A Master's Thesis

The effect of fish oil on allergic skin inflammation in mice

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The effect of fish oil on allergic skin inflammation in mice

Sang-Chul Han

(Supervised by professor **Eun-Sook Yoo**)

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ABSTRACT

Allergic skin inflammation such as atopic dermatitis (AD) is characterized by pruritus and inflammation. Histamine and various cytokines are the main pruritogens causing edema and itching. Increased levels of serum immunoglobulin E (IgE) and infiltration of inflammatory cells in skin lesions also mark allergic skin inflammation. Thymic stromal lymphopoietin (TSLP), in particular, strongly activates the maturation of dendritic cells within the epidermis, and can regulate the allergic inflammation reaction. Regulatory T cells (Tregs) play a key role in various immune responses and prevent or suppress the differentiation, proliferation and function of various immune cells, including cluster of differentiation 4 $(CD4)^+$ T cells. Moreover, since all Tregs express CD25 at the cell surface and have forkhead box P3 (Foxp3) as a transcription factor, CD25 and Foxp3 have been identified as makers of Tregs. Fish oil (FO) contains eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) called omega-3 fatty acids polyunsaturated fatty acids (n-3 PUFAs). The n-3 PUFAs in fish oil have been studied in a variety of diseases and are known to reduce inflammation responses in allergic patients. Recently, some studies have explored the effects of fermentation on the characteristics of various foods such as wine, yogurt and cheese. Fermentation has tremendous abilities to transform the chemical structure of constituents or to create new substances. In the present study, we investigated the effects of the fermentation of fish oil using fermented fish oil (FFO) and natural fish oil (NFO) on the modulation of the immune system. Results reveal that, FFO alleviates various immune disorder symptoms and up-regulates $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs). In comparison with the induction group, the administration of FFO or NFO decreased scratching behavior, cutaneous edema, inflammatory cell infiltration, expression of TSLP, serum IgE and histamine levels. Both FFO and NFO treatment regulated the expression

of AD-associated pro-/anti-inflammatory cytokines (IL-4, -13, IFN-γ and TGF-β) and transcription factors (Foxp3, T-bet and GATA3) at the sites of inflammation. However, FFO-treated group showed stronger inhibitory effects on various experimental AD symptoms than NFO-treated group. Although FFO or NFO treatment did not increase the Foxp3 level and there was no difference in the $CD4^+CD25^+$ Treg population compared with the normal group, the FFO treatment increased expression of Treg-associated factors (TGF-β and IL-10), resulting in significantly elevated suppressor activity compared with NFO-treated group. In addition, ingestion of FFO increased Foxp3 level and $CD4^+CD25^+Foxp3^+$ Treg population in anti-CD3&28-stimulated $CD4^+$ T cells compared with NFO. These results suggest that the anti-allergic effect of FFO is associated with enrichment of $CD4^+CD25^+Foxp3^+$ Treg at the inflamed sites and FFO may be effective source for improving the allergic symptoms of AD.

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1. Introduction

Atopic dermatitis (AD), an immune-mediated inflammation of the skin, primarily occurs among children in developed countries, and affects approximately 3% of infants, 10~20% of children and 1~3% of adults worldwide (Leung *et al.* 2004). AD is a chronic skin disease that mainly appears together with a variety of other diseases, such as eczema, asthma, hay fever and allergic conjunctivitis (47 Nystad, W. 2005). The symptoms of AD include peripheral eosinophilia, epidermal hyperplasia and tissue remodeling, such as spongiosis. These systemic disorders are caused by skin barrier dysfunction, severe dehydration, and mutation of filaggrin that is essential for the modulation of epidermal homeostasis. In addition, the skin lesions of AD patients are generally characterized by various inflammatory cells infiltration, such as mast cells, basophils, eosinophils and T cells (Nystad *et al.* 2005, Li *et al.* 2005, Li *et al.* 2010).

Pruritus (itch), a frequent and unpleasant symptom of dermatological disease, such as AD, often causes repetitive scratching behavior. Moreover, itching and scratching are critical factors in the maintenance of AD, and affect the skin edema or increasingly exacerbate AD (Wahlgren 1999).

Immunoglobulin E (IgE), the antibody found in the skin, blood and mucous membranes, plays a critical role in the allergy response, and is also an important target in the treatment of allergy and AD (Levin *et al.* 2006). IgE is an essential component for the activation of eosinophils and mast cells. Moreover, its concentration in the serum within the general population is the lowest among the five immunoglobulin (Ig) subtypes (IgA, IgD, IgE, IgG, IgM), but the majority of the patients with AD generally have high IgE levels among the various Ig subtypes (Stone *et al.* 2010).

The mast cell or hematopoietic cell plays a critical role as the effector cell in various

inflammatory responses of allergic diseases. It expresses a high-affinity receptor I (FcεRI) on its surface membrane, and the interaction between IgE and FcεRI causes the degranulation of the mast cell (Kitamura *et al.* 2005). The mast cell, a granule-containing secretory cell, has a variety of inflammatory factors for allergic responses. Mast cells activated, by diverse compounds or an allergen, rapidly secrete three classes of substances into the extracellular space. These are (1) chemical mediators, such as histamine, tryptase, serotonin, heparin and thromboxane; (2) lipid mediators, such as prostaglandin D_2 and leukotriene; and (3) synthesized growth factors and cytokines, such as tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-2, -3, -4, -5, -13, and so on (Leitges *et al.* 2002, Kawakami *et al.* 2009).

Among the variety of inflammatory mediators released from the mast cell, histamine, usually considered a marker of mast cell degranulation in immediate allergic reaction, is a violent inducer of itching. Moreover, it is the typical major mediator in storage granules of the mast cell, and directly triggers type I allergic responses (Schwartz 2004).

Thymic stromal lymphopoietin (TSLP) is a cytokine that regulates T cell homeostasis through the activation of antigen presenting cells (APCs) (Al-Shami *et al.* 2005). TSLP, mainly produced by fibroblasts and keratinocytes in the skin lesions of acute and chronic AD, strongly activates the maturation of dendritic cells (DCs) in the epidermis, and can initiate and regulate allergic inflammation reaction (Bogiatzi *et al.* 2007). Moreover, TSLP can stimulate naive T cell to express pro-inflammatory cytokines (IL-4, -5 and -13) while decreasing the expression of interferon (IFN)-γ and IL-10: however, the expression of these factors is caused by TSLP-activated DCs rather than TSLP-stimulated T cell (Ebner *et al.* 2007, Soumelis *et al.* 2002).

Increased numbers of activated $CD4^+$ or $CD8^+$ T cells and enormous numbers of infiltrated $CD4^+$ T cells in the dermis, which are primarily associated with the dysfunction of the body's

immune system by an imbalance between T helper (Th) 1- and Th2-type cells, generally characterize the majority of patients with AD (Werfel 2009). The initial phase of AD has a significantly increased Th2 subtype. Th2 cells produce a variety of cytokines, such as IL-4, -5 and -13, and express discriminatively more of the transcription factor GATA-binding protein 3 (GATA3), which activates the secretion of cytokines and is important for the differentiation from naive T cell to Th2 cell. IL-4 plays a crucial role in allergic responses and the differentiation from naive T cell to Th2 cell, and it induces the isotype switching of B cells to produce IgE. Compared with acute AD, chronic AD skin lesions generally have fewer Th2 cells, and express a number of Th1 cells producing IFN-γ to inhibit Th2 cell function. The transcription factor T-box-expressed-in-T-cell (T-bet) is a critical for the differentiation of Th1 cells (Zheng *et al.* 1997, Szabo *et al.* 2000).

Regulatory T cells (Tregs) that play a key role in various immune responses, including Th2 cells-mediated diseases, such as AD, and are found in lymph nodes, skin lesions, spleen or peripheral blood, maintain the peripheral immune homeostasis and tolerance to allergens. They also prevent or suppress differentiation, proliferation and function of various immune cells including $CD4^+$ or $CD8^+$ T cells (Robinson 2005, Ziegler 2006, Tadokoro *et al.* 2006). Exhaustion of the cluster of differentiation (CD) 25^+ cells can aggravate the Th2 cell-mediated inflammation stimulated by various antigens (Saito *et al.* 2008). Since all Tregs express high CD25 levels at the cell surface, CD25 has been identified as a marker of Tregs. However, a $CD25^{+hi}$ expression is regarded as an activation marker rather than a normal marker of Treg because CD25 expression on Tregs increases after sensitization with antigens (Finney *et al.* 2010). AD is characterized by an allergic reaction with dermatitis, and results from mutations of forkhead box protein 3 (Foxp3). Expressed Foxp3 to develop the Treg function has been identified as a specific Treg marker, and $Foxp3^+$ T cells can inhibit the activation or proliferation

of various immune cells, such as Th1/2/17, and so on (Zheng *et al.* 2007, Ziegler 2006b). Treg can be roughly categorized into two types: natural Treg (nTregs) and inducible Treg (iTregs). Naturally occurring $CD4^+CD25^+FoxP3^+$ nTreg that are generated in the thymus are self-reactive and suppress the activation and proliferation of effector T cells through a cell-contact-dependent, IL-10, or TGF-β-independent manner (Sakaguchi 2004, Shevach 2002). Induced iTreg from naive T cells that are specific for antigens arise during an immune response, and suppress the activation of helper T cells in an IL-10- or TGF-β-dependent manner (Buckner *et al.* 2004).

Several studies on the anti-allergic effects of fish oil have been carried out. Fish oil intake during pregnancy can reduce the sensitization to allergens, alleviate the severity of AD, eczema, and asthma, and down-regulate the expression of IL-1, -4, -13 and IFN-γ during pregnancy (Kremmyda LS. *et al.* 2011, Krauss-Etschmann *et al.* 2008). Omega-3 (n-3) and omega-6 (n-6) are typical polyunsaturated fatty acid (PUFA). Fish oil intake maintains higher n-3 PUFA status and lower n-6 PUFA status in the body. In addition, fish oil contains various n-3 PUFAs, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Seki *et al.* 2010). EPA inhibits the production of the inflammatory cytokines (IL-2, -12 or IFN-γ), up-regulates the expression of the anti-inflammatory cytokines (IL-10), and increases the number of $Foxp3^+$ Tregs (Hara *et al.* 2001). DHA diminishes the proliferation of effector T cells by increasing the mRNA expression of Foxp3, TGF-β and IL-10 (Yessoufou *et al.* 2009).

Recently, some studies have combined various foods with fermentation, such as wine, yogurt, cheese, soybean paste, etc., and investigated the anti-allergic effects of the fermented products on a variety of allergic reactions and some cancers. Fermentation can transmute the chemical structure of some constituents to create new substances. The beneficial effects of natural fish oil (NFO) have been described in many diseases, but the mechanism and anti-allergic effect by which fermented fish oil (FFO) modulates the immune system is poorly understood. In this

study, we produced a special fermented fish oil and identified suppression of various allergic reactions/factors and the up-regulation of the differentiation of $CD4^+CD25^+Foxp3^+$ Treg from $CD4^+$ T cells.

2. Materials and Methods

2-1. Experimental animals.

BALB/c mice (female, 7 weeks old) were purchased from Orient Bio (Korea) and were maintained for 1 week before the start of any experiments. These mice were housed in the animal facility of Jeju National University under controlled temperature (23±1°C), humidity (60±10 %), light (lights on from 08:00 to 20:00 hours) and pathogen-free conditions. All animal experiments were approved by the Animal Care and Use Committee at Jeju National University.

2-2. Fermentation process of fish oil

Fermented fish oil was provided by Fermentec Inc. (Jeju, Korea). Pulverized fish by-product was mixed with water, raw sugar, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* under anaerobic fermentation conditions. After 15 days, hexane was added to the fermented fish liquid and then fermented fish oil (FFO) was separated from the hexane extract by rotary evaporation.

2-3. Fatty acids composition of natural fish oil and fermented fish oil

The fatty acid compositions of natural fish oil and fermented fish oil were analyzed by the Feeds & Foods Nutrition Research Center (Pukyong National University, Korea) (Table 2).

2-4. Determination of optimal dosage.

To determine the optimal dose of FFO, we tested the anti-allergic effect of FFO in the Compound 48/80 (Sigma-Aldrich Chemical Co., St. Louis, MO, USA)-induced pruritus mouse model. Various doses of FFO (20 and 100 mg/kg) were orally administered 1 h before the subcutaneous injection of Compoud 48/80 (5 mg/kg). After the injection of stimulator, the amounts of IgE and histamine released in mouse serum were measured, and a dosage of 100 mg/kg of FFO showed the anti-allergic effect. To determine the minimum duration of FFO treatment, the experiment was conducted as reported previously (Albers, R., Bol, M., Bleumink, R., Willems, A., Blonk, C., and Pieters, R. 2002) with modifications. Results indicated that more than 2 weeks of FFO treatment was required to acquire anti-allergic effects in the dinitrochlorobenzene (DNCB, Tokyo chemical industry, Japan)-stimulated experimental AD mouse model.

2-5. DNCB-stimulated experimental AD mouse model.

For the induction of experimental AD in mice, the mice were divided into 5 groups (normal, induction, painted hydrocort, orally administered NFO (100 mg/kg/day) and orally administered FFO (100 mg/kg/day); n = 8/group). 100 μL of 1% DNCB dissolved in acetone/olive oil solution (3:1 acetone/olive oil ratio) was painted on their abdomens as the first sensitization. 7 days after the DNCB painting, the mice received a second sensitization with 100 μL of 0.5% DNCB on each ear surface for 31 days every other day. On day 12, the mice in the NFO and FFO groups were given 100 mg/kg of NFO or FFO in drinking water, daily, respectively and were painted with 2 mg/g HYDROCORT cream (Green Cross, Korea) on their ear surface every other day. On day 32, all mice were sacrificed after all experiments were terminated.

2-6. Non-stimulated mouse model.

To confirm the Foxp3 level and the differentiation of Treg by NFO or FFO treatment, normal mice were given 100 mg/kg/day of FFO or NFO in drinking water, everyday, or painted with hydrocort on each ear surface every other day for 20 days without DNCB stimulation.

2-7. Scratching behavior, macroscopic edema and histological evaluation.

In the experimental AD mouse model, DNCB stimulation elicited scratching of the ears by using the hind paws around the painted site. The mice in each group were placed in an observation chamber,- scratching behaviors were videotaped for 10 min; we then counted the number of scratching behaviors. Ear thickness was measured using a Digital Thickness Gauge (Mitutoyo, Japan). Ear tissues were fixed with 10% formalin and then embedded in paraffin. Paraffin sections (3 mm each) were stained with hematoxylin and eosin (H&E) solution to reveal a thickened epidermis or infiltration of various inflammatory cells.

2-8. Immunohistochemistry (IHC) assay for detection of TSLP in ear tissue.

To analyze the expression of TSLP in each ear, paraffin sections (3 mm, each) of ear tissue were prepared. IHC assay was performed with anti-rabbit TSLP (Novus Biologicals, USA) and all IHC experiments were analyzed using a Rabbit specific HRP/DAB detection IHC kit (Abcam, UK) according to the manufacturer’s instructions.

2-9. Western blot for detection of TSLP in ear tissue.

Total protein was isolated from the ear tissue of each group using lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 % Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaNO_3$, 10 mM NaF, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 25 μg/mL leupeptin]. Ear tissue lysates were centrifuged at 15,000 rpm for 15 min at 4°C and supernatants were used for western blotting. The total protein concentration of each sample was quantified by the Bio-Rad assay method (Bio-Rad, Hercules, CA). Extracts containing 30 μg of protein were loaded next to a prestained protein-mass ladder (Bio-Rad) on a NuPAGE 4-12 % bis-Tris gel (Invitrogen). The proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane using an iBlot gel transfer device (Invitrogen, San Diego, CA). The membrane was blocked with blocking buffer (5 % skim milk in TTBS) for 2 hr at room temperature, followed by incubation with primary antibodies (1:500) for 2 hr and overnight at 4°C. Antibody was diluted in 1 % BSA in TTBS buffer. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-primary Ab host IgG (1:5000) for 1 hr at RT. After washing again, the result was visualized with a western blot detection system (iNtRON Biotechnology, Korea) according to the manufacturer's instructions.

2-10. Enzyme-linked immunosorbent assay (ELISA)

Production of IgE (Biolegend, San Diego, CA) and histamine (Labor Diagnostika Nord, Nordhorn, Germany) proteins in mouse serum or of IL-4 and IFN-γ (R&D Systems, St. Louis, MO) in the supernatant of cultured cells were measured using ELISA kits according to the manufacturer's instructions.

2-11. Splenocyte culture in BALB/c mouse

Mice from each group were sacrificed by cervical dislocation and their spleens were removed aseptically. To obtain single-cell suspensions, spleens were forced through a wire gauze (70 μm pore size) using the plunger of a 5-mL syringe. Red blood cells (RBC) were removed using RBC lysis buffer. After washing, splenocytes were seeded (1.0×10^6 cells/mL) using trypan blue assays and incubated in the presence or absence of anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) (eBioscience, San Diego, CA) for 3 days. Following incubation, the supernatants were collected to determine levels of IL-4 and IFN-γ and cells were immediately analyzed by flow cytometric analysis (BD Biosciences, San Diego, CA).

2-12. Isolation of $CD4^+$ T cells in splenocyte

A single-cell suspension was isolated from the spleen of each group. $CD4^+$ T cells were isolated with anti-Mouse CD4 Purified (eBioscience) and DSB-X^{TM} Biotin Protein Labeling Kit (Invitrogen) according to the manufacturer's instructions. Briefly, cells were incubated with DSB-X labeled antibody for 10 min. The bead-bound cells in the FlowComp release buffer were incubated for 10 min. The supernatant containing the bead-free cells was transferred to a new tube and the cell pellet in preferred cell medium was resuspended and briefly stored at 2~8^{o}C until further use.

2-13. Flow cytometric analysis (FACS)

To analyze Foxp3 level, cells were permeabilized with the Foxp3 fixation/permeabilization kit

(BD Biosciences) and stained with anti-Foxp3-FITC (eBioscience) according to the manufacturer's instructions. Briefly, to block mouse Fc receptors, the cell suspension was reacted with CD16/CD32 (BD Biosciences) for 15 min, fixation/permeabilization buffer for 20 min and anti-Foxp3-FITC for 30 min. To measure the differentiation of $CD4^+CD25^+$ T cells, $CD4^+$ T cells were stained with anti-CD4-FITC (eBioscience) and anti-CD25-PE (eBioscience). Briefly, to block mouse Fc receptors, the cell suspension was reacted with CD16/CD32 (BD Biosciences) for 15 min, anti-CD4-FITC for 30 min and anti-CD25-PE for 30 min.

2-14. Extraction of total RNA and real-time PCR

Total RNA was isolated using TRI reagent (Molecular Research Center, INC., Cincinnati, OH) according to the manufacturer's instructions. Reverse transcription was performed using a First-Strand cDNA Synthesis kit (Promega). Briefly, total RNA (1 μg) was incubated with oligo $(dT)_{18}$ primer at 70°C for 5 min and cooled on ice for 5 min. After RT premix, the reaction ingredients were incubated at 42°C for 60 min. Reactions were terminated by raising the temperature to 70°C for 15 min.

The real-time quantitative PCR was performed with TaqMan®Universial Master Mix II or Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, La Jolla, CA) with a StepOnePlus™Real-Time PCR (Applied Biosystems, Piscataway, NJ). Real-time PCR results were analyzed using the StepOne™ software that measures the amplification of the target and of the endogenous control in samples and in a reference sample. Measurements were normalized using the endogenous control.The mRNA level was normalized to the gene expression of GAPDH. The primers used in these experiments are shown in Table 1.

Table 1. The sequence of primers used in real-time PCR analysis

Gene	Sequences
mIL-4-F	5'– ACAGGAGAAGGGACGCCAT – 3'
mIL-4-R	5'– GAAGAACTACAGACGAGCTCA – 3'
mIL-13-F	5'– GCAACATCACACAGGACCAGA – 3'
mIL-13-R	5'– GTCAGGGAATCCAGGGCTAC – 3'
mIFN-γ-F	5'– TCAAGTGGCATAGATGTGGAAGAA – 3'
mIFN-γ-R	5'– TGGCTCTGCAGGATTTTCATG – 3'
mTGF-β-F	5'– GAAGGCAGAGTTCAGGGTCTT – 3'
mTGF-β-R	5'– GGTTCCTGTCTTTGTGGTGAA – 3'
mIL-10-F	5'– ATAACTGCACCCACTTCCCA – 3'
mIL-10-R	5'– TCATTTCCGATAAGGCTTGG – 3'
mFoxp3-F	5'– CCCATCCCCAGGAGTCTTG – 3'
mFoxp3-R	5'– CCATGACTAGGGGCACTGTA – 3'

2-15. Statistical analysis

Quantity One version 4.2.1 and Image-Pro plus version 4.5 software were used to transform images into numerical values. Student's t-test and two-way analysis of variance were used to determine the statistical significance of differences between experimental and control group values. Data represent the mean ± standard deviation.

3. Results

3-1. Induction of experimental atopic dermatitis by topical stimulation with DNCB.

For the induction of experimental AD in BALB/c mice, the mice were first painted with 1% DNCB or vehicle on their abdoments as the first sensitization (day-7). On day 0, the mice received the second sensitization 0.5 % DNCB on their ears every other day for up to 24 days. Starting on day 12, the mice were given 100 mg/kg of FFO or NFO orally everyday, and mice were painted with hydrocort on their ears every other day. We repetitively conducted the experiments as above and, on day 31, all mice were sacrificed (Figure 1)

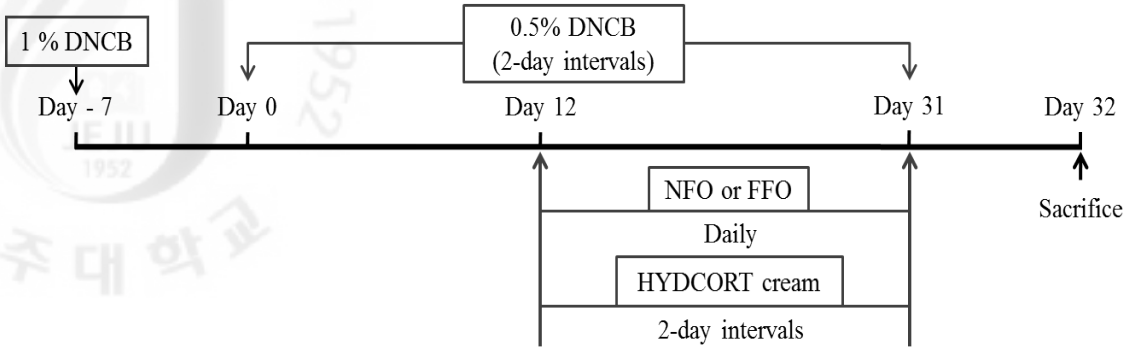


Figure 1. Induction of experimental atopic dermatitis by topical stimulation with DNCB. As shown, the mice were first painted with 1% DNCB or vehicle on their abdomen as the first sensitization (day-7). On day 0, the mice received the second sensitization with 0.5 % DNCB on their ears every other day for up to 24 days. On day 12, mice were given FFO or NFO (100 mg/kg) in drinking water, daily, and were painted with hydrocort on their ears every other day. On day 31, the mice were sacrificed.

3-2. Effect of fish oil on the scratching behaviors and cutaneous edema in DNCB-challenged mice.

Itching is a critical factor in the maintenance of AD symptoms, and affects skin edema. Therefore, we tested whether FFO or NFO has a therapeutic effect on pruritus and cutaneous edema. An experimental AD model was established by DNCB challenge. 12 days after a second sensitization with DNCB, mice were given FFO or NFO (100 mg/kg) everyday, and the mice were painted with hydrocort every other day. We measured the number of scratches in a 10-min period three times (0, 10 and 31 days). In the induction group, the number of scratches increased in a time-dependent manner, and on day 31, FFO and hydrocort treatment significantly decreased ($P < 0.05$) scratching behaviors compared with NFO treatment (Figure 2). Cutaneous edema as a measure of AD progression was also measured. Administration of FFO ($P < 0.001$) or NFO ($P < 0.05$) reduced ear thickness and the FFO-treated group was not appreciably different from the hydrocort treatment group. Moreover, the FFO treatment showed stronger inhibitory effects on cutaneous edema than the NFO treatment (Figure 3.).

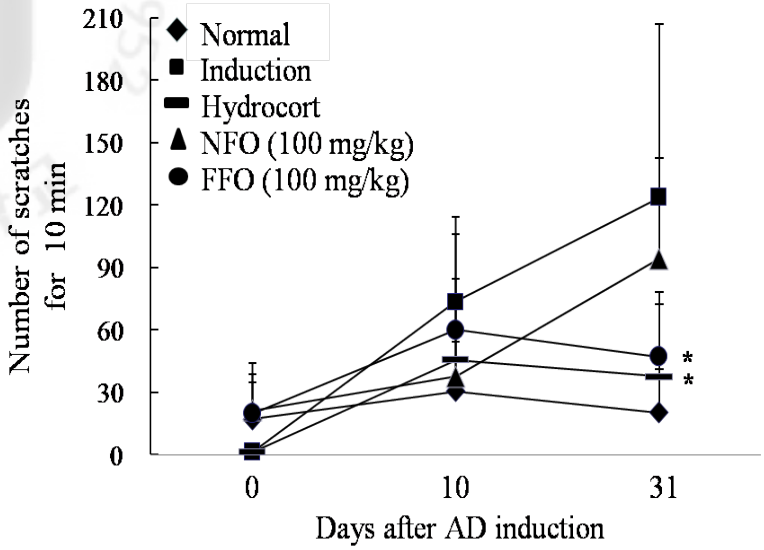


Figure 2. Effect of fish oil on the scratching behaviors in DNCB-challenged mice. BALB/c mice were stimulated with DNCB for up to 31 days at 2-day intervals. Mice were given 100 mg/kg FFO (●) or NFO (▲) daily and painted with hydrocort (▬) every other day. The spontaneous scratching behaviors of the mice were videotaped for 10 min with a digital camera on Days 0, 10 and 31. Values are mean the ± S.D (n=8 mice per group). * compared to mice stimulated with DNCB alone (Induction group). *$P < 0.05$.

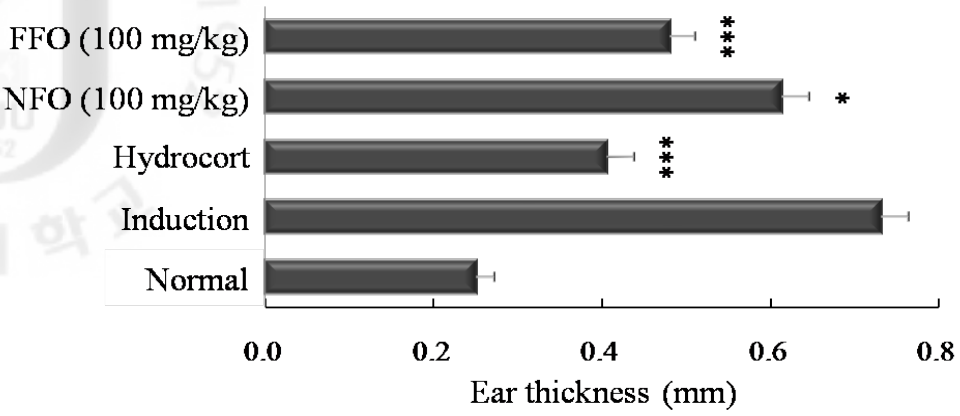


Figure 3. Effect of fish oil on the cutaneous edema in DNCB-challenged mice. BALB/c mice were stimulated with DNCB for up to 31 days at 2-day intervals. Mice were given 100 mg/kg FFO or NFO daily and painted with hydrocort every other day. Ear thickness was measured with a thickness gauge on Day 31. Values are mean the ± S.D (n=8 mice per group). *, *** compared to mice stimulated with DNCB alone (Induction group). *$P < 0.05$; ***$P < 0.001$.

3-3. Histological features of ear tissue in DNCB-stimulated BALB/c mice.

The skin lesions of AD are characterized by infiltration of various inflammatory cells, such as lymphocytes, or granulocytes. We tested whether application of FFO or NFO alleviates the infiltration of various inflammatory cells in the ears tissues of the DNCB-challenged mice. Mice stimulated by DNCB were fed with 100 mg/kg FFO or NFO daily for 20 days, and the anti-infiltration effect on various inflammatory cells in the ear tissues by FFO or NFO treatment was monitored by H&E staining. As a result, the inflammatory cell infiltration and epidermal thickness decreased in the FFO- or NFO-treated group, but FFO-treated group showed stronger inhibitory effects on various experimental AD symptoms than NFO (Figure 4).

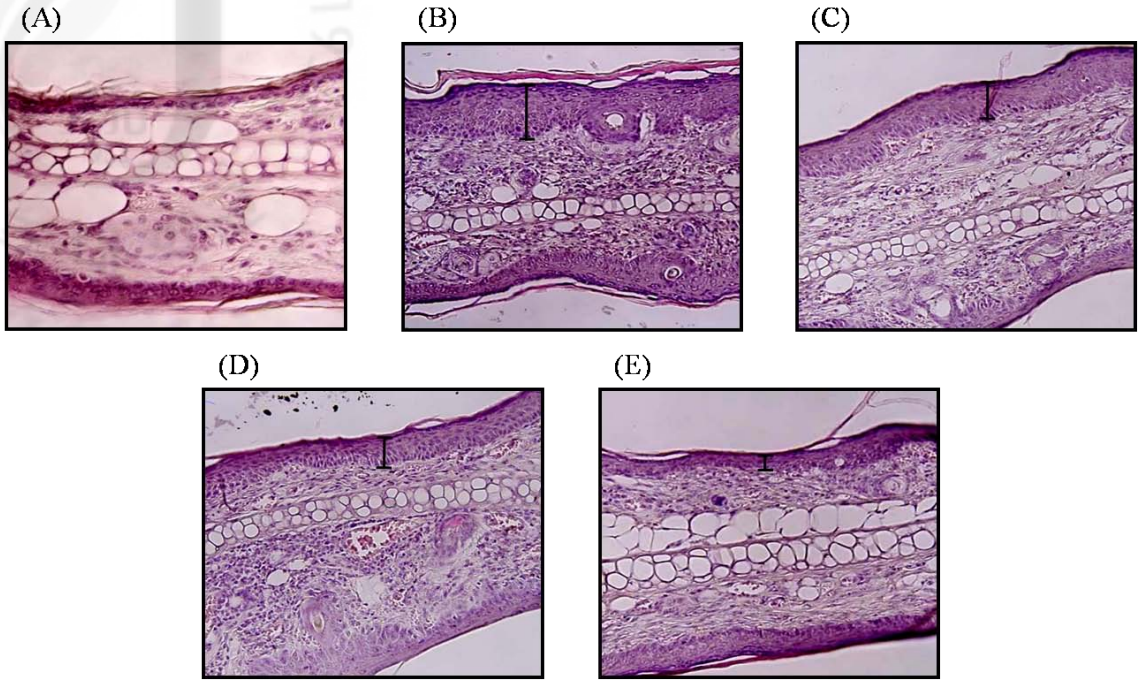


Figure 4. Histological features of ear tissue in DNCB-stimulated BALB/c mice. Stimulated mice by DNCB were given 100 mg/kg FFO or NFO everyday and were painted with hydrocort every other day. Paraffin sections of ear tissues were stained with hematoxylin and eosin (H&E, x200). (A): Normal; (B): Induction; (C): Hydrocort; (D): NFO 100 mg/kg; (E): FFO 100 mg/kg.

3-4. Effect of fish oil on serum IgE and histamine hyperproduction in DNCB-challenged mice.

IgE is an important target in treatment for allergy and AD as it, is essentially the component that activate mast cells to release histamine (Levin *et al.* 2006). Therefore, we measured serum IgE and histamine levels in the DNCB-stimulated mice. Mice with dermatitis were fed with 100 mg/kg FFO or NFO daily for 20 days, and release of IgE and histamine in mouse serum was measured by ELISA. The FFO or NFO-treated group showed significantly decreased IgE (both $P < 0.005$) and histamine (each $P < 0.001$ or $P < 0.005$) levels compared with the induction group, and FFO treatment showed stronger inhibitory effects on various allergic symptoms than NFO (Figure 5 and 6).

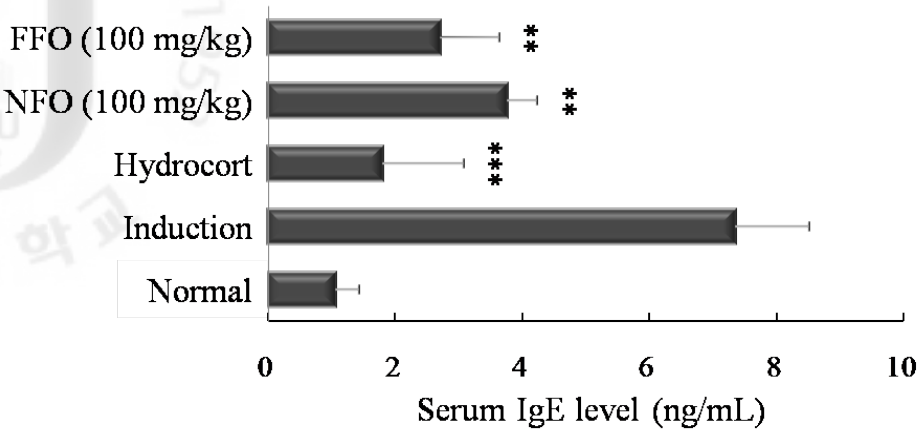


Figure 5. Effect of fish oil on serum IgE hyperproduction in DNCB-challenged mice. Mice stimulated with DNCB were given 100 mg/kg FFO or NFO daily and painted with hydrocort every other day. After their sacrifice, the release of IgE in mouse serum was measured by ELISA. Values are mean the ± S.D (n=8 mice per group). **, *** compared to mice stimulated with DNCB alone (Induction group). ** $P < 0.005$; *** $P < 0.001$.

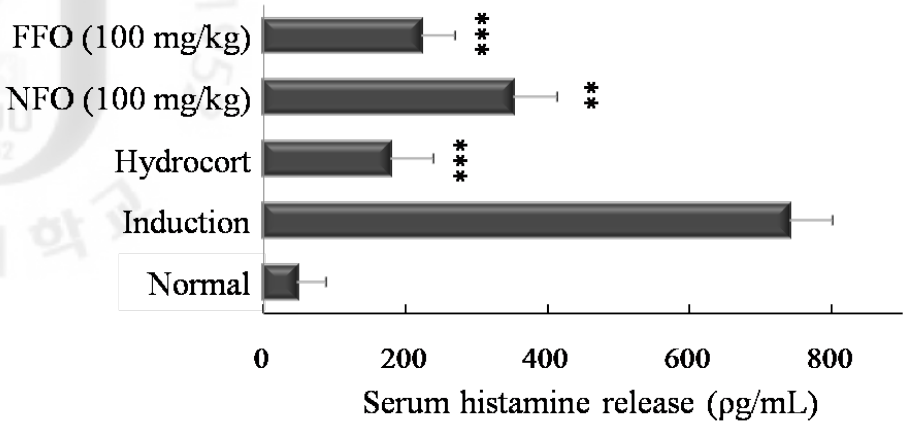


Figure 6. Effect of fish oil on serum histamine hyperproduction in DNCB-challenged mice. Mice stimulated with DNCB were given 100 mg/kg FFO or NFO daily and painted with hydrocort every other day. After their sacrifice, the release of histamine in mouse serum was measured by ELISA. Values are mean the ± S.D (n=8 mice per group). **, *** compared to mice stimulated with DNCB alone (Induction group). ** $P < 0.005$; *** $P < 0.001$.

3-5. Effect of fish oil on the expression of TSLP in ear tissues of DNCB-stimulated mice.

TSLP strongly activates the maturation of dendritic cells in the epidermis, and can regulate an allergic inflammation reaction (Bogiatzi *et al.* 2007). Therefore, we measured the expression of TSLP at the sites of AD. Mice stimulated with DNCB were each treated with 100 mg/kg FFO or NFO, and the expression of TSLP at the inflammatory sites (ear) was determined by IHC and western blot. DNCB-stimulated histological abnormalities, such as increased TSLP, were significantly inhibited in the group, were given FFO compared with those given NFO (Figure 4 A~E). FFO significantly diminished the expression of TSLP compared with the induction or NFO-treated ear tissues of DNCB-challenged mice (Figure 4 G).

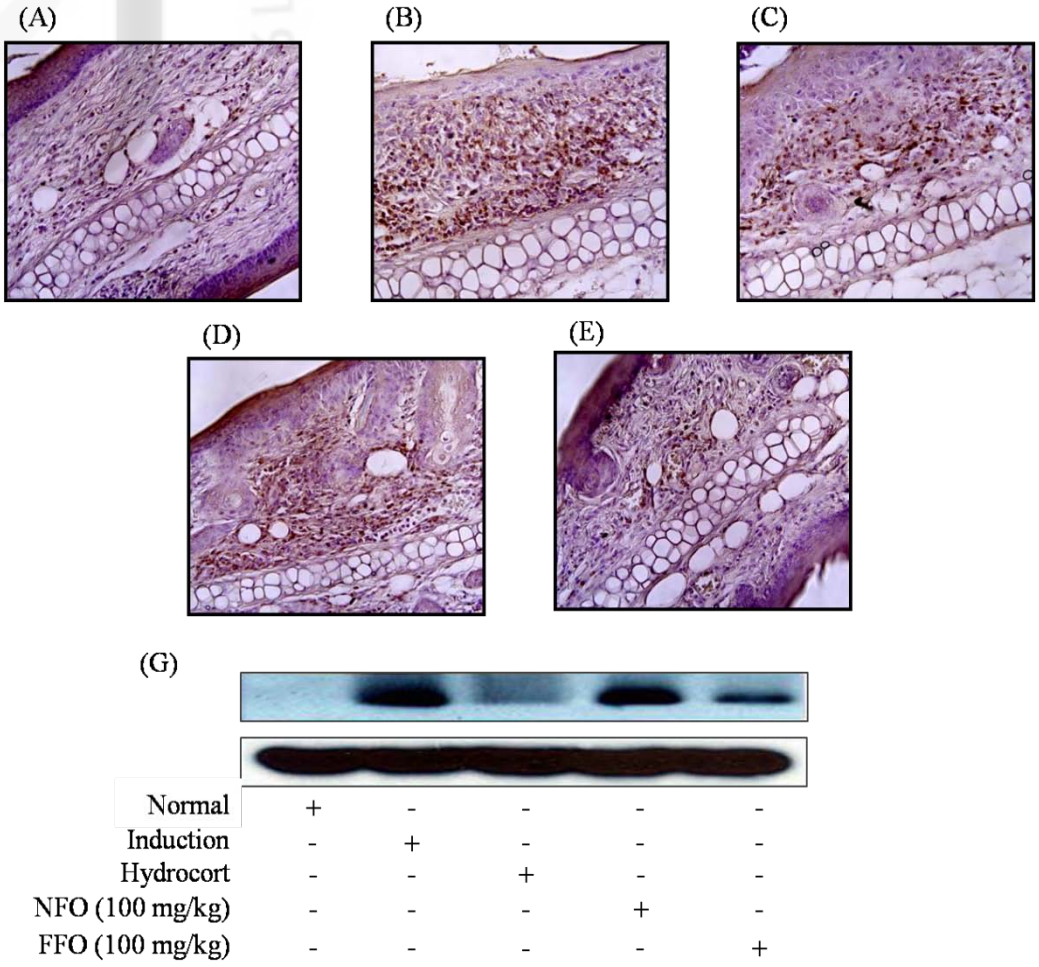


Figure 7. Effect of fish oil on the expression of TSLP in ear tissues of DNCB-stimulated mice. Mice stimulated with DNCB were given 100 mg/kg FFO or NFO daily and painted with hydrocort every other day. Paraffin sections of ear tissues were stained with immunohistochemistry (IHC, x200; A~E) and (G) were used with western blot by utilizing TSLP-specific mouse antibody. (A): Normal; (B): Induction; (C): Hydrocort; (D): NFO 100 mg/kg; (D): FFO 100 mg/kg.

3-6. Effect of fish oil on the expression of AD-associated cytokines and transcription factor in the ear tissues of DNCB-challenged mice.

Next, we measured whether FFO has an anti-inflammatory effect on DNCB-induced dermatitis, which is a typical Th2 cell-mediated immune disorder. Mice with dermatitis by DNCB stimulation were given 100 mg/kg FFO or NFO, and ear tissues were used to measure the expression of AD-associated inflammatory factors, such as Th2 (IL-4, -13) and Th1 (IFN-γ) cytokines, by real-time PCR. In the induction group, the IL-4 level was extremely elevated, and FFO or NFO (both $P < 0.005$) decreased the expression of IL-4 compared with the induction group, FFO was not appreciably different from the NFO treatment group (Figure 8. A). Ingestion of FFO reduced IL-13 ($P < 0.001$) and IFN-γ ($P < 0.001$) levels compared with the NFO-treated group (Figure 8. B). In addition, FFO treatment increased the expressions of Treg-associated factors (TGF-β and Foxp3), resulting in significantly (each $P < 0.001$ and $P < 0.05$) elevated suppressor activity compared with NFO-treated group (Figure 9).

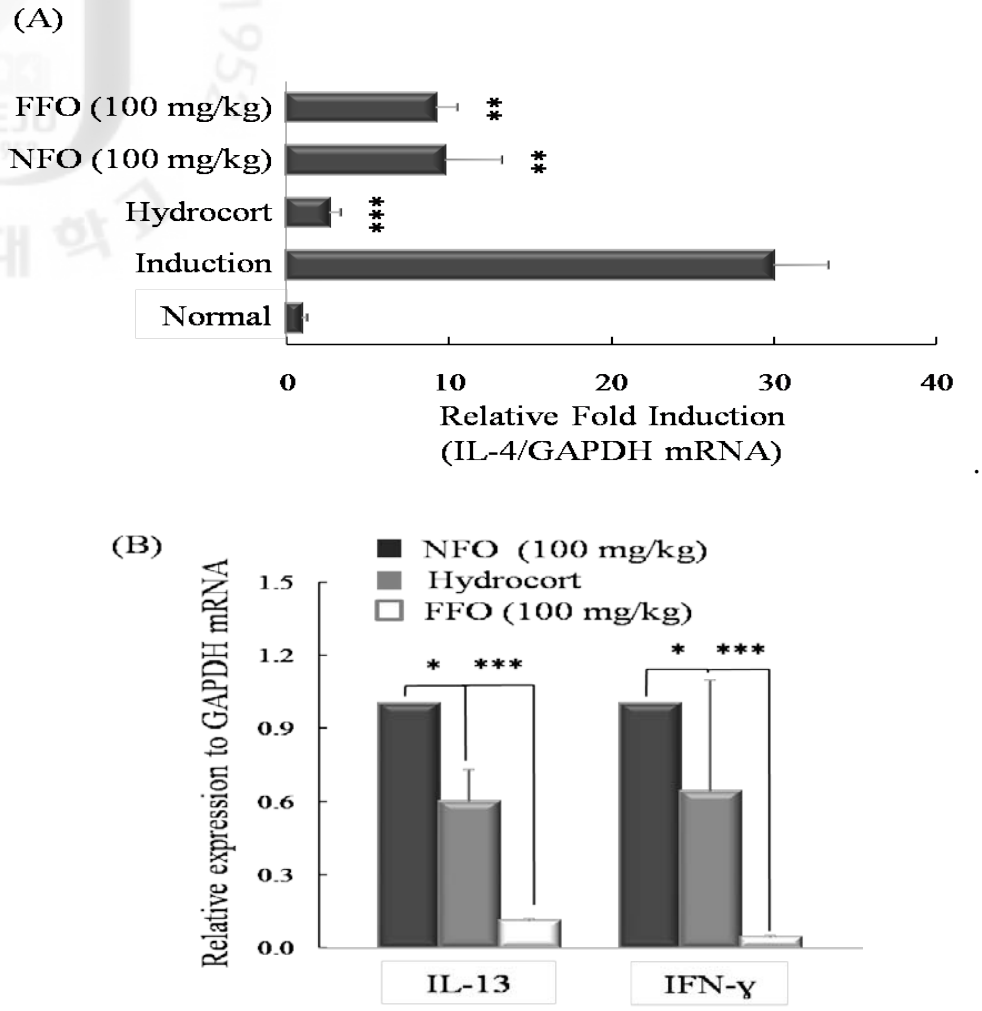


Figure 8. Effect of fish oil on the expression of AD-associated inflammatory cytokines in the ear tissues of DNCB-challenged mice. Mice stimulated with DNCB were given 100 mg/kg FFO or NFO daily and painted with hydrocort every other day. After their sacrifice, ear tissues were isolated and used to measure the expressions of inflammatory cytokines mRNA such as IL-4 (A), IL-13 and INF-ɣ (B) by real-time PCR. Values are mean the ± S.D (n=8 mice per group). *, **, *** compared to mice stimulated with Induction group (A) or NFO-treated group (B). *$P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

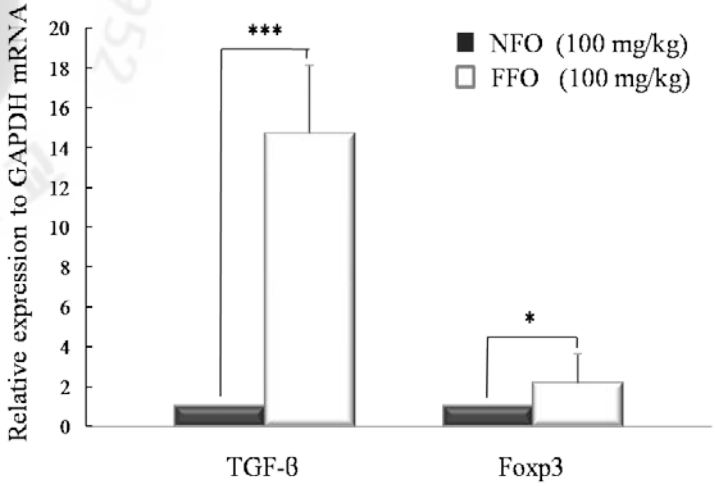


Figure 9. Effect of fish oil on the expression of AD-associated anti-inflammatory cytokine and transcription factor in the ear tissues of DNCB-challenged mice. Mice stimulated with DNCB were given 100 mg/kg FFO or NFO daily. After their sacrifice, ear tissues were isolated and used to measure the expressions of anti-inflammatory cytokine (TGF-β) and transcription factor (Foxp3) mRNA by real-time PCR. Values are mean the ± S.D (n=8 mice per group). *, *** compared to mice stimulated with NFO-treated group. *$P < 0.05$; *** $P < 0.001$.

3-7. Macroscopic observation of spleen features in DNCB-challenged mice.

The spleen plays important roles in the active immune system through cell-mediated pathway, and produces various immune cells, especially T and B cells. Therefore, we observed the morphologic features of the spleen in DNCB-challenged mice. The induction group showed an excessively bloated spleen but the FFO or NFO-treated group showed a smaller spleen size with reduced the thickness and length, and the FFO-treated group had a smaller spleen than the NFO-treated group (Figure 10).

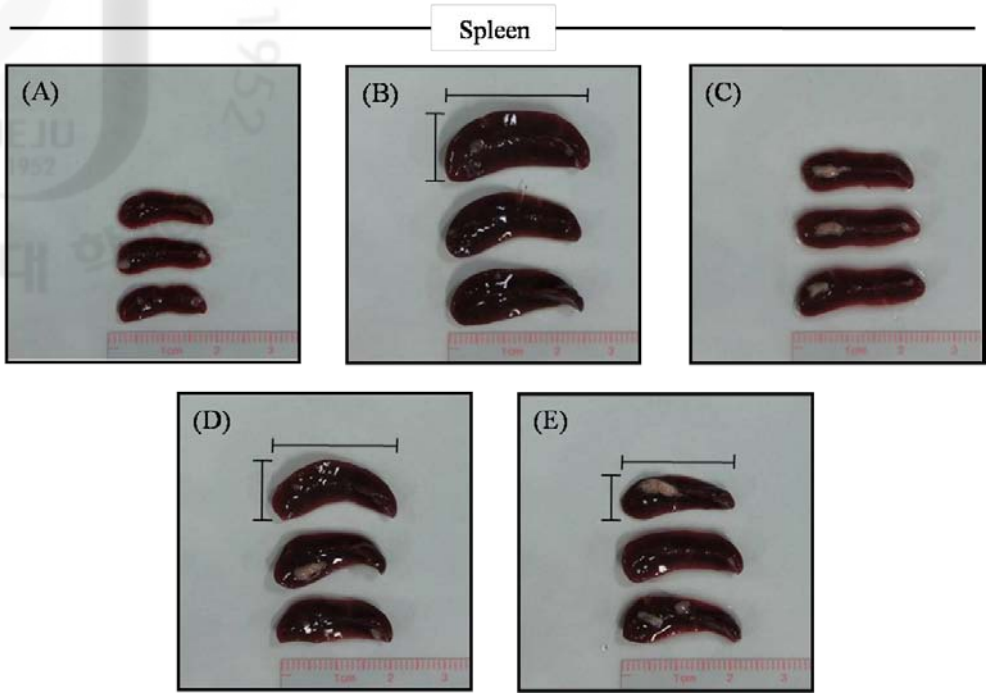
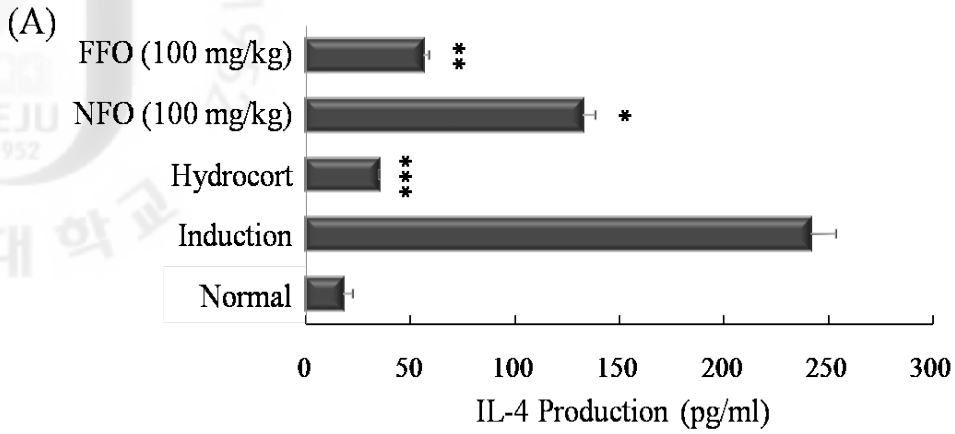


Figure 10. Macroscopic observation of spleen features in DNCB-challenged mice. Mice were stimulated with DNCB for up to 31 days at 2-day intervals. Mice were given 100 mg/kg FFO or NFO everyday and were painted with hydrocort every other day. After their sacrifice, spleen was isolated and photographed using digital camera to confirm morphologic alteration. (A): Normal; (B): Induction; (C): Hydrocort; (D): NFO 100 mg/kg; (E): FFO 100 mg/kg.

3-8. Effect of fish oil on the expression of inflammatory cytokines and transcription factors in splenocytes of DNCB-challenged mice.

We measured whether FFO or NFO has an anti-inflammatory effect on the DNCB-stimulated experimental AD. 100 mg/kg FFO or NFO was fed to DNCB-challenged mice for 20 days. Splenocytes isolated from the spleens of each group were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) for 3 days. The relative transcript levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines and the expressions of Th1 (T-bet) and Th2 (GATA3) transcription factors were measured by ELISA or FACS. The administration of FFO or NFO significantly ($P < 0.005$) inhibited the levels of IL-4 and INF-γ compared with the induction group in terms of splenocytes-stimulated with anti-CD3 and anti-CD28 (Figure 11). In addition, ingestion of FFO or NFO decreased T-bet expression from 44.6 % (induction) to 33.9 % (F.F.O) or 38.3 % (NFO), GATA3 expression, like T-bet expression, was also diminished in the FFO- or NFO-treated group from 28.8 % (induction) to 18.7 % (FFO) or 21.7 % (NFO) but the FFO-treated group had stronger anti-inflammatory effects on effector T cell-related inflammatory factors than the NFO-treated group (Figure 12 and 13).



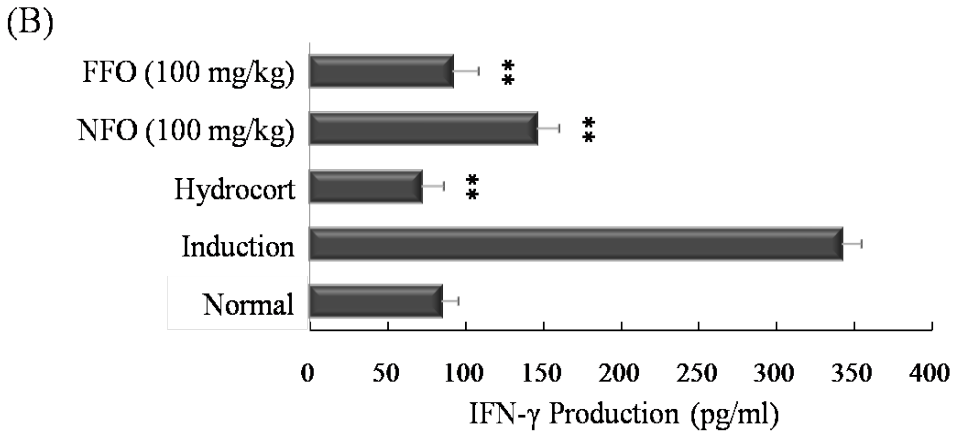


Figure 11. Effect of fish oil on the IL-4 and IFN-γ productions in splenocytes of DNCB-challenged mice. Splenocytes of stimulated mice with DNCB were isolated from spleens of each group. And then cells (1.0 x 10^6 cells/mL) were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) for 3 days. Mouse IL-4 (A) and IFN-γ (B) amounts were determined from the culture supernatant by ELISA method. The measurements were made in triplicate and the values are mean the ± S.D (n=8 mice per group). *, **, *** compared to mice stimulated with DNCB alone (Induction group). *$P < 0.01$; **$P < 0.005$; *** $P < 0.001$.

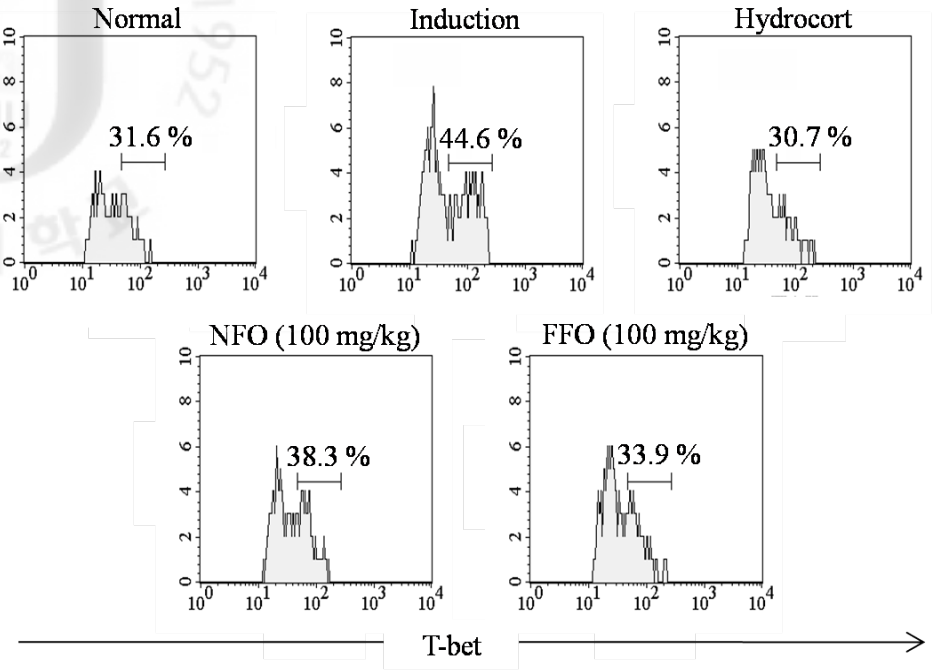


Figure 12. Effect of fish oil on the expression of transcription factor in splenocytes of DNCB-challenged mice. Splenocytes of stimulated mice with DNCB were isolated from spleens of each group. And then cells (1.0 x 10^6 cells/ml) were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) for 3 days. Cells were permeabilized with T-bet fixation/permeabilization buffer and stained with anti-T-bet-Alexa Fluor® 488. T-bet levels were analyzed by FACS. Data are from 8 mice per group.

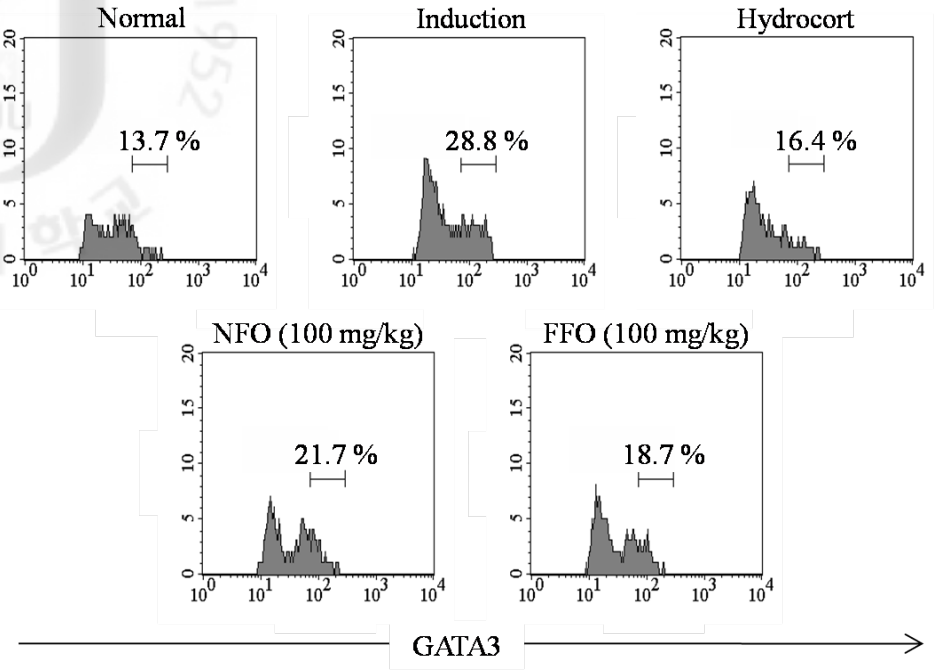


Figure 13. Effect of fish oil on the expression of transcription factor in splenocytes of DNCB-challenged mice. Splenocytes of stimulated mice with DNCB were isolated from spleens of each group. And then cells (1.0 x 10^6 cells/ml) were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) for 3 days. Cells were permeabilized with GATA3 fixation/permeabilization buffer and stained with anti-GATA3-PE. GATA3 levels were analyzed by FACS. Data are from 8 mice per group.

3-9. Effect of fish oil on the expression of Treg-associated factors and the differentiation of $CD4^+CD25^+$Treg in splenocytes of normal mice.

We demonstrated that the administration of FFO significantly reduced the progression of DNCB-induced experimental AD by inhibiting scratching behaviors, cutaneous edema, inflammatory cell infiltration, expression of TSLP and various cytokines. Based on recent reports, Tregs play a key role in various immune responses and prevent or suppress differentiation, proliferation and the function of various immune cells, including $CD4^+$ T cell (Tadokoro *et al.* 2006). Therefore, we analyzed whether administration of FFO or NFO affects the increase of factors related to differentiation of Treg in the absence of any stimulation. 100 mg/kg FFO or NFO was fed to normal BALB/c mice for 20 days, and splenocytes isolated from the spleens of each group were not stimulated. The administration of FFO did not affect the Foxp3 levels (Figure 14) and $CD4^+CD25^+$ Treg population (Figure 15 A) compared with the normal or NFO-treated group. Although FFO treatment did not increase the Foxp3 level and no difference in the $CD4^+CD25^+$ Treg population was observed compared with the NFO treatment group, the FFO-treated group showed increased expression of Treg-associated factors (TGF-β or IL-10), resulting in significantly ($P < 0.001$ or $P < 0.005$) elevated suppressor activity compared with the NFO-treated group (Figure 15. B).

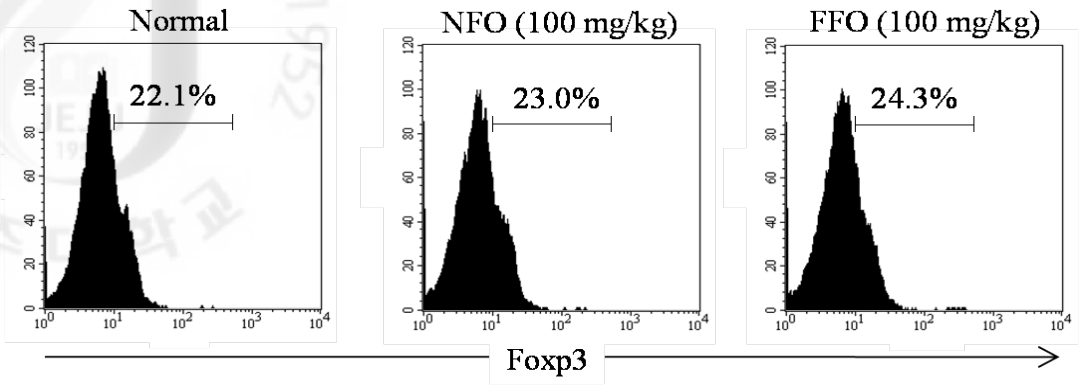


Figure 14. Effect of fish oil on the expression of transcription factor in splenocytes of normal mice. Normal mice were given 100 mg/kg FFO or NFO daily for 20 days. Splenocytes were isolated from the spleens of each group. Anti-CD3 and anti-CD28-nonstimulated cells (1.0 x 10^6 cells/mL) were permeabilized with Foxp3 fixation/permeabilization buffer and stained with anti-Foxp3-FITC. Foxp3 levels were analyzed by FACS. Data are from 5 mice per group.

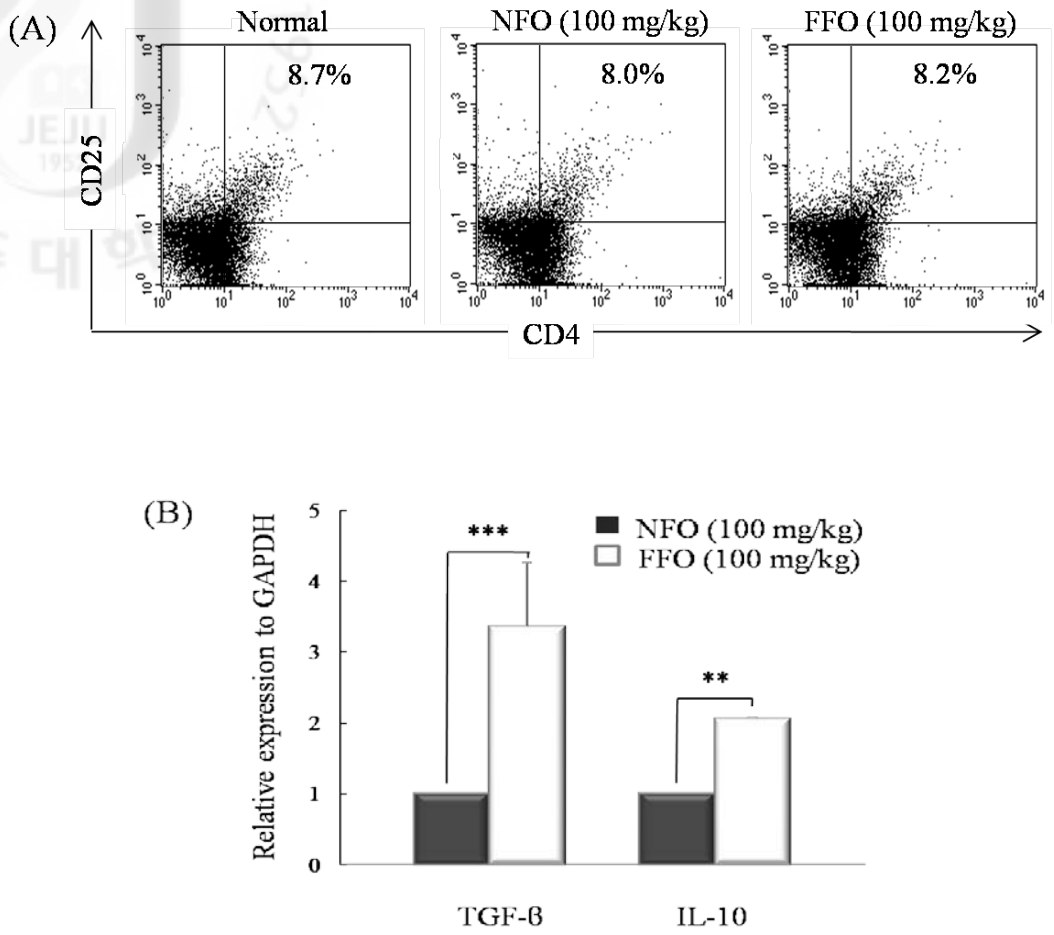


Figure 15. Effect of fish oil on the differentiation of $CD4^+CD25^+$Treg and the expression of anti-inflammatory cytokines in splenocytes of normal mice. Normal mice were given 100 mg/kg FFO or NFO daily for 20 days. Splenocytes were isolated from the spleens of each group. (A) Anti-CD3 and anti-CD28-nonstimulated cells (1.0 x 10^6 cells/mL) were stained with anti-CD4-FITC and anti-CD25-PE. The $CD4^+CD25^+$ Treg population was analyzed by FACS. Data are derived from 5 mice per group. (B) The spleen was used to measure the expression of anti-inflammatory cytokines (TGF-β and IL-10) mRNA by real-time PCR. Values are mean the ± S.D (n=5 mice per group). **, *** compared to mice treated with NFO. $^{**}P < 0.005$; $^{***}P < 0.001$.

3-10. Effect of fish oil on the differentiation of $CD4^+CD25^+Foxp3^+$ Treg in anti-CD3 and –CD28-stimulated splenocytes of normal BALB/c mice.

Our earlier studies showed that administration of FFO or NFO did not elevate the Foxp3 level and $CD4^+CD25^+$ Treg population in the absence of stimuli; only FFO treatment increased TGF-β and IL-10 levels (Figure 14, 15). Therefore, we analyzed whether the administration of FFO or NFO increases the expression of factors related to the differentiation of Treg in the presence of stimuli. FFO (100 mg/kg) or NFO (100 mg/kg) was fed to normal BALB/c mice for 20 days, and a single-cell suspension was prepared from the spleens of each group. $CD4^+$ T cells were isolated from splenocytes with $CD4^+$ T cell isolation beads and stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) for 3 days. The administration of FFO increased Foxp3 levels (14.2 %) and the $CD4^+CD25^+$ Treg population (16.0 %) more than double in comparison with that in the NFO-treated group (each 7.1 % and 8 %), (Figure 16 and 17). In addition, to demonstrate that the cells were Treg, we analyzed whether the administration of FFO increases the $CD4^+CD25^+Foxp3^+$ Treg population in the presence of stimuli. As a result, FFO treatment increased the $CD4^+CD25^+Foxp3^+$ Treg population (11.5 %) to more than twice that of the NFO-treated group (5.5 %), (Figure 18).

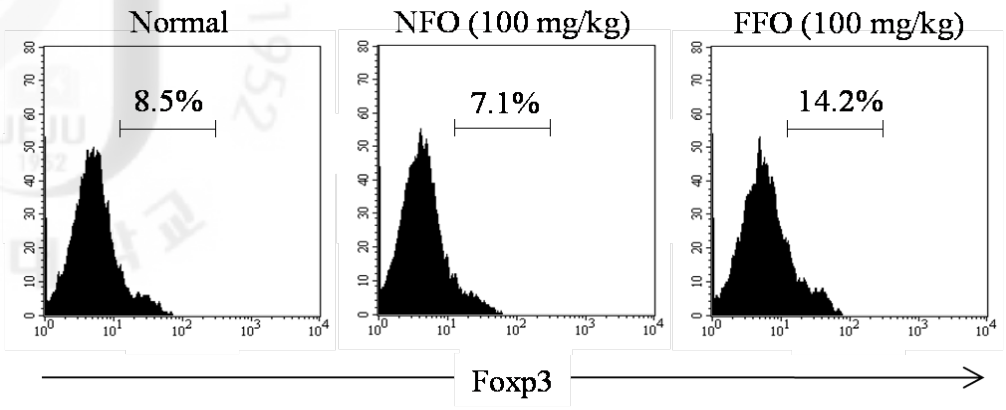


Figure 16. Effect of fish oil on the expression of transcription factor in anti-CD3 and –CD28-stimulated splenocytes of normal BALB/c mice. Normal mice were given 100 mg/kg FFO or NFO daily for 20 days. A single-cell suspension was prepared from the spleens of each group. $CD4^+$ T cells were isolated with $CD4^+$ T cell isolation bead. And then $CD4^+$ T cells (1.0 x 10^6 cells/mL) were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) for 3 days. $CD4^+$ T cells were permeabilized with Foxp3 fixation/permeabilization buffer and stained with anti-Foxp3-FITC. Foxp3 levels were analyzed by FACS. Data are derived from 5 mice per group.

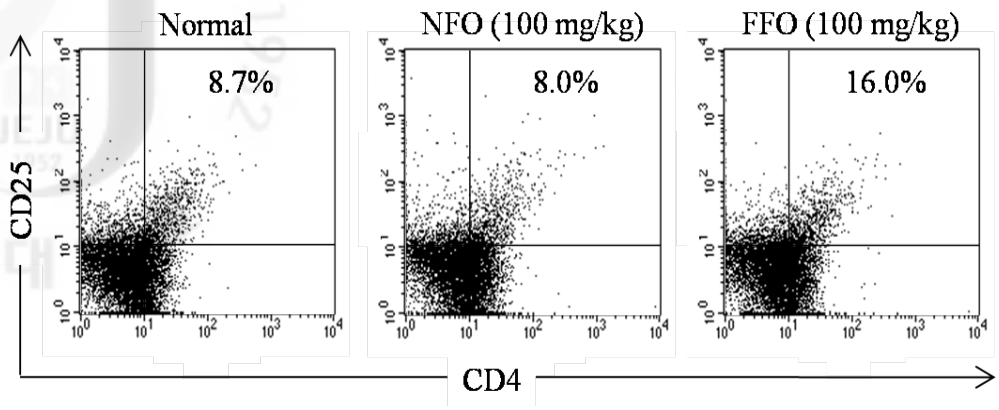


Figure 17. Effect of fish oil on the differentiation of $CD4^+CD25^+$Treg in anti-CD3 and –CD28-stimulated splenocytes of normal BALB/c mice. Normal mice were given 100 mg/kg FFO or NFO daily for 20 days. A single-cell suspension was prepared from the spleens of each group. $CD4^+$ T cells were isolated with $CD4^+$ T cell isolation bead. And then $CD4^+$ T cells (1.0 x 10^6 cells/mL) were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) for 3 days. $CD4^+CD25^+$ T cells were stained with anti-CD4-FITC and anti-CD25-PE. $CD4^+CD25^+$ Treg population was analyzed by FACS. Data are derived from 5 mice per group.

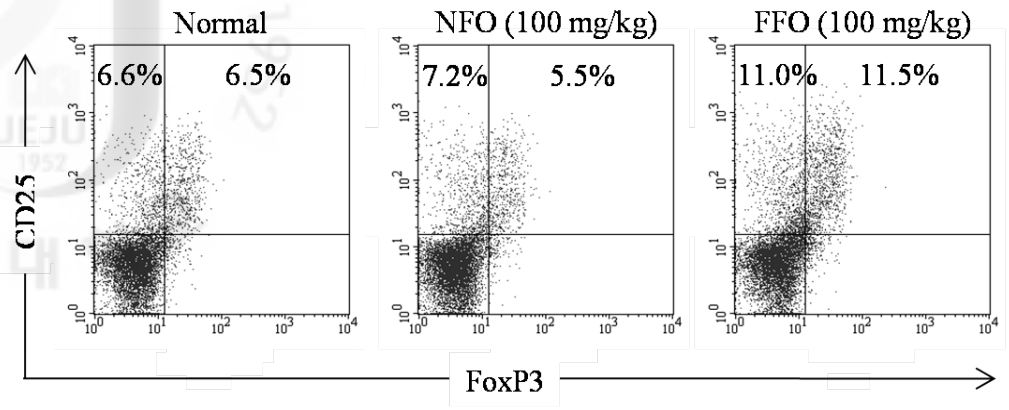


Figure 18. Effect of fish oil on the differentiation of $CD4^+CD25^+Foxp3^+$ Treg in anti-CD3 and –CD28-stimulated splenocytes of normal BALB/c mice. Normal mice were given 100 mg/kg FFO or NFO daily for 20 days. A single-cell suspension was prepared from the spleens of each group. $CD4^+$ T cells were isolated with $CD4^+$ T cell isolation bead. And then $CD4^+$ T cells (1.0×10^6 cells/ml) were stimulated with anti-CD3 (1 μg/mL) and anti-CD28 (0.5 μg/mL) for 3 days. $CD4^+$ T cells were permeabilized with Foxp3 fixation/permeabilization buffer and stained with anti-Foxp3-FITC and anti-CD25-PE. The $CD4^+CD25^+Foxp3^+$ Treg population was analyzed by FACS. Data are derived from 5 mice per group.

Table 2. Fatty acids composition of natural fish oil and fermented fish oil

constituent	Natural fish oil (%)	Fermented fish oil (%)
Myristic acid C14:0	3.430	2.954
Stearic acid C18:0	3.417	2.992
Eicosatrienoic acid C20:3	0.523	0.497
Linolenic acid C18:3n3	0.638	0.847
Eicosapentaenoic acid (EPA) C20:5n3	2.657	4.350
Docosahexaenoic acid (DHA) C22:6n3	8.366	13.614

4. Discussion

In this study, we fermented natural fish oil and compared the differences between fermented fish oil (FFO) and natural fish oil (NFO) on the modulation of the immune system. Moreover, we developed the FFO and NFO and investigated their regulation efficacy in experimental immune disorders. Administration of FFO potently induced the generation of $CD4^{+}CD25^{+}$Foxp3+ Treg in the spleen compared with NFO treatment. The enrichment of TGF-β or Foxp3 at sites of inflammation is associated with elevated $CD4^{+}CD25^{+}Foxp3^{+}$ Treg populations, which down-regulate the progression of experimental immune disorders. The administration of fermented fish oil generates regulatory T cell populations.

Atopic dermatitis (AD), an immune-mediated inflammation of the skin, primarily occurs among children in developed countries, and mainly appears together with a variety of diseases such as eczema, asthma and allergic conjunctivitis (Leung *et al.* 2004, Nystad *et al.* 2005).

Fish oil can decrease the risk of deteriorating clinical manifestations of allergic diseases and is associated with a lower expression of IL-4, -13 and IFN-γ during pregnancy (Krauss-Etschmann *et al.* 2008). Fish oil, contains an abundance of omega-3 polyunsaturated fatty acids (n-3 PUFAs), and its intakes leads to in higher n-3 PUFA status and lower n-6 PUFA status in the body. It also contains various n-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Seki *et al.* 2010). EPA and DHA inhibit the production of inflammatoty cytokines and increase the expression of anti-inflammatory cytokines, they also diminish the proliferation of effector T cells by increasing the number of $Foxp3^{+}$ Tregs (Hara *et al.* 2001, Yessoufou *et al.* 2009).

In the present study, we used the DNCB-induced experimental AD mouse model to investigate the anti-inflammatory effect of fermented fish oil or natural fish oil. Scratching behaviors due to

pruritus aggravates unpleasant symptoms of dermatological diseases such as AD and affects the skin edema. Therefore, we tested whether FFO or NFO can suppress pruritus and skin edema. The administration of FFO or NFO decreased scratching behavior and ear thickness in DNCB-challenged mice (Figure 2 and 3). Moreover, base on H&E staining to confirm the histological features of ear tissue in DNCB-stimulated mice, and ingestion of FFO or NFO alleviated the inflammatory cell infiltration and epidermal thickness compared with the induction group (Figure 4). IgE is an important target in treatment of allergy and AD, and histamine released from activated mast cells is a violent inducer of pruritus (Schwartz 2004). Therefore, we tested whether FFO or NFO treatment decreases serum IgE and histamine hyperproduction in DNCB-stimulated mice. The administration of FFO or NFO significantly decreased IgE (both $P < 0.005$) and histamine ($P < 0.001$ or $P < 0.005$) levels compared with the induction group (Figure 5 and 6). Based on the above results, we assumed that decreased IgE and histamine levels by FFO or NFO treatment leads to alleviation of pruritus or cutaneous edema (Figure 1, 5 and 6), furthermore, the FFO treatment showed stronger inhibitory effects on various allergic symptoms than the NFO treatment group (Figure 1~6). TSLP is mainly expressed in the lesions of acute and chronic AD, it strongly activates the maturation of dendritic cells in the epidermis, and can stimulate naive T cells to express pro-inflammatory cytokines, such as IL-4, -5, -13 and IFN-γ (Bogiatzi *et al.* 2007, Ebner *et al.* 2007). Therefore, we measured whether the administration of FFO or NFO decreases TSLP hyperproduction at the sites of disease progression in DNCB-stimulated mice. The ingestion of FFO significantly diminished the expression of TSLP compared with the induction or NFO-treated group in the ear tissue of DNCB-challenged mice (Figure 4). In the majority of patients, AD is primarily associated with a dysfunction of the body's immune system and an imbalance between Th1- and Th2-type cells. Chronic AD skin lesions generally have fewer Th2 cells, and IL-4 plays a crucial role in the allergic responses

and the differentiation from naive T cells to Th2 cells (Werfel 2009). Thus, we measured whether FFO or NFO has an anti-inflammatory effect on DNCB-induced dermatitis, which is a typical Th2 cell-mediated immune disorder. The induction group using DNCB stimulation had an extremely elevated IL-4 level, but the administration of FFO or NFO decreased the expression of IL-4 (both $P < 0.005$), in particular, ingestion of FFO down- regulated IL-13 ($P < 0.001$) and IFN-ɣ ($P < 0.001$). There was also significantly increased expression of Treg-associated factors, TGF-β ($P < 0.001$) and Foxp3 ($P < 0.05$) compared with those in the NFO-treated group (Figure 8 and 9). Based on the above results, we assumed that FFO and NFO have potent anti-inflammatory properties; however, FFO showed stronger inhibitory effects on various inflammatory reactions than NFO. An enrichment of TGF-β or Foxp3 by FFO treatment at the sites of inflammation could affect the increase in regulatory T cell populations, which would down-regulated the progression of experimental immune disorders (Figure 8 and 9).

The spleen plays important roles in regulating the immune system and comprises various immune cells, especially T and B cells. We observed the morphologic features of the spleen in DNCB-challenged mice, and the induction group had an excessively bloated spleen; the FFO or NFO-treated group showed smaller spleen sizes by reduction of the thickness and length. The FFO-treated group in particular showed a smaller sized spleen than the NFO-treated group (Figure 10). Next, we measured whether FFO or NFO regulates the expression of AD symptom-associated factors in the splenocytes of normal mice. The administration of FFO or NFO decreased the relative transcript levels of IFN-γ (both $P < 0.005$) and IL-4 (each $P < 0.005$ or $P < 0.05$) cytokines and the expression of T-bet (each 33.9 % or 38.3 %) and GATA3 (each 18.7 % or 21.7 %) transcription factors compared with the induction group, which is splenocyt-stimulated with both anti-CD3 and anti-CD28 (Figure 11~13). How does fermented fish oil suppress a variety of immunological symptoms in DNCB-stimulated experimental immune

disorders? Regulatory T cells (Tregs) play a key role in various immune responses including Th2 cell-mediated disease, such as AD, and prevent or suppress differentiation, proliferation, and function of various immune cells, including $CD4^+$ T cells (Robinson 2005, Tadokoro *et al.* 2006). Moreover, since all Treg cells express CD25 at the cell surface and have Foxp3 as the transcription factor, these proteins have been identified as the markers of Tregs. Treg can be roughly categorized into two types; natural Tregs (nTregs) and inducible Tregs (iTregs). Naturally occurring nTreg, which is generated in the thymus, is self-reactive and suppresses in a TGF-β-independent manner (Sakaguchi 2004). Induced iTreg from naive T cells is specific for antigens arising during an immune response, and suppresses the activation of helper T cells in a TGF-β-dependent manner (Buckner *et al.* 2004). Therefore, we analyzed whether the administration of FFO affects the increase of factors related to Treg differentiation without any stimuli. Although the FFO treatment did not increase the Foxp3 level and no difference in the $CD4^+CD25^+$ Treg population was observed between the NFO treatment group (Figure 14 and 15 A), the FFO-treated group showed increased expressions of Treg-associated factors (TGF-β and IL-10). This resulted in significantly (each $P < 0.001$ or $P < 0.005$) elevated suppressor activity compared with the NFO-treated group (Figure 15. B). Based on the findings, ingestion of FFO or NFO did not increase the $CD4^+CD25^+$ Treg population (Figure 15 A) but elevated its suppressor capacity (Figure 15 B). Interestingly, the administration of FFO up-regulated the expression of Treg-associated factors (TGF-β and Foxp3) by more than double at the sites of inflammation (Figure 9). Therefore, we analyzed whether the administration of FFO or NFO increases the expression of factors related to Treg differentiation in the presence of stimuli. The administration of FFO increased more than two-fold the Foxp3 levels (14.2 %) and $CD4^+CD25^+$ Treg population (16.0 %) compared with NFO in $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 (Figure 16 and 17). In addition, we analyzed whether the administration of FFO or

NFO increases the $CD4^+CD25^+Foxp3^+$ Treg population in the presence of stimuli. Findings indicate that the FFO treatment increased more than two-fold the $CD4^+CD25^+Foxp3^+$ Treg population (11.5 %) compared with NFO treatment (5.5 %), (Figure 18). As a result, ingestion of FFO significantly increased the Foxp3 level and induced the differentiation of $CD4^+CD25^+Foxp3^+$ Treg from $CD4^+$ T cells with stimulation.

Based on our results, fermented fish oil showed more potent disease suppression efficacy and $CD4^+CD25^+Foxp3^+$ Treg induction capacity than natural fish oil. Why does fermented fish oil show more potent anti-allergic effects than natural fish oil? Fish oil intake maintains a higher Omega-3 (n-3) PUFA, such as eicosapentaenoic acid (EPA), which inhibits the production of the pro-inflammatory cytokines or increases the number of Foxp3+ Tregs, and docosahexaenoic acid (DHA) diminishes the proliferation of effector T cells in the body (Hara *et al.* 2001, Yessoufou *et al.* 2009) Fermented fish oil contains lower than omega-6 PUFAs levels, but more than double the concentration of EPA and DHA compared with natural fish oil (Table 2), thus increasing the immune-modulatory activities by which $CD4^+CD25^+Foxp3^+$ Tregs are generated.

In summary, fermented fish oil showed stronger inhibitory effects on various experimental AD symptoms than natural fish oil, and had potent immunomodulatory effects on immune disorders, such as histological disorders or increased inflammatory cytokines by up-regulating the generation of Tregs in spleen. However, it is not clear why ingestion of FFO has stronger immunomodulatory effects than NFO. We are currently trying to identify the interrelationships between FFO, Treg, and regulatory dendritic cell. Our results suggest that the anti-allergic effect of fermented fish oil is associated with the enrichment of $CD4^+CD25^+Foxp3^+$ Treg in the inflamed sites and fermented fish oil may be an effective source for improving the allergic symptoms of AD.

5. References

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