Anti-inflammatory effects and enhancing immune response of freshwater hybrid catfish oil in RAW264.7 cells

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Abstract. The present study assessed the effect of freshwater hybrid catfish oil (FFO) on the inflammatory status of lipopolysaccharide (LPS)-stimulated RAW264.7 cells and investigated the underlying mechanisms. RAW264.7 cells were supplemented with various concentrations [0.125-2% in 0.5% propylene glycol (v/v)] of FFO with or without LPS (1 μg/ml) for 24 h. Inflammatory cytokines and mediators were quantified using ELISA and reverse transcription-quantitative PCR. The results revealed that FFO treatment inhibited the secretion and mRNA expression of the pro-inflammatory cytokines IL-6, IL-1β, TNF-α. In line with this, FFO suppressed the expression and secretion of the inflammatory mediators cyclooxygenase-2 and prostaglandin E2. FFO also reduced apoptotic body formation and DNA damage. Correspondingly, FFO enhanced the immune response by modulating the cell cycle regulators p53, cyclin D2 and cyclin E2. Accordingly, FFO may be developed as a nutraceutical product to prevent inflammation.

Introduction

Inflammation is one of the first lines of defense against harmful stimuli, such as pathogens, damaged cells, trauma, bacteria and irritants (1). Macrophages detect and react to certain pathogens and consequently regulate the inflammatory response (2). Lipopolysaccharide (LPS) is an endotoxin derived from the outer membrane of Gram-negative bacteria and also a powerful mediator of systemic inflammation and a driver of septic shock (3). LPS is able to activate macrophages to release several inflammatory cytokines (4). Activation of the inflammatory pathway may be induced by pro-inflammatory mediators and cytokines being secreted, including nitric oxide (NO), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and prostaglandin (PG)E2 (5). Inflammation is one cause of increased morbidity and mortality in intensive care units, also resulting in elevated hospital-related costs (6,7). Nowadays, several anti-inflammatory drugs are available, such as non-steroidal anti-inflammatory drugs (NSAIDs) (8). However, a previous study suggested that NSAIDs may induce gastrointestinal tract bleeding (9). Safe and effective strategies to prevent and treat inflammation and its associated diseases are thus urgently required.

The freshwater hybrid catfish (*Pangasius* sp.) belongs to the freshwater catfish family. It has become one of the most popular freshwater fish species and has a high demand, particularly on the European and US markets. Fish contains 2-30% fat and ~50% of its body weight is discarded as waste during the fish processing operation (10). One of the fish processing byproducts is fish oil (FO). FO is a source of long-chain polyunsaturated fatty acid (e.g. omega-3 fatty acids), particularly fish oil extracted from marine fish, which is mainly composed of cis-5,8,11,14,17-eicosapentaenoic acid (EPA) and cis-4,7,10,13,16,19-docosahexaenoic acid (11). As a component in FO, omega-3 fatty acids have several benefits, including protection against atherosclerosis, arrhythmias and chronic obstructive pulmonary diseases (12). They also reduce blood pressure, blood glucose and symptoms of asthma and cystic fibrosis (13-15). However, a previous study by our group demonstrated that fish oil from freshwater hybrid catfish contains a high level of monounsaturated omega-9 fatty acid (MUFA) (16). Furthermore, freshwater hybrid catfish oil (FFO) was indicated to have anti-diabetic effects by improving insulin resistance and adipokine imbalance in a rat model of type 2 diabetes and also suppress pro-inflammatory cytokine protein expressions in the skeletal muscle tissues of those rats (17). The omega-9 fatty acid increased of high-density lipoprotein-cholesterol and decreased low-density lipoprotein-cholesterol (17). However, the effect of FFO on the

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inflammatory condition and the underlying mechanisms have remained elusive. In the present study, the anti-inflammatory effects of FFO on RAW264.7 macrophages stimulated by LPS were examined and the associated mechanism was investigated.

Materials and methods

Chemicals. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. β-nicotinamide adenine dinucleotide phosphate and LPS were purchased from Merck KGaA. All other chemicals with high purity were purchased from commercial sources.

Preparation of FFO. FO of freshwater hybrid catfish (*Pangasius gigas* x *Pangasianodon hypophthalmus*) was purchased from a private company, Me Natural Co., Ltd., which cooperated and received the adipose tissue from the Center of Excellence in Giant Catfish and Buk Siam Catfish, Faculty of Fisheries Technology and Aquatic Resources, Maejo University (Chiang Mai, Thailand). FO was extracted as previously described, which exhibited a high omega-9 content and biological activity (18). In brief, frozen adipose tissues were purified by cleaning and steaming at 90°C for 30 min. The liquid oil was subsequently filtered through a filter sack and squeezed using a screw compressor. The squeezed liquid was centrifuged at 2,268 x g for 10 min at 25°C to separate the solid particles from the oil and the supernatant FFO was separated. Solvent-free extraction was used to obtain FFO. As previously, adipose tissue was extracted and partially purified as aforementioned, resulting in FFO at a yield of 300 ml per 1 kg of adipose tissue.

Determination of fatty acids, fat-soluble vitamins and heavy metal levels of FFO. The chemical compounds, including the fatty acids and fat-soluble vitamins, were sent for analysis at a certified lab with international standardization in the field of information technology (ISO172025), the Central Laboratory (Thailand) Co. Ltd., Chiang Mai Branch, following the TE-CH 260 in-house method of the Association of Official Analytical Chemists 996.06 (19). Heavy metal contamination of FFO was also detected according to this in-house method.

Cell culture. RAW264.7 cells were purchased from the American Type Culture Collection. Cells at passage 2-22 were maintained in DMEM (Thermo Fisher Scientific, Inc) containing 3.7 g/l $NaHCO_3$ supplemented with 10% FBS (Thermo Fisher Scientific, Inc) and 1% penicillin/streptomycin in a humidified atmosphere at 37°C with 5% CO_2 and sub-cultured every 4-5 days using 0.05% trypsin-EDTA in PBS (Thermo Fisher Scientific, Inc.). Cells were seeded at a density of $1x10^5$ cells/well and cultured in 6-, 12- and 96-well plates for 3 days until subsequent experimentation. The medium was replaced every 2 days during culture.

Determination of cell viability. The MTT assay was performed to assess the effect of FFO on cell viability. Cells were incubated with serum-free medium with FFO at 0, 0.125, 0.25, 0.5, 1 or 2% in 0.5% propylene glycol (v/v). Subsequently, serum-free

medium containing 0.5 mg/ml of MTT (Thermo Fisher Scientific, Inc.) was added to each well, followed by incubation at 37°C for 4 h. The MTT solution was then aspirated and cells were washed once with ice-cold PBS. The purple formazan crystals were dissolved in DMSO for 30 min and cell viability was subsequently analyzed by measuring the absorption at a wavelength of 570 nm using an M965 AccuReader microplate reader (Metertech, Inc.). The lysed cells were detected at a wavelength of 680 nm was used as a reference. Cell viability was calculated as follows: Cell viability (%) = [(Absorbance value-reference value) x100]/[mean of (absorbance value-reference value) in untreated cells].

ELISA. RAW264.7 cells were seeded into 12-well plates at a density of $1x10^5$ cells/ml and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was removed and cells were treated with different concentrations of FFO [0.125-2% in 0.5% propylene glycol (v/v)] with or without LPS (1 µg/ml) in fresh medium for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Subsequently, the cells were homogenized and lysed cells were centrifuged at 2,000 x g for 10 min at 4°C. The supernatant was collected and stored at -80°C for quantification of IL-6 (cat. no. BIOL-431304), IL-1β (cat. no. BIOL-432604), TNF-α (cat. no. BIOL-430904), NO (cat. no. 780001) and PGE2 (cat. no. ABBK-KTE70765-96T) concentrations using commercial kits (BioLegend, Inc.) according to the manufacturer's protocols.

NO assay. The nitrate/nitrite concentration was determined using a colorimetric assay kit (Cayman Chemical Co.). In brief, cells were treated with different concentrations of FFO [0.125-2% in 0.5% propylene glycol (v/v)] with or without LPS (1 µg/ml) for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Treated cells were centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was subsequently collected to measure the NO concentration at a wavelength of 540 nm using an M965 AccuReader microplate reader (Metertech, Inc.).

Hoechst 33342 staining. To confirm the effect of FFO on LPS-induced DNA damage, RAW264.7 cells were seeded into 8-well cell culture slides and treated with different concentrations of FFO [0.125-2% in 0.5% propylene glycol (v/v)] with or without LPS (1 µg/ml) for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Treated cells were fixed with 4% paraformaldehyde for 10 min at room temperature and subsequently stained with Hoechst 33342 (5 µg/ml) for 10 min at room temperature. Cells were washed twice with PBS and observed under a Nikon Eclipse Ni-U fluorescent microscope (original magnification, x40; Nikon Corporation).

DNA damage assay. To further determine the protective effect of FFO on LPS-induced DNA damage, the effect of FFO on DNA impairment was investigated via ELISA. RAW264.7 cells were seeded into 12-well plates at a density of $1x10^5$ cells/ml and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. The culture medium was removed and cells were treated with different concentrations of FFO [0.125-2% in 0.5% propylene glycol (v/v)] with or without LPS (1 µg/ml) for 24h at 37°C in a humidified atmosphere with 5% CO_2.

Table I. Primer sequences and expected amplicon sizes for gene amplification.

cDNA	GenBank accession no.	Forward primer	Reverse primer	Amplicon size (bp)
TNF-α	NM013693.3	5'-ACCTGGCCTCTCTACCTTGT-3'	5'-CCCGTAGGGCGATTACAGTC-3'	161
IL-1β	NM008361.4	5'-GCCACCTTTTGACAGTGATGAG-3'	5'-AGTGATACTGCCTGCCTGAAG-3'	165
IL-6	NM031168.2	5'-CAACGATGATGCACTTGCAGA-3'	5'-TCTCTCTGAAGGACTCTGGCT-3'	201
COX-2	NM011198.4	5'-CCACTTCAAGGGAGTCTGGA-3'	5'-AGTCATCTGCTACGGGAGGA-3'	197
Cyclin D2	NM009829.3	5'-ACCTCCCGCAGTGTTCCTATT-3'	5'-CACAGACCTCTAGCATCCAGG-3'	93
Cyclin E2	NM001037134.2	5'-TCTGTGCATTCTAGCATCGACTC-3'	5'-AAGGCACCATCGTCTACACATTC-3'	149
p27	NM009875.4	5'-GCGGTGCCTTTAATTGGGTCT-3'	5'-GGCTTCTTGGGCGTCTGCT-3'	230
p53	NM011640.3	5'-ACCGCCGACCTATCCTTACC-3'	5'-TCTTCTGTACGGCGGTCTCTC-3'	118
GAPDH	NM001289726.1	5'-TGTGTCCGTCGTGGATCTGA-3'	5'-TTGCTGTTGAAGTCGCAGGAG-3'	150

TNF-α, tumor necrosis factor-α; IL, interleukin; COX-2, cyclooxygenase-2.

Treated cells were centrifuged at 10,000 x g for 20 min at 4˚C. The supernatant was collected and stored at -80˚C for quantification of 8-hydroxy-2'-deoxyguanosine (8-OHdG; cat. no. AB-EIADNAD), a DNA damage marker, using commercial kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted and purified from RAW264.7 cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and reverse transcribed into cDNA using the SensiFAST™ cDNA synthesis kit (Bioline). qPCR was subsequently performed using SYBR Real-Time PCR Master Mix (Bioline) on a CFX Touch real-time PCR system (Bio-Rad Laboratories, Inc.). PCR amplifications were performed at a 20-μl volume with the following thermocycling conditions: A polymerase enzyme activation step at 95˚C for 2 min; followed by 40 cycles of denaturation at 95˚C for 5 sec, 10 sec of annealing at 60˚C depending on primers, and 10 sec of elongation at 72˚C. The primer sequences used for qPCR were purchased from Macrogen, Inc. and used at a final concentration of 0.4 μM. The primer sequences for mouse TNF-α, IL-1β, IL-6, COX2, p53, p27, cyclin D2, cyclin E2 and GAPDH are presented in Table I (20-25). Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (26) and normalized to GAPDH. Data were reported as the relative fold change. qPCR amplification was performed in duplicate for each synthesized cDNA set.

Statistical analysis. Statistical analysis was performed using SPSS version 23 software (IBM Corp.). Values are expressed as the mean ± standard error of the mean. One-way ANOVA followed by Dunnett's test was used to compare differences between multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Fatty acids and vitamins in FFO. As presented in Table II, FFO contained several fatty acids, including saturated, unsaturated, MUFAs and polyunsaturated fatty acid (PUFAs)

Table II. Fatty acid composition and vitamin content of freshwater hybrid catfish oil.

Chemical component	Amount
Saturated fatty acids, g/100 g	40.38±2.94
Unsaturated fatty acids, g/100 g	55.80±0.64
Monounsaturated fatty acids, g/100 g	46.74±2.24
Oleic acid, g/100 g	42.07±1.79
Omega-9	42.27±1.76
Polyunsaturated fatty acids, g/100 g	12.75±1.04
Omega-3	1.17±0.27
Omega-6	10.95±1.03
Vitamins	
Vitamin A (retinol), μg/100 g	1.80±0.12
Vitamin E (α-tocopherol), mg/100 g	0.69±0.06

Values are expressed as means ± standard error of the mean (n=3).

at 40.38, 55.80, 46.74 and 12.75 g/100 g of FFO, respectively. Among the detected MUFAs, the predominant fatty acid was omega-9 (42.27±1.76 g/100 g of FFO). In addition, FFO also contained PUFAs and the predominant fatty acids were omega-3 (1.17±0.39 g/100 g of FFO) and omega-6 (10.95±1.46 g/100 g of FFO). In addition, vitamin A was present at 1.80±0.12 μg/100 g of FFO and vitamin E was present at 0.69±0.06 mg/100 g of FFO.

Heavy metal content profiles of FFO. The concentrations of arsenic, copper, lead, mercury, tin and zinc in FFO are presented in Table III. The results demonstrated that FFO contained copper and lead at much lower concentrations, while arsenic, mercury, tin and zinc were not detected. Moreover, the USA established a recommended dietary allowance of copper for adults at 0.9 mg/day (27).

FFO decreases the secretion of pro-inflammatory cytokines in RAW264.7 cells. To determine the anti-inflammatory effect of FFO in RAW264.7 cells, the levels of pro-inflammatory cytokines were detected via ELISA. As presented in Fig. 1A, LPS
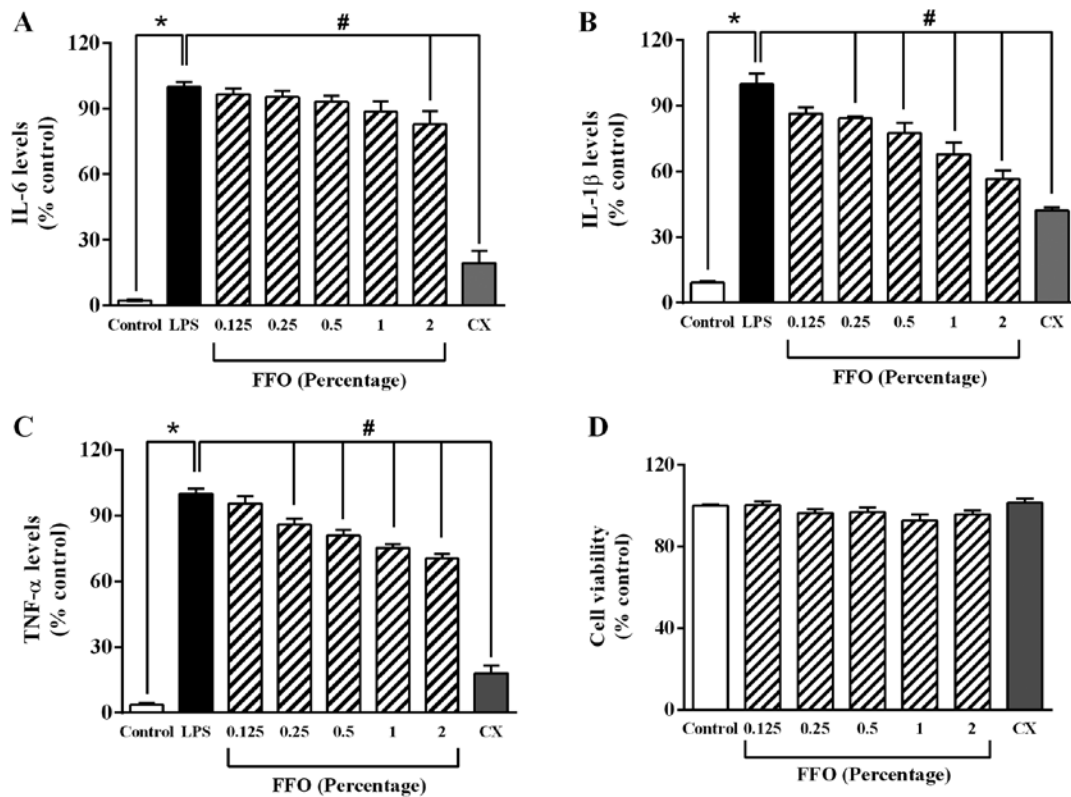
Figure 1. Inflammatory response was inhibited by treatment with FFO in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-treated with different concentrations of FFO in the presence or absence of LPS for 24 h. The production of (A) IL-6, (B) IL-1β and (C) TNF-α was measured by ELISA. (D) Viability of RAW264.7 cells after exposure to different concentrations of FFO with or without LPS for 24 h. Values are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. control; #P<0.05 vs. LPS. IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; FFO, freshwater hybrid catfish oil; CX, celecoxib.

at 1 μg/ml significantly increased the levels of pro-inflammatory cytokines, while added FFO at 2% significantly decreased IL-6 production compared with LPS-treated cells. Added FFO at 0.25-2% also markedly decreased TNF-α and IL-1β expression compared with LPS-treated cells (Fig. 1B and C). Similarly, celecoxib (CX), an NSAID (28), significantly decreased IL-1β expression. However, in the absence of LPS, there was no significant effect of FFO (0.25-2%) and CX on the viability of RAW264.7 cells compared with the control cells (Fig. 1D). Taken together, these results suggested that FFO exerts an anti-inflammatory effect without a cytotoxic effect.

FFO decreases the mRNA expression levels of pro-inflammatory cytokines in RAW264.7 cells. To confirm the inhibitory effect of FFO on inflammation, RT-qPCR analysis was performed to detect the mRNA expression levels of the pro-inflammatory cytokines IL-6, IL-1β and TNF-α in RAW264.7 cells. A single dose (2%) was selected, as it significantly decreased all inflammatory cytokines (Fig. 1). The results demonstrated that the expression levels of the pro-inflammatory cytokines significantly decreased following additional treatment with FFO and CX compared with LPS-treated cells (Fig. 2). Collectively, these results suggested that FFO inhibits the synthesis of pro-inflammatory cytokines in activated macrophages.

FFO suppresses molecules involved in inflammatory signaling. The present study further investigated the molecular

mechanisms by which FFO reduces inflammation and thus, signalling molecules of the inflammatory pathway were assessed. As presented in Fig. 3A, LPS significantly enhanced NO production compared with the control. However, the addition of FFO had no effect on NO production compared with LPS-treated cells. Furthermore, the effects of FFO on the production of PGE2, a principal mediator of inflammation, and COX-2, a prostaglandin-endoperoxide synthase (29), were investigated in the present study. The results demonstrated that FFO significantly decreased COX-2 mRNA expression, which in turn decreased PGE2 production (Fig. 3B and C). Taken together, these results suggested that FFO exerts anti-inflammatory effects on LPS-stimulated RAW264.7 cells.

FFO prevents apoptosis and DNA damage. To validate the inhibitory effect of FFO on cell apoptosis, RAW264.7 cells treated with FFO, with or without LPS for 24 h, were stained with Hoechst 33342. Microscopic observation demonstrated that treatment with LPS increased the rate of cell apoptosis featuring nuclear fragmentation, chromatin condensation and apoptotic body formation compared with the control cells (Fig. 4A and B). However, these features were reduced in FFO- and CX-treated cells (Fig. 4C and D).

In addition, it was investigated whether FFO is able to prevent DNA damage. As presented in Fig. 4E, treatment with LPS markedly increased the production of the 8-OHdG adduct, an oxidative stress-induced DNA damage marker (30), compared with the control cells. It was observed that the production of 8-OHdG induced by LPS significantly decreased
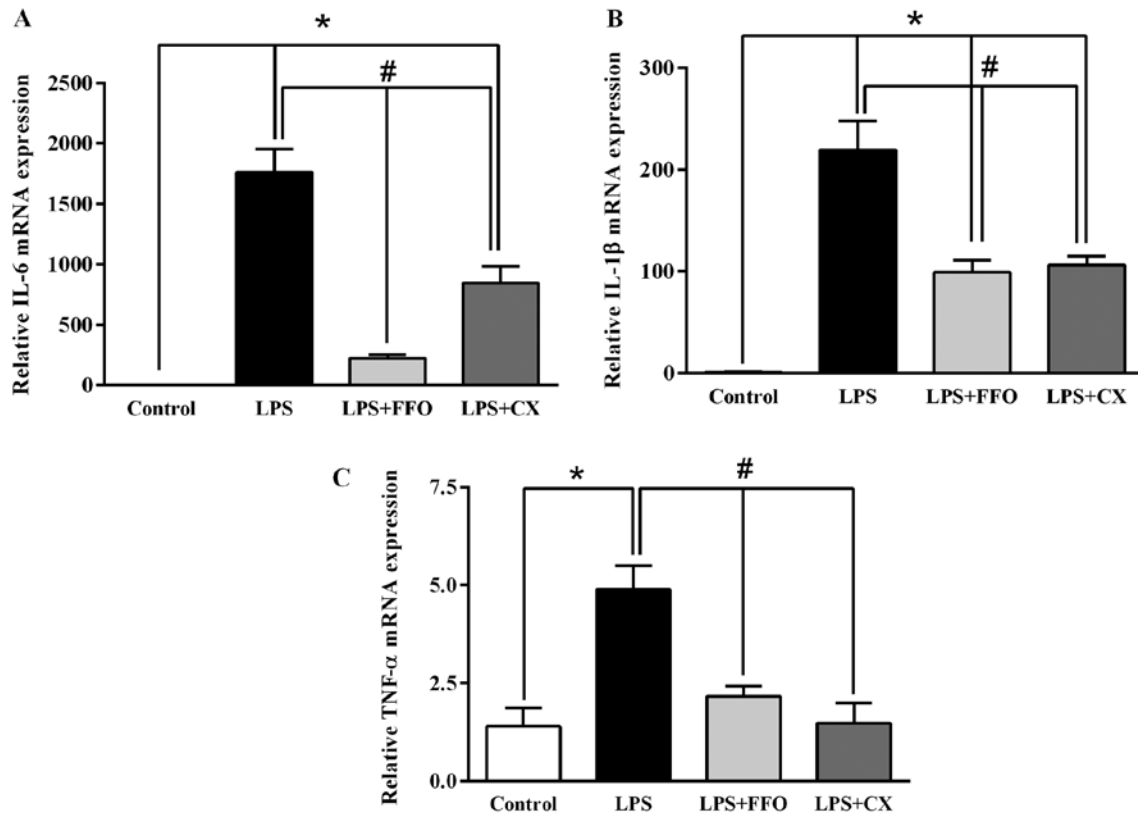
Figure 2. Gene expression of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-treated with different concentrations of FFO in the presence or absence of LPS for 24 h. The expression of (A) IL-6, (B) IL-1β and (C) TNF-α was measured by reverse transcription-quantitative PCR. Values are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. control; #P<0.05 vs. LPS. IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; FFO, freshwater hybrid catfish oil; CX, celecoxib.
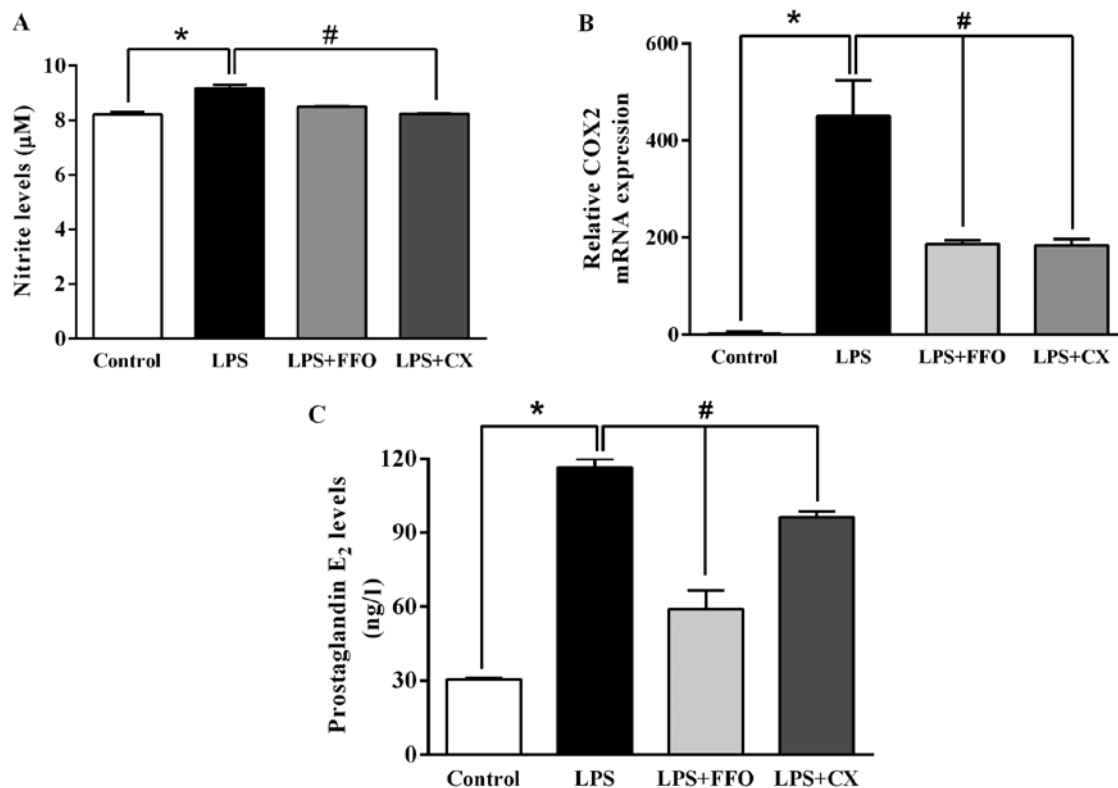

Figure 3. FFO attenuates NO production, COX-2 mRNA expression and PGE2 production in LPS-stimulated RAW264.7 cells. RAW264.7 cells were supplemented with FFO or CX in the presence or absence of LPS for 24 h. The production of (A) NO, (B) expression of COX-2 and (C) PGE2 levels were evaluated. Values are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. control; #P<0.05 vs. LPS. COX-2, cyclooxygenase-2; PGE2, prostaglandin E2 LPS, lipopolysaccharide; NO, nitric oxide; FFO, freshwater hybrid catfish oil; CX, celecoxib.
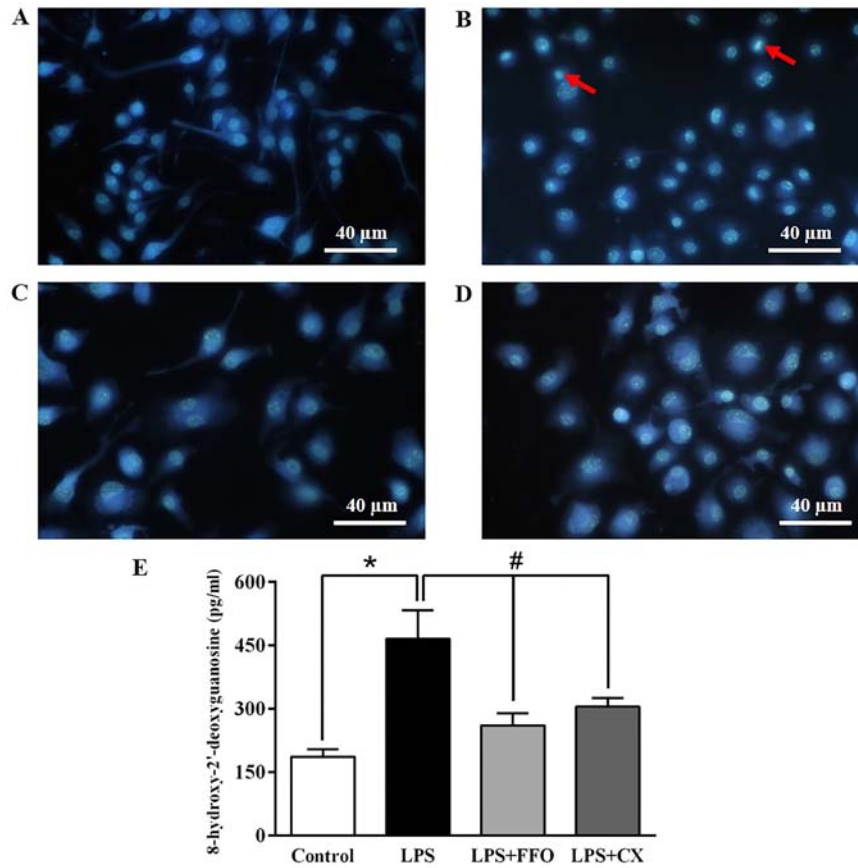
Figure 4. Cytoprotective effect of FFO in LPS-stimulated RAW264.7 cells. Cells were seeded in 12-well plates and treated with FFO or CX in the presence or absence of LPS. After 24 h, cells were treated with Hoechst 33342 at 5 μg/ml for 10 min and then observed under an inverted fluorescence microscope (original magnification, x40; total magnification, x400; scale bar, 40 μm). The cell nucleus changes of apoptotic cells are indicated by red arrows. (A) Control, (B) LPS group, (C) LPS+FFO group and (D) LPS+CX group. (E) The amount of 8-OHdG in the DNA was determined using an 8-OHdG-EIA kit. Values are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. control; #P<0.05 vs. LPS. LPS, lipopolysaccharide; FFO, freshwater hybrid catfish oil; CX, celecoxib.
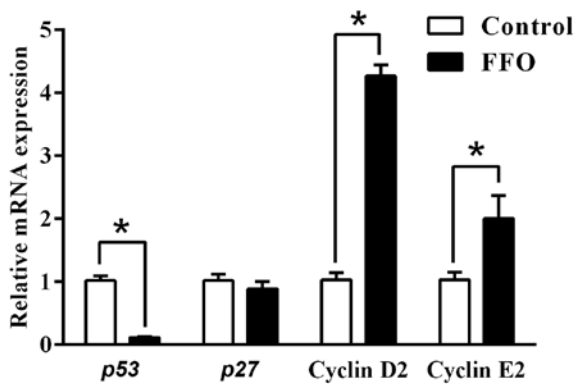

Figure 5. FFO promotes the proliferation of RAW264.7 cells by modulating cell cycle regulators. The gene expression of cell cycle regulators was determined by reverse transcription-quantitative PCR after treatment with FFO for 24 h. Values are expressed as the mean ± standard error of the mean (n=5). *P<0.05 vs. control. LPS, lipopolysaccharide; FFO, freshwater hybrid catfish oil.

Table III. Heavy metal content in freshwater hybrid catfish oil.

Element (symbol)	Amount (mg/kg)
Arsenic (As)	Not detected
Copper (Cu)	<0.50
Lead (Pb)	<0.050
Mercury (Hg)	Not detected
Tin (Sn)	Not detected
Zinc (Zn)	Not detected

in cells co-treated with FFO. Similarly, CX also significantly decreased 8-OHdG levels. Collectively, these results suggested that FFO has a cytoprotective effect.

FFO enhances immune response by modulating cell cycle regulators. A previous study reported that programmed cell death serves an important role in the regulation of inflammation (31). Thus, the current study also investigated the effect of FFO on cell cycle regulators. To identify the effect of FFO that are responsible for enhancing the immune response, the present study also investigated the expression of cell proliferation markers in RAW264.7 cells. As presented in Fig. 5, the gene expression of the cell cycle inhibitors p27 and p53 (32-35) decreased in FFO-treated cells compared with the control. Furthermore, gene expression of the cell cycle inducers cyclin D2 and cyclin E2 increased in RAW264.7 cells treated with FFO. Taken together, these results suggested that FFO improves inflammatory status by modulating cell cycle regulators.

Discussion

To the best of our knowledge, the present study was the first to demonstrate that FFO rich in omega-9 exerts anti-inflammatory effects *in vitro* by decreasing the expression and secretion of pro-inflammatory cytokines and mediators, preventing DNA damage via reduction of apoptotic body formation and 8-OHdG, and also promotes an immune response. A previous study demonstrated that the activation of tissue macrophages releases various pro-inflammatory cytokines, including TNF-α, IL-1 and IL-6, resulting in autoimmune and inflammatory diseases. In addition, n-3 polyunsaturated fatty acids (PUFAs) serve anti-inflammatory effects by reducing the production of TNF-α, IL-1β, IL-6 and tissue factors by stimulated monocytes (36). Thus, inhibiting the synthesis of these cytokines may prove useful for the treatment of autoimmune and inflammatory diseases. The results of the present study demonstrated that FFO markedly decreased the production of IL-6, IL-1β and TNF-α and mRNA expression levels in RAW264.7 cells, similar to NSAIDs. These results suggested that FFO exerts an anti-inflammatory effect by downregulating pro-inflammatory cytokines at both the transcriptional and translational levels, without any cell toxicity. Similarly, oleic acid, one of the most representative monounsaturated omega-9 fatty acids, was reported to mediate anti-inflammatory effects by inhibiting reactive oxygen species, p38 MAPK and Akt signaling pathways/IKK/NF-κB in BV2 cells (37).

Macrophages are associated with acute and chronic inflammatory responses by stimulating NO generation, resulting in an increment of macrophage activity (38). NO and PGE2 production are critical immune-regulatory biomarkers for chronic inflammatory diseases, such as hepatic dysfunction and pulmonary disease (39). The results of the present study demonstrated that FFO decreased PGE2 and its synthase enzyme COX-2, but not the NO level, similar to the action of NSAIDs. Previous studies have reported that natural products, including coumarin, Indonesian cassia extract and *Halocynthia aurantium* or docosahexaenoic acid-omega-3, decrease PGE2 and NO expression levels, which suggests that they have potential as anti-inflammatory agents (40-42). Conversely, it has been demonstrated that omega 3 increases the production of PGE2 (43). The increment of the PGE2 concentration may be inhibited by the NF-κB signaling pathway and EP4 receptor, resulting in anti-inflammatory effects (44). There are controversial data on the effect of PGE2 in inflammation. The results of the present study demonstrated that FFO contains several fatty acids, including omega-3, -6 and -9. Consistently, previous studies have demonstrated that omega-3 fatty acids decrease PGE2 by decreasing the catalytic monomer of COX-1 dimer by arachidonic acid and inhibiting COX-1 oxygenation (45,46). In addition, omega-9 exerts anti-inflammatory effects in inflammation via a PPAR-γ expression-dependent mechanism (47).

It is well-known that there is a close association between inflammation and DNA damage (48). NO generated by inflammatory cytokine stimulation is sufficient to induce oxidative DNA damage (49). The results of the present study demonstrated that LPS induced DNA damage by nuclear fragmentation, chromatin condensation and apoptotic body formation, the effects of which were reversed following treatment with FFO and NSAIDs. Consistently, n-3 polyunsaturated fatty acids attenuate oxidative stress-induced DNA damage

in vascular endothelial cells through upregulation of nuclear factor-mediated antioxidant response and the decrease in intracellular reactive oxygen species (50). In addition, the present study demonstrated that the expression of cell cycle regulators, including cyclin D2 and cyclin E2, increased following treatment with FFO, while p53 expression was inhibited. A previous study reported that cyclin D2 deficiency suppresses immune activity (51). On the other hand, hyperactive cyclin D2 expression promotes autoimmune disease or allograft rejection (52). Other natural products merely promote immune responses by regulating cell cycle regulators. For instance, *A. asphodeloides* enhances the immune response of RAW264.7 cells by extending the cell cycle S-phase, suppressing p27 and increasing cyclin D2 and cyclin E2 gene expression (53).

In conclusion, the results of the present study demonstrated that FFO improved inflammation by suppressing the mRNA expression and secretion of pro-inflammatory cytokines and their mediators, and inhibiting apoptotic body formation and DNA damage. FFO also enhanced the immune response by modulating cell cycle regulators. Thus, FFO may be used as a natural anti-inflammatory supplement. Moreover, future *in vivo* studies and clinical trials are required to elucidate whether FFO has an overall anti-inflammatory effect in autoimmune or inflammatory diseases.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BT performed the experiments, collected and analysed the data, and wrote the first draft of the manuscript. AO designed the experiments, collected and analysed the data and wrote the manuscript. NL and KM provided, analyzed and interpreted the data. DA designed and verified the experiments, analysed the data, and wrote and provided critical feedback for the manuscript. DA and AO confirm the authenticity of all the raw data. All authors read and approved the final version of the study.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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