HOSTED BY
ELSEVIER

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2017.07.019

A single administration of fish oil inhibits the acute inflammatory response in rats

Laura Lícia Milani de Arruda[1], Franciele Queiroz Ames[1], Damila Rodrigues de Morais[2], Renata Grespan[1], Ana Paula Maziero Gil[1], Maria Angélica Raffaini Covas Pereira Silva[1], Jesuí Vergílio Visentainer[2], Roberto Kenji Nakamura Cuman[1], Ciomar Aparecida Bersani-Amado[1]✉

[1]*Department of Pharmacology and Therapeutics, State University of Maringá, Maringá, PR, Brazil*

[2]*Department of Chemistry, State University of Maringá, Maringá, PR, Brazil*

ARTICLE INFO

Article history:
Received 13 May 2017
Received in revised form 18 Jun 2017
Accepted 16 Jul 2017
Available online 22 Aug 2017

Keywords:
Omega-3 fatty acid
Polyunsaturated fatty acid
Pleurisy
Leukocyte migration
EPA
DHA

ABSTRACT

Objective: To investigate the antiinflammatory effects of a single administration of fish oil (FO) on the acute inflammatory response.
Methods: The paw edema and pleurisy models were used to evaluate the effects of FO dissolved in olive oil (FOP) orally administered in a single dose in rats. Nitric oxide (NO) concentrations in the pleural exudate were performed according to the Griess method and the cytokine concentrations were determined by Luminex bead-based multiplex assay.
Results: FOP treatment (30 and 300 mg/kg) significantly reduced paw edema. FOP treatment at 18.75, 37.5, 75.0, 150.0, and 300 mg/kg decreased both the volume of pleural exudate and cellular migration into the pleural cavity and each of these doses presented the same effectiveness. Treatment with FOP (300 mg/kg) reduced NO, TNF-α, IL-1β, and IL-6 concentrations in the pleural exudate.
Conclusions: The present data provide evidence that FO has inhibitory effects on the acute inflammatory response when administered in a single dose in rats. This effect might be attributable to a direct inhibitory effect of FO on the production or release of inflammatory mediators that are involved in the pathological processes evaluated herein.

1. Introduction

Inflammation involves a complex response of vascularized living tissue to harmful stimuli and is often associated with pain. This process involves such characteristic events as increases in vascular permeability, local blood flow, and the migration of leukocytes in an attempt to eliminate the offending agent and heal tissue lesions. However, if these events are not properly resolved, then inflammation can develop into more serious conditions, with consequent increases in the proliferation of granulomatous tissue and loss of organ function [1,2].

The currently available drugs that are used for the clinical treatment of inflammatory diseases include nonsteroidal antiinflammatory drugs (NSAID), steroidal antiinflammatory drugs, biological agents (*e.g.* tumor necrosis factor inhibitors) and immunosuppressive drugs [3]. Unfortunately, these treatments are often ineffective and require high doses or prolonged periods of treatment, thus causing adverse effects on the gastrointestinal tract, kidneys, and liver [4,5]. Thus, the use of natural products or traditional medicines that have favorable therapeutic effects but fewer adverse effects has gained interest in the treatment of inflammatory diseases [6].

Polyunsaturated fatty acids (PUFA) participate in the formation of membrane phospholipids and are important in the prevention and treatment of inflammatory diseases, autoimmune diseases, coronary diseases, hypertension, and arthritis [7–9]. One group of PUFA has a first double bond that is located on the third or sixth carbon atom from the terminal methyl carbon. These groups are referred to as omega-3 (n-3) and omega-6 (n-6), respectively [7].

The animal body lacks desaturase enzymes, which insert double bonds between carbons 3 and 4 and between carbons 6 and 7. Therefore, they are essential for mammals and must be obtained through the diet [7].

First author: Laura Lícia Milani de Arruda, Department of Pharmacology and Therapeutics, State University of Maringá, Maringá, PR, Brazil.
✉Corresponding author: Ciomar Aparecida Bersani-Amado, Department of Pharmacology and Therapeutics, State University of Maringá, Maringá, PR, Brazil.
Tel: +55 44 30115166
E-mail: cabamado@uem.br
Peer review under responsibility of Hainan Medical University.
Foundation project: This study was financially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

The main representatives of the n-3 family are α-linolenic acid (ALA; 18:3n-3), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3). The main representatives of the n-6 family are linoleic acid (LNA; 18:2n-6) and arachidonic acid (ARA; 20:4n-6) [7]. The fatty acids of both families, n-3 and n-6, are found in vegetable oils, whereas the oil that is extracted from certain cold-water fish (*e.g.* sardines, salmon, anchovies) is the richest source of n-3, mainly EPA and DHA.

Omega-3 PUFA have well-established antiinflammatory properties [10,11]. They have also been proposed to have antioxidant actions, especially DHA [12]. Double-blind studies in patients with rheumatoid arthritis who used diet supplementation with n-3 PUFA for a long period of time reported significant improvements in symptoms, with a reduction of the number of swollen joints, pain relief, and a reduction of traditional antiinflammatory drugs use [11,13,14].

However, to our knowledge, although the antiinflammatory actions of n-3 are well established, few studies have been conducted to evaluate the antiinflammatory activity of n-3 PUFA with acute treatment without supplementation in the diet for an extended period of time. Research on the effectiveness of fish oil (FO) is justified because it is the richest natural source of DHA and EPA, with commercially available standards containing high concentrations of n-3 fatty acid formulations. The present study evaluated the effects of a single administration of FO on the acute inflammatory response.

2. Materials and methods

2.1. Animals

A total of 142 male Wistar rats, weighing 200–220 g (7–8 weeks old), were kept in an environment under controlled temperature (22 ± 2) °C and a 12 h/12 h light/dark cycle with free access to a standard pellet diet and water. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the State University of Maringa (ECAE/UEM 045/2012).

2.2. Fish oil preparation

The FO that was used in the present study was commercially obtained from Naturalis (São Paulo, Brazil). The OMEGA 3 DHA 250 product (triglycerides form) was chosen because of its high DHA content. According to the manufacturer, each 500 mg capsule contains 250 mg DHA and 50 mg EPA. For the experiments, the contents of the FO capsule were diluted in olive oil (OO).

Initially, the fatty acids that were present in the FO capsule were quantified to validate the aforementioned information from the manufacturer. The composition of the fatty acids that were present in the FO preparations (FOP) and OO as a diluent was also evaluated. In this assay, two FOP that contained FO (10 and 100 mg/mL) diluted in OO were used. For the calculations that were used for the preparations, we considered the amount of DHA that was contained in the FO capsule (250 mg/capsule).

2.3. Chemical evaluation of lipid profile

Fatty acid methyl esters (FAMEs) were prepared by transesterification of the total lipids as described by Maia and Rodriguez-Amaya [15]. The FAMEs were then separated by gas chromatography with a Thermo 3 300 gas chromatograph fitted with a flame ionization detector and fused-silica CP-7420 (SELECT FAME) capillary column (100 m × 0.25 mm internal diameter and 0.25 μm of cyanopropylpolysiloxane). The operation parameters were the following: detector temperature, 230 °C; injection temperature, 220 °C; column temperature, 185 °C for 10 min, programmed to increase at 4 °C/min up to 235 °C, maintained for 5 min; carrier gas, hydrogen (1.2 mL/min; ultrapure; White Martins, Rio de Janeiro, RJ, Brazil); makeup gas, nitrogen (30 mL/min); split injection at a 1:80 ratio. The percentages were determined by integration of the peak areas using CHROMQUEST 5.0 software (Thermo Fisher Scientific, Inc., Waltham, USA). Individual FAME were identified by comparing the retention times of the samples with standards from Sigma (St. Louis, MO, USA) and coelution with standards of known compositions. The FAMEs were expressed as mole% of fatty acids.

2.4. Induction of paw edema

This experiment was performed to determine the amount of FO that effectively inhibited the inflammatory response. For this assay, FOP (30 and 300 mg/kg; n = 7/group, respectively), OO (n = 5), indomethacin (Indo; 5 mg/kg; n = 5), and water (n = 5) were orally administered in a single dose in different groups of rats, which were fasted for 15 h. The treatment was realized 1 h before induction of paw edema.

Paw edema was induced by intradermally injecting 0.1 mL of a suspension of carrageenan (Cg; 200 μg/paw, dissolved in sterile saline) in one of the hind paws of the rats. In the contralateral paw, an equal volume of saline was injected according to Winter *et al* [16]. The paw volume (in microliters) to the tibial-tarsal joint was measured 1, 2, and 4 h after the carrageenan injection using a digital plethysmograph (Ugo Basile). Paw edema was expressed in terms of the increase in paw volume by subtracting the volume of the paw that received saline (control paw) from the volume of the paw that received carrageenan.

2.5. Induction of pleurisy

In the pleurisy model, the rats were treated with different amounts of FOP to determine whether the difference in intensity of the antiinflammatory effect depended on the amount of FOP that was administered. FOP (18.75, 37.5, 75, 150, and 300 mg/kg; n = 8, 8, 6, 6, and 8/group, respectively), pure fish oil (FO; 300 mg/kg; n = 7), OO (n = 7), indomethacin (5 mg/kg; n = 6), and water (n = 8) were administered orally in a single dose in the different groups of rats, which were fasted for 15 h. A normal animals group (n = 6) without receiving pleural injection of carrageenan was also evaluated.

Pleurisy was induced by injecting 0.25 mL of a suspension of carrageenan (200 μg) into the intrapleural cavity 1 h after the respective treatments according to the technique described by Vinegar *et al* [17]. Carrageenan was diluted in phosphate-buffered saline (PBS; pH 7.4). Four hours later, the animals were anesthetized and sacrificed for the collection of pleural inflammatory exudate. The collected material was transferred to centrifuge tubes, and the total volume was determined. After centrifugation at 2 500 rotations per minute (rpm) for 10 min, the

material was used to determine the number of total and differential leukocytes that were recruited to the pleural cavity using a standard optical microscope.

2.6. Composition of fatty acids in plasma

Rats that were fasted for 15 h were given an oral administration of a single dose of FOP (30 and 300 mg/kg). After 30 min, 1 h, or 2 h, the animals (n = 5 animals in each group) were anesthetized with a solution of 10% ketamine and 2% xylazine at a dose of 40 mg/kg body weight, and blood was collected by puncture of the inferior vena cava using a heparinized syringe. The composition of the fatty acids that were present in the plasma of rats (n = 5) without treatment (zero time) was also evaluated. The blood was centrifuged at 5000 rpm for 15 min at 4 °C, and the plasma was separated and frozen at −80 °C. Subsequently, total lipid (TL) were extracted from the plasma according to the technique described by Folch *et al* [18], and the TL content was determined gravimetrically. The same techniques described in chemical evaluation of lipid profile were used to assess the fatty acids composition in the plasma of rats.

2.7. Measurement of nitric oxide and cytokine concentrations

To determine nitric oxide (NO) and cytokine concentrations in the pleural exudate, groups of rats that were fasted for 15 h were treated orally with FOP (30–300 mg/kg, n = 6 and 8/group, respectively), FO (300 mg/kg; n = 7), OO (n = 7), indomethacin (5 mg/kg; n = 6), or water (n = 8) in a single dose 1 h before pleurisy induction.

Four hours after the induction of pleurisy, the exudates were collected and centrifuged at 2500 rpm for 10 min at 4 °C. The supernatants were stored at −70 °C for the subsequent determination of NO and cytokine concentrations. The determination of the NO concentration was performed according to the Griess method, which determines nitrite production as a measure of gas production [19]. A 50 μL sample of the exudate was placed in a 96-well microplate in quadruplicate, and Griess solution was added (1% sulfanilamide in 5% phosphoric acid and 0.1% dihydrochloride *N*-1-naftiletilonodiamine in water) at room temperature. After 10 min, an enzyme-linked immunosorbent assay plate reader was used at a wavelength of 550 nm. Nitric oxide concentrations were calculated based on a standard curve for sodium nitrite. The results are expressed in μM.

Cytokine concentrations [tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukine-6 (IL-6), and interleukine-10 (IL-10)] were determined in the pleural exudates of the different groups of rats [FOP (300 mg/kg; n = 8), FO (300 mg/kg; n = 7), OO (n = 7), indomethacin (5 mg/kg; n = 6), or water (n = 8)] by Luminex bead-based multiplex assay (Luminex, Austin, TX, USA) according to the manufacturer's instructions.

2.8. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). The data were subject to GraphPad Prism software (version 5.0) and analyzed using analysis of variance (ANOVA) followed by Tukey's test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Composition of fatty acids in fish oil capsule

As shown in Table 1, each capsule of 500 mg FO contained 249.13 and 65.50 mg of DHA and EPA, respectively. These results validate the information that was provided by the manufacturer (Naturalis), in which each 500 mg capsule contained 250 mg DHA and 50 mg EPA.

Table 1
Fatty acid composition (%) present in OO, FO capsule and FOP.

Fatty acids	OO	FO capsule		FOP	
		mg/500 mg	%	10 mg/mL	100 mg/mL
14:0 (myristic acid)	0.00	1.18	0.35	1.89	0.00
14:1n-9 (physeteric acid)	0.00	0.05	0.01	0.00	0.00
15:0 (pentadecylic acid)	0.00	0.28	0.08	0.00	0.00
15:1 (pentadecenoic acid)	0.00	0.01	0.00	0.00	0.00
16:0 (palmitic acid – PAM)	13.16	9.56	2.50	16.13	11.51
16:1n-9 (palmitoleic acid)	0.12	0.23	0.06	0.05	0.08
16:1n-7 (hexadecenoic acid)	1.11	2.04	0.53	1.37	1.02
16:1n-5 (hexadecenoic acid)	0.00	0.43	0.11	0.25	0.00
17:0 (margaric acid)	0.08	2.02	0.50	0.22	0.22
17:1 (heptadecenoic acid)	0.21	0.50	0.13	0.12	0.24
18:0 (stearic acid – STA)	3.17	18.99	4.47	5.36	3.42
18:1n-9 (oleic acid – OLA)	69.25	33.38	7.91	62.37	61.53
18:1n-7 (vaccenic acid)	2.59	7.88	1.86	2.27	2.51
18:2n-6 (linoleic acid – LNA)	7.45	4.34	1.03	6.58	6.25
18:3n-3 (α-linolenic acid – ALA)	0.74	0.65	0.16	0.67	0.65
20:0 (arachidonic acid)	0.42	0.52	0.11	0.61	0.50
20:4n-6 (a acid – ARA)	0.00	9.79	2.15	0.08	0.53
22:0 (behenic acid)	0.00	1.71	0.33	0.18	0.17
20:5n-3 (eicosapentaenoic acid -EPA)	0.00	65.50	14.50	0.28	2.19
24:0 (lignoceric acid)	1.69	12.38	2.25	0.27	0.69
22:5n-6 (docosapentaenoic acid – DPAn-6)	0.00	33.72	6.83	0.00	0.00
22:5n-3 (docosapentaenoic acid – DPAn-3)	0.00	16.44	3.33	0.43	0.42
22:6n-3 (docosahexaenoic acid – DHA)	0.00	249.13	50.79	0.86	8.08

Values were represented as mole % of fatty acids.

3.2. Profile of fatty acids in olive oil and fish oil preparation

The analysis of the FOP that were used to treat the animals (10 and 100 mg/mL) confirmed the presence of EPA and DHA in amounts that were consistent with each preparation. None of these n-3 fatty acids were detected in OO. A high percentage of n-9 oleic acid (OLA) was found in all three of the analyzed samples (Table 1).

3.3. Effect of fish oil on paw edema

The intradermal injection of carrageenan in one of the hind paws in rats (control; treated orally with water) caused a local inflammatory response, with a maximum intensity of edema 4 h after application of the phlogistic agent. Treatment with FOP (30 and 300 mg/kg) reduced paw edema 2 and 4 h after the carrageenan injection [30 mg/kg FOP: 44.6% at 2 h ($P < 0.01$) and 52.4% at 4 h ($P < 0.001$); 300 mg/kg FOP: 39.9% at 2 h ($P < 0.01$) and 50.8% at 4 h ($P < 0.001$)]. Treatment with indomethacin (*i.e.* the antiinflammatory reference drug) at a dose of 5 mg/kg also significantly reduced paw edema 2 and 4 h after the carrageenan injection [58.0% at 2 h ($P < 0.001$) and 73.0% at 4 h ($P < 0.001$)]. Treatment with OO did not alter the development of the inflammatory response compared with the control group (Cg; Figure 1).

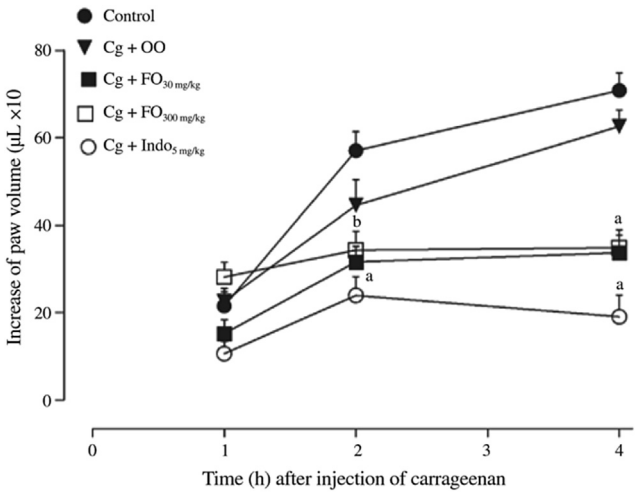

Figure 1. Effect of fish oil on the development of edema induced by intraplantar injection of carrageenan (200 µg) in rats (n = 5–7/group). Each point represents the mean ± SEM paw volume, 1, 2, and 4 h after injection of carrageenan, $^{a}P < 0.001$ and $^{b}P < 0.01$ when compared with the control group (ANOVA, Tukey's test).

3.4. Effect of fish oil on pleurisy

The intrapleural injection of carrageenan induced an acute inflammatory response in rats (control; treated orally with water), characterized by an increase in the volume of pleural exudate and the recruitment of leukocytes into the pleural cavity compared with normal rats that did not receive carrageenan injection. Treatment with FOP at 18.75, 37.5, 75, 150, and 300 mg/kg reduced both the volume of pleural inflammatory exudate (34.3%, 35.4%, 43.3%, 38.1%, and 37.0%, respectively; Figure 2) and the number of leukocytes that were recruited to the pleural cavity (24.4%, 17.3%, 20.8%, 18.9%, and 20.3%, respectively; Table 2). These doses of FOP presented similar effectiveness. Treatment with pure FO at 300 mg/kg also reduced the volume of pleural inflammatory exudate (47.8%) and the recruitment of leukocytes to the pleural cavity (21.7%). Treatment with indomethacin only reduced the volume of pleural exudate by 62.2% (Figure 2), and OO did not affect the inflammatory response compared with the control group (Cg; Figure 2 and Table 2).

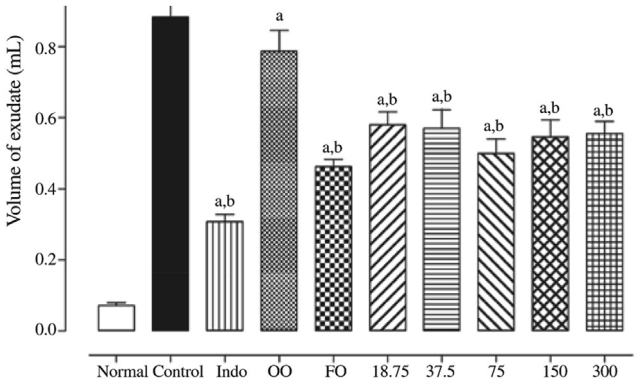

Figure 2. Effect of fish oil on the volume of pleural inflammatory exudate. Each point represents the mean ± SEM the volume of exudate, 4 h after injection of carrageenan in rats (n = 6–8/group). $^{a}P < 0.001$ when compared with the normal group; $^{b}P < 0.001$ when compared with control group (ANOVA, Tukey's test).

Table 2

Effect of fish oil on the total number of leukocytes (cells/mm^3) and differential pleural inflammatory exudate of rats.

Group	n	LEU	PMN	MN
Normal	6	5 200 ± 230	1 560 ± 121	3 640 ± 159
Control	8	73 471 ± 2 536	63 232 ± 2 406	10 238 ± 989
Cg + $Indo_5$	6	71 800 ± 5 530	59 722 ± 6 316	12 078 ± 1 606
Cg + OO	7	74 000 ± 4 145	61 638 ± 4 402	12 362 ± 1 669
Cg + FO_{300}	7	57 528 ± 3 245[a]	47 173 ± 1 552[a]	10 355 ± 1 883
Cg + $FOP_{18.75}$	8	55 528 ± 1 009[a]	48 694 ± 7 962[a]	6 833 ± 716
Cg + $FOP_{37.5}$	8	60 775 ± 2 477[a]	48 494 ± 3 493[a]	12 282 ± 1 913
Cg + FOP_{75}	6	58 167 ± 7 965[a]	50 903 ± 7 288	7 263 ± 679
Cg + FOP_{150}	6	59 600 ± 5 221[a]	49 854 ± 4 872[a]	9 746 ± 1 944
Cg + FOP_{300}	8	58 556 ± 3 118[a]	47 937 ± 2 308[a]	10 619 ± 1 294

Each point represents the mean ± SEM white blood cell count (cells/mm^3), 4 h after injection of carrageenan (Cg). LEU = total leukocytes; PMN: polymorphonuclear leukocytes; MN: mononuclear leukocytes. $^{a}P < 0.05$ when compared with the control group (ANOVA, Tukey's test).

Based on these findings, one issue is whether the antiinflammatory activity of FOP was the same regardless of the amount administered. Another issue was whether the absorption profile of FO would be the same if it was administered at an amount that is 10-times higher. To address these issues, we performed the following experiments.

3.5. Fatty acid profile in plasma

As shown in Table 3, in plasma of rats without treatment we detected the presence of various fatty acids, and ARA and LNA were the most abundant, followed by PAM, and OLA. The high percentage of ARA (n-6 fatty acid) at zero time may have been attributable to the effect of the diet, which was rich in n-6 fatty acids [20,21].

Table 3
Fatty acid profile in plasma of rats.

Fatty acids	Zero-time	FOP 30 mg/kg			FOP 300 mg/kg		
		30 min	1 h	2 h	30 min	1 h	2 h
14:0 (myristic acid)	0.59	0.53	0.60	0.49	0.59	0.54	0.61
14:1n-9 (physeteric acid)	0.00	0.01	0.01	0.04	0.01	0.02	0.04
15:0 (pentadecylic acid)	0.48	0.45	0.50	0.56	0.42	0.39	0.34
15:1 (pentadecenoic acid)	0.00	0.02	0.03	0.02	0.04	0.04	0.03
16:0 (palmitic acid – PAM)	22.70	18.92	20.14	21.23	19.57	18.41	20.49
16:1n-9 (palmitoleic acid)	1.25	0.38	0.29	0.39	0.32	0.30	0.55
16:1n-7 (hexadecenoic acid)	0.25	0.07	0.02	1.36	1.78	1.43	1.78
16:1n-5 (hexadecenoic acid)	0.00	0.00	0.00	0.03	0.04	0.06	0.07
17:0 (margaric acid)	0.47	0.54	0.41	0.46	0.51	0.40	0.48
17:1 (heptadecenoic acid)	0.00	8.31	9.31	7.41	6.61	5.89	12.57
18:0 (stearic acid – STA)	8.39	8.97	8.77	8.16	8.36	7.85	8.62
18:1n-9 (oleic acid – OLA)	14.00	12.21	18.46	24.27	12.30	17.58	21.22
18:1n-7 (vaccenic acid)	1.80	1.66	1.84	1.72	1.46	1.76	1.73
18:2n-6 (linoleic acid – LNA)	24.20	19.95	19.87	19.63	20.82	21.13	15.38
18:3n-3 (α-linolenic acid – ALA)	1.09	0.88	0.81	0.73	1.21	1.00	1.07
20:0 (arachidic acid)	0.00	0.18	0.27	0.35	0.28	0.21	0.36
20:4n-6 (arachidonic acid – ARA)	22.20	23.06	16.38	10.80	21.34	18.98	10.19
22:0 (behenic acid)	0.00	0.05	0.05	0.05	0.07	0.08	0.02
20:5n-3 (eicosapentaenoic acid – EPA)	0.38	0.53	0.35	0.40	0.99	0.76	1.14
24:0 (lignoceric acid)	0.34	0.56	0.29	0.23	0.44	0.37	0.15
22:5n-3 (docosapentaenoic acid – DPAn-3)	0.39	0.83	0.34	0.25	0.79	0.66	0.24
22:6n-3 (docosahexaenoic acid – DHA)	1.46	1.88	1.23	1.41	2.06	2.13	2.91

Rats (n = 5/group) were treated with FOP, in amounts of 30 and 300 mg/kg body weight. The evaluation was performed 30 min, 1 h, and 2 h after oral administration of FOP. Zero time represent composition of the fatty acids present in the plasma of rats without treatment. The values are expressed as mole % of fatty acids.

The profiles of the n-3 fatty acids showed that although the 300 mg amount was 10-times larger than 30 mg, the percentage of EPA was only two-times larger, and the DHA content was the same 30 min after the respective treatments. However, in rats those were treated with the highest amount of FOP, the percentage of these fatty acids in plasma remained higher at the first and second hour after administration (Table 3).

3.6. Effect of fish oil on nitric oxide concentration in pleural exudates

The carrageenan injection in the intrapleural cavity in rats led to an increase in nitrite (NO_2) levels compared with normal rats that did not receive carrageenan injection in the pleural cavity. The increase in NO_2 levels indirectly reflects the production of NO in the inflammatory exudate. Four hours after induction of the inflammatory response, treatment with FO (300 mg/kg), FO diluted in OO (FOP – 30 and 300 mg/kg), OO, and indomethacin reduced the levels of NO_2 in the exudates compared with control group –Cg (Figure 3).

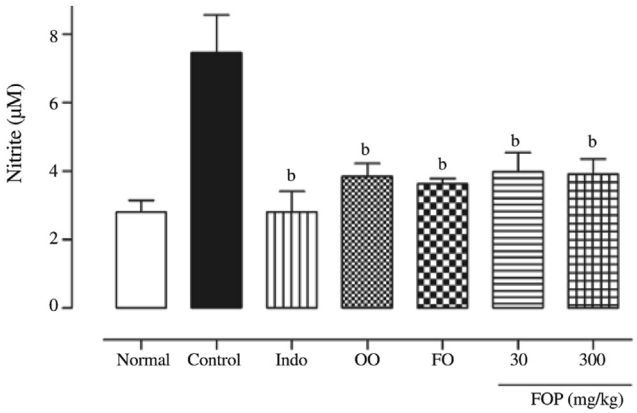

Figure 3. Effect of fish oil on nitrite concentration in the pleural exudate of rats (n = 6–8/group).
Each column represents the mean ± SEM of nitrite concentration, 4 h after injection of carrageenan. [a]$P < 0.001$ when compared with the normal group, [b]$P < 0.001$ when compared with control group (ANOVA, Tukey's test).

3.7. Effect of fish oil on cytokine concentrations in pleural exudates

The concentrations of TNF-α, IL-1β, IL-6, and IL-10 were significantly elevated in the inflammatory exudate in rats that received an intrapleural injection of carrageenan compared with normal rats that did not receive carrageenan injection in the pleural cavity (Figure 4). Treatment with FO and FO diluted in OO (FOP) at a dose of 300 mg/kg did not alter the levels of IL-10 in the exudates (Figure 4D). Treatment with FO and FOP at a dose of 300 mg/kg reduced TNF-α, IL-1β, and IL-6 concentrations in the pleural inflammatory exudates 4 h after the intrapleural injection of carrageenan (Figure 4A, 4B and 4C). The reference antiinflammatory drug indomethacin reduced the concentrations of IL-1β and IL-6 (Figure 4B, 4C) but did not alter the concentration of TNF-α or IL-10 (Figure 4A, 4D). OO only reduced the concentration of IL-1β (Figure 4B).

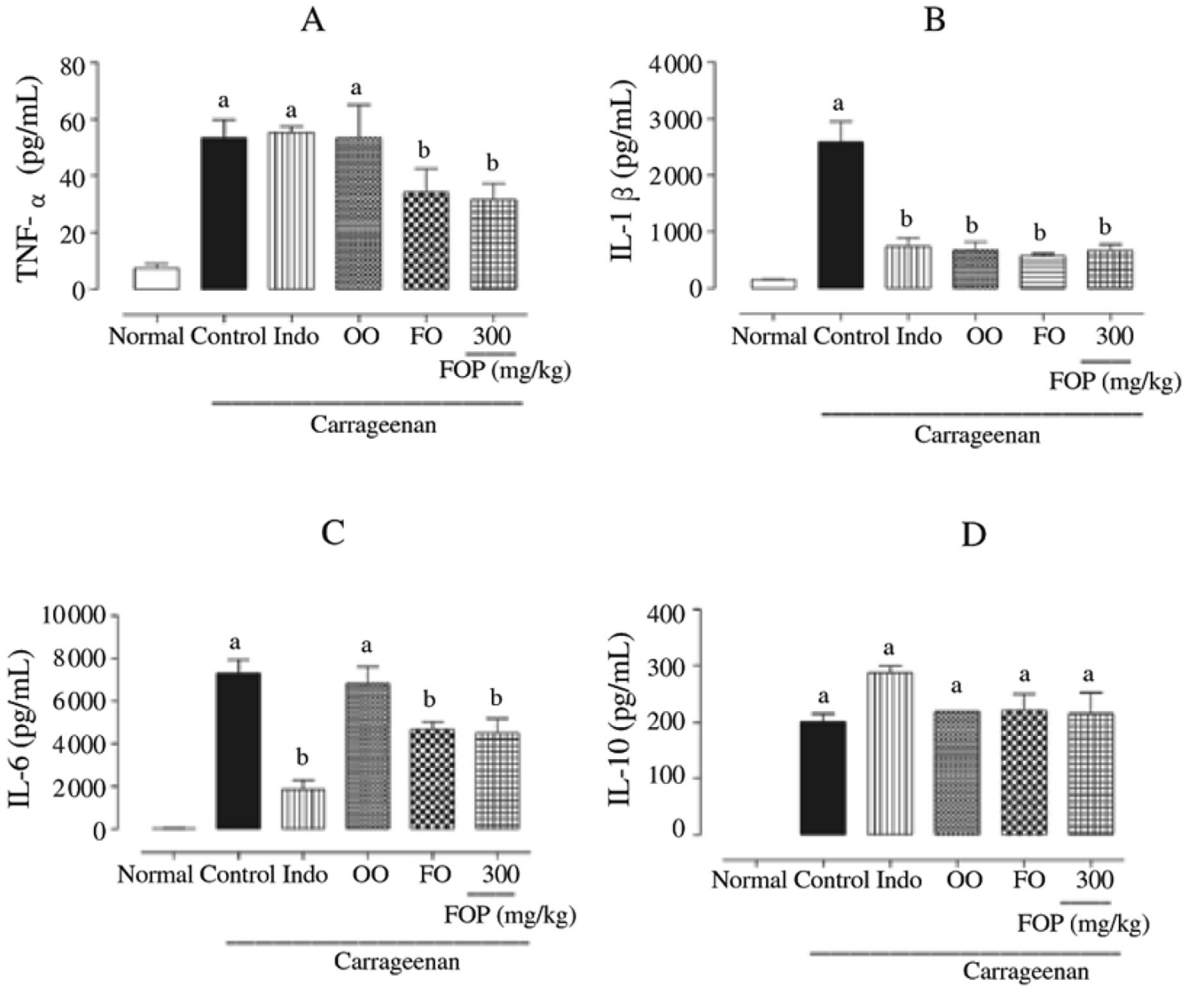

Figure 4. Effect of fish oil on the levels of TNF-α (A), IL-1β (B), IL-6 (C) and IL-10 (D) in pleural exudate of rats (n = 6–8/group). Each point represents the mean levels of TNF-α, IL-1β, IL-6 and IL-10 ± SEM, 4 h after injection of carrageenan. [a]$P < 0.001$ when compared with the normal group. [b]$P < 0.001$ when compared with the control group (ANOVA, Tukey's test).

4. Discussion

The present study showed that oral FOP administration in rats in a single dose reduced edema and leukocyte recruitment in models of paw edema and pleurisy. These effects may be at least partially related to a significant decrease in the inflammatory mediators concentrations (*e.g.* NO, TNF-α, IL-1β, and IL-6), as noted in pleural exudates obtained from FOP-treated animals.

Carrageenan-induced paw edema is an effective model that has often been used to evaluate the anti-edematous effects of compounds that act on the acute phase of inflammation [22]. In this model, treatment with FOP at doses of 30 and 300 mg/kg similarly reduced the formation of edema. We also investigated the effects of treatment with FOP at doses of 18.75, 37.5, 75, 150, and 300 mg/kg on other experimental model of acute inflammation (*i.e.* pleurisy) to determine whether a similar response pattern would be observed. Carrageenan-induced pleurisy is a model has been well characterized and allows the determination of edema formation, cell migration, and inflammatory mediators that are involved in this process [23]. We found that all of the FOP doses tested (18.75, 37.5, 75, 150, and 300 mg/kg) similarly inhibited edema and cellular recruitment. This effect may result from inhibitory actions of FOP on some mediators involved in the inflammatory response, such as histamine, bradykinin, lipid mediators derived from ARA, NO, and cytokines [22,24].

The reduction of the number of leukocytes that were recruited to the pleural cavity following FOP treatment was an important finding because the migration of cells to the site of inflammation is a crucial step in the organism's defense. If such leukocyte mobilization intensifies, then the accumulation of these cells can damage tissue, depending on the activity of metalloproteinases and generation of reactive oxygen/nitrogen species. Thus, antiinflammatory agents that limit neutrophil migration to the site of injury are often important to obtain clinical benefits [25].

Previous studies have demonstrated the ability of n-3 PUFA to decrease adhesion molecule expression on leukocytes and endothelial cells [26,27]. Dietary FO has also been shown to reduce leukocyte migration that is caused by the chemotactic agents leukotriene B4 (LTB4) and formyl-methionyl-leucyl-phenylalanine (FMLP) [28]. Thus, the inhibitory effects of FOP on leukocyte recruitment might be related to its ability to reduce adhesive interactions with leukocytes and the production of chemotactic mediators, such as NO and cytokines, or through direct effects on the signaling cascade of these mediators.

The present results showed that FOP effectively inhibited the levels of NO, TNF-α, IL-1β, and IL-6 in pleural exudates. In turn, indomethacin reduced the concentrations of NO, IL-1β and IL-6, but did not alter the concentration of TNF-α. These data suggest differences in the action of FOP and indomethacin on the mediators involved in the carrageenan-induced pleurisy. NO is

involved in the acute inflammatory process through its ability to increase vascular permeability and modulate the adhesion and migration of leukocytes [29]. Therefore, the reductions of NO levels may also partially could explain the inhibitory effects of FOP on exudate formation and cellular recruitment. During inflammation, cytokines such as TNF-α and IL-1β, mediate an intercellular interaction between infiltrating leukocytes and endothelium promoting leukocyte recruitment. These cytokines act on endothelial cell receptors to induce NO production/release, thus initiating a cascade that induces the production of other cytokines and chemokines and the expression of cellular adhesion molecules in neutrophils [25]. The decreases in TNF-α and IL-1β levels that were observed in the present study may explain the reductions of the levels of NO and other cytokines, such as IL-6. In turn, the cytokine IL-6 regulates neutrophil clearance during acute inflammation through the specific down-regulation of the production of neutrophil-attracting chemokines [30]. The observed inhibitory effect of FOP on the production of proinflammatory cytokines (*i.e.* TNF-α, IL-1β, and IL-6) may have led to a decrease in the influx of neutrophils and consequently the extent of injury. The increases in the IL-10 levels (anti-inflammatory cytokine) observed in the inflammatory exudate of the rats that received an intrapleural injection of carrageenan can be explained as a regulatory mechanism on the production of proinflammatory mediators (cytokines, chemokines, nitric oxide and prostaglandins) [31]. Treatments with FO, FOP and indomethacin did not change the increased IL-10 levels, which can contribute to the observed anti-inflammatory effect.

The inhibitory effects of FOP on carrageenan-induced paw edema and pleurisy occurred independently of the dose administered. These data associated with those showing the plasmatic fatty acid profile at different time points (30 min, 1 h, and 2 h) after oral FO administration (doses of 30 and 300 mg/kg), suggest that FOP may exert a maximal effect, and highest doses may not necessary.

The choice of OO to dilute the FO was based on a series of studies that evaluated the benefits of FO in rheumatoid arthritis [32,33]. Although the results of the some studies highlighted the beneficial effects of OO to ameliorate the symptoms of rheumatoid arthritis [34,35], in the present study it was evidenced that the OO utilized in FOP preparation is not responsible for the antiinflammatory effect observed. Such evidence was supported by an pleurisy assay performed with a group of rats orally pretreated with FO (300 mg/kg) without the OO addition. The observed effect was of the same magnitude to that effect of the FOP preparation at the same dose (300 mg/kg).

The literature suggests that EPA and DHA (*i.e.* n-3 PUFA that are abundant in FO) are able to inhibit the inflammatory response [7,10]. However, to our knowledge, most studies that have reported the antiinflammatory activity of FO, both in humans and in animals, were performed using a dietary supplement in higher quantities for an extended period of time (several days to months). In these studies, antiinflammatory activity was generally explained by the fact that the increase in consumption of these fatty acids results in incorporation of the cellular membrane into phospholipids [36,37], thus lowering the levels of ARA and consequently the amount of available substrate for eicosanoid synthesis, including prostaglandin E_2 (PGE_2), thromboxane A_2 (TXA_2), leukotriene B_4 (LTB_4), and 5-hydroxyeicosatetraenoic acid (HETE) [7,10]. Additionally, EPA is able to act as a substrate for the enzymes cyclooxygenase (COX) and 5-lipoxygenase (5-LOX), giving rise to eicosanoids with different structures (PGE_3, TXA_3, LTB_5, LTE_5) from those that are formed from ARA [7,10]. Generally, mediators that are produced from n-3 PUFA via COX and 5-LOX are considered less potent with regard to inflammatory and chemoattractant activity. However, one possibility is that the formation of these mediators reduces the effective concentration of ARA-derived eicosanoids, resulting in a less-favorable inflammatory environment [7,10]. Massaro [38] suggested that phospholipids that are derived from EPA and DHA can be metabolized by a second pathway, leading to the formation of compounds that stimulate the resolution of inflammation (E-series resolvins and D, derived from EPA and DHA, respectively and protectins derived only from DHA).

Thus, these studies cannot fully explain the present data, in which the inhibitory effect on acute inflammation occurred after treating the animals with a single dose of FO. It is considered that the incorporation of n-3 PUFA into membrane phospholipids of cells involved in inflammation occurs in a dose and time dependent fashion, with a steady-state composition reached within about 4 weeks [10].

Other studies corroborate our results, showing that n-3 PUFA interfere with the synthesis of peptide mediators (*e.g.* cytokines), as observed in the present study. The antiinflammatory effects observed thereafter may not necessarily be related to the profile of lipid mediators that are produced, but the changes in gene expression caused by a direct effects the fatty acids in the signaling pathways, leading to activation of the transcription of these mediators [39,40].

In summary, the present study provided evidence that FO has significant antiinflammatory activity when orally administered in a single dose in animals. This activity may be at least partially attributable to a direct inhibitory effect of FO on the production and/or release of mediators that are involved in the inflammatory process.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

The authors are grateful for (a) the technical assistance of Jaílson Araujo Dantas and Célia Regina Miranda, (b) the financial support of the Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

References

[1] Nathan C. Points of control in inflammation. *Nature* 2002; **420**(6917): 846-852.
[2] Wallach D, Tae-Bong K, Kovalenko A. Concepts of tissue injury and cell death in inflammation: a historical perspective. *Nat Rev Immunol* 2014; **14**(1): 51-59.
[3] Kumar DA, Chopra B, Dass R, Kumar MS. An update on anti-inflammatory compounds: a review. *Antiinflamm Antiallergy Agents Med Chem* 2015; **14**(2): 81-97.
[4] Batlouni M. Nonsteroidal anti-inflammatory drugs: cardiovascular, cerebrovascular and renal effects. *Arq Bras de Cardiol* 2010; **94**(4): 522-563.
[5] McCarberg BH, Cryer B. Evolving therapeutic strategies to improve nonsteroidal anti-inflammatory drug safety. *Am J Ther* 2014; **22**: e167-e178.

[6] Koeberle A, Werz O. Multi-target approach for natural products in inflammation. *Drug Discov Today* 2014; **19**(12): 1871-1882.
[7] Yates CM, Calder PC, Rainger GE. Pharmacology and therapeutics of omega-3 polyunsaturated fatty acids in chronic inflammatory disease. *Pharmacol Ther* 2014; **141**(3): 272-282.
[8] Tortosa-Caparrós E, Navas-Carrillo D, Marín F, Orenes-Piñero E. Anti-inflammatory effects of omega 3 and omega 6 polyunsaturated fatty acids in cardiovascular disease and metabolic syndrome. *Crit Rev Food Sci Nutr* 2017; **57**(16): 3421-3429.
[9] Moro K, Nagahashi M, Ramanathan R, Takabe K, Wakai T. Resolvins and omega three polyunsaturated fatty acids: clinical implications in inflammatory diseases and cancer. *World J Clin Cases* 2016; **4**(7): 155-164.
[10] Calder PC. Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance. *Biochim Biophys Acta* 2015; **1851**(4): 469-484.
[11] Ellulu MS, Khaza'ai H, Abed Y, Rahmat A, Ismail P, Ranneh Y. Role of fish oil in human health and possible mechanism to reduce the inflammation. *Inflammopharmacology* 2015; **23**(2–3): 79-89.
[12] Di Nunzio M, Valli V, Bordoni A. PUFA and oxidative stress. Differential modulation of the cell response by DHA. *Int J Food Sci Nutr* 2016; **67**(7): 834-843.
[13] Proudman SM, James MJ, Spargo LD, Metcalf RG, Sullivan TR, Rischmueller M, et al. Fish oil in recent onset rheumatoid arthritis: a randomised, double-blind controlled trial within algorithm-based drug use. *Ann Rheum Dis* 2015; **74**(1): 89-95.
[14] Veselinovic M, Vasiljevic D, Vucic V, Arsic A, Petrovic S, Tomic-Lucic A, et al. Clinical benefits of n-3 PUFA and ɤ-linolenic acid in patients with rheumatoid arthritis. *Nutrients* 2017; **9**(4): 325.
[15] Maia EL, Rodriguez-Amaya DB, Franco MRB. Fatty acids of the total, neutral, and phospholipids of the Brazilian freshwater fish *Prochilodus scrofa*. *J Food Comp Anal* 1994; **7**(4): 240-251.
[16] Winter CA, Risley EA, Nuss GV. Carragenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc Soc Exp Biol* 1962; **111**: 544-547.
[17] Vinegar R, Truax JF, Selph JL. Some quantitative temporal characteristics of carrageenin-induced pleurisy in the rat. *Proc Soc Exp Biol Med* 1973; **143**(3): 711-714.
[18] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 1957; **226**(1): 497-509.
[19] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate and nitrite [^{15}N] nitrate in biological fluids. *Anal Biochem* 1982; **126**(1): 131-138.
[20] Kasdallah-Grissa A, Nakbi A, Koubaa N, El-Fazaâ S, Gharbi N, Kamoun A, et al. Dietary virgin olive oil protects against lipid peroxidation and improves antioxidant status in the liver of rats chronically exposed to ethanol. *Nutr Res* 2008; **28**(7): 472-479.
[21] Cintra DE, Costa AV, Peluzio Mdo C, Matta SL, Silva MT, Costa NM. Lipid profile of rats fed high-fat diets based on flaxseed, peanut, trout, or chicken skin. *Nutrition* 2006; **22**(2): 197-205.
[22] Morris CJ. Carrageenan-induced paw edema in the rat and mouse. *Methods Mol Biol* 2003; **225**: 115-121.
[23] Moore AR. Pleural models of inflammation: immune and non-immune. *Methods Mol Biol* 2003; **225**: 123-128.
[24] Wohlers M, Xavier RA, Oyama LM, Ribeiro EB, do Nascimento CM, Casarini DE, et al. Effect of fish or soybean oil-rich diets on bradykinin, kallikrein, nitric oxide, leptin, corticosterone and macrophages in carrageenan stimulated rats. *Inflammation* 2005; **29**(2–3): 81-89.
[25] Fattori V, Amaral FA, Verri WA. Neutrophils and arthritis: role in disease and pharmacological perspectives. *Pharmacol Res* 2016; **112**: 84-98.
[26] Huang CY, Sheu WHH, Chiang AN. Docosahexaenoic acid and eicosapentaenoic acid suppress adhesion molecule expression in human aortic endothelial cells via differential mechanisms. *Mol Nutr Food Res* 2015; **59**(4): 751-762.
[27] Yoshihara T, Shimada K, Fukao K, Sai E, Sato-Okabayashi Y, Matsumori R. Omega 3 polyunsaturated fatty acids suppress the development of aortic aneurysms through the inhibition of macrophage-mediated inflammation. *Circ J* 2015; **79**(7): 1470-1478.
[28] Estevão-Silva CF, Ames FQ, de Souza Silva-Comar FM, Kummer R, Tronco RP, Cuman RKN, et al. Fish oil and adjuvant-induced arthritis: inhibitory effect on leukocyte recruitment. *Inflammation* 2016; **39**(1): 320-326.
[29] Cirino G, Distrutti E, Wallace JL. Nitric oxide and inflammation. *Inflamm Allergy Drug Targets* 2006; **5**(2): 115-119.
[30] Fielding CA, McLoughlin RM, McLeod L, Colmont CS, Najdovska M, Grail D, et al. IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. *J Immunol* 2008; **181**(3): 2189-2195.
[31] Cuzzocrea S, Mazzon E, Calabro G, Dugo L, De Sarro A, Van De Loo FA, et al. Inducible nitric oxide synthase-knockout mice exhibit resistance to pleurisy and lung injury caused by carrageenan. *Am J Respir Crit Care Med* 2000; **162**(5): 1859-1866.
[32] Kremer JM, Lawrence DA, Jubiz W, DiGiacomo R, Rynes R, Bartholomew LE, et al. Dietary fish oil and olive oil supplementation in patients with rheumatoid arthritis: clinical and immunologic effects. *Arthritis Rheum* 1990; **33**(6): 810-820.
[33] Soeken KL, Miller SA, Ernst E. Herbal medicines for the treatment of rheumatoid arthritis: a systematic review. *Rheumatol Oxf* 2003; **42**(5): 652-659.
[34] Aparicio-Soto M, Sánchez-Hidalgo M, Rosillo MÁ, Castejón ML, Alarcón-de-la-Lastra C. Extra virgin olive oil: a key functional food for prevention of immune-inflammatory diseases. *Food Funct* 2016; **7**(11): 4492-4505.
[35] Bahadori S, Salamzadeh J, Kamalinejad M, Ardekani MRS, Keshavarz M, Ahmadzadeh A. Study of the effect of an oral formulation of fig and olive on rheumatoid arthritis (RA) remission indicators: a randomized clinical trial. *Iran J Pharm Res* 2016; **15**(3): 537-545.
[36] Flock MR, Skulas-Ray AC, Harris WS, Gaugler TL, Fleming JA, Kris-Etherton PM. Effects of supplemental long-chain omega-3 fatty acids and erythrocyte membrane fatty acid content on circulating inflammatory markers in a randomized controlled trial of healthy adults. *Prostagl Leukot Essent Fat Acids* 2014; **91**(4): 161-168.
[37] Healy DA, Wallace FA, Miles EA, Calder PC, Newsholme P. The effect of low-to-moderate amounts of dietary fish oil on neutrophil lipid composition and function. *Lipids* 2000; **35**(7): 763-768.
[38] Massaro M, Scoditti E, Carluccio MA, De Caterina R. Basic mechanisms behind the effects of n-3 fatty acids on cardiovascular disease. *Prostagl Leukot Essent Fat Acids* 2008; **79**(3–5): 109-115.
[39] Huang Q, Wang T, Wang HY. Ginsenoside Rb2 enhances the anti-inflammatory effect of ω-3 fatty acid in LPS-stimulated RAW264.7 macrophages by upregulating GPR120 expression. *Acta Pharmacol Sin* 2017; **38**(2): 192-200.
[40] Inoue T, Tanaka M, Masuda S, Ohue-Kitano R, Yamakage H, Muranaka K, et al. Omega-3 polyunsaturated fatty acids suppress the inflammatory responses of lipopolysaccharide-stimulated mouse microglia by activating SIRT1 pathways. *Biochim Biophys Acta* 2017; **1862**(5): 552-560.