INTRODUCTION

Immune system has the function to fight against the external antigen or hazardous stimuli for protecting our body and maintaining the homeostasis. Macrophages are one of the component which play a critical role in the innate and adaptive immune responses. The phagocytic activity of activated macrophages would be augmented during inflammation, and they would kill pathogens by the secretion of the pro-inflammatory cytokines, such as tissue necrotic factor (TNF)-α, Interleukin (IL)-1 or IL-6, Granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF) reactive oxygen species (ROS), nitric oxide (NO), and any other mediators like cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), against pathogenic microorganisms to defense the harmful stimuli that cause inflammation. Activation of macrophages is a key point in innate and adaptive immunities for the initiation and propagation of defensive reactions against pathogens. Thus, the identification of agents that can modulate macrophages is of significant interest.

Hizikia fusiformis (harvey) Okamura (H. fusiformis), a brown sea vegetable growing wild on rocky coastlines around in temperate seaside areas of Korea, Japan, and China. Since it is rich in dietary fiber and essential minerals such as calcium, iron, and magnesium, it has been used as a healthy

Background and Objectives: Hizikia fusiformis is widely used in oriental health food in Japan, China, and Korea, and is known for its anti-oxidation properties.

Materials and Method: In this study, we investigated the anti-inflammatory and immune-modulatory effects and mechanisms of Hizikia fusiformis (H. fusiformis) extracts in lipopolysaccharide (LPS)-treated RAW 264.7 cells. RAW 264.7 cells were incubated in the presence of different concentrations of the viscozyme component of H. fusiformis (1, 2, 5, and 10 μg/mL), and changes in expression of pro-inflammatory cytokines (GM-CSF, iNOS, VEGF, and COX-2) were evaluated by real-time PCR and immunoblotting. In addition, the associated signaling pathway including phospho (p)-pNF-κB 65, p-pIkBa, p-p38, and p-p44/42 was also evaluated.

Results: The viscozyme component of H. fusiformis downregulated the expression of GM-CSF, iNOS, VEGF, and COX-2 mRNA. The augmented NO and ROS production was decreased by administration of H. fusiformis. The signal intensity of p-pNF-κB 65, p-pIkBa, p-p38, and p-p44/42 protein activated by LPS was ameliorated by administration of the viscozyme fraction in RAW 264.7 cells.

Conclusion: These results suggest that H. fusiformis has potential as a therapeutic agent for inflammatory diseases.

KEY WORDS: Sargassum · Polysaccharides · NF-kappa B · Inflammation · Immunity.

Polysaccharide from Hizikia Fusiformis Enhances the Immunomodulatory Activity of Macrophages

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food complement for centuries in East Asian countries. Recently, several studies have reported that extract of *H. fusiformis* shows the immune-modulatory, anti-cancer, anti-atopic and anti-oxidant activities. The anti-inflammatory activity of *H. fusiformis* suggests applications for diverse disorders including allergic and auto-immune, and sinonasal relative disease. Allergic disease is immunoglobulin E mediated disease characterized by the hyper-reactivity to environmental allergens that are finally evoked the chronic inflammation in nose, lung or skin etc. which usually treated with antihistamine and intranasal/oral steroids. Many concerns have been reported when used these agents in long-term therapy because of the adverse effects. Since macrophages have an important role in allergic disease, the identification of agents that can modulate the function of macrophages is of significant interest. The potential therapeutic effects of *H. fusiformis* in allergic disease have not been studied. Thus, we evaluated anti-inflammatory mechanisms of the *H. fusiformis* extract using LPS-induced RAW 264.7 cells.

**MATERIALS AND METHODS**

Materials

RAW 264.7 macrophage cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Neutral red assay kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharides (LPS), sulfanilamide, phosphoric acid, and naphyl ethylene-diamine dihydrochloride (NED) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). DMEM with the supplement of 100 IU/mL penicillin, 100 IU/mL streptomycin was purchased from Gibco (BRL, Gnd Island, NY). The other chemicals and solvents were of analytical reagent grade and purchased from Sigma.

Preparation of *Hizikia fusiformis* extract

The natural *H. fusiformis* was extracted and purified in the Department of Biochemical re-engineering in Chosun University. The procedure was similar to the previous work. After grinding freeze dried *H. fusiformis* in a mixer, 2 g of powder was digested and extracted at 50°C with 100 uL of viscozyme (in 100 mL of 0.1 M sodium acetate buffer solution pH 4.5) for six hours in a shaking incubator (200 rpm). After digestion, the viscozyme was denatured at 100°C for 15 min and supernatant was harvested with centrifuge (10,000 g for 10 min). The supernatant solution was concentrated in a rotary evaporator and the extract powder was prepared using lyophilizer. The viscozyme component of *H. fusiformis* was used in all experiments.

Cell cultures

RAW 264.7 macrophage cells were maintained in DMEM media supplemented with 1% penicillin/streptomycin (gibco/Invitrogen, Carlsbad, CA) and 10% FBS (Capricorn) (w/v). The cells were grown to 60% confluence in 60 mm culture plates at 37°C in a 5% CO2 humidified atmosphere. Cells were pre-treated with different concentration of viscozyme component of *H. fusiformis* (1–10 μg/mL) for 1h and then incubated with LPS (100 ng/mL) for additional 23 h.

Cell viability analysis

Cell proliferation was measured by a colorimetric assay using MTT. In brief, macrophage RAW 264.7 cells diluted into 5×10⁴ cells/well with DMEM medium were seeded in 96-wells plates and cultivated at 37°C in an atmosphere of 5% CO2 for overnight. On next day, the cells were cultured with viscozyme at various concentrations (1, 2, 5 and 10 μg/mL) for 23 h. 10 μL of MTT stock solution (5 mg/mL) were added to each well and incubated for additional 4 h. Blue formazans were eluted from cells by the additional of 100 μL of DMSO with gentle shaking for 30 min at room temperature. The absorbance at 570 nm was measured by microplate reader (Epoch, BioTek, VT, USA). Cell viability was calculated by the following equation. Cell viability (%) = A2/A1×100, where A1 is the absorbance of the control group, and A2 is the absorbance of the test samples group. Experiments were performed in triplicate and repeated two times.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. The RNA was reverse transcribed into cDNA with the Reverse Transcription kit (Qiagen, Venlo, NLD). 10 μL of SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), 4 pmol of forward/reverse primers each, and 1 μL of cDNA in a final volume of 20 μL were included in each reaction tube. qRT-PCR was performed using a 7500 FAST qRT-PCR System (Applied Biosystems). Target mRNA expression was normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression.
phosphate dehydrogenase (GAPDH) expression. Primers used were shown in Table 1. Experiments were repeated more than three times.

**Measurement of nitrite oxide**

NO level in the macrophage RAW 264.7 cells was measured as follows. Briefly, RAW 264.7 cells were diluted to a density of $5 \times 10^5$ cells/well and incubated for overnight. Various concentrations (1, 2, 5 and 10 µg/mL) of viscozyme component of *H. fusiformis* were put into each well for 1 h and then, LPS (100 ng/mL) was added for additional 23 h. DMEM medium and LPS (100 ng/mL) were used as a normal control and positive control, respectively. After incubation for 23 h, the culture media was collected and incubated with Griess reagent (5% phosphoric acid containing 0.1% NED and 1% sulfanilamide) for 15 min. NO was assayed at 540 nm using automated microplate reader (Epoch). Experiments were repeated more than three times.

**Phagocytosis assay**

Neutral red uptake method was used to measure the phagocytic capacity of macrophages of *H. fusiformis*. After RAW 264.7 macrophages incubated in a 96 well with *H. fusiformis* for 24 h, 200 µL of neutral red solution was added into each well and incubated for another 2 h. After discarding supernatant, the cells were rinsed with fixative fastly and washed with phosphate buffered saline (PBS, pH 7.4) twice to remove the neutral red which wasn’t phagocytized by macrophages. Then, cells were lysed by adding 200 µL neutralization solution for lysis for 30 min at room temperature. The cells were washed with PBS twice, again. Cells treated LPS (100 ng/mL) were selected as a positive control. The absorbance at 540 nm was assayed by microplate reader (Epoch). Experiments were repeated more than three times.

**DCF-HA assay**

Intracellular ROS levels were measured by the 2’7’-di-chlorofluorescein diacetate (DCF-DA) assay. After RAW 264.7 macrophages incubated in a 96 well with *H. fusiformis* for 1 h plus additional 23 h with LPS treatment, the medium was removed and the cells were washed twice with PBS. Then, the cells were incubated with 5 µL DCF-DA diluted in PBS for 30 min at room temperature. The H$_2$O$_2$ was used as a positive control. Fluorescence was measured using a fluorescence plate reader (BMG LABTECH GmbH, Offenberg, Germany). Experiments were repeated more than three times.

**Immunoblot analysis**

After 24 h incubation with LPS (100 ng/mL), the medium was removed and cells were washed with PBS and lysed with 1% NP-40 lysis buffer. After centrifuging at 13,000×g for 10 min at 4°C, the supernatant was collected. Equal amounts of protein were separated by electrophoresis on 10–12% Tris-HCl gels and then processed to transfer to a PVDF membrane. After blocking with TBS-T (20 mMTris, 500 nM NaCl, and 0.1% Tween-20) containing 5% (w/v) skim milk, the membrane was incubated with a specific primary antibody for phospho(p)-NF-κB p65, p-IκBα, p-p38,p38, p-p44/42 (Cell Signaling Technology, Danvers, MA, USA) or GAPDH (Santa Cruz Biotechnology) followed by peroxidase-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, USA). The membranes were developed using the enhanced chemiluminescent analysis system (ChemiDoc MP, Bio Rad, Hercules, USA). Results were obtained from three independent experiments (p-p44/42, p-p38); and obtained from two independent times (p-pIκBα:p-pNF-kB 65).

**Statistical analysis**

All experimental data were presented as mean ± standard deviation (SD). It was analyzed by Mann-Whitney U test using SPSS (version 20.0, IBM Corp., Armonk, NY, USA). The difference was considered to be statistically significant at a level of p<0.05.

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**Table 1. Primers for real-time polymerase chain reaction**

<table>
<thead>
<tr>
<th>Primers</th>
<th>iNOS F: 5'-ACATTGATCAGAAGCTGTCCCAC-3'</th>
<th>R: 5'-CAAAGGCTGTGAGTCCTGCAC -3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td>F: 5'-CTCTGACCATGATGGCCCG-3'</td>
<td>R: 5'-TGGAAGGGCCAGTGCTGTITG-3'</td>
</tr>
<tr>
<td>VEGF</td>
<td>F: 5'-CTACCTCCACCATGCAAGA-3'</td>
<td>R: 5'-GCAGTAGCCTCGGTATAGA-3'</td>
</tr>
<tr>
<td>COX-2</td>
<td>F: 5'-TGTAGTCACCATCCTGCGCCTGCAG-3'</td>
<td>R: 5'-GTGGGGGCAAGTCGCTGTATC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5'-ATCATCTCGGCCTCTACGTCCAC-3'</td>
<td>R: 5'-GTGAGGTACCCACCCACTGACAC-3'</td>
</tr>
</tbody>
</table>

F: forward, R: reverse
RESULTS

Effect of *H. fusiformis* on the cell viability of RAW 264.7 cells

RAW 264.7 cells viability in the presence of *H. fusiformis* is shown in Fig. 1. As shown in figure, the viability of RAW 264.7 cells did not decrease significantly with the increase of polysaccharide concentrations from 1 ug/mL to 100 ug/mL, the results indicate that viscozyme extract of *H. fusiformis* does not affect the viability of RAW 264.7 cells at the concentration of 1–100 ug/mL.

Effect of *H. fusiformis* on the phagocytic activity of RAW 264.7 cells

As shown in Fig. 2, phagocytic activity of *H. fusiformis*-treated RAW 264.7 cells all exceeded the control group and with the increase of the concentration of *H. fusiformis*, the phagocytosis of macrophage RAW 264.7 cells was significantly increased in a dose-dependent manner, which indicated that *H. fusiformis* had abilities to enhanced phagocytic activity of RAW 264.7 cells. The phagocytic activity at the concentrations of 1–10 ug/mL of *H. fusiformis* extract was significantly higher than the control group (p<0.05).

Effect of *H. fusiformis* on the production of NO

To evaluate the effects of *H. fusiformis* on the production of NO in RAW 264.7 cells, Griess assay was performed to determine the nitrite accumulation. LPS (100 ng/mL) was used as a positive control. As shown in Fig. 3A, NO production of macrophage RAW 264.7 cells was increased after treatment with LPS (100 ng/mL). The increased NO production was decreased with the *H. fusiformis* treatment in a dose-dependent manner. It had statistical significance in high concentration (p<0.05).

Effect of *H. fusiformis* on the production of ROS

DCF-DA can be used for detection the intracellular ROS due to oxidative stress, since it can react with radicals and then converted to a fluorescent product. As shown in the Fig. 3B, ROS level was increased in response to the H2O2 and LPS treatment (positive control). Compared to positive control group, the ROS level was decreased in *H. fusiformis* pre-treated group. However, it did not reach to the statistical significance.

Effect of *H. fusiformis* on the various mRNAs expression

In terms of pro-inflammatory cytokines such as iNOS, GM-CSF, VEGF and COX-2, the mRNA expression was augmented by the LPS treatment. The augmented iNOS mRNA expressions were decreased significantly after *H. fusiformis* pre-treatment from at a concentration. The elevated levels of GM-CSF and VEGF mRNA by LPS treatment were significantly decreased by the high concentration of *H. fusiformis* (p<0.05) (Fig. 4). However, there was...
Fig. 3. Effect of H. fusiformis on the NO & ROS production in RAW 264.7 cells. A: RAW 264.7 cells were treated with various concentrations of H. fusiformis for 1 h and LPS for 24 h. Nitrite concentration in the supernatant was detected by Griess reagent, and NO production was measured by absorbance at 540 nm. LPS (100 ng/mL) was used as the positive control. Values are expressed as the mean ± SD (n=3, p < 0.05). B: Compared to positive control group, ROS level was decreased in H. fusiformis pre-treated group. NO: nitric oxide, ROS: reactive oxygen species, HF: Hiziki fusiformis, LPS: lipopolysaccharides.

Fig. 4. Effect of H. fusiformis on the various mRNA expression. The augmented iNOS mRNA expressions by LPS administration were decreased significantly after H. fusiformis pre-treatment. The elevated levels of GM-CSF and VEGF mRNA by LPS treatment were significantly decreased by H. fusiformis (HF: 10 ug/mL) (p < 0.05). HF: Hiziki fusiformis, LPS: lipopolysaccharides.
no detectable change in COX-2 mRNA expression.

**Effect of *H. fusiformis* on the ERK, MAP kinase and NF-KB cell signaling pathway.**

The expression of p-p44/42 and p-p38 protein were markedly increased by the treatment of LPS in RAW 264.7 cells, however, pre-treatment of the 10 µg/mL of *H. fusiformis* decreased their signal intensity. As for NF-κB cell signaling pathway, the signal intensity of p-pNF-κB 65 and p-pIkBα was increased by the LPS treatment and their intensity was decreased by the *H. fusiformis* administration (Fig. 5).

**DISCUSSION**

*H. fusiformis* are widely consumed in Japan for centuries, however, it had not been yet developed any kind of health-related commercial product. Since, plant-derived natural product have priority over the medication, several efforts to find out the bioactive material from nature product are still ongoing. Until now, fucoidan and laminarin are known to be the major constituents of *H. fusiformis* with published bioactive effects.12) Here, we reported the anti-inflammatory and anti-oxidant effect of *H. fusiformis* extract. In this study, results showed that *H. fusiformis* could improve immune function by enhancing the phagocytic activity of macrophage and down-regulating the pro-inflammatory cytokine mRNA expressions. Also, LPS-activated MAPK and NF-κB signal pathway were also de-activated by the administration of the viscozyme component of *H. fusiformis*.

Macrophage play a significant defensive role in immunity. They have the ability of phagocytosis of parasites and microbes and to activation process of T-and B-lymphocyte by antigen.13) Phagocytosis of pathogens not only participates in activating the innate and adaptive immune system, but also in the regulation of immune regulation, thus, one could escape from foreign invasion by using this mechanism and improve the organism’s immune function. Present study showed that *H. fusiformis* significantly enhanced the phagocytosis activity of the macrophage RAW264.7 cells (Fig. 2). This results suggest that *H. fusiformis* has the potential to be developed as an immune booster or immuno-potentiator.

Macrophages are also an important source of pro-inflammatory cytokine (TNF, IL-1, IL-6, IL-12), VEGF, NO and chemokines, leukotrienes, prostaglandins, and complement, which are the main effectors in the inflammatory process.13) TNF and GM-CSF can stimulate human macrophage to kill some microbes. iNOS produced by macrophage produce NO by oxidation of L-arginine, which act as a cytotoxin. The generated NO also contributed to kill the infectious microorganism,14) and increased iNOS protein and NO level have been found in chronic inflammatory or autoimmune disease, such as asthma, rheumatoid arthritis and diabetes.15-17) In this study, *H. fusiformis* extract has proved to down regulate their expression. These finding are consistent with the previous studies results.12) Hence, given that allergic disease has a background of several kinds of chronic inflammation, this iNOS/NO reducing potential of *H. fusiformis* extract would might have a clinical importance. Moreover, NO has the potential as cell signaling mediator and it has been seen to activate NF-κB and AP-1 transcription factors,18-19) the
ability of reducing NO of H. fusiformis is of useful in that many inflammatory mechanisms are related with those transcription factors. In this experiment, other cytokines such as IL-1B, IL-8 and TNF were also evaluated, however, consistent aRT-PCR results had failed to get. And, while previous study had shown that H. fusiformis reduce the COX-2 protein expression, the reduced level of COX-2 mRNA expression by administration of H. fusiformis could not show the statistical significance in our study. This difference might have originated from and related to the preparation process the H. fusiformis.

ROS is generated under not only physiological but also pathological condition and related with many age-related, neurodegenerative, cardiovascular and allergic disease. Antioxidant can scavenge the toxic ROS and have beneficial health effect via protecting against some type of disease like cancers and coronary heart disease. Any naturally available anti-oxidative compounds are welcomed in daily dietary food. In present study, ROS level was decreased by the administration of the H. fusiformis. H2O2 is a product derived of normal metabolism and converted to singlet oxygen which can oxidize others. The activity of H. fusiformis observed in the present study implies that it has antioxidant potential to decrease the pro-oxidants like H2O2, and further study are needed to draw a clear conclusion.

NF-κB is a key inflammatory transcription factor that regulates the expression of genes encoding inflammatory cytokines and modulators during inflammation. It forms a cytoplasmic complex under normal physiological conditions, but when activated by LPS, it is released from the inactive complex and translocated into the nucleus to activate different pro-inflammatory target genes, such as iNOS and COX-2. Since MAPKs regulate NF-κB transcriptional activity by phosphorylation of its IkB subunit and mediate cell signal transduction from the cell surface to the nucleus, we measured its effect on MAPKs, such as JNK and p38 activation in RAW 264.7 cells.

In summary, H. fusiformis could markedly improve the immune-modulatory activity of macrophage RAW 264.7 cells by stimulating the phagocytic activity of macrophages and significantly ameliorating the production of NO/ROS in macrophage. Also, H. fusiformis improve immunomodulatory activity through decreasing mRNA levels of pro-inflammatory cytokines (iNOS, GM-CSF and VEGF). The underlying mechanisms of H. fusiformis on the regulation of immune response on activating RAW 264.7 may be mediated by the p38, p44/42 and NF-κB signaling pathway. Taken together, H. fusiformis has the potential to be an immuno-modulating agent, and also can be used as functional food and pharmaceutical therapy ingredients.

Acknowledgments
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Declaration of Interests
The authors declare that there is no conflict of interest.

Author Contributions
Moon Hee Choi: acquisition of data, Eun-su Jang: acquisition of data, Hyun-Jae Shin: study concept and design, Jung Heon Lee: study concept and design, Jieun Lee: acquisition of data, analysis and interpretation of data, drafting of the manuscript, revision of the manuscript, statistical analysis, study supervision.

REFERENCES


