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The immune-enhancing activity of *Cervus nippon mantchuricus* extract (NGE) in RAW264.7 macrophage cells and immunosuppressed mice

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ABSTRACT

Chemotherapeutics are often used to inhibit the proliferation of cancer cells. However, they can also harm healthy cells and cause side effects such as immunosuppression. Especially traditional oriental medicines long used in Asia, may be beneficial candidates for the alleviation of immune diseases. *Cervus nippon mantchuricus* extract (NGE) is currently sold in the market as coffee and health drinks. However, NGE was not widely investigated and efficacy remain unclear and essentially nothing is known about their potential immune-regulatory properties. As a result, NGE induced the differentiation of RAW264.7 macrophage cells. NGE-stimulated RAW264.7 macrophage cells elevated cytokines levels and NO production. NGE-stimulated RAW264.7 macrophage cells activated MAPKs and NF-κB signaling pathways. NGE encouraged the immuno-enhancing effects in immunosuppressed short-term treated with NGE mice model. NGE or Red ginseng encouraged the immuno-enhancing effects in immunosuppressed long-term treated with NGE mice model. Our data clearly show that NGE contains immune-enhancing activity and can be used to treat immunodeficiency.

1. Introduction

The regulation of immune response plays a pivotal role in preventing and treating diseases, and increasing attention has been poured to the investigation of immunomodulation and immunostimulation induced by active substance. Previous reports indicated that immunomodulators could help the host defense responses, which was an efficient way to enhance resistance to disease (Yu, Kong, Zhang, Sun, & Chen, 2016). Many investigators have shown that the increase of physical immunity could efficiently defense against disease. Immunotherapy has been proposed for more than a century and has made spectacular progress in recent years.

Lipopolysaccharide (LPS) interacts with the cell-surface protein Toll-like receptor 4 (TLR4) and intracellular connector protein MyD88 (Takeda, Kaisho, & Akira, 2003). The TLR4 is a member of the TLR superfamily, involved in the innate immune system and triggers LPS response (Aderem & Ulevitch, 2000). LPS is known to have ability to activate macrophages (Poltorak et al., 1998). Macrophages play essential role in immunity and inflammatory processes, being induced by TLR ligands, and they secrete various cytokines including tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-1β, IL-6, IL-12, granulocyte macrophage colony stimulating factor (GM-CSF), nitric oxide (NO), inducible NO synthase (iNOS) and prostaglandin E2 (PGE2) (Agarwal, Piesco, Johns, & Riccelli, 1995). Various transduction pathways are related with differentiation, cell activation and cytokine secretion of macrophages. The mitogen activated protein kinase (MAPK) and Nuclear factor (NF)-κB signaling pathways are the most important intracellular transduction cascades for macrophages (Varin & Gordon, 2009).

Extract from *Cervus nippon mantchuricus*, deer bone extract, called nok-gol (NGE) and Red ginseng (RG), is the most commonly used traditional remedies in oriental medicine. They have been widely used to invigorate Qi. Previous works have indicated that the RG is well known immune modulator (Lee, Hwang, et al., 2014; M. J. Lee et al., 2016; Lee, Han, et al., 2014). RG could be heat steamed and dried. As a consequence of this process, RG undergoes certain biochemical changes and acquires certain pharmacological properties such as anti-aging, anti-viral, anti-memory loss, anti-obesity and anti-cancer activities (T. H. Kang et al., 2009; Kim, Hahm, Yang, Lee, & Shim, 2005; M. H. Lee, Seo, Kang, Oh, & Choi, 2014; Y. Lee & Oh, 2015; C. Z. Wang, Anderson,

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Du, He, & Yuan, 2016). NGE, supplied by Nongshim Corporation is currently sold in the market as coffee and health drinks. In recent study, it was found that NGE oral administration is useful for the treatment of various inflammatory diseases, memory loss, bone resorption and neutropenia (Choi et al., 2013; Du et al., 2015; S. K. Kang et al., 2006; H. Lee, Choi, et al., 2014; Lee, Park, et al., 2014). However, NGE was not widely investigated and efficacy remains unclear and essentially nothing is known about their potential immune-regulatory properties. In this study, we evaluated the immune-enhancing activity of NGE *in vitro* measuring leukocytes levels, NO levels, and cytokines levels in RAW264.7 macrophage cells. The intervention of MAPK pathway and NF-κB pathway is also investigated. Moreover, we evaluated the immune-enhancing activity of NGE *in vivo* using immunosuppressed mice. Our data clearly show that NGE contains immune-enhancing activity and can be used to treat immunodeficiency.

2. Materials and methods

2.1. Cervus nippon mantchuricus extract (NGE) and Red ginseng (RG) preparation

Cervus nippon mantchuricus extract (NGE) was provided by Nongshim Corporation (Seoul, Korea). NGE contained 88.8% of crude protein, 1.9% of crude fat, 2.2% of crude ash, and 3.0% of moisture. Total ganglioside content of the extract as sialic acid was 0.09%. Total amino acid and free amino acid amount of extract were 922.5 and 8.21 mg/g, respectively. NGE is white powder extracted by water and dissolved in water for experiments. Red ginseng (RG) was purchased from Korea Ginseng Corporation (KGC, Daejeon, Korea). RG product is made of concentrated six-years-grown Korean Red ginseng extract. More information on NGE (http://nongshim.co.kr) and RG (http://www.kgc.co.kr) production processes could be requested to the company.

2.2. Cell culture

The RAW264.7 murine macrophage cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Welgene, Daegu, Korea) and 1% antibiotics (Ab, Welgene, Daegu, Korea) at 37 °C in a 5% CO_2 humidified incubator.

2.3. Cell morphology

The RAW 264.7 cells (5×10^5 cells/mL) were cultured in the presence of LPS and NGE (25, 100, 500 μg/mL) for 24 h in 60 mm culture dish. Morphological changes were analyzed by taking images using a camera connected to a light microscopy (Olympus, Tokyo, Japan).

2.4. WST assay

Cell viability was determined using WST assay (Dogen, Seoul, Korea). RAW264.7 cells (1×10^4 cells/well) were seeded into 96-well plates and incubated overnight. Cells were then treated with different concentrations of NGE and incubated for another 24 h. 10 μL of WST solution was added to 100 μL cell culture medium, and plates were incubated for 2 h. Optical density (OD) was determined at 450 nm using a ELISA reader (Versa Max, Molecular Devices, Sunnyvale, CA, USA).

2.5. Griess assay

NO production was measured in RAW 264.7 culture supernatant using Griess reagent kit (Promega, Madison, WI, USA). In detail, 150 μL of supernatant from each well was transferred to 96-well plate and then mixed with 150 μL of Griess reagent solution. Mixtures were then incubated for 30 min at room temperature. OD was determined at 570 nm using a ELISA reader (Versa Max, Molecular Devices, Sunnyvale, CA, USA).

2.6. Western blot analysis

Harvested cells were lysed with buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 1 mM Na3VO4, 1 mM DTT, 1 mM NaF, 1 mM PMSF, and PI cocktail on ice for 30 min. The lysates were cleared by centrifugation at 13,000 rpm for 20 min at 4 °C. The supernatant was stored at − 70 °C until use. The protein concentration was quantified using a Bio-Rad Bradford protein assay (Bio-Rad, Hercules, CA, USA). Next, total proteins (15–20 μg) were electrophoresed using 6–15% reducing SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 0.1% Tween-20 in PBS (PBST) containing 1% skim milk and 1% BSA for 1 h, the membranes were incubated overnight at 4 °C with the indicated primary antibodies. After washing in 1 × PBST for 15 min (3 times × 5 min), the membranes were incubated with diluted enzyme-linked secondary antibodies. After washing in 1 × PBST for 1 h (4 times × 15 min), the protein bands were detected using the EZ-western chemiluminescent detection kit and visualized by exposing the membranes to X-ray films. In a parallel experiment, cytoplasmic and nuclear proteins were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Each protein was blotted by appropriate antibodies as follows: anti-NF-κB, p-EKR1/2, p-P38, p-JNK and Lamin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-anti-iNOS, COX-2, p-NF-κB and GAPDH antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-α-tubulin antibody was purchased from Sigma (St. Louis, MO, USA).

2.7. Animals

Six-week-old male BALB/c mice (20 ± 2 g) were purchased from Orient (Sungnam, Korea). The mice were randomized into eight (no treatment, cyclophosphamide, G-CSF, NGE 25 mg/kg, NGE 50 mg/kg, NGE 100 mg/kg, NGE 200 mg/kg, NGE 400 mg/kg) and seven groups (no treatment, cyclophosphamide, G-CSF, NGE pre-treatment, RG pre-treatment, NGE post-treatment, RG post-treatment), each comprising six mice. All mice were kept under pathogen-free environment and allowed free access to the diet and water. All methods were carried out in accordance with relevant guidelines and regulations and procedures involving mice were approved by the animal care center of Kyung-Hee University (Approval Number KHUASP (SE)-14-014). At the end of the experiment, mice were sacrificed by CO_2 inhalation, and cardiac blood was collected.

2.8. Sensitization and treatment

To induce immunosuppression, mice were intraperitoneally injected with 100 mg/kg of cyclophosphamide twice in one week and then treated with cyclophosphamide or G-CSF (1 μg/kg) two times in next week. Mice were rested for one week with no treatment when treatments were switched. NGE and RG (200 mg/kg) pre-treatment group was orally administered every day before the induction with cyclophosphamide or G-CSF. NGE and RG (200 mg/kg) post-treatment group was orally administered every day for the last procedure. At the end of the experiment, mice were sacrificed by CO_2 inhalation, and cardiac blood was collected.

2.9. Spleen weight

The Spleens from all mice were removed and weighed immediately after sacrifice.