

Immune Enhancement Effect of *Asterias amurensis* Fatty Acids through NF- κ B and MAPK Pathways on RAW 264.7 Cells

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Asterias amurensis is a marine organism that causes damage to the fishing industry worldwide; however, it has been considered a promising source of functional components. The present study aimed to investigate the immune-enhancing effects of fatty acids from three organs of *A. amurensis* on murine macrophages (RAW 264.7 cells). *A. amurensis* fatty acids boosted production of immune-associated factors such as nitric oxide (NO) and prostaglandin E2 in RAW 264.7 cells. *A. amurensis* fatty acids also enhanced the expression of critical immune-associated genes, including *iNOS*, *TNF- α* , *IL-1 β* , and *IL-6*, as well as *COX-2*. Western blotting showed that *A. amurensis* fatty acids stimulated the NF- κ B and MAPK pathways by phosphorylation of NF- κ B p-65, p38, ERK1/2, and JNK. *A. amurensis* fatty acids from different tissues resulted in different levels of NF- κ B and MAPK phosphorylation in RAW 264.7 cells. The results increase our understanding of how *A. amurensis* fatty acids boost immunity in a physiological system, as a potential functional material.

Keywords: Immune enhancement, fatty acid, NF- κ B pathway, MAPK pathway, starfish

Introduction

Immune cells are provided with sufficient fuel to sustain energy homeostasis and cellular processes under normal homeostatic conditions [1]. Diet and its components are important in human health and are associated with the immune system, secretion of adipokines, and metabolic pathways [2]. Lipids containing fat-soluble vitamins and fatty acids are notable nutritional components for human health, and are important to prevent diseases associated with their compositional alteration [3]. In particular, free fatty acids have been reported to be associated with immune function and modulation [1]. Diverse fatty acids, including saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids (PUFAs), are associated with chronic diseases such as cardiovascular disease, cancers, and diabetes [4–8].

Omega-6 PUFAs, including arachidonic acid (ARA, 20:4n-

6), and omega-3 PUFAs, such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are associated with inflammation and the immune system. These fatty acids, which include more than 20 carbon atoms containing more than two double bonds, are the precursors of lipid signaling molecules, such as eicosanoids and docosanoids, catalyzed by cyclooxygenases (COX) and lipoxygenases [9, 10]. They are crucial cellular signaling factors related to inflammation and immune regulation in physiological systems [11]. ARA and EPA are precursors of pro-inflammatory and anti-inflammatory eicosanoids [11, 12]. DHA is a precursor of anti-inflammation-associated lipid signaling molecules known as docosanoids, such as resolvins and protectins [13], which are anti-lipid mediators for cancer therapy [14]. In addition, long-chain omega-3 PUFAs, EPA, and DHA were reported to provide beneficial effects in diseases such as arthritis and asthma via positive immune regulation [11, 12].

The starfish *Asterias amurensis* is an important harmful organism that causes damage to the fishing industry, and is found in the North Pacific Ocean as well as the east coast of Korea [15]. However, starfish, including *A. amurensis*, have also been considered a potential source of various bioactive natural products, including steroids, saponins, steroidal glycosides, anthraquinones, alkaloids, phospholipids, and peptides [16]. These compounds show antitumor, anti-inflammatory [17], immunomodulatory, anti-allergy, antifungal, hemolytic [18], antimutagenic [19], neuritogenic [20], cytotoxic, and antiviral [21] effects. In particular, *A. amurensis* possesses phosphatidylcholine that contains large amounts of EPA [22] and glycosylceramide [15]. The fatty acid profiles of *A. amurensis* and their anti-inflammatory regulation against murine macrophage cells have been confirmed. However, no study has reported the immune regulation in murine macrophages, such as RAW 264.7 cells, by fatty acids from *A. amurensis*. The present study shows that fatty acids from *A. amurensis* induce immunity-related cytokines and signaling proteins in RAW 264.7 cells.

Materials and Methods

Starfish Sample

To reduce the variation of starfish lipid content, *A. amurensis* samples were obtained from the Eastern Sea near Gangwon province, South Korea, in April 2015 [23, 24]. Three organs (skin, gonad, and the digestive gland (d-gland)) were identified and dissected to extract fatty acids.

Fatty Acid Preparation

Fatty acids were extracted according to a modified version of the one-step method described by Garces and Mancha [25]. Fatty acids were purified using a one-step hydrolysis and extraction method, as modified previously [26].

Nitric Oxide Production

Murine macrophages (RAW 264.7 cells; Korean Cell Line Bank, Korea) were incubated in RPMI-1640 medium (Gibco Laboratories, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The macrophages were cultured with different concentrations (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, or 4.0% (v/v); volume of fatty acids / total culture volume of cells) of *A. amurensis* fatty acids or with 1 µg/ml of lipopolysaccharide (LPS; Sigma-Aldrich, USA) as a positive control. Nitric oxide (NO) in the macrophage culture supernatant induced by treatment with *A. amurensis* fatty acids was analyzed using the Griess reagent (Sigma-Aldrich) [27, 28] at an optical density of 540 nm.

Total RNA Preparation and Expression Analysis of Immune-Associated Genes

Total RNA was extracted from the macrophages, using the Tri reagent (Molecular Research Center, Inc., USA), and the nucleotide concentration was evaluated using a nanophotometer (Implen, Germany). Complementary DNA (cDNA) was prepared using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

The transcription of five immune-associated genes (*iNOS*, *COX-2*, *IL-1β*, *IL-6*, and *TNF-α*) as well as β-actin (as a control) was analyzed using the QuantStudio 7 FlexReal-Time PCR System (Thermo Fisher Scientific, USA), in a 96-well format, in a total reaction volume of 20 µl/well, using SYBR Premix Ex Taq II (Takara Bio Inc., Japan). The specific primer pairs used are shown in Table 1.

Western Blotting

RAW 264.7 cells were cultured with different concentrations (0.5%, 1.0%, 1.5%, or 2.0%) of *A. amurensis* fatty acids. After 24 h, protein samples were prepared using radioimmunoprecipitation assay buffer (Tech & Innovation, China) and the protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Western blotting was performed using polyvinylidene difluoride membranes, according to the

Table 1. Sequences of oligonucleotide primers used to amplify immune-associated genes.

Gene name	Accession No.	Nucleotide sequence	Product size (bp)
<i>iNOS</i>	BC062378.1	F: TTCCAGAATCCCTGGACAAG R: TGGTCAAACCTTGGGGTTC	180
<i>IL-1β</i>	NM_008361.4	F: GGGCCTCAAAGGAAAGAATC R: TACCAGTTGGGAACTCTGC	183
<i>IL-6</i>	NM_031168.2	F: AGTTGCCTTCTTGGGACTGA R: CAGAATTGCCATTGCACAAC	191
<i>COX-2</i>	NM_011198.4	F: AGAAGGAAATGGCTGCAGAA R: GCTCGGCTTCCAGTATTGAG	194
<i>TNF-α</i>	D84199.2	F: ATGAGCACAGAAAGCATGATC R: TACAGGCTTGCTCACTCGAATT	276
β-Actin	NM_007393.5	F: CCACAGCTGAGAGGAAATC R: AAGGAAGGCTGGAAAAGAGC	193

method of Narayanan *et al.* [29]. The membranes were incubated with specific antibodies recognizing p (phosphorylated)-NF- κ B p65 (R&D Systems, USA), p-p38 (R&D Systems, USA), p-ERK1/2 (R&D Systems), p-JNK (R&D Systems), and β -actin (Santa Cruz Biotechnology, Inc., USA). After incubation with the appropriate secondary antibodies, the immunoreactive proteins were detected using the Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific).

Quantification of Prostaglandin E₂ (PGE₂)

PGE₂ production was analyzed using a PGE₂ enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, USA) according to the manufacturer's instructions. PGE₂ production was also analyzed using *p*-nitrophenyl phosphate as a substrate, at an absorbance of 405 nm. The experiment was carried out in duplicate, and PGE₂ production was measured on the basis of a standard curve.

Statistical Analysis

The results were analyzed using Statistix 8.1 statistics software (Statistix, USA). NO production was evaluated using one-way analysis of variance followed by comparison with the RPMI medium-only group. Differences were considered significant at $p < 0.01$ and highly significant at $p < 0.001$.

Results

A. amurensis Fatty Acids Enhanced NO Production in RAW 264.7 Cells

In general, *A. amurensis* fatty acids were not toxic to RAW 264.7 cells; however, the fatty acids from the gonad and d-gland were toxic to the cells at high concentrations (2.5–4.0%), as assessed using a macrophage proliferation assay (data not shown). NO is a critical cellular signaling

molecule that participates in immune regulation in physiological systems and is an important biomarker as an inflammatory mediator. NO production is stimulated by natural products, such as ginseng, to boost immunity [30]. To analyze the potential immune-enhancing effect of *A. amurensis* fatty acids, RAW 264.7 cells, which can produce NO, were used. As shown in Fig. 1, NO production in RAW 264.7 cells increased in a dose-dependent manner in response to 0.5–4.0% fatty acids from the three *A. amurensis* organs. In particular, the fatty acids from the d-gland induced substantial increases in NO production of more than 300% compared with that induced by skin and gonad fatty acids.

A. amurensis Fatty Acids Enhanced Immune-Associated Gene Expression in RAW 264.7 Cells

In RAW 264.7 cells, the expression of most immune-associated genes was enhanced in a dose-dependent manner by the *A. amurensis* fatty acids. However, the expression of *IL-6* was not affected by the different concentrations of *A. amurensis* fatty acids. The expression of *COX-2* was highly increased by *A. amurensis* fatty acids. The expression of *TNF- α* was increased markedly by *A. amurensis* fatty acids from the skin and d-gland; however, the increase in *TNF- α* expression induced by the gonad fatty acids was lower than that induced by the other two organs. Inducible nitric oxide synthase (*iNOS*), a key enzyme that generates NO and plays an important role in immune regulatory conditions [31, 32], was highly expressed in response to gonad- and d-gland-derived fatty acids (Fig. 2), indicating that *iNOS* expression did not correlate completely with the results of NO production.

A. amurensis Fatty Acids Enhanced PGE₂ Production in RAW 264.7 Cells

The immune-enhancing effects of *A. amurensis* fatty acids on RAW 264.7 cells were also determined by measuring the level of PGE₂, which is an important pro-inflammatory factor (Fig. 3). The result showed that the production of PGE₂ increased dose-dependently in RAW 264.7 cells treated with skin, gonad, and d-gland fatty acids. In addition, the levels of PGE₂ in macrophage cells induced by d-gland and gonad fatty acids were higher than those induced by skin fatty acids, which was slightly different from the results for NO production. The produced PGE₂ levels were much lower than those stimulated by LPS as a positive control. By contrast, the NO level in cells treated with d-gland fatty acids was similar to the level induced by the LPS control.

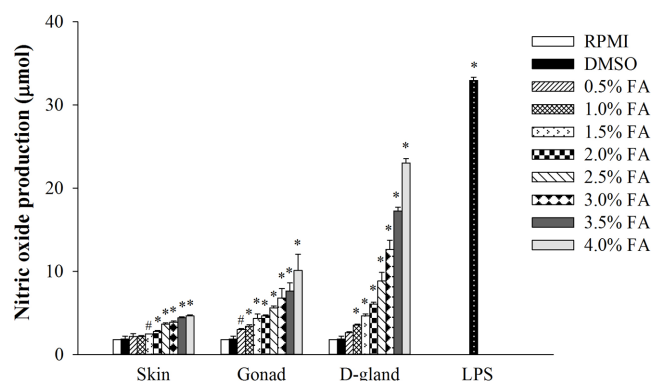


Fig. 1. Effects of fatty acids from three organs of *Asterias amurensis* on nitric oxide production.

and * indicate a significant difference at $p < 0.01$ and $p < 0.001$, respectively, compared with cells treated with RPMI medium only. FA, fatty acids; DMSO, dimethyl sulfoxide.

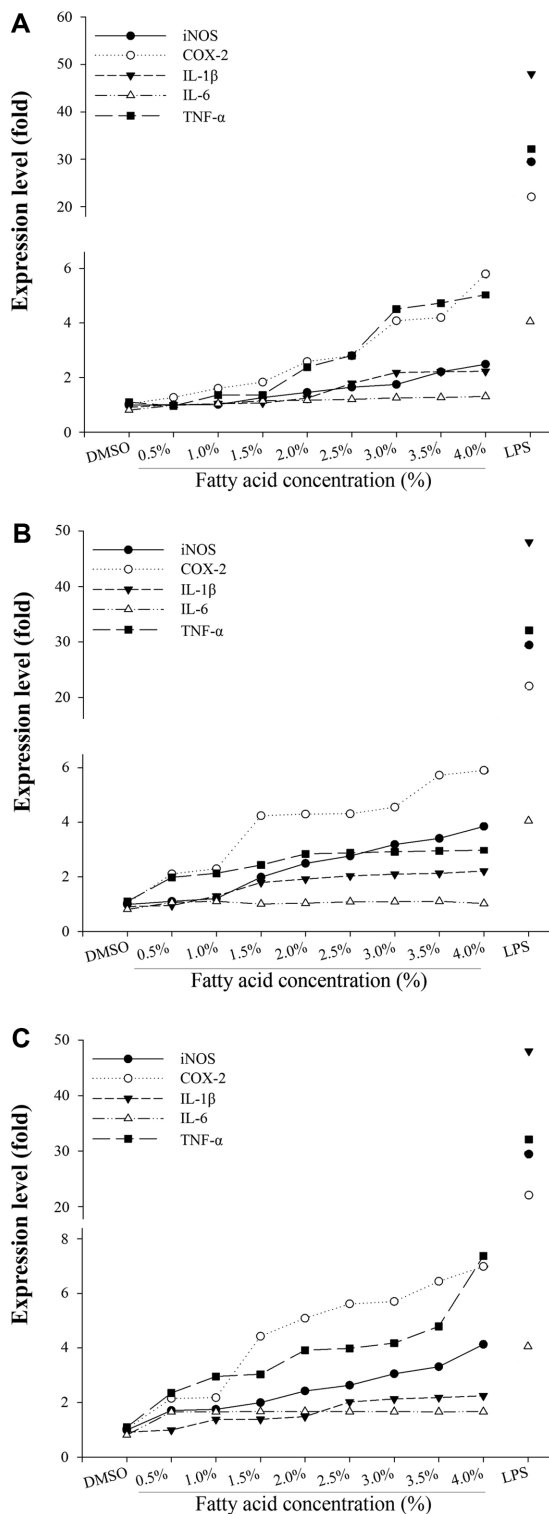


Fig. 2. Quantification of immune-associated mRNAs by relative expression (fold) in RAW 264.7 cells treated with *Asterias amurensis* (A) skin-derived fatty acids, (B) gonad-derived fatty acids, and (C) d-gland-derived fatty acids.

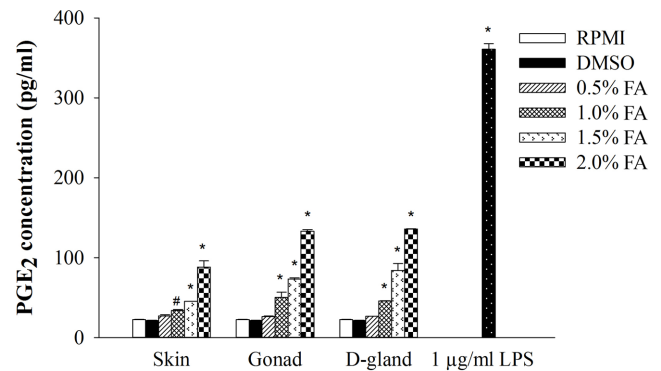


Fig. 3. Quantification of PGE₂ in RAW 264.7 cells treated with fatty acids from three organs of *Asterias amurensis*.

and * indicate a significant difference at $p < 0.01$ and $p < 0.001$, respectively, compared with cells treated with RPMI medium only. FA, fatty acids; DMSO, dimethyl sulfoxide.

A. amurensis Fatty Acids Enhanced Immunity through the MAPK and NF- κ B Signaling Pathways in RAW 264.7 Cells

To investigate which immune signaling pathways contribute to *A. amurensis* fatty acid-induced immune enhancement in RAW 264.7 cells, the levels of NF- κ B- and MAPK-associated proteins were analyzed via western blotting after treatment of RAW 264.7 cells with fatty acids from the three organs. Fig. 4 shows that *A. amurensis* fatty acids dose-dependently stimulated the phosphorylation of NF- κ B p-65 in the NF- κ B signaling pathway, whereas the level of I κ B α was dose-dependently decreased. *A. amurensis* fatty acids also increased the phosphorylation of ERK1/2, JNK, and p38 in a dose-dependent manner. Interestingly, phosphorylation of NF- κ B p-65 in RAW 264.7 cells was more pronounced after treatment with gonad and d-gland fatty acids, compared with that after treatment with skin fatty acids. Phosphorylation of p-38, ERK1/2, and JNK also increased to different levels depending on the source organ of the fatty acids. Fatty acids from d-glands induced the largest increase in phosphorylation of p-38, whereas those from the gonads caused the largest increase in JNK phosphorylation. Phosphorylation of ERK1/2 was increased to a greater extent by gonad and d-gland fatty acids compared with that induced by skin fatty acids.

Discussion

A. amurensis and other starfish have been studied because of their diverse biological functional compounds [16], even though *A. amurensis* attacks mollusks and other echinoderms

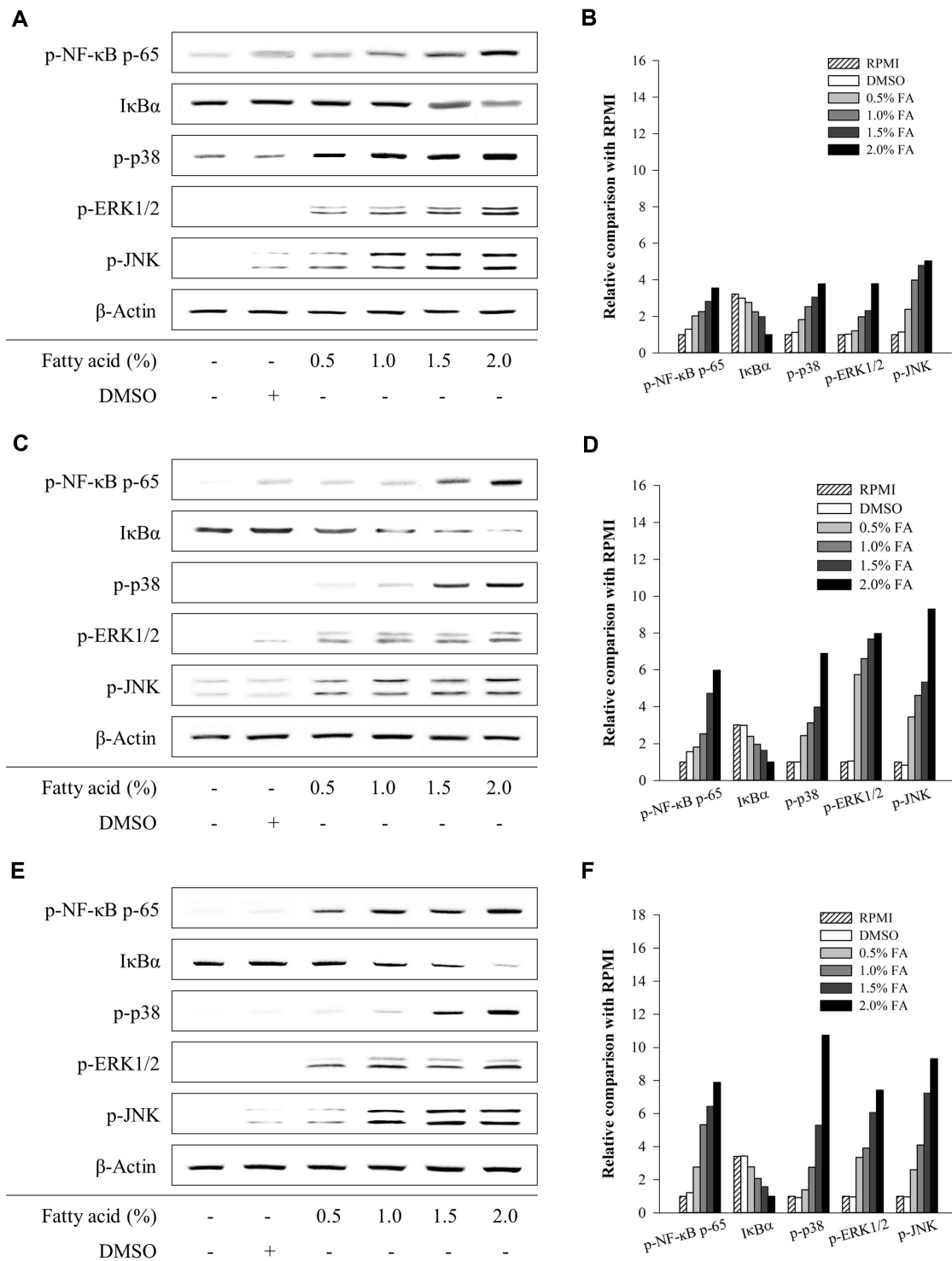


Fig. 4. Effect of *Asterias amurensis* fatty acids on the NF-κB and MAPK pathways in RAW264.7 cells, (A) Immunoblot of proteins from cells treated with *Asterias amurensis* skin-derived fatty acids. (B) Relative band intensity of proteins from cells treated with *Asterias amurensis* skin-derived fatty acids. (C) Immunoblot of proteins from cells treated with *Asterias amurensis* gonad-derived fatty acids. (D) Relative band intensity of proteins from cells treated with *Asterias amurensis* gonad-derived fatty acids. (E) Immunoblot of proteins from cells treated with *Asterias amurensis* d-gland-derived fatty acids. (F) Relative band intensity of proteins from cells treated with *Asterias amurensis* d-gland-derived fatty acids. FA, fatty acids; DMSO, dimethyl sulfoxide.

in the fishery industry [15]. However, to date, there has been no report about the immune-enhancing effect of fatty acids extracted from specific organs of *A. amurensis* on macrophages.

Immune cells such as macrophages are supplied with sufficient levels of glucose, amino acids, and fatty acids to maintain energy homeostasis and cellular processes [1]. These immune-associated cells are involved in acute and chronic inflammatory responses, including pain, fever, swelling, and tenderness [33, 34]. We confirmed the fatty acid profiles of three *A. amurensis* tissues and the cytotoxicity of these fatty acids in RAW 264.7 cells (data not shown). Our current results demonstrated immune-enhancing effects of the fatty acids on macrophages. The levels of NO and PGE₂, which are important immune-regulatory biomarkers in macrophages, were increased according to the concentration of the fatty acids from all three organs (Figs. 1 and 3). The fatty acids from the three organs induced less PGE₂ compared with that induced by the LPS positive control. By contrast, only the fatty acids from the d-gland induced NO secretion at a level comparable to that induced by LPS. These results suggested that the *A. amurensis* d-gland is a potentially important source of factors that can enhance NO production in macrophages.

We also investigated the expression of genes encoding immune-associated proteins (*iNOS* and *COX-2*) as well as those encoding cytokines (*IL-1β*, *IL-6*, and *TNF-α*), which are involved in proinflammatory regulation in activated macrophages [35]. The results showed that *COX-2* expression increased markedly according to the concentration of *A. amurensis* fatty acids, and *TNF-α* expression was stimulated even at low concentrations of *A. amurensis* skin-derived fatty acids; however, its expression was further increased at high concentrations of fatty acids from the d-gland and gonad. In contrast, the expression of *IL-6* did not show any difference depending on the concentration of the fatty acids. These results indicated that molecular regulation to activate the transcription of immune-associated genes might vary according to the source organ of the fatty acids in *A. amurensis*.

To regulate immune enhancement in RAW 264.7 cells, NF-κB p-65 and MAPK/ERK signaling molecules, such as ERK1/2, JNK, and p38, are coordinated via protein phosphorylation. NF-κB activity is modulated according its phosphorylation level and the degradation of associated proteins, such as p-65 and IκBα [36]. MAPK/ERK-associated proteins take part in the regulation of cell growth, differentiation, and cellular responses to cytokines and stresses [37]. Furthermore, MAPK is believed to control the

NF-κB pathway and modulate the expression of inflammatory cytokines and the inflammatory process [38, 39]. The results of the present study demonstrated that fatty acids from *A. amurensis* organs activated the phosphorylation of the members of the NF-κB p-65 and MAPK pathways, such as ERK1/2, JNK, and p38, and thus promoted an immune response. These results indicated that fatty acids from *A. amurensis* enhanced immunity through the MAPK and NF-κB signaling pathways in RAW 264.7 cells. Moreover, the response varied depending on the source organ of the fatty acids, which suggested that the fatty acids from the three *A. amurensis* tissues might enhance the immune system through different cellular signaling pathways in RAW 264.7 cells [40, 41].

The results confirmed that *A. amurensis* contains potential immunity-enhancing fatty acids. The results will also aid our understanding of the mechanism of immune enhancement induced by *A. amurensis* fatty acids in macrophages.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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