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Abstract

Background: Cheong-Hwa-Bo-Um-Tang (CHB) has been traditionally used to treat pharyngolaryngitis and throat inflammation in Korea. However, the anti-inflammatory activity of CHB and the exact component proportion ratio of CHB have not been fully reported.

Materials and Methods: This study was conducted to optimize the component proportion ratio of CHB on the basis of experimental evidence using multiplex cytokine profiling assay in LPS-stimulated RAW 264.7 mouse macrophages.

Results: The optimized CHB (named Chunhwabun-Cheong-Hwa-Bo-Um-Tang) inhibited the production of NO, IL-6, MCP-1, GM-CSF, IL-10, IP-10, VEGF, M-CSF, RANTES, MIP-1 β , LIF, LIX, and TNF- α in LPS-stimulated RAW 264.7 mouse macrophages without decreasing cell viability ($P < 0.05$). Chunhwabun-Cheong-Hwa-Bo-Um-Tang consisted of roots of *Scrophularia buergeriana*, *Trichosanthes kirilowii*, *Anemarrhena asphodeloides*, *Phellodendron amurense*, *Angelica gigas*, *Rehmannia glutinosa*, *Paeonia japonica*, *Cnidium officinale*, and *Glycyrrhiza uralensis* (in the ratio of 8: 3: 3: 3: 3: 4: 4: 3: 3).

Conclusion: The results of this study indicate that the multiplex cytokine profiling assay could be one of the effective methods for evidence-guided optimization of the component proportion ratio of a traditional herbal formula used for treating excessive inflammation such as the cytokine storm.

Key words: Cheong-Hwa-Bo-Um-Tang; Lipopolysaccharide; Inflammation; Macrophages; Cytokine; Nitric Oxide

Introduction

Differential use of cellular and molecular components shapes immune responses, but understanding of how these are regulated to promote defense and health during infections is still incomplete (Kallal and Biron, 2013). Innate immune responses are evolutionarily conserved processes that provide crucial protection against invading organisms (Dantoft et al., 2013). The inflammasome, an intracellular multiprotein complex, has emerged as playing a key role in innate immunity (Im and Ammit, 2014).

Recent studies have identified the critical role of inflammasome activation in host defense and inflammation (Yuk and Jo, 2013). Inflammation, the process aimed at restoring homeostasis after an insult, can be more damaging than the insult itself if uncontrolled, excessive, or prolonged (dos Santos et al., 2012). Uncontrolled inflammation leads to extensive tissue damage and manifestation of pathological states like sepsis, autoimmune diseases, metabolic diseases, and cancer (Lacatus 2013).

Cytokines are known to control the initiation, perpetuation, execution, and resolution of an immune response, which involves temporally and spatially orchestrated interactions between different immune cells (Ilangumaran et al., 2004). Although chemokines, chemotactic cytokines that control the migratory patterns and positioning of all immune cells, were initially appreciated as important mediators of acute inflammation, it is suggested that this complex system of approximately 50 endogenous chemokine ligands and 20 G protein-coupled seven-transmembrane signaling receptors is also critical for the generation of primary and secondary adaptive cellular and humoral immune responses (Griffith et al., 2014).

Cells of the innate immune system detect pathogen-associated molecular patterns or endogenous molecules released as a result of tissue injury or inflammation through various innate immune receptors, collectively termed pattern-recognition receptors (Ibrahim et al., 2013).

Macrophages play an important role in inflammatory disease through the release of factors such as nitric oxide (NO), reactive oxygen species, intracellular calcium, inflammatory cytokines, chemokines, growth factors, prostaglandin mediators, and transcription factors involved in the immune response to pathogens such as bacteria and viruses (Medina et al., 2010).

Lipopolysaccharide (LPS) is a potent bacterial effector triggering the activation of the innate immune system following binding with the complex CD14, myeloid differentiation protein 2, and Toll-like receptor 4 (Paciello et al., 2013).

A traditional herbal formula, Cheong-Hwa-Bo-Um-Tang (CHB), which was introduced in *Donguibogam*, has been used to treat pharyngolaryngitis and throat inflammation in Korea. However, the anti-inflammatory activity of CHB and the exact component proportion ratio of CHB have not been fully reported.

This study was conducted to optimize the component proportion ratio of CHB on the basis of experimental evidence using multiplex cytokine profiling assay in LPS-induced RAW 264.7 mouse macrophages.

Materials and Methods

Materials

DMEM, FBS, penicillin, streptomycin, PBS, and other tissue culture reagents were purchased from Gibco BRL (Grand

Island, NY, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), indomethacin, Griess reagent, and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The multiplex bead-based cytokine assay kits used for the determination of cytokine concentration were purchased from Millipore (Billerica, MA, USA).

Cell Viability Assay

RAW 264.7 mouse macrophages were obtained from the Korea Cell Line Bank (Seoul, Korea). RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS containing 100 U/mL of penicillin and 100 µg/mL of streptomycin at 37°C in a 5% CO₂ humidified incubator. Cell viability was evaluated with the modified MTT assay.

Preparation of CCH, GCH, SCH, and LCH Derived from CHB

CHB has been reported to consist of roots of *Scrophularia buergeriana*, *Trichosanthes kirilowii*, *Anemarrhena asphodeloides*, *Phellodendron amurense*, *Angelica gigas*, *Rehmannia glutinosa*, *Paeonia japonica*, *Cnidium officinale*, and *Glycyrrhiza uralensis*. But the component proportion ratio of CHB has been differently reported in traditional medical classics. Hence, firstly, four kinds of herbal formulae derived from CHB were prepared on the basis of traditional medical classics and articles. These four herbal formulae are Chunhwabun-Cheong-Hwa-Bo-Um-Tang (CCH), Guaruin-Cheong-Hwa-Bo-Um-Tang (GCH), Hwangkeum-Cheong-Hwa-Bo-Um-Tang (SCH), and Indongdeung-Cheong-Hwa-Bo-Um-Tang (LCH). The compositions of CCH, GCH, SCH, and LCH are shown in TABLE 1. All herbs were obtained from Omniherb (Daegu, Korea) and deposited at the Department of Pathology, College of Korean Medicine, Gachon University. Because herbal formulae have been traditionally extracted using water, in the present study, CCH, GCH, SCH, and LCH were extracted with boiling water for 2 hr, filtered, and then lyophilized (yield: 38.44% ; 35.63% ; 32.12% ; 29.2%, respectively). The powdered extract was dissolved in saline and then filtered through a 0.22 µm syringe filter.

Quantification of NO Production

NO concentration in culture medium was determined by the Griess reaction assay. Specifically, after incubation of cells with materials for 24 hr, 100 µL of the supernatant from each well was mixed with 100 µL of Griess reagent in wells of a 96-well plate. After incubation for 15 min at room temperature, OD was determined at 540 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

TABLE 1: The contents of CCH, GCH, SCH, and LCH

| Herbal medicines | Ratio | | | |
|--|-------|-----|-----|-----|
| | CCH | GCH | SCH | LCH |
| Root of <i>Scrophularia buergeriana</i> | 8 | 8 | 6 | 6 |
| Root of <i>Trichosanthes kirilowii</i> | 3 | | 3 | 3 |
| Semen of <i>Trichosanthes kirilowii</i> | | 3 | | |
| Root of <i>Anemarrhena asphodeloides</i> | 3 | 3 | 3 | 3 |
| Root of <i>Phellodendron amurense</i> | 3 | 3 | 4 | 4 |
| Root of <i>Angelica gigas</i> | 3 | 3 | 2 | 2 |
| Root of <i>Rehmannia glutinosa</i> | 4 | 4 | 4 | 4 |
| Root of <i>Paeonia japonica</i> | 4 | 4 | 2 | 2 |
| Root of <i>Cnidium officinale</i> | 3 | 3 | 2 | 2 |
| Root of <i>Glycyrrhiza uralensis</i> | 3 | 3 | 3 | 3 |
| Root of <i>Scutellaria baicalensis</i> | | | 4 | |
| Root of <i>Coptis chinensis</i> | | | | 3 |
| Stem of <i>Lonicera japonica</i> | | | | 3 |

Multiplex Bead-Based Cytokine Assay

After 24 hr treatment with materials, cytokines released by treated cells were measured in cell culture supernatants using a Luminex assay based on xMAP technology. This assay was performed using Milliplex kits (Millipore) and Bio-Plex 200 suspension array system (Bio-Rad) as described previously (Yoon et al., 2009; Yuk et al., 2010). Standard curves for each cytokine were generated using the kit-supplied reference cytokine samples. Production of the following cytokines was analyzed: interleukin (IL)-6, IL-10, interferon inducible protein-10 (IP-10), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF; IL-6 class cytokine), lipopolysaccharide-induced CXC chemokine (LIX; CXCL5), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , MIP-2, RANTES/CCL5, tumor necrosis factor (TNF)- α , and vascular endothelial growth factor (VEGF).

Statistical Analysis

The results presented are summarized from three independent experiments and are expressed as the mean \pm SD. Significant differences were examined using a Student's *t*-test with SPSS 11.0 software (SPSS, Chicago, IL, USA). In all cases, a *P* value < 0.05 was considered statistically significant.

Results

Effects of CCH, GCH, SCH, and LCH on Cell Viability

Cell viability of RAW 264.7 mouse macrophages in the presence of CCH, GCH, SCH, and LCH is shown in Figure 1. Cytotoxicity of these formula extracts (up to a concentration of 200 μ g/mL) was not obvious after 24 hr incubation. Based on this result, concentrations of these formula extracts up to 200 μ g/mL were chosen for subsequent experiments.

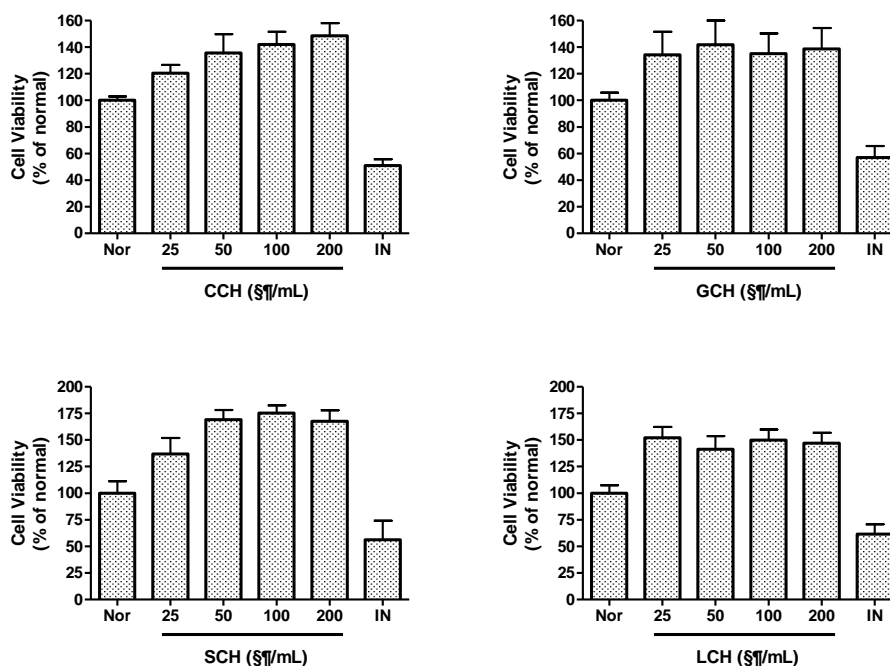


Figure 1: Effects of CCH, GCH, SCH, and LCH on cell viability of RAW 264.7 mouse macrophages. After 24 hr treatment, cell viability was evaluated by a modified MTT assay. Normal group (Nor) was treated with media only. IN denotes indomethacin (0.5 μ M). Values are expressed as the mean \pm SD of more than three independent experiments.

Effects of CCH, GCH, SCH, and LCH on NO Production

The effects of CCH, GCH, SCH, and LCH on NO production in LPS-stimulated RAW 264.7 mouse macrophages are shown in Figure 2. Both CCH and LCH significantly decreased NO production in LPS-stimulated RAW 264.7 mouse macrophages at concentrations of 25, 50, 100, and 200 μ g/mL, whereas SCH significantly decreased NO production at concentrations of 25 and

200 µg/mL. GCH significantly decreased NO production at a concentration of 50µg/mL.

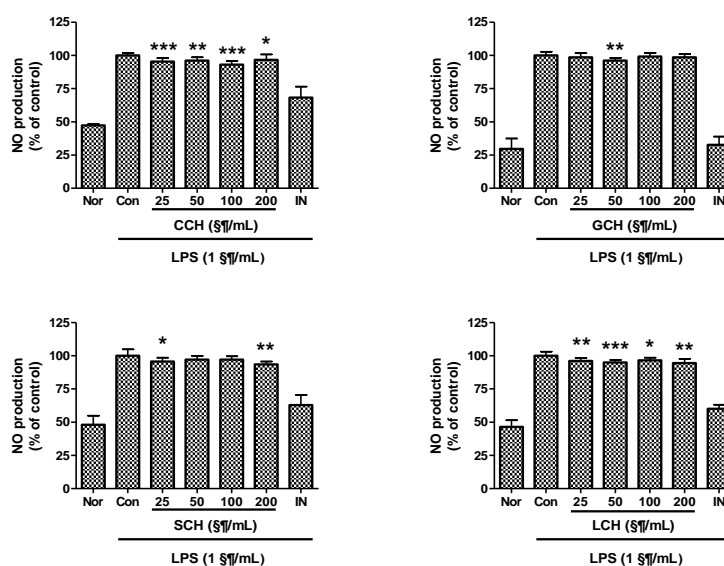


Figure 2: Effects of CCH, GCH, SCH, and LCH on NO production in LPS-stimulated RAW 264.7 mouse macrophages. After 24 hr treatment, NO production was evaluated by Griess reagent assay. Normal group (Nor) was treated with media only. Control group (Con) was treated with LPS (1 µg/mL) only. IN denotes indomethacin (0.5 µM). Values are expressed as the mean ± SD of more than three independent experiments. * $P < 0.05$ vs. Con; ** $P < 0.01$; *** $P < 0.001$.

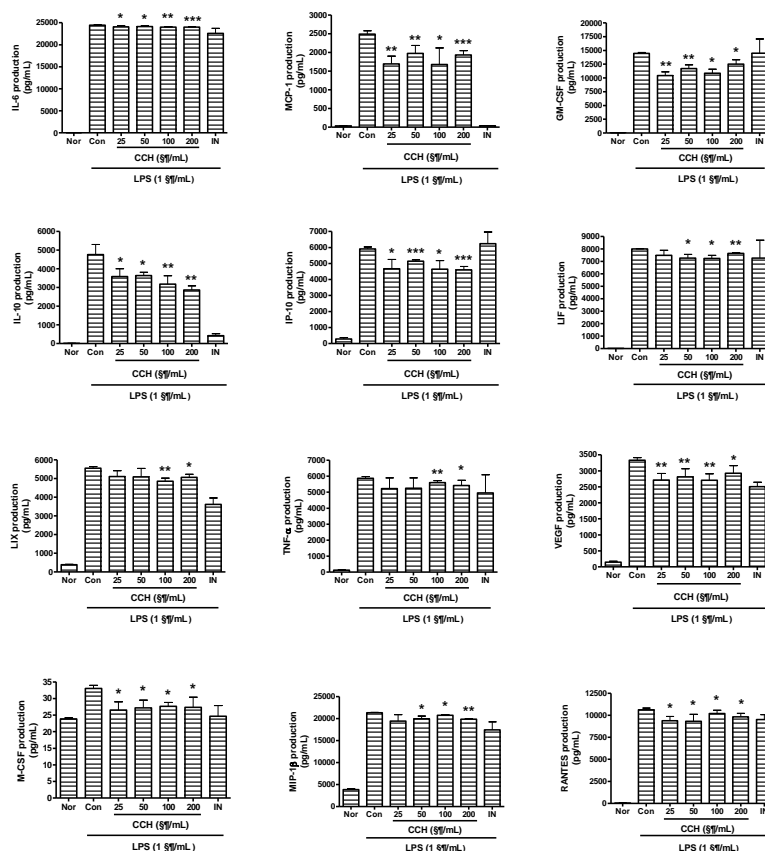


Figure 3: Effects of CCH on production of cytokines such as IL-6, MCP-1, GM-CSF, IL-10, IP-10, LIF, LIX, TNF-α, VEGF, M-CSF, MIP-1β, and RANTES in LPS-stimulated RAW 264.7 mouse macrophages. Fluorescence intensity of each cytokine in the culture medium was measured by a Multiplex bead-based cytokine assay after 24 hr incubation. Normal group (Nor) was treated with media only. Control group (Con) was treated with LPS (1 µg/mL) alone. IN denotes indomethacin (0.5 µM). Values are expressed as the mean ± SD of more than three independent experiments. * $P < 0.05$ vs. Con; ** $P < 0.01$; *** $P < 0.001$.

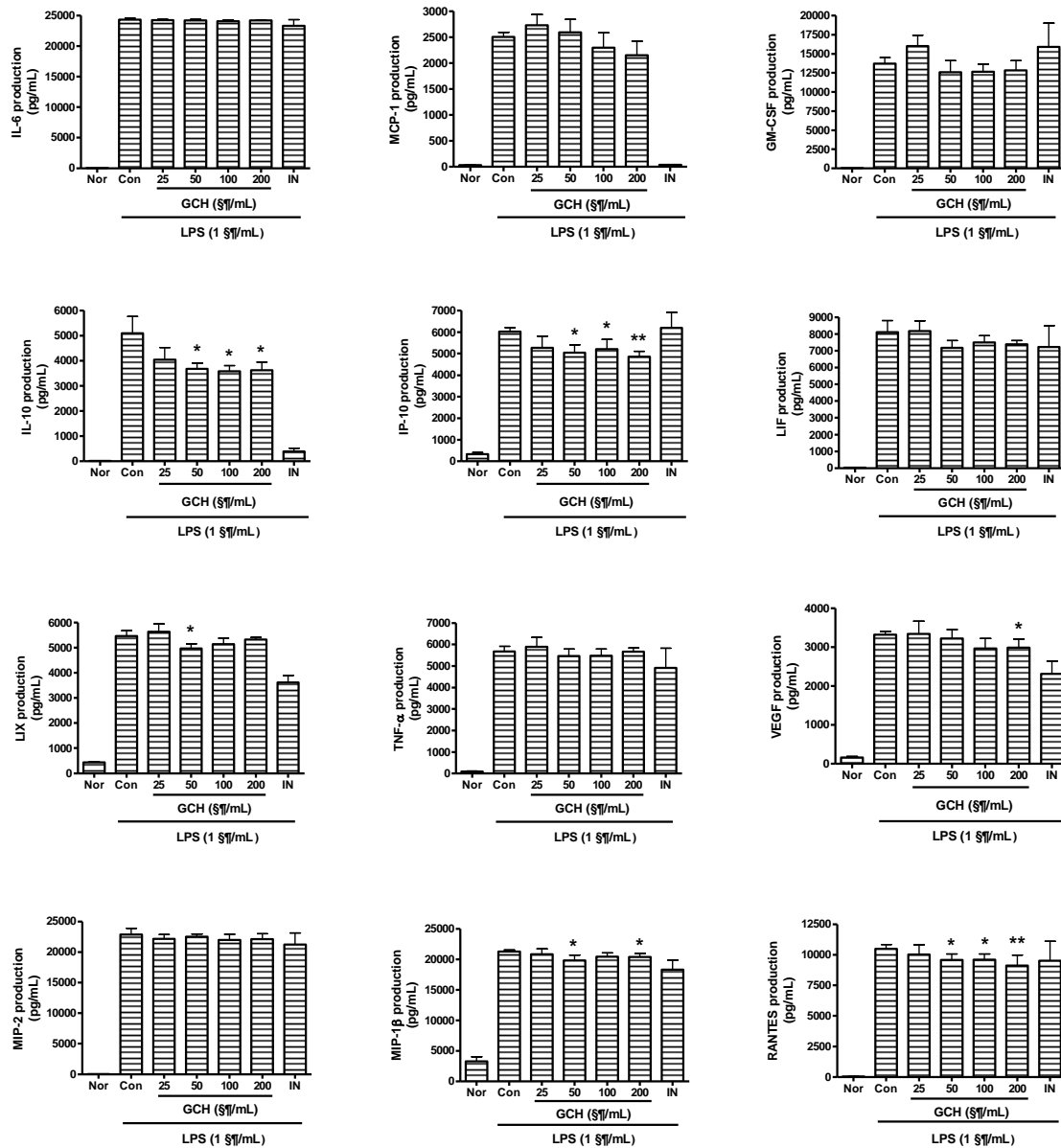


Figure 4: Effects of GCH on production of cytokines such as IL-6, MCP-1, GM-CSF, IL-10, IP-10, LIF, LIX, TNF- α , VEGF, MIP-2, MIP-1 β , and RANTES in LPS-stimulated RAW 264.7 mouse macrophages. Fluorescence intensity of each cytokine in the culture medium was measured by a Multiplex bead-based cytokine assay after 24 hr incubation. Normal group (Nor) was treated with media only. Control group (Con) was treated with LPS (1 μ g/mL) alone. IN denotes indomethacin (0.5 μ M). Values are expressed as the mean \pm SD of more than three independent experiments. * $P < 0.05$ vs. Con; ** $P < 0.01$.

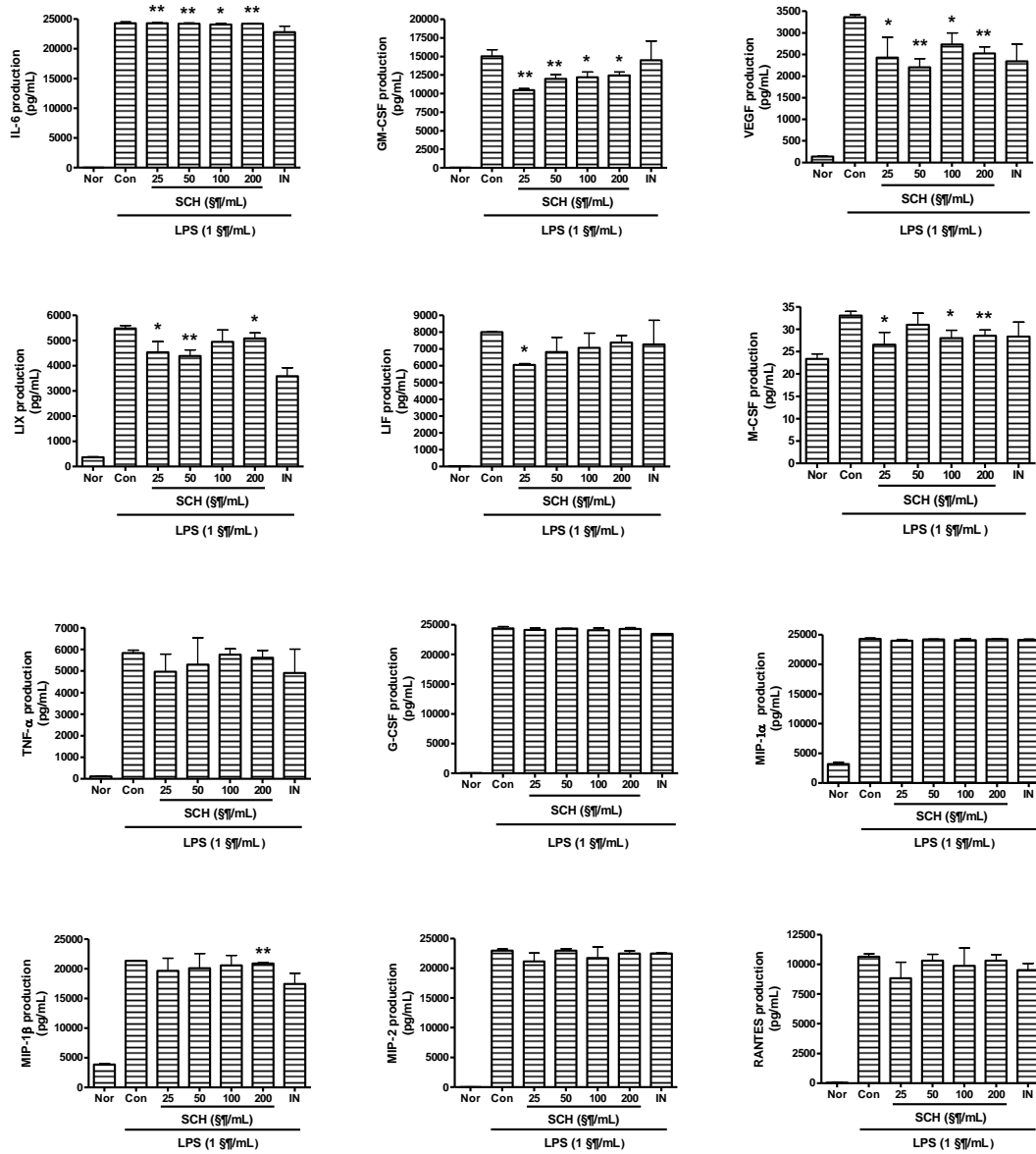


Figure 5: Effects of SCH on production of cytokines such as IL-6, GM-CSF, VEGF, LIX, LIF, M-CSF, TNF- α , G-CSF, MIP-1 α , MIP-1 β , MIP-2, and RANTES in LPS-stimulated RAW 264.7 mouse macrophages. Fluorescence intensity of each cytokine in the culture medium was measured by a Multiplex bead-based cytokine assay after 24 hr incubation. Normal group (Nor) was treated with media only. Control group (Con) was treated with LPS (1 μ g/mL) alone. IN denotes indomethacin (0.5 μ M). Values are expressed as the mean \pm SD of three independent experiments. * $P < 0.05$ vs. Con; ** $P < 0.01$.

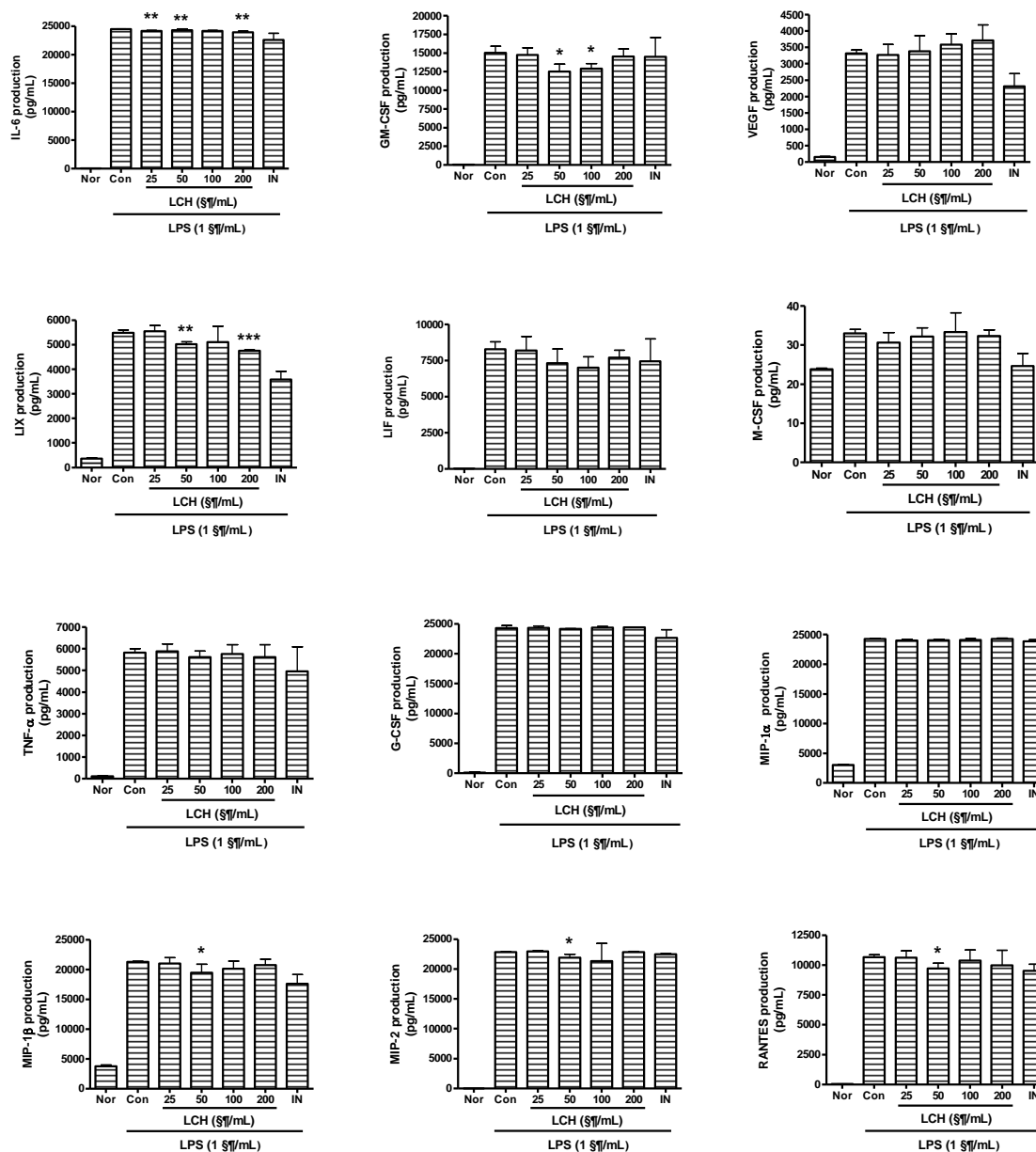


Figure 6: Effects of LCH on production of cytokines such as IL-6, GM-CSF, VEGF, LIX, LIF, M-CSF, TNF- α , G-CSF, MIP-1 α , MIP-1 β , MIP-2, and RANTES in LPS-stimulated RAW 264.7 mouse macrophages. Fluorescence intensity of each cytokine in the culture medium was measured by a Multiplex bead-based cytokine assay after 24 hr incubation. Normal group (Nor) was treated with media only. Control group (Con) was treated with LPS (1 μ g/mL) alone. IN denotes indomethacin (0.5 μ M). Values are expressed as the mean \pm SD of three independent experiments. * $P < 0.05$ vs. Con; ** $P < 0.01$; *** $P < 0.001$.

Discussion

Physical barriers along with immune defense systems which have evolved to protect the host from microbial invasion and acute inflammation is a response to infection or cellular disturbances by other means such as trauma (Bauernfeind and Hornung, 2013). But the uncontrolled inflammatory phenomena could be a turning point for the development of acute or chronic inflammatory diseases (dos Santos et al., 2012). Among the many immuno-inflammatory leukocytes, macrophages and monocytes are of great importance (Kang et al., 2010).

Macrophages play a central role in the inflammatory response and serve as an essential interface between innate and adaptive immunity by modulating NO, various cytokines, prostaglandins, calcium, and hydrogen peroxide (Maruotti et al., 2007).

NO is a major component in the host innate immune reaction to various pathogens including bacteria, viruses, fungi, and other parasites (Bogdan et al., 2000). Hernansanz-Agustín et al. have also reported that, in the immune system, NO has been mainly associated with antibacterial defenses exerted through oxidative, nitrosative, and nitrative stress and signal transduction through cyclic GMP-dependent mechanisms (Hernansanz-Agustín et al., 2013). But excessive NO production is related to the development

of septic shock, neuropathological diseases, rheumatoid arthritis (RA), and other autoimmune disorders (O'Shea et al., 2002).

Cytokines are small proteins which play important roles in cell signaling and they are secreted by a variety of cellular sources acting either on the cell producing them (autocrine) or on the surrounding cells (paracrine) (Burska et al., 2014). Cytokines also control the initiation, perpetuation, execution, and resolution of an immune response, which involves temporally and spatially orchestrated interactions between different immune cells (Ilangumaran et al., 2004).

However, excessive and uncontrolled production of inflammatory mediators such as cytokines, NO, and PGE₂ may lead to not only autoimmune disorders but also serious systemic complications such as microcirculatory dysfunction, tissue damage, and septic shock with a resulting high mortality (Schoettler and Brahn, 2009; Ulevitch and Tobias, 1995).

Schultzberg et al. have already reported that the activation of microglia and astrocytes with subsequent release of cytokines and other inflammatory factors such as NO are common features of both acute and chronic neurodegenerative disorders (Schultzberg et al., 2007).

Hu et al. reported that the dysregulation of inflammatory/immune responses in sepsis is responsible for multiple organ failure, for which overexpression of pro-inflammatory cytokines (i.e. cytokine storm) is a major mechanism (Hu et al., 2014). It is well known that both viral and bacterial infections contribute to the pathogenesis of severe sepsis, which is characterized by an overwhelming production of NO and proinflammatory cytokines, such as IL-1 and IL-6 (Ulloa and Tracey, 2005). Despite the recent discovery of antibiotics, sepsis remains a clinical challenge, with high mortality rates and increasing prevalence (Sessler and Shepherd, 2002).

Ballara et al. have suggested that increased levels of IL-6, TNF- α , and VEGF often correlate with several inflammatory autoimmune diseases including RA, systemic-onset juvenile chronic arthritis, osteoporosis, psoriasis, polyclonal plasmacytosis, malignant plasmacytoma, Crohn's disease, and experimental autoimmune encephalomyelitis (Ballara et al., 2001). It has already been demonstrated that LIX/CXCL5 expression in bone cells has implications for inflammatory bone diseases such as arthritis and periodontal disease (Ruddy et al., 2004).

Recently, it has been reported by Srivastava et al. that various chemokines such as MCP-1, M-CSF, G-CSF, GM-CSF, and IP-10 are increased in bronchoalveolar fluid and the lung tissue of pneumococcal pneumonia during lung inflammation (Srivastava et al., 2005). Wareing et al. have also suggested that the expression level of MIP-1 α , MIP-1 β , MIP-2, and RANTES is increased in lung tissue after influenza infection (Wareing et al., 2004).

IL-10, although traditionally considered as an anti-inflammatory cytokine that modulates the function of adaptive immune-related cells, has also been implicated in promoting abnormal angiogenesis in the eye and in the pathobiology of autoimmune diseases such as lupus and encephalomyelitis (Dace et al., 2009). In psoriasis, a chronic immune-mediated skin disease, the central role of cytokines and their functional interaction with adhesion molecules in the recruitment of tissue-specific lymphocytes has been clearly shown, mainly involving IL-10 and LIF (Schon and Boehncke, 2005). With respect to asthma, a chronic inflammatory disease of the airway, it has already been reported that bronchial epithelial cell changes in asthma are induced by LIF which promotes the expression of neurokinin-1 receptor, and JAK/STAT pathway and MAPK/ERK pathway may participate in the process (Hu et al., 2006).

There is an increasing interest in herbal drugs especially for the treatment of various inflammatory diseases (Hsieh et al., 2008). As a traditional herbal formula, CHB has been used to treat pharyngolaryngitis and throat inflammation in Korea. However, the anti-inflammatory activity of CHB and the exact component proportion ratio of CHB have not been fully reported.

This study was conducted to optimize the component proportion ratio of CHB on the basis of experimental evidence using multiplex cytokine profiling assay in LPS-stimulated RAW 264.7 cells.

Initially, four kinds of herbal formulae (CCH, GCH, SCH, and LCH) derived from CHB were prepared on the basis of traditional medical text and articles. Multiplex assay with MTT and Griess assay was performed to evaluate the anti-inflammatory activity of these formulae. Among these four formulae, CCH turned out to be the most effective formula which significantly inhibited the production of inflammatory mediators such as NO, IL-6, MCP-1, GM-CSF, IL-10, IP-10, VEGF, M-CSF, RANTES, MIP-1 β , LIF, LIX, and TNF- α without decreasing cell viability in LPS-stimulated RAW 264.7 cells. GCH was found to significantly inhibit the production of IP-10, RANTES, IL-10, and MIP-1 β , whereas SCH was found to significantly inhibit the production of NO, IL-6, GM-CSF, VEGF, LIX, and M-CSF in LPS-stimulated RAW 264.7 cells. LCH significantly inhibited the production of NO, IL-6, GM-CSF, and LIX in LPS-stimulated RAW 264.7 cells.

In conclusion, the current study demonstrates that CCH has an anti-inflammatory property related to the inhibition of NO, IL-6, MCP-1, GM-CSF, IL-10, IP-10, VEGF, M-CSF, RANTES, MIP-1 β , LIF, LIX, and TNF- α in LPS-stimulated RAW 264.7 mouse macrophages without decreasing cell viability. The actual effect of CCH in acute and chronic inflammatory diseases deserves further study. The multiplex cytokine profiling assay may be one of the effective methods for evidence-guided optimization of the component proportion ratio of a traditional herbal formula used for treating excessive inflammation such as the cytokine storm.

Acknowledgments

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