Modulatory Effects of Chrysanyhemi Flos Pharmacopuncture on Nitric-oxide (NO) Production in Murin Macrophagy Cells

Hwa-Young Shin, Hyun-Jong Lee, Yun-Kyu Lee, Seong-Chul Lim, Jae-Soo Kim
Department of Acupuncture & Moxibution, Daegu Haany University College of Oriental Medicine, Daegu, Korea

1. Introduction

Chrysanthemi Flos (CF), the flowers of Chrysanthemum indicum or C. morifolium, is rich in volatile oils, flavonoids and so on [1]. It is used to treat warm pathogen disease with fever and headache. It also clears eyes and dizziness [1,2].

Macrophages are main players in the immune function as antigen-presenting cells in initiating adaptive immune responses and in the innate immune response [3]. Many studies have demonstrated that Chrysanthemum cinerari-aefolium, same genus as Chrysanthemum, increases innate immunity [4]. These immune responses are reported to be stimulated not only by intact bacterial cells but also by their components, including peptidoglycan, lipoteichoic acids, and intra-extracellular polysaccharide products, and cell wall (CW) and cell free extract (CFE) fractions [5-8].

Much evidence exists that herbs have effective immunomodulatory activities in both preclinical and clinical researches [9-13], and studies of the modulatory effects on nitric-oxide (NO) production have been conducted [14-16]. Also, medicinal herbs, Compositae, have been studied for immune regulation [17], but few studies have addressed Chrysanyhemi Flos regulating the production of nitric-oxide (NO). Therefore, the aim of this study was to use the lipopolysaccharide (LPS)-treated RAW 264.7 murine macrophage model to further explore the potential immunomodulation of CF, especially for the regulation of NO production.

2. Material and methods

2.1. Sample preparation

Chrysanthemi Flos was purchased from Omniherb (Korea). CF was prepared as follows: Chrysanthemi Flos, 100 g, was...
added to 2,000 ml of distilled water and boiled in a heating extractor for 3 hours. The extract was filtered and concentrated by using a rotary evaporator and was lyophilized by using a freeze dryer [17.9 g]. The lyophilized extract was dissolved in water and filtered three times through microfilter paper (Whatman no. 2, 0.45–0.2 μm). Then, it was placed in a disinfected vial and sealed for further study.

2.2. Cell culture
Mouse monocyte/macrophage cell line RAW 264.7 (ATCC TIB-71) was grown in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/ml) and penicillin [100 U/ml], at 37°C with 5% CO2. Cells were transferred to 96-well culture plates at a density of 1 x 10⁵ cells/well and were allowed to adhere for 24 hours prior to sample treatment. The culture supernatant was collected and stored at -80°C until it was analyzed for nitric-oxide (NO), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α).

2.3. Cell viability
The general viability of cultured cells was determined by reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan. MTT in living cells is reduced to formazan crystals, and the amount of formazan dissolved in dimethyl sulfoxide (DMSO), as measured by using the by spectroscopic method, shows the growth of cells. The cell viability was calculated as follows:

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\text{Cell viability} (\%) = \left( \frac{\text{optical density}_{\text{sample}}}{\text{optical density}_{\text{control}}} \right) \times 100
\]

2.4. Phagocytosis analysis
RAW 264.7 cells were seeded in triplicates at a density of 5 x 10⁵ cells/well in 96-well plates with complete DMEM and were allowed to adhere for 24 hours. Cells were cultured with LPS (1 μg/ml) as control. Various concentrations of CF were incubated with cells for 24 hours. After that, the cells were washed three times with phosphate buffered saline, (pH 7.2), and the phagocytic ability of the macrophages was measured by using a phagocytosis assay kit (Cayman Chemical, Michigan, USA) according to the procedure described by the manufacturer.

2.5. NO determination
The NO concentration was determined by measuring the amount of released nitrite with Griess reagent (Sigma-Aldrich, Missouri, USA) according to the Griess reaction [19]. Griess reagent produced a chemical reaction with nitrite and formed a purple azo salt that was consistent with the NO concentration. Briefly, 50 μl of cell culture supernatant was added to new 96-well plates and mixed with 50 μl of modified Griess reagent. After incubation at room temperature for 15 min, the absorbance was measured in a plate reader at 540 nm. Nitrite concentrations were calculated on the basis of a NaNO₂ standard curve.

2.6. Cytokine measurement
The concentrations of IL-6 and TNF-α in the culture supernatant were measured by using a IL-6 kit (Thermo scientific, Illinois, USA) and a TNF-α kit (R&D systems, Minnesota, USA), respectively, according to the procedure described by the manufacturer.

2.7. Statistical analysis
The results were expressed as means ± standard deviations (SD). Significant changes were evaluated by using the one-way ANOVA with Dunnett’s post-hoc test. Values of p < 0.05 were considered significant.

3. Results
3.1. Cytotoxicity on RAW 264.7 cells
In order to evaluate the cytotoxicity of CF, samples were prepared at various concentrations and used to treat RAW 264.7 cells. The results of this evaluation are shown in Fig. 1 at concentrations of 50, 100 and 200 μg/ml. The cell viability was recalculated into 100% of LPS-treated control group. The cell’s viabilities treated with LPS, both LPS and CF 50 μg/ml, both LPS and CF 100 μg/ml, both LPS and CF 200 μg/ml, vehicle, CF 50, CF 100 and CF 200 μg/ml, respectively 100.0 ± 1.6, 128.7 ± 1.0, 128.5 ± 2.7, 136.7 ± 5.5, 75.3 ± 2.2, 78.0 ± 3.2, 87.3 ± 1.3 and 75.2 ± 2.6 %. LPS with or without CF treatments significantly increased the cell’s viabilities compared to vehicle with or without CF treatments. Moreover all groups treated with both LPS and CF (LPS(+) CF(50, 100 and 200)) showed significantly increased cell viabilities compared to control group treated with LPS.

![Figure 1 Cell viability of CF on RAW 264.7 cells. LPS(+) CF(50, 100 and 200 μg/ml): control group treated with LPS. LPS(–) CF(50, 100 and 200 μg/ml): experimental groups treated with both LPS and CF (50, 100 and 200 μg/ml). LPS(–) CF(50, 100 and 200 μg/ml): experimental groups treated with vehicle. Data were expressed as the mean ± SD of the three experiments. * significantly different from LPS(+) CF(50, 100 and 200 μg/ml), p < 0.05.](image-url)

3.2. Phagocytic activity of RAW 264.7 cells
In order to evaluate the phagocytic activity of CF, we prepared samples at various concentrations and used them to treat RAW 264.7 cells. The results of this evaluation are shown in Fig. 2 at concentrations of 50, 100 and 200 μg/ml. The phagocytic activity was normalized to 100% for the LPS-treated control group. The
phagocytic activities treated with LPS, both LPS and CF (50 μg/ml), both LPS and CF (100 μg/ml), both LPS and CF (200 μg/ml), vehicle, CF (50 μg/ml), CF (100 μg/ml) and CF (200 μg/ml) were, respectively, 100.0 ± 6.4, 99.4 ± 2.1, 106.7 ± 8.2, 119.0 ± 6.7, 92.0 ± 3.4, 92.3 ± 3.0, 93.8 ± 2.5 and 86.5 ± 5.9%.

Both LPS and CF treatments increased the phagocytic activities compared to LPS treatments in a dose-dependent manner. Especially both LPS and CF (200 μg/ml) treatments showed statistical difference compared to the LPS-treated control group (p < 0.05, Fig. 2). However, groups treated without LPS showed no changes at all.

In order to evaluate the NO production of CF, we prepared samples at various concentrations and used them to treat RAW 264.7 cells. The results of this evaluation are shown in Fig. 3 at concentrations of 50, 100 and 200 μg/ml. The NO productions treated with LPS, both LPS and CF (50 μg/ml), both LPS and CF (100 μg/ml), both LPS and CF (200 μg/ml), vehicle, CF (50 μg/ml), CF (100 μg/ml) and CF (200 μg/ml) were, respectively, 14.6 ± 0.4, 14.8 ± 0.2, 14.8 ± 0.2, 13.0 ± 0.4, 1.2 ± 0.0, 1.3 ± 0.0, 1.2 ± 0.1 and 2.8 ± 1.9 μM.

LPS treatment significantly increased the NO production compared to vehicle treatment (p < 0.05), but both LPS and CF (200 μg/ml) treatment could significantly reduce the NO production induced by LPS treatment (p < 0.05, Fig. 3).

3.4. IL-6 production of RAW 264.7 cells

In order to evaluate the IL-6 production of CF, we prepared samples at various concentrations and used them to treat RAW 264.7 cells. The results of this evaluation are shown in Fig. 4 at concentrations of 50, 100 and 200 μg/ml. The IL-6 production treated with LPS, both LPS and CF (50 μg/ml), both LPS and CF (100 μg/ml), both LPS and CF (200 μg/ml), vehicle, CF (50 μg/ml), CF (100 μg/ml) and CF (200 μg/ml) were, respectively, 1097.1 ± 88.0, 1045.7 ± 0.3, 1198.7 ± 1.4, 1081.4 ± 0.3, 5.1 ± 3.3, 6.2 ± 55.0, 3.9 ± 95.0 and 3.2 ± 14.3 pg/ml.

LPS treatment significantly increased the IL-6 production compared to vehicle treatment (p < 0.05). However, CF treatment did not change the IL-6 production induced by LPS treatment (Fig. 4).

3.5. TNF-α production of RAW 264.7 cells

In order to evaluate the TNF-α production of CF, we prepared samples at various concentrations and used to treat RAW 264.7 cells. The results of this evaluation are shown in Fig. 5 at concentrations of 50, 100 and 200 μg/ml. The TNF-α production treated with LPS, both LPS and CF (50 μg/ml), both LPS and CF (100 μg/ml), both LPS and CF (200 μg/ml), vehicle, CF (50 μg/ml), CF (100 μg/ml) and CF (200 μg/ml) were, respectively, 452.7 ± 3.0, 448.3 ± 2.5, 441.3 ± 3.4, 440.7 ± 5.4, 1.9 ± 1.1, 1.5 ± 0.7,
TNF-α production of CF on RAW 264.7 cells. LPS(+) CF(-): control group treated with LPS. LPS(+ CF): 50, 100 and 200 μg/ml): experimental groups treated with both LPS and CF (50, 100 and 200 μg/ml). LPS(- CF(-): experimental group treated with vehicle. LPS(- CF): 50, 100, and 200 μg/ml): experimental groups treated with CF (50, 100 and 200 μg/ml). Data are expressed as the mean ± SD of the three experiments.

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