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Original Article

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

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Key Words

CF; herb; immunomodulatory; macrophage; nitric-oxide (NO); RAW 264.7

Abstract

Objectives: Much evidence exists that herbs have effective immunomodulatory activities. Chrysanthemi Flos (CF) is effective in clearing heat, reducing inflammation, dropping blood pressure and treating headache and is used as a pharmaceutical raw material for an immune enhancer. The purpose of this study was to investigate the modulatory effect of Chrysanthemi Flos pharmacopuncture on nitric-oxide (NO) production in activating macrophages.

Methods: After a murine macrophage cell line, RAW 264.7, was cultured in the presence of lipopolysaccharide (LPS), immune-modulating abilities of CF were evaluated by using NO, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) production and phagocytic activity of macrophages.

Results: CF enhanced the activities of macrophages by increasing the phagocytic activity and decreasing NO production. Especially, both LPS and CF, 200 μg/ml, treatment could significantly reduce the NO production, but did not change the production of IL-6 and TNF-α.

Conclusion: The results of this study indicate that CF may be of immunomodulatory value, especially for adverse diseases due to increased NO production. It may have potential for use as immunoenhancing pharmacopuncture.

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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 cinerariaefolium,* 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

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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% CO_2. Cells were transferred to 96-well culture plates at a density of 1 x 10^5 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). Samples were incubated with cells for 24 hours, and cells cultured with LPS from *Escherichia coli* serotype 0127:B8 (Sigma-Aldrich, Missouri, USA) at a concentration of 1 μg/ml [18] were used as a control.

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 were seeded in 96-well plates at a density of 1 x 10^5 cells/well and were cultured at 37° C in 5% CO_2. Cells were pretreated with the sample at concentration, of 0, 50, 100 and 200 μg/ml with or without 1 μg/ml of LPS for 24 hours. Then, cells were treated with 0.05 mg/ml (final concentration) of MTT and incubated at 37° C for an additional 4 hours. The medium containing MTT was discarded, and the MTT formazan that had been produced was dissolved in 200 μl of DMSO. The absorbance was read at 595 nm with a reference wavelength of 690 nm. The cell viability was calculated as follows:

Cell viability (%) = [(optical Density $_{595}$ of sample)/
(optical density $_{595}$ of control)] × 100.

2.4. Phagocytosis analysis

RAW 264.7 cells were seeded in triplicates at a density of 5 x 10^4 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 $NaNO_2$ 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 were respectively 100.0 ± 1.6, 126.7 ± 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.

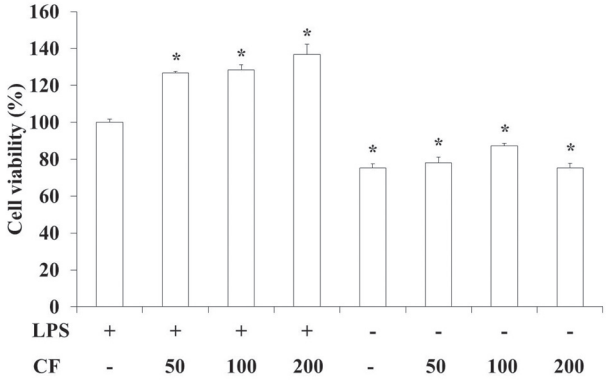

Figure 1 Cell viability of CF on RAW 264.7 cells.
LPS(+) CF(-): control group treated with LPS.
LPS(+) CF (50, 100 and 200 μg/ml): experimental groups treated with both LPS and CF (50, 100 and 200 μg/ml).
LPS(-) CF(-): experimental groups treated with vehicle.
LPS(-) CF (50, 100 and 200 μg/ml): experimental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

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.

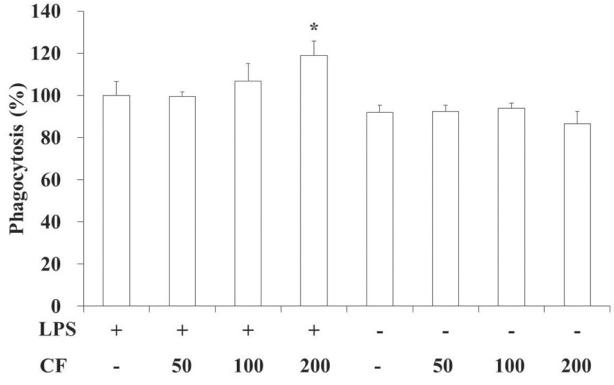

Figure 2 Phagocytic activity of CF on RAW 264.7 cells.
LPS(+) CF(-): control group treated with LPS.
LPS(+) CF (50, 100 and 200 μg/ml): experimental groups treated with both LPS and CF (50, 100 and 200 μg/ml).
LPS(-) CF(-): experimental group treated with vehicle.
LPS(-) CF (50, 100 and 200 μg/m): experimental groups treated with CF (50, 100 and 200 μg/ml).
Data are expressed as the mean ± SD of the three experiments.
* significantly different from LPS(+) CF(-), $p < 0.05$.

3.3. NO production of RAW 264.7 cells

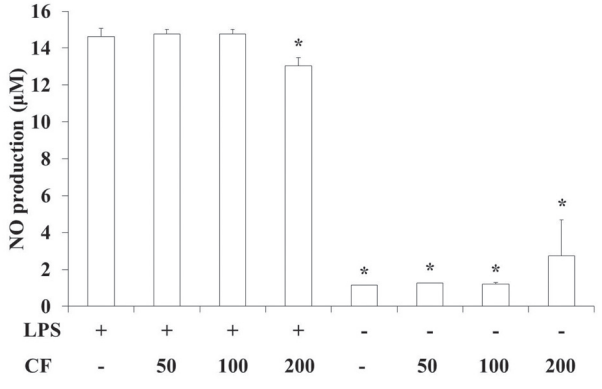

Figure 3 NO production of CF on RAW 264.7 cells.
LPS(+) CF(-): control group treated with LPS.
LPS(+) CF (50, 100, and 200 μg/ml): experimental groups treated with both LPS and CF (50, 100 and 200 μg/ml).
LPS(-) CF(-): experimental group treated with vehicle.
LPS(-) CF(50, 100 and 200 μg/ml): experimental groups treated with CF (50, 100 and 200 μg/ml).
Data are expressed as the mean ± SD of the three experiments.
* significantly different from LPS(+) CF(-), $p < 0.05$.

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, 1185.7 ± 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).

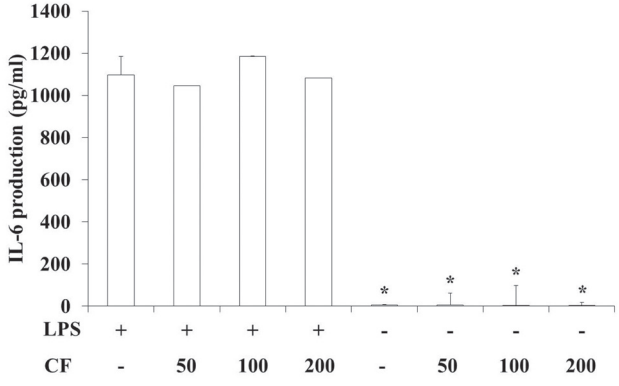

Figure 4 IL-6 production of CF on RAW 264.7 cells.
LPS(+) CF(-): control group treated with LPS.
LPS(+) CF (50, 100 and 200 μg/ml): experimental groups treated with both LPS and CF (50, 100 and 200 μg/ml).
LPS(-) CF(-): experimental group treated with vehicle.
LPS(-) CF (50, 100 and 200 μg/ml): experimental groups treated with CF (50, 100 and 200 μg/ml).
Data are expressed as the mean ± SD of the three experiments.
* significantly different from LPS(+) CF(-), $p < 0.05$.

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,

1.0 ± 0.6 and 2.4 ± 0.6 pg/ml.

LPS treatment significantly increased the TNF-α production compared to vehicle treatment ($p < 0.05$). However, CF treatment did not change the TNF-α production induced by LPS treatment (Fig. 5).

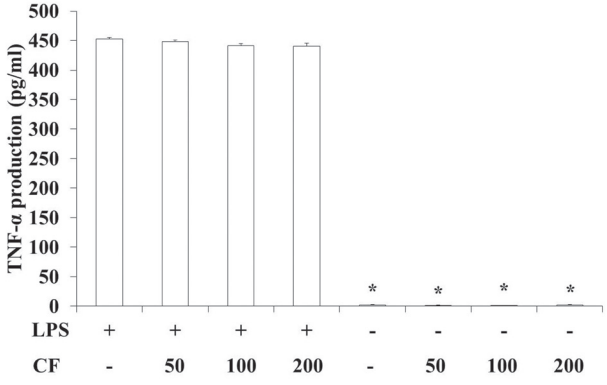

Figure 5 TNF-α production of CF on RAW 264.7 cells.
LPS(+) CF(-): control group treated with LPS.
LPS(+) CF (50, 100 and 200 μg/ml): experimental groups treated with both LPS and CF (50, 100 and 200 μg/ml).
LPS(-) CF(-): experimental group treated with vehicle.
LPS(-) CF (50, 100, and 200 μg/ml): experimental groups treated with CF (50, 100 and 200 μg/ml).
Data are expressed as the mean ± SD of the three experiments.
* significantly different from LPS(+) CF(-), $p < 0.05$

4. Discussion and conclusion

Chrysanthemi Flos, the flowers of *Chrysanthemum indicum* or *C. morifolium,* is rich in borneol, camphor, chrysanthenone, cosmosiin, apigenin, quercitrin, luteolin, thymol, heneicosane, tricosane and hexacosane [1]. It is traditionally used to disperse wind and clear heat. Also, it is used clinically for elimination of wind-heat patterns associated with a common cold or warm pathogen disease with fever and headache. It is also used to calm the liver and extinguish wind, whose symptoms are dizziness, headache and deafness due to ascendant liver yang. In addition, it is effective in clearing red, swollen, dry and painful eyes from wind-heat in the liver channel or ascendant liver yang. It can be used for spots in front of the eyes, blurry vision, or dizziness due to liver and kidney yin deficiency [1,2,20].

LPS is present in the cell's outer membrane of gram negative bacteria and augments pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, at macrophages or monocytes [21]. One important role of the macrophage is the phagocytosis of pathogens and necrotic cellular debris. When a macrophage ingests a pathogen, the pathogen becomes trapped in a phagosome, which then fuses with a lysosome. Within the phagolysosome, enzymes and toxic peroxides digest the pathogen. Macrophages can digest more than 100 bacteria before they finally die due to their own digestive compounds [22].

LPS as endotoxin [23] causes an immune response, but excessive production of inflammatory mediators can destroy normal tissues and cause shock [24]. Thus, herbs that regulate the immune response are important. Many studies concerning the immunomodulatory effects of herbs, such as immunomodulatory effects on healthy volunteers, anti-tumor immune response, splenocyte proliferation, macrophage function, NK anti-tumor activities and so on [9-13], have been performed.

To investigate the in this study, CF on LPS-stimulated RAW 264.7 macrophages were examined immunomodulatory effects by measuring the cytotoxicity, the phagocytic activity and the amount of NO, IL-6 and TNF-α. No cytotoxicity was observed for all CF-treated concentrations. However, LPS treatment increased the cell viability, and both LPS and CF treatment augmented the cell viability which was increased by LPS treatment. Although a high-concentration LPS treatment (e.g., 100 μg/ml) showed cytotoxicity in RAW 264.7 cells, the low concentration used in this study could stimulate the proliferation of macrophage cells [25]. CF treatment could significantly augment the stimulation of proliferation only in the presence of LPS. For the phagocytic activity of CF, although CF treatment without LPS did not change the phagocytic activity compared to the vehicle-treated group, CF treatment with LPS could significantly augment the phagocytic activity compared to the LPS-treated control group in a dose-dependent manner.

NO is generated by macrophages as part of the human immune response. The immune system may regulate the armamentarium of the phagocytes that play a role in the inflammation and the immune responses with NO. NO secreted as an immune response acts as a free radical and is toxic to bacteria. The mechanisms for this include DNA damage [26-28] and degradation of iron-sulfur centers into iron ions and iron-nitrosyl compounds [29]. NO shows an anti-cancer or an anti-microbial effect, but excessive secretion can kill normal cells or promote inflammation [30]. In this study, LPS treatment significantly increased the NO production compared to vehicle treatment ($p < 0.05$). However, both LPS and CF (200 μg/ml) treatment significantly reduced the NO production induced by LPS treatment ($p < 0.05$).

IL-6 is an interleukin that acts as both a pro-inflammatory and an anti-inflammatory cytokine. It is secreted by macrophages and T cells to stimulate immune response, e.g., during infection, but excessive secretion causes rheumatoid arthritis and inflammatory diseases, such as Crohn's disease [31]. TNF-α is a cytokine involved in systemic inflammation and is a member of cytokine groups that stimulate an acute phase reaction. It is produced chiefly by activated macrophages. The effect of TNF-α is to stimulate phagocytosis of macrophages, but excessive secretion causes decreased myocardial contractility, low blood pressure and metabolic process damage [32].

LPS treatment significantly increased the IL-6 and the TNF-α productions compared to vehicle treatment ($p < 0.05$). However, CF treatment did not change the IL-6 and the TNF-α productions induced by LPS treatment. The results for IL-6 and TNF-α may reveal the characteristics of that CF immunomodulatory effect; that is, it does not affect IL-6 and TNF-α-induced immune responses.

In conclusion, CF regulates the production of NO and can prevent the pathological phenomena caused by excessive production of NO, such as expansion of blood vessels, mutation and damage to organization and nerve by inflammation. CF may play an important role in intensity modulated NO-induced immune responses and may have potential for use as an immunoenhancing pharmacopuncture. Further studies will be needed to unravel the characteristics of the effects of CF and the mechanisms of its functions.

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