose of 3 mg/kg or receiving a dose of 6 mg/kg (Figure 3.A). Receiving peiminine at a dose of 1 m/kg increased the production of IL-10 from peritoneal macrophages stimulated with LPS compared to macrophages isolated from untreated mice (Figure 3.B). Receiving peiminine at a dose of 3 m/kg increased the production of IL-10 from peritoneal macrophages stimulated with LPS compared to macrophages isolated from untreated mice or macrophages obtained from mice receiving peiminine at a dose of 1 mg/kg (Figure 3.B). The situation in the group receiving peiminine with a dose of 6 mg/kg was different, so that IL-10 production decreased compared to the group receiving peiminine with a dose of 3 mg/kg (Figure 3 B). Statistically, the amount of IL-10 production in this group was similar to the group receiving peiminine with a dose of 1 mg/kg (Figure 3.B).

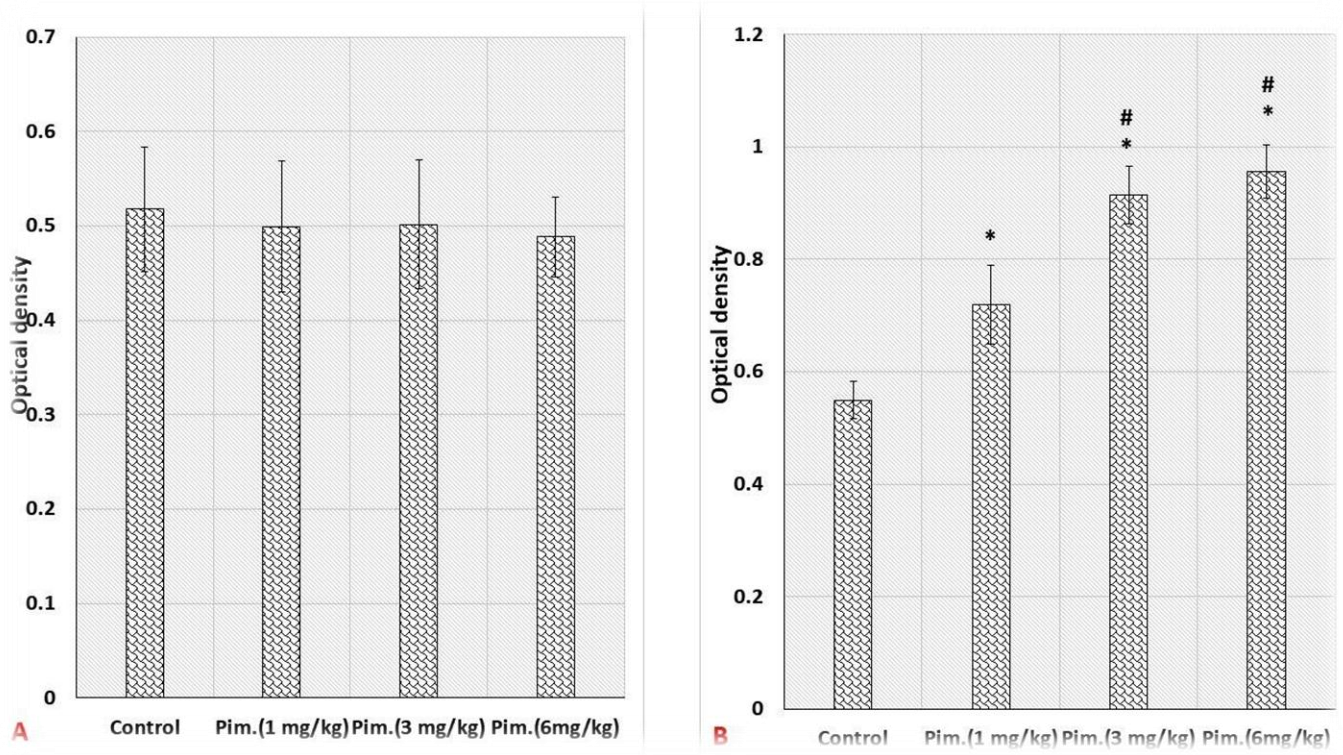

Figure 1. A) Modulation of neutral red uptake and **B)** phagocytosis of neutral red stained zymosan by peritoneal macrophage isolation from mice orally received peiminine for 14 consecutive days. *: P<0. 05 versus control mice; #: P<0.05 versus mice who received 1 mg/kg of peiminine; $: P<0.05 versus mice who received 3 mg/kg of peiminine.

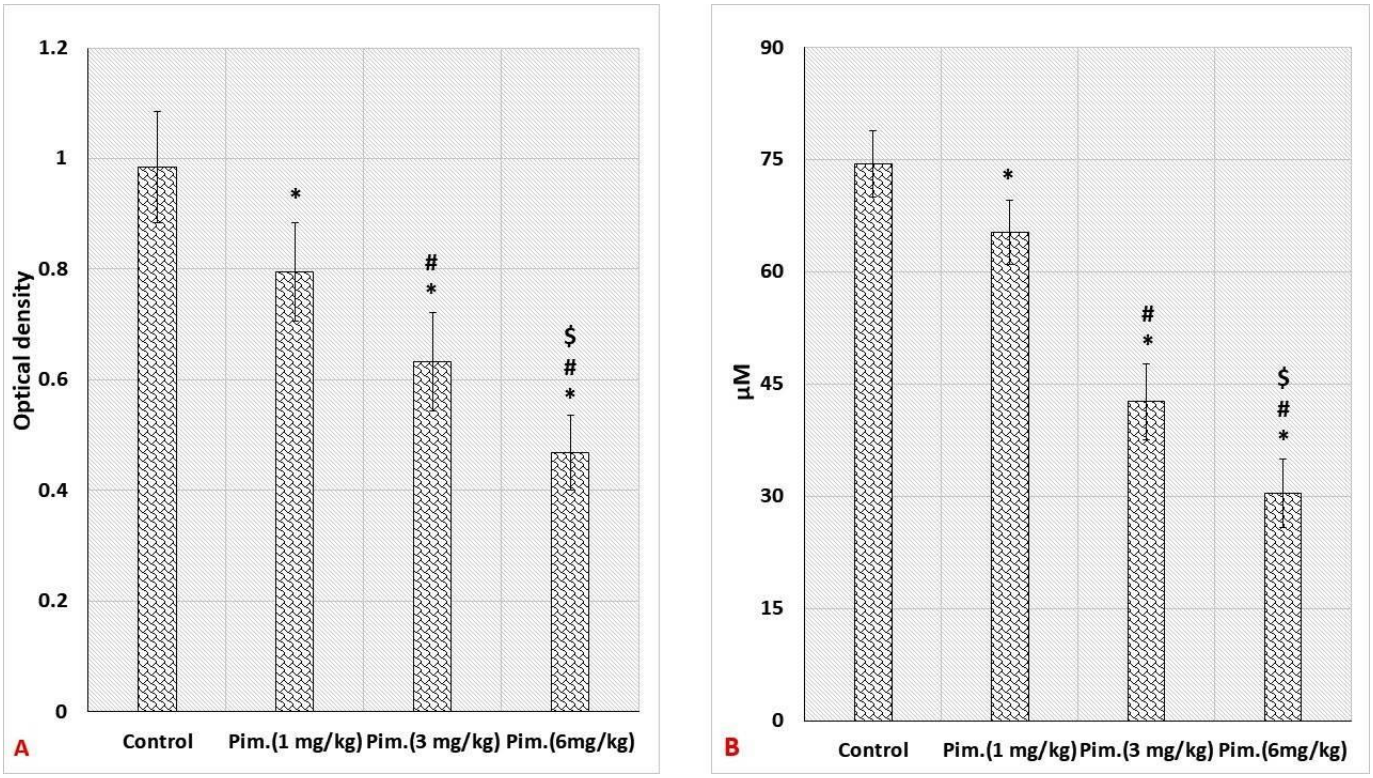

Figure 2. **A)** Assessment of macrophage respiratory burst after activation by tetradecanoylphorbol acetate (TPA) and **B)** nitric oxide production of macrophages after challenge. *: P<0. 05 versus control mice; #: P<0.05 versus mice who received 1 mg/kg of peiminine; $: P<0.05 versus mice who received 3 mg/kg of peiminine.

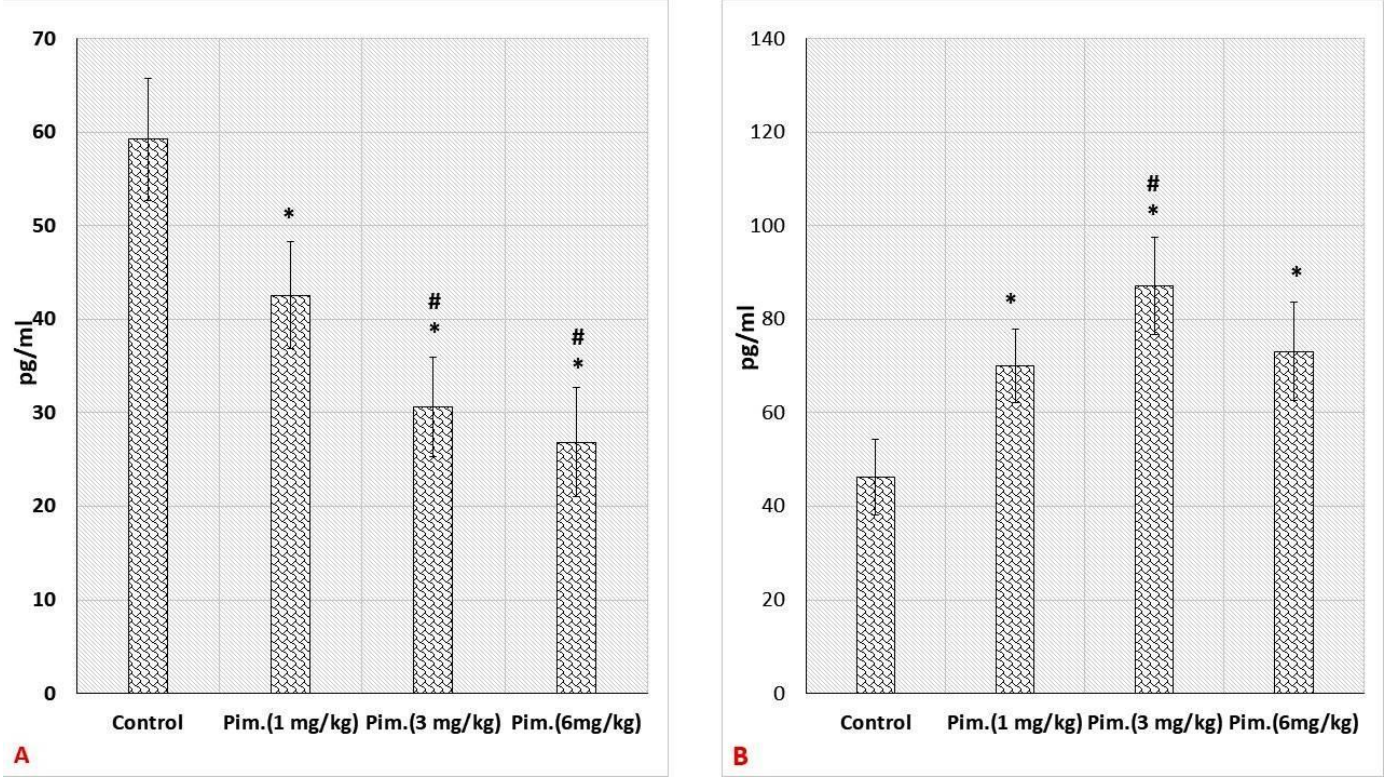

Figure 3. The effect of oral administration of peiminine (Pim.) on cytokine production by peritoneal macrophages after stimulation with LPS. Then, we removed the conditioned media and stimulated the macrophages with lipopolysaccharide (LPS) for 24 hours. Cell-free supernatants were isolated and monitored for levels of IL-12 (**A**) and IL-10 (**B**) by ELISA. *: P<0. 05 versus control mice; #: P<0.05 versus mice who received 1 mg/kg of peiminine; $: P<0.05 versus mice who received 3 mg/kg of peiminine.

Discussion

In addition to antioxidant effects, past studies have demonstrated well that peiminine has profound effects on body cells through changes in gene expression. For example, it has been shown that peiminine declined colorectal cancer through up-regulating miR-760 via regressing the expression of long noncoding RNA LINC00659.[8] In the present study, we confirmed that peiminine effectively induces an anti-inflammatory phenotype in macrophages .In our study, the animals were under the influence of peiminine for one month, so part of the anti-inflammatory phenotype in the macrophage population was due to the effects of peiminine on the expression of macrophage genes. The objective manifestation of this case can be seen in the changes in the production of IL-12 and IL-10 cytokines after the ex vivo stimulation of isolated macrophages with LPS. The anti-inflammatory benefits of peiminine have been discussed in several documents. Peiminine decreased pulmonary inflammation and fibrosis in the rat model of bleomycin-induced lung injury, via inhibiting circulating IFN-γ levels and reducing signal transduction pathways, including ERK1/2, NF-κB, TGF-β, FasL, and TGF-β.[14] Treatment with peiminine promoted a protective effect on lipopolysaccharide-induced mastitis by regressing signaling pathways like NF-κB, MAPKs, and Akt.[15]

Signal transduction is essential for the macrophages to respond effectively to respond to environmental conditions effectively. This is partially achieved by compartmentalized structures involved in signaling pathways similar to lipid raft structures.[16] Peiminine was found to disrupt lipid rafts formation by attenuating the cholesterol content.[17]

Nowadays. It is clear that macrophages are not uniform and are significantly diverse.[11, 18, 19] Based on the factors in their environments, these cells can plan and transform into cells with different functions. Classically activated macrophage cells, or M1 macrophages, have inflammatory solid properties and play an essential role in removing intracellular infections. On the other hand, due to their inflammatory capabilities, they can participate in causing tissue damage, so M1 macrophages act as the primary executive cells in tissue-specific autoimmune diseases. M2 macrophages or macrophages activated by the alternative method produce less inflammatory cytokines and mainly play an essential role in ending inflammation and tissue repair through trophic factors.[11,19] According to the explanations that will come below, it

seems that the oral intake of peiminine was effective in inducing the M2 phenotype in macrophages.

IL-12 is a potent pro-inflammatory cytokine produced mainly by M1 macrophages.[20] This cytokine can instruct T cell-dependent immunity. IL-10 is the famous anti-inflammatory cytokine that may produce by M2 macrophages. This cytokine plays vital role in terminating inflammatory reactions and, eventually inhibiting tissue destruction.[21] According to our data, *ex vivo* secretion of IL-12 by LPS-stimulated macrophages significantly down-regulated in a dose-dependent manner compared to macrophages isolated from mice without treatment. Also, the production of IL-10 by LPS-stimulated macrophages isolated from mice received low to moderate doses of peiminine significantly increased compared to macrophages alone. Meanwhile, receiving the peiminine at a high doses has inhibited the ability of macrophages to produce both inflammatory and anti-inflammatory cytokines.

Macrophage cells produce a high level of proteases and oxygen, and nitrogen free radicals. These mediators play an important role in eliminating pathogen.[11, 22, 23] However, when the amount of production of these radicals is excessive, or their production takes place in inappropriate conditions, these free radicals will lead to the creation and spread of tissue damage.[23] The production of free radicals by macrophage cells occurs as a result of various stimuli, such as effective phagocytosis of opsonized microbial agents or some cytokines and mediators released following tissue damage or inappropriate immune regulation.[22] In the present study, to evaluate the amount of non-specific production (in the absence of infectious agents) of oxygen and nitrogen free radicals and the reconstruction of immunopathological conditions by macrophage cells, N-formyl methionine leucine phenylalanine was used to stimulate ~~of~~ isolated peritoneal macrophages. Our results showed that receiving peiminine by mice dose-dependently has reduced the production of oxygen and nitrogen free radicals by macrophages. Reducing the production of these substances is one of the characteristics of M2 macrophages.[11]

Phagocytosis is an essential role of macrophages to eliminate pathogens. Furthermore, phagocytosis of apoptotic bodies and debris is essential for tissue resolution and termination of inflammatory reactions.[24] Our results indicated an increase in phagocytosis in macrophages obtained from mice treated with peiminine in a non-dose-dependent manner. Increased phagocytic power is characteristic of M2 macrophages compared to M1 macrophages.[11]

The cationic dye, like neutral red, can be uptake and accumulated in the lysosome compartment of macrophages according to the level of cell membrane activation. Neutral red ingestion by macrophages is related to factors like cell viability, activity, and membrane integrity.[24] Our results have confirmed that NR uptake did not show any change between the vitality of macrophages isolated from different studying groups. Therefore, the decrease in the production of oxygen and nitrogen substances and IL-10 that was reported in the present study was not due to a simple inhibition of the cells, because, in this case, the vitality of the isolated macrophages from the treated groups should have decreased.

Collectively, these findings proposed that the macrophage isolated from mice orally received peiminine had an anti-inflammatory (M2-like) phenotype due to preservation of the vitality of macrophages, increased phagocytosis, reduced production of potential harmful ROS and NO by macrophages, decreased inflammatory cytokine IL-12 and conversely increased anti-inflammatory cytokine IL-10.

Research Highlights

What Is Already Known?

The review of literature showed that peiminine had potent pharmacological effects and could suppress neuroinflammation by inhibiting the NF-κB pathways and extracellular-regulated protein kinase (ERK1/2).

What Does This Study Add?

This study proposed that peiminine had profound immunomodulatory benefits and that the macrophage isolated from mice that orally received peiminine had an anti-inflammatory phenotype.

Authors' Contributions

SMAF and ND designed and supervised the study. BM performed the experiments and collected the data. SMAF analyzed the data and prepared the final manuscript.

Conflict of Interest Disclosures

The authors have no conflict of intrest.

Ethical Approval

Animal procedures were done in accordance with the Ethics Committee of the Faculty of Veterinary Medicine of Urmia University, Urmia, Iran.

Funding/ Support

Urmia University, Urmia, Iran.

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