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Eicosapentaenoic acid and docosahexaenoic acid exert anti-inflammatory and antinociceptive effects in rodents at low doses

Maria Elizabeth Pereira Nobre[a,b], *Alyne Oliveira Correia*[a], *Marília de Brito Borges*[a], *Thayga Maria Araújo Sampaio*[a], *Solon Arcoverde Chakraborty*[a], *Danilo de Oliveira Gonçalves*[c], *Gerly Anne de Castro Brito*[c], *Luzia Kalyne Almeida Moreira Leal*[c], *Cícero Francisco Bezerra Felipe*[a], *Daniel Luna Lucetti*[a], *Ricardo Mário Arida*[b], *Glauce Socorro de Barros Viana*[a,c,*]

[a] *Faculty of Medicine Estácio of Juazeiro do Norte – FMJ, Juazeiro do Norte, Brazil*
[b] *Federal University of São Paulo – UNIFESP, São Paulo, Brazil*
[c] *Federal University of Ceará – UFC, Fortaleza, Brazil*

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ABSTRACT

In the present study, we evaluated omega-3 polyunsaturated fatty acid (PUFA) (consisting of 20:5n-3 and 22:6n-3) properties on inflammation and nociception. Among the in vivo tests, writhing, formalin, and hot plate tests were conducted in mice, and carrageenan-induced paw edema, peritonitis, and Hargreaves tests were performed in rats. Following the carrageenan-induced edema, immunohistochemistry for tumor necrosis factor-α (TNF-α) was also carried out. We found that omega-3 PUFA treatment significantly decreased acetic acid–induced abdominal contortions as well as the first and second phases of the formalin test, which were reversed by naloxone. The carrageenan-induced rat paw edema was significantly reduced, along with neutrophil migration to the peritoneal cavity in the omega-3 PUFA treatment. In addition, there was a decrease in TNF-α immunostained cells in the inflamed paw with the omega-3 treatment compared with no omega-3. Withdrawal threshold in response to the thermal stimulation was significantly increased by the omega-3 treatment in the Hargreaves and hot plate tests. The in vitro studies (myeloperoxidase, lactate dehydrogenase, MTT cell viability and lipid peroxidation assays) were performed in human neutrophils. These studies showed that omega-3 treatment significantly decreased myeloperoxidase release, presented no cytotoxicity, and did not alter lipid peroxidation. Our study suggests that omega-3 PUFA anti-inflammatory and antinociceptive actions may involve inhibition of cyclooxygenases and microglial activation, leading to a reduced release of proinflammatory cytokines such as TNF-α, among other factors. The omega-3 PUFAs are potential candidates used alone or in combination with conventional nonsteroidal anti-inflammatory drugs, for the treatment of diseases where inflammation plays an important role.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MPO, myeloperoxidase; MTT, 3-(4 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; PMA, phorbol myristate acetate; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid reactive substances; TNF-α, tumor necrosis factor-α.

* *Corresponding author.*
E-mail address: gviana@live.com (G.S.B. Viana).

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1. Introduction

Omega-3 refers to a family of polyunsaturated fatty acids (PUFAs) among which the most nutritionally notable ones are α-linolenic acid (18:3n-3), eicosapentaenoic acid (EPA) (20:5n-3), and docosahexaenoic acid (DHA) (22:6n-3). The body converts α-linolenic acid to EPA and then to DHA. The formulation used in the present work is a combination of EPA to DHA, that is, a direct and efficient way to increase long-chain omega-3 PUFA body levels (eg, by consuming EPA- and DHA-rich foods or supplements) [1]. Eicosapentaenoic acid is converted to prostaglandins (PGs) in the body to regulate cell activity and maintain healthy cardiovascular functions. Docosahexaenoic acid is one of the products of omega-3 PUFA metabolism and the main n-3 PUFA in the brain and nervous tissues [2].

The anti-inflammatory properties of EPA as well as DHA have been supported by numerous clinical studies, such as those focusing on maintaining normal blood cholesterol levels, controlling depression, and preventing stroke and cancer [3]. Docosahexaenoic acid is another long-chain omega-3 PUFA, found in abundance in fish and some algae. It is the predominant omega-3 PUFA, not only in the brain, but also in the retina, and an adequate supply of DHA is essential for proper brain, eye, and nerve functions. Low levels of DHA have been associated with Alzheimer disease and dementia [4].

Anti-inflammatory drugs consist of nonsteroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids. Nonsteroidal anti-inflammatory drugs include nonselective cyclooxygenase inhibitors (COX-1 and COX-2 inhibitors) and COX-2 selective inhibitors, also referred to as coxibs. Although COX-2 inhibitors present less upper gastrointestinal toxicity than traditional NSAIDs, both selective and nonselective COX inhibitors (excluding aspirin) can exert deleterious effects on the cardiovascular system [5,6]. Continued studies on inflammatory diseases are needed because the side effects of available anti-inflammatory drugs pose a major problem for clinical use. The development of newer and more powerful anti-inflammatory drugs with fewer side effects is greatly needed.

Most of the literature on omega-3 PUFA deals with their anti-inflammatory effects and actions on cardiovascular diseases and diabetes [7-9]. Some literature states their effects on inflammatory bowel diseases and rheumatoid arthritis [10-13]. Recent studies also report omega-3 PUFA actions on inflammation in rodents [14-19], but few deal with nociception [20,21]. Furthermore, with few exceptions that administered omega-3 PUFA intravenously [17,19], most were carried out by adding omega-3 to the diet [15,16,18,21,22].

Based on these observations, we hypothesized that long-chain omega-3 PUFA exerts anti-inflammatory properties in the rat. To test this hypothesis, EPA and DHA were administered orally to rodents at low doses to determine the actions on nociception. In addition, our specific research objectives included conducting immunohistochemistry assays for TNF-α in rat paws with carrageenan-induced edema as well as myeloperoxidase (MPO), lactate dehydrogenase (LDH), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and thiobarbituric acid reactive substances (TBARS) assays in human neutrophils in vitro, to examine the extent of omega-3 PUFA anti-inflammatory and antioxidant actions.

2. Methods and materials

2.1. Drugs

The omega-3 supplement used for this study was obtained from Proepa, Aché Laboratórios Farmacêuticos, São Paulo, Brazil, and each 1000-mg capsule contained 180 mg EPA, 120 mg DHA, and 2 mg tocopherol. Carrageenan (λ type IV), naloxone, and indomethacin were purchased from Sigma Chemical (St Louis, MO). Dexamethasone was obtained from Aché Laboratories; heparin, from Wyeth (São Paulo, Brazil); and morphine, from Cristália (São Paulo, Brazil). The omega-3 supplement (administered orally) was dissolved in an aqueous suspension prepared with 0.4% Tween 80 (as vehicle and always used as control) immediately before use. All other reagents were of analytical grade.

2.2. Animals

Male Swiss mice (25-30 g) or male Wistar rats (180-200 g) were provided by the Animal House of the Federal University of Ceará, Brazil. The animals were housed in plastic cages with sawdust as bedding and kept in a room with controlled temperature (25°C ± 2°C) under a 12 hours/12 hours light/dark cycle. Food and water were provided ad libitum. The experiments were carried out according to the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services (NIH publication no. 85-23, revised 1985). The project was approved by the Animal Ethics Committee of the Faculty of Medicine of the Federal University of Ceará.

2.3. Pharmacological testing in vivo

2.3.1. Acetic acid–induced abdominal contortions (writhing test) in mice

This method is used to evaluate peripheral analgesic actions. Acetic acid causes analgesia by liberating endogenous substances, including serotonin, histamine, PGs, bradykinin, and substance P that stimulate pain in nerve endings. Local peritoneal receptors (acetylcholine and histamine receptors) and their mediators are postulated to be partly involved in the abdominal constriction (writhing) response [23]. The method has been associated with increased levels of PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products. Male Swiss mice (8-12 animals per group) were used in this experiment. The animals were treated with the omega-3 supplement (1, 2.5, and 5 mg/kg, orally) 60 minutes before receiving a 0.6% acetic acid injection (10 mL/kg, IP), and the number of contractions was recorded for 20 minutes after a 10-minute interval.

2.3.2. Formalin test in mice

The formalin test is a tonic model of continuous pain resulting from formalin-induced tissue injury. It is a useful model for the screening of new drugs thought to act on inflammatory, neurogenic, and central mechanisms of nociception [24]. Twenty microliters of 1% formalin was administered (subcutaneous) in the mouse's right hind paw, and the licking time

was recorded from 0 to 5 minutes (phase 1, neurogenic) and from 20 to 25 minutes (phase 2, inflammatory) after the formalin injection. The animals (8-15 per group) were treated with saline (0.1 mL/10 g, IP), morphine (4 mg/kg, IP), omega-3 supplement (0.5, 1, and 5 mg/kg, orally), morphine + naloxone (4 and 1 mg/kg, IP, respectively), or naloxone + omega-3 (5 mg/kg, orally, respectively), 30 minutes before the formalin injection. Naloxone was injected 15 minutes before morphine or the omega-3 administrations. Morphine was used as the reference drug, whereas naloxone, an opioid antagonist, was used to assess a possible participation of the opioid system in the omega-3 PUFA effect.

2.3.3. *Carrageenan-induced paw edema in rats*

Paw swelling or footpad edema is a convenient method for assessing inflammatory responses to irritants such as carrageenan. Typically, the drugs are assessed for acute anti-inflammatory activity by examining their ability to reduce or prevent the development of carrageenan-induced paw swelling. This model has long been used to evaluate the anti-inflammatory properties of agents such as NSAIDs that inhibit PG production [25]. The animals were randomly selected and divided into groups of 5 to 17 animals. The omega-3 supplement was dissolved in 1% Tween 80 and orally administered at the doses of 1, 2.5, and 5 mg/kg. The other groups were injected with either the reference drug (indomethacin, 20 mg/kg, orally) or vehicle (Tween 80). Thirty minutes later, edema was induced by the injection of 0.1 mL of a 1% carrageenan solution into the animal's right hind paw. Measurements of the paw volume were done by means of a plethysmometer (Ugo Basile, Comerio, VA, Italy), immediately before the carrageenan injection and 1, 2, 3, 4, and 24 hours after. The paw edema volume was determined by the difference between the final and initial volumes.

2.3.4. *Immunohistochemistry analyses for tumor necrosis factor-α*

Tumor necrosis factor-α (TNF-α) is a central regulator of inflammation, and its antagonists may be effective in treating inflammatory disorders in which TNF-α plays an important pathogenic role [26]. For immunohistochemistry assays of the TNF-α, the streptavidin-biotin-peroxidase method was used. Three groups of rats were treated with either the vehicle (0.4% Tween 80 in distilled water as normal controls) or omega-3 (2.5 or 5 mg/kg, orally). After 30 minutes, an intraplantar injection of 1% carrageenan was administered to the animals, and 3 hours later, all animals were euthanized, and 5-mm plantar region sections were immersed in a buffered formalin solution for 24 hours. The sections were then deparaffinized, dehydrated in xylol and ethanol, and immersed in 0.1 M citrate buffer (pH 6) under microwave heating for 18 minutes to allow for antigen recovery. After cooling at room temperature for 20 minutes, the sections were washed with a phosphate-buffered saline (PBS) and followed by a 15-minute blockade of endogenous peroxidase with a 3% H_2O_2 solution. The sections were incubated overnight (4°C) with rabbit primary antibodies (anti–TNF-α) as 1:200 in PBS–bovine serum albumin (BSA). The next day, the sections were washed in PBS and incubated for 30 minutes with the secondary biotinylated rabbit antibody (anti-IgG), 1:200 dilution in PBS-BSA. After washing in PBS, the sections were incubated for 30 minutes, with the conjugated streptavidin peroxidase complex (ABC Vectastain complex; Vector Laboratories, Burlingame, CA). After another washing with PBS, the sections were stained with 3,3′-diaminobenzidine-peroxide (DAB) cromophore, counterstained with Mayer hematoxylin, dehydrated, and mounted in microscope slides for analyses.

2.3.5. *Carrageenan-induced peritonitis in rats*

The carrageenan-induced peritonitis is a well-characterized experimental model of acute inflammation, largely used to test new anti-inflammatory drugs that permit the quantification of the leukocytes migration into the peritoneal cavity [27]. Groups of 5 animals were treated with either the omega-3 supplement (2.5, 5, and 10 mg/kg, orally), dexamethasone (5 mg/kg, IP), or vehicle, 30 minutes before the induction of inflammation by means of a 2% carrageenan (500 μg/mL) intraperitoneal injection. All drugs were administered at a 10 mL/kg volume, and then the animals were returned to their cages with free access to water. After 5 hours, the peritoneal fluid was collected by abdominal laparoscopy. For this, all animals were pretreated with a heparinized saline (5 IU/mL, IP). A sample of the peritoneal fluid was diluted (1:10) in Turk liquid for quantification of cell numbers, using a Neubauer chamber. For differential counting of leukocytes, the exudate was centrifuged at 1000 rpm for 5 minutes, and 3% BSA (200 μL) was added to the pellet for the preparation of slides. The cells were stained by a conventional fast pigment, and the results were expressed by the number of cells/mm^3 (total and differential leukocyte counts in the wash fluid).

2.3.6. *Hargreaves test in rats*

The plantar test (Hargreaves' method) enables the researcher to discern the drug effect on a peripherally mediated response to thermal stimulation in the unrestrained rat. It measures the response to an infrared heat stimulus by focusing the infrared source below the rat plantar surface [28]. By pressing a button, the latency to paw withdrawal and infrared intensity are recorded automatically. The rats (180-200 g) were distributed into the following groups (5-6 animals per group): normal controls (animals received only distilled water, orally), carrageenan group (animals received water, orally, and subsequently an intraplantar injection of 100 μL 1% carrageenan into the right hind paw), Indo20 (animals received 20 mg/kg indomethacin, orally, before the injection of carrageenan), and 3 groups were treated with the omega-3 supplement (1, 5, and 10 mg/kg, orally). With the exception of normal controls, the treatments of all other groups were immediately followed by the administration of carrageenan. One hour after treatments, the animals were submitted to a focused thermal stimulation for provoking peripheral hyperalgesia. The withdrawal threshold (time in seconds to move the injected paw) was measured. The data were analyzed by analysis of variance (ANOVA) and the Student-Newman-Keuls test, with $P < .05$ considered significant.

2.3.7. *Hot plate test in rats*

The hot plate test measures the response to a noninflammatory acute nociceptive input and is a model normally used for studying central nociceptive activities [29]. This test measured

the nociceptive responses of mice when placed on a warmed metal plate, at a standard constant temperature. The latency to a nociceptive response was recorded at the baseline and 30, 60, 90, and 120 minutes after administration of the tested drugs. It was defined as the time elapsed until the subject licked or flicked its hind paw. The omega-3 supplement was orally administered at doses of 2.5 and 5.0 mg/kg, and morphine (4 mg/kg, IP) was used as reference.

2.4. *In vitro studies*

2.4.1. *Myeloperoxidase release from human neutrophils*

Myeloperoxidase is a hemeprotein abundantly expressed in polymorphonuclear leukocytes (neutrophils) and secreted during their activation [30]. The MPO release test from human neutrophils was performed according to a previously described method [31]. Myeloperoxidase is widely used as a biomarker for inflammation. In the present work, human leukocytes (2.5×10^6 cells/mL) were suspended in buffered Hank's balanced solution with calcium and magnesium. The preparations predominately contained neutrophils (85.0% ± 2.8%), and the cell viability, as determined by the trypan blue test, was 97.7% ± 0.94%. The cells were preincubated with the omega-3 supplement (1, 10, 50, and 100 μg/mL) for 15 minutes at 37°C. Indomethacin (Indo, 35.7 μg/mL) was used as the positive control and reference drug. Human neutrophils were stimulated by the addition of phorbol myristate acetate (PMA) (0.1 μg/mL) for 15 minutes at 37°C. The suspension was centrifuged for 10 minutes at 2000*g*, 4°C. Aliquots (50 μL) of the supernatants were added to PBS (100 μL), phosphate buffer (50 μL, pH 7.0), and H_2O_2 (0.012%). After 5 minutes at 37°C, 3,3′,5, 5′-tetramethylbenzidine (1.5 mM, 20 μL) was added as substrate, and the reaction was stopped by 30-μL sodium acetate (1.5 M, pH 3.0). The absorbance was determined spectrophotometrically at 620 nm.

2.4.2. *Lactate dehydrogenase assay in human neutrophils*

The assay was performed according to the manufacturer instructions (Labtest, Minas Gerais, Brazil). The LDH is a simple and accurate colorimetric assay for dead and plasma membrane damaged cells. Lactate dehydrogenase present in human neutrophils (due to plasma membrane damage) participates in a coupled reaction, which converts a yellow tetrazolium salt into a red formazan dye measured at 492 nm. The concentrations of the omega-3 supplement used were 1, 10, 50, and 100 μg/mL. The amount of formazan formed in the reaction is directly proportional to the amount of LDH present in the samples, which, in turn, is directly proportional to the number of dead or damaged cells [32].

2.4.3. *MTT assay for cell viability measurements in human neutrophils*

Another parameter used for cytotoxicity is the metabolic activity of viable cells. Tetrazolium salts are reduced only by metabolically active cells. Thus, the yellow salt MTT is reduced by the mitochondrial enzymatic system succinate-tetrazole reductase to a blue-purple–colored formazan salt. The absence of MTT reduction is an indication of cytotoxicity [33]. Human neutrophils (2.5×10^6 cells/mL) were incubated in plaques of 96 wells for 30 minutes at 37°C under a 5% CO_2 atmosphere, in the presence of omega-3 (1-100 μg/mL), Hanks (untreated cells), or Triton X-100 (0.2% as reference). The plaques were then centrifuged at 2000 rpm for 15 minutes at 25°C, and the supernatants discarded. A 10% MTT solution (200 μL, 10 mg/mL) was added to each well. After 3 hours, the cells were centrifuged again under the same conditions, and the supernatants discarded. Then, dimethyl sulfoxide (150 μL) was added, the plaques were shaken for 15 minutes, and the absorbance was measured in the plaque reader at 540 nm. The experiments were carried out fivefold and repeated on 3 different days.

2.4.4. *Determination of TBARS levels in human neutrophils*

Oxidative stress in the cellular environment results in the formation of highly reactive and unstable peroxides derived from PUFAs. Their decomposition results in the formation of malondialdehyde, which can be colorimetrically quantified, following its reaction with thiobarbituric acid. The measurement of TBARS is a well-established method for screening and monitoring lipid peroxidation. This method was previously described [34]. Briefly, a suspension of neutrophils (2.5×10^6 cells/mL) was incubated with omega-3 (1, 10, 50, and 100 μg/mol), vitamin E (50 μg/mL), distilled water (vehicle), or Hanks' solution [Hanks' balanced salt solution (HBSS) untreated cells]. Next, 10-μM citochalasin B was added, followed by 100-nM N-formyl-methionyl-leucyl-phenyl-alanine. After 20 minutes, the mixture was then centrifuged, and trichloroacetic (20% in HBSS) and tiobarbituric (1.2%) acids were added to the supernatant (500 μL). The reaction medium was heated for 30 minutes at 100°C, and the absorbance determined at 560 nm.

2.5. *Statistical analyses*

All results are presented as means ± SEM. A One-way ANOVA followed by the Student-Newman-Keuls test were used for

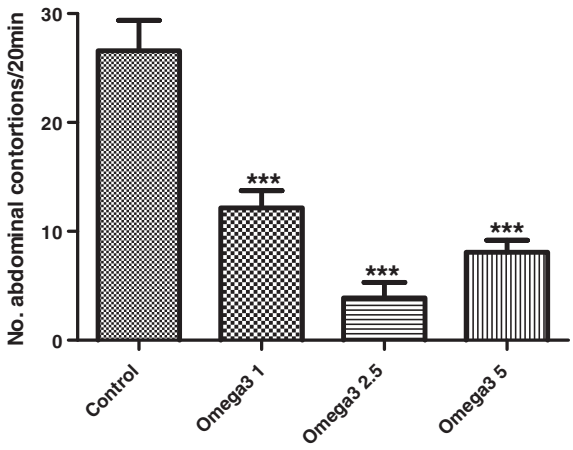

Fig. 1 – Omega-3 PUFA (1, 2.5, and 5 mg/kg, orally) dose dependently decreased the abdominal contortions (writhings) induced by acetic acid in mice. Values are expressed as means ± SEM from 8 to 13 animals per group. ***P < 0.001 vs control (One-way ANOVA followed by the Student-Newman-Keuls as the post hoc test).

comparing the results among treatments. However, in the case of tests evaluating effects at different time points (carrageenan-induced paw edema and hot plate tests), the Two-way ANOVA and $P<0.05$.

3. Results

3.1. *Pharmacological testing in vivo*

3.1.1. *Writhing test*

The acetic acid–induced writhing test in mice is used for detecting both central and peripheral analgesia. The intraperitoneal administration of acetic acid provokes a stereotype behavior characterized by abdominal contractions, among other effects. In the writhing test, the oral administration of the omega-3 supplement decreased the acetic acid-induced abdominal contortions by 48%, 85%, and 71% at doses as low as 1, 2.5, and 5 mg/kg, respectively (Fig. 1).

3.1.2. *Formalin test*

The omega-3 supplement doses at 1 and 5 mg/kg decreased the response of the first phase of the formalin test by 30% and 46%, respectively, whereas no effect was seen with the 0.5 mg/kg dose. However, inhibitions of the order of 29%, 54%, and 95% were observed with the doses of 0.5, 1, and 5 mg/kg in the second phase of the test. Morphine, as expected, significantly decreased both phases of the formalin test (77% and 94% for the first and second phases, respectively) (Fig. 2A). On the other hand, the naloxone (1 mg/kg, IP, 15 minutes before omega-3 administration) pretreatment significantly reversed the effect of the omega-3 supplement at both phases of the test, suggesting the involvement of the opioid system in the antinociceptive effect of the fatty acids (Fig. 2B).

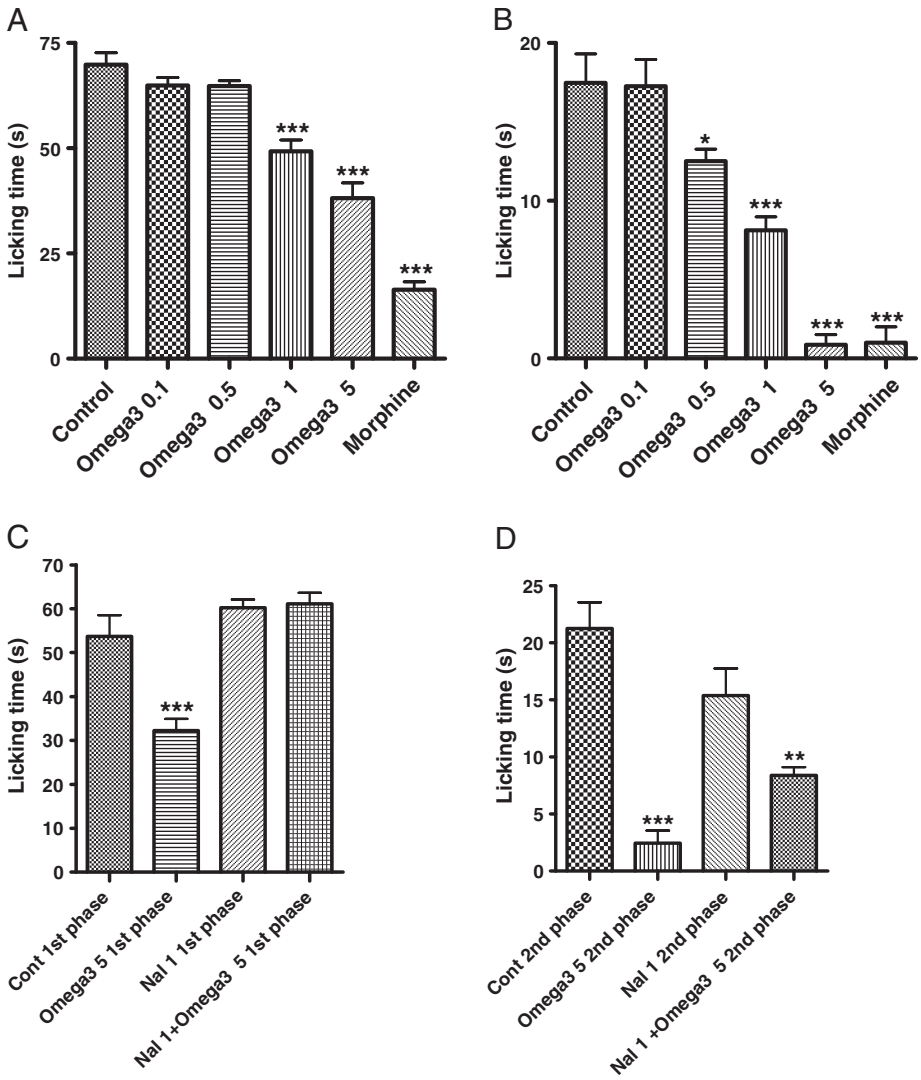

Fig. 2 – Omega-3 PUFA (0.1, 0.5, 1, and 5 mg/kg, orally) preferentially decreased the second phase (neurogenic) in the formalin test in mice. Morphine (4 mg/kg, IP) was used as reference. Values are expressed as means ± SEM from 7 to 21 animals per group. A and B, *P < 0.05 and ***P < 0.001 vs control. C, ***P < 0.001 vs control or omega-3. Comparisons were made within the same phases (One-way ANOVA followed by the Student-Neuman-Keuls as the post hoc test).

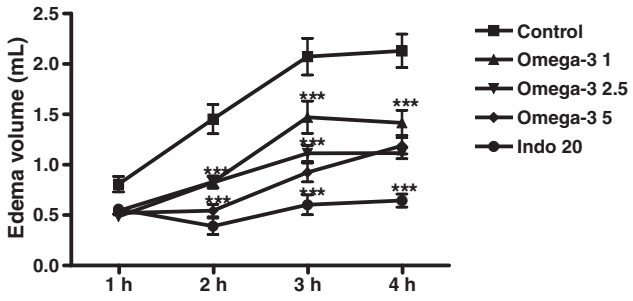

Fig. 3 – Omega-3 PUFA (1, 2.5, 5, and 10 mg/kg, orally) reduced the carrageenan-induced paw edema in rats. Indomethacin (Indo, 20 mg/kg, orally) was used as reference. Values are expressed as means ± SEM from 5 to 17 animals per group. A and B, Edema volumes measurements performed at 1, 2, 3 and 4 hours after carrageenan administration. ***P < 0.001 vs control. Comparisons were made within the same time period (Two-way ANOVA followed by the Bonferroni test).

3.1.3. *Carrageenan-induced paw edema in rats*

The omega-3 supplement at doses of 1, 2.5, and 5 mg/kg, orally, significantly decreased the edema volume at all periods of observation, with percentage inhibitions of 42, 46, and 63 (third hour) and 65, 48, and 40 (fourth hour), at the 3 doses studied, respectively. Indomethacin, which was used as the reference drug, decreased the edema volume by 75% and 63%, at the third and fourth hours, respectively (Fig. 3).

3.1.4. *Immunohistochemistry for TNF-α in the carrageenan-induced rat paw edema*

In Fig. 4A, an intense immunostaining was observed, indicating a massive expression of TNF-α–positive cells in rat paws injected with 1% carrageenan (inflamed paw from the untreated group). It may also be inferred that TNF-α plays an important role in this inflammation model. It is possible to observe a strong brown staining in the cytoplasm of neutrophils, eosinophils, and macrophages because TNF-α is a well-known proinflammatory cytokine involved in carrageenan mechanisms. In the groups treated with omega-3 (2.5 and 5 mg/kg, orally), the immunostaining for TNF-α was markedly reduced (Fig. 4B and C). The leukocytes infiltration was also reduced, and the small quantity of neutrophils present did not show any immunostaining. Therefore, it could be assumed that, at the doses tested, the omega-3 supplement reduces the expression of TNF-α in the model of paw edema induced by carrageenan. Fig. 4D shows the reduction in immunostained mononuclear cells after omega-3 treatment, as compared with the carrageenan-treated group (vehicle).

3.1.5. *Carrageenan-induced peritonitis in rats*

Although carrageenan significantly increased the neutrophil migration to the rat peritoneal cavity by 82%, as compared with negative controls, this increase was only 51% with omega-3 at the dose of 2.5 mg/kg, orally. The values returned to normal at omega-3 supplement doses of 5 and 10 mg/kg, and a similar effect was observed with dexamethasone (10 mg/kg, orally), used as the reference drug (Fig. 5).

3.1.6. *Hargreaves test in rats*

The omega-3 supplement at doses of 1, 5, and 10 mg/kg, orally, increased more than 2-fold the latency to withdrawal from the thermal stimulus (eg, withdrawal threshold), as compared with controls injected with carrageenan only. These effects were very similar to those observed after indomethacin administration, which was used as reference drug (2.4 times increase) (Fig. 6).

3.1.7. *Hot plate test in mice*

The omega-3 supplement significantly increased the withdrawal threshold in a dose-dependent manner compared with controls. Thus, 30 minutes after omega-3 administration (2.5 and 5.0 mg/kg, orally), the latency time increased 1.1 and 1.9 times compared with controls at the same period of observation. After 90 minutes, the increases were 1.2 and 2 times for the 2 doses. Morphine (4 mg/kg, IP), used as the reference, increased around 3 times and near to the cutoff time (45 seconds) of the animal's withdrawal threshold to thermal stimuli (Fig. 7).

3.2. *In vitro testing*

3.2.1. *PMA-stimulated MPO release from human neutrophils*

The cells incubated in the presence of positive controls (0.4% Tween 80) significantly had increased MPO release, a biomarker for inflammation in human neutrophils, by more than 2 times when compared with cells exposed to Hanks' solution (negative controls). The cells exposed to the omega-3 PUFA, at concentrations of 1, 10, 50, and 100 μg/mL, brought MPO release values close to or even lower (with the higher concentration) than those observed with the negative control. Similar results were observed for indomethacin that was used as a reference drug (Fig. 8).

3.2.2. *Lactate dehydrogenase assay in human neutrophils*

Although 0.2% Triton used as a cytotoxic and positive control increased the LDH release 6-fold, indicating a cell membrane damage, values observed in the presence of omega-3 PUFA at concentrations ranging from 1 to 100 μg/mL were close to and not significantly different from those of the negative controls (HBSS solution) (Fig. 9).

3.2.3. *MTT assay in human neutrophils*

The omega-3 PUFA did not alter cell viability as evaluated by the MTT assay in human neutrophils in vitro. The observed values were close to those observed with the negative control (HBSS solution). The percentage of viable cells in the presence of 0.2% Triton (positive control) was very low (Fig. 10).

3.2.4. *Determination of TBARS levels in human neutrophils (lipid peroxidation assay)*

The results presented in Fig. 11 show that the omega-3 PUFA at a low concentration range (1-50 μg/mL) did not affect lipid peroxidation levels. However, at higher concentrations (100 μg/mL), it increased TBARS levels, which is indicative of lipid peroxidation as compared with the negative control (HBSS solution). Use of 0.2% Triton significantly increased lipid peroxidation levels 9-fold. Vitamin E (50 μg/mL) presented values close to those of negative controls.

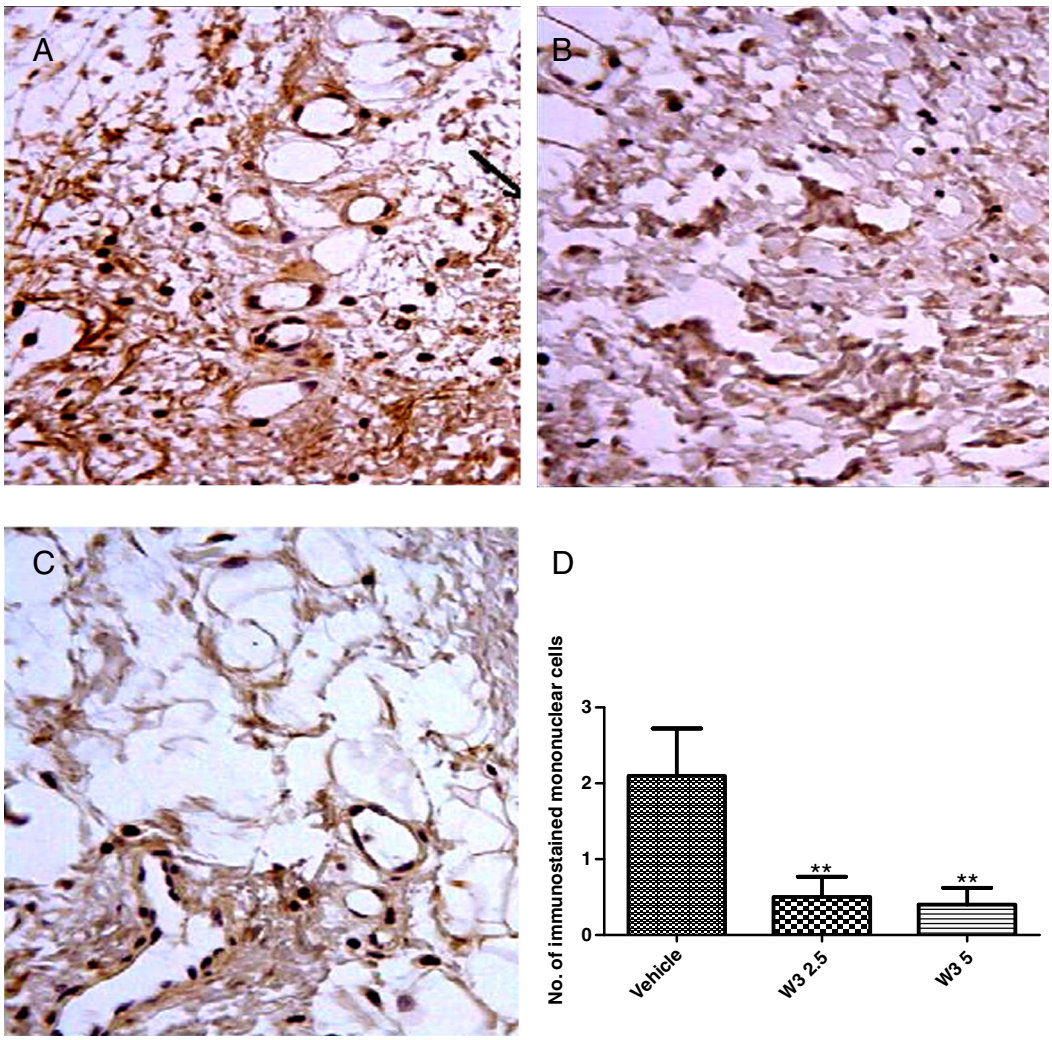

Fig. 4 – Omega-3 PUFA decreased TNF-α immunostainings in the rat inflamed paw, as evaluated by the carrageenan-induced edema model. The animals were treated with distilled water or the omega-3 supplement (2.5 and 5 mg/kg, PO) and 60 minutes later injected with carrageenan at the right hind paw. The animals were euthanized 3 hours later, and their paws processed for immunohistochemistry studies. A, Inflammation positive control (TNF-α). B, Omega-3 (2.5 mg/kg). C, Omega-3 (5 mg/kg). The brown staining indicates interaction of primary and secondary antibodies and, as a consequence, the presence of TNF-α. The dark arrow indicates immunostained cells (original magnification ×400). D, Number of immunostained mononuclear cells (original magnification ×1000). Columns represent means ± SEM of the number of stained cells (n = 3) counted in 10 different fields. **P < 0.001 vs vehicle (One-way ANOVA followed by Student-Newman-Keuls as the post hoc test).

4. Discussion

Dietary omega-3 PUFA are known to present anti-inflammatory and immunomodulating effects that may be of relevance to several diseases (such as atherosclerosis and stroke) as well as chronic diseases involving the inflammatory processes [12,13,35,36]. Previously, omega-3 PUFA anti-inflammatory actions were attributed mainly to their suppressive effect on the formation of arachidonic acid–derived PGs and leukotrienes. However, more recent studies demonstrated that those effects are due to omega-3–derived lipid mediators, resolvins and protectins, which present anti-inflammatory and inflammation-resolving properties [37,38].

Other findings have demonstrated that some resolvins, such as those belonging to the D and E series, can dampen pain of inflammatory and postoperative origin [39]. Chemically, these compounds are hydroxylated derivatives of EPA (for E-resolvins) and of DHA (for D-resolvins and protectin D1) [40]. A recent review [41] discussed the mechanisms by which resolvins act as antinociceptives on their receptors in immune and neuronal cells by regulating inflammatory mediators, transient receptor potential ion channels, and spinal cord synaptic transmission.

Nonsteroidal anti-inflammatory drugs are the most widely prescribed drugs for the treatment of pain and inflammation. Conventional NSAIDs inhibit COX-1 and COX-2 isoforms of the COX enzyme. The inhibition of COX-1 up-regulates COX-2

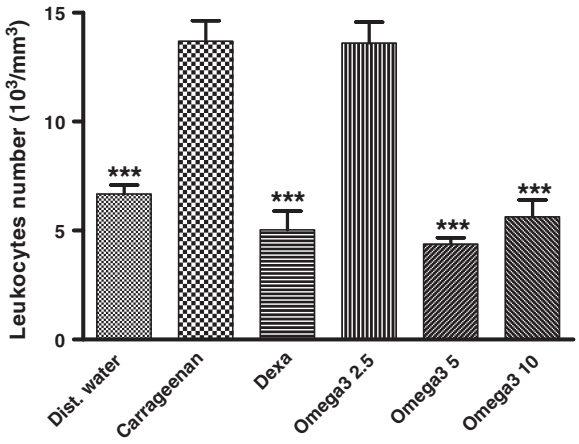

Fig. 5 – Omega-3 PUFA (2.5, 5, and 10 mg/kg, orally) reduced leukocytes migration into the peritoneal cavity, as evaluated by the carrageenan-induced peritonitis model in rats. Dexamethasone (Dexa, 10 mg/kg, orally) was used as reference. Values are expressed as means ± SEM from 3 to 5 animals per group. Cell counting was performed 5 hours after the intraperitoneal injection of carrageenan. ***P < 0.001 vs carrageenan only (One-way ANOVA followed by Student-Newman-Keuls as the post hoc test).

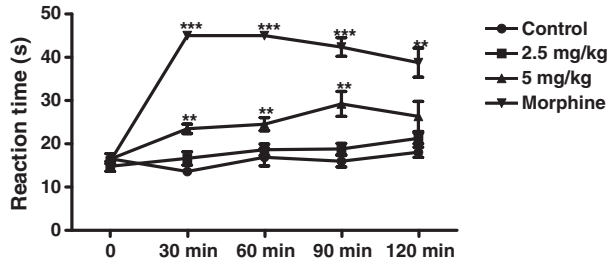

Fig. 7 – Omega-3 PUFA (2.5 and 5 mg/kg, orally) increased the reaction time (seconds) only at the higher dose, as evaluated by the hot plate test in mice. Morphine (4 mg/kg, IP) was used as reference. Measurements were performed at indicated times and the cutoff time was 45 seconds. Values are expressed as means ± SEM from 7 to 8 animals per group. *P < 0.05 and **P < 0.01 vs control, at the same period (Two-way ANOVA followed by the Bonferroni test).

expression in association with gastric hypermotility, and PGs produced by COX-2 counteract the deleterious effect of COX-1 inhibition [42]. In addition, the suppression of COX-2–derived prostacyclin (PGI_2) is sufficient to explain most adverse cardiovascular effects of NSAIDs, which are likely to be augmented by secondary mechanisms such as suppression of nitric oxide production [43]. Newer NSAIDs have been introduced in recent years, and although they present better safety, efficacy, and tolerability, the full spectrum of adverse reactions of these drugs is yet to be known [44]. Furthermore, there is a need for safer and more tolerable drugs. Natural products, alone or in combination with NSAIDs, would amplify the potency of anti-inflammatory drugs and reduce their side effects as already suggested [45].

Although the literature presents several studies on the inflammatory effects of omega-3 PUFA in humans [46-53], there are only a few based on their antinociceptive effects [20] and almost none in rodents. Such findings induced us to conduct this work, relating the anti-inflammatory activity of omega-3 PUFA to their antinociceptive effects on experimental models of inflammation and nociception, at low doses. Although we did not perform pharmacokinetic experiments, there are several earlier and more recent works [54-57] on the

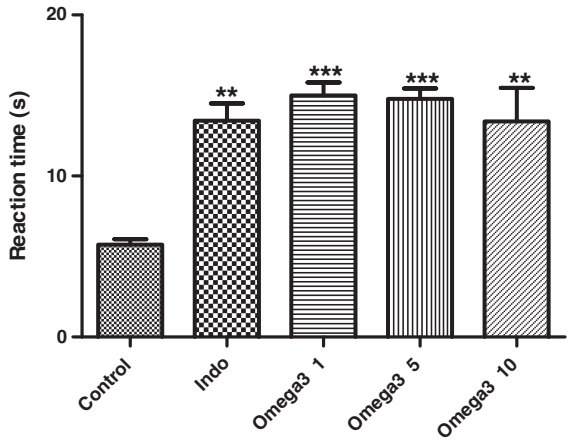

Fig. 6 – Omega-3 PUFA (1, 5, and 10 mg/kg, orally) increased the latency to withdrawal (seconds) from the thermal stimuli, in the Hargreaves method in rats. Indomethacin (Indo 20 mg/kg, orally) was used as reference. Measurements were performed 1 hour after administration of carrageenan at the right hind paw. Values are expressed as means ± SEM from 5 to 6 animals per group. **P < 0.01 and ***P < 0.001 vs control (One-way ANOVA followed by Student-Newman-Keuls as the post hoc test).

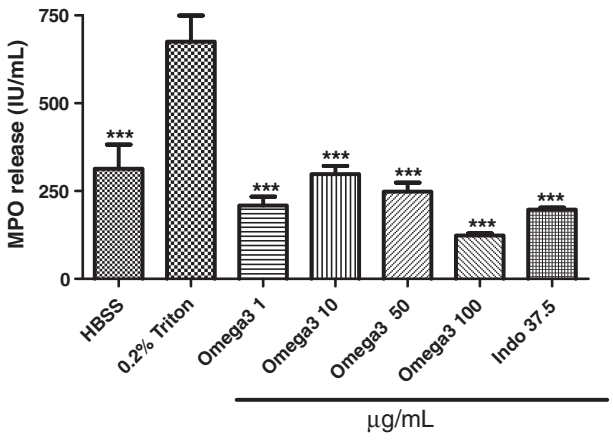

Fig. 8 – Omega-3 PUFA (1–100 µg/mL) inhibited PMA-stimulated MPO (a biomarker for inflammation) release from human neutrophils in vitro. Indomethacin (Indo 37.5 µg/mL) was used as reference. Triton 0.2% was the positive control. Values are expressed as means ± SEM. ***P < 0.001 vs 0.2% Triton (One-way ANOVA followed by the Student-Newman-Keuls as the post hoc test).

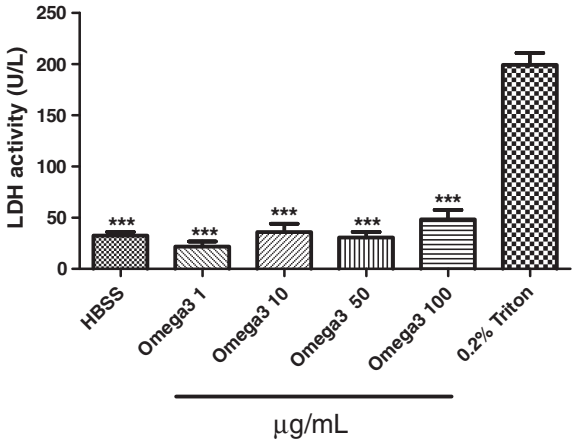

Fig. 9 – Omega-3 PUFA (1-100 µg/mL) effects evaluated by the LDH assay in human neutrophils in vitro. HBSS and 0.2% Triton are negative and positive controls, respectively. Values are expressed as means ± SEM. ***P < 0.001 vs 0.2% Triton (One-way ANOVA followed by the Student-Newman-Keuls as the post hoc test).

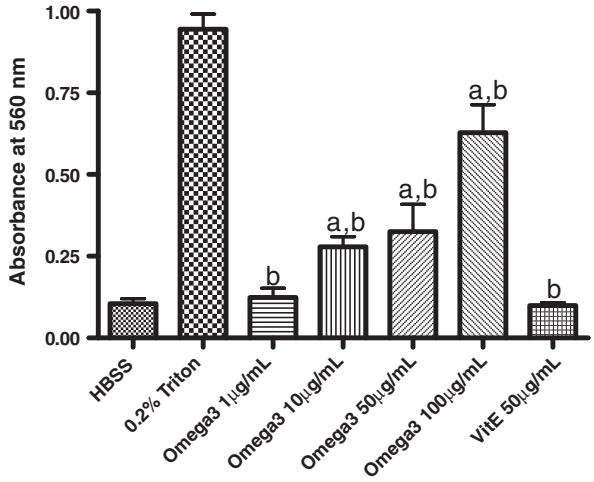

Fig. 11 – Omega-3 PUFA (1-100 µg/mL) effects assessed by the TBARS assay in human neutrophils in vitro. HBSS and 0.2% Triton are the negative and positive controls, respectively. Vitamin E (Vit E, 50 µg/mL) was used as reference. Values are expressed as means ± SEM; vs HBSS (a) and vs 0.2% Triton (b), at P < 0.001 to 0.05 (One-way ANOVA followed by the Student-Newman-Keuls as the post hoc test).

bioavailability of omega-3 PUFA in humans, and some in rodents as well [58,59]. We observed, even at very low doses, that omega-3 PUFAs were tolerated and absorbed in rats, but we cannot assume that the effects are entirely due to EPA and DHA or from their lipid metabolites. However, the evidence for the safety and efficacy of omega-3 PUFA are compelling [60] and appear to be beneficial in the current study.

In the writhing test, we found that omega-3 PUFA decreased mice writhes values significantly and dose dependently. Different nociceptive mechanisms are known to be involved in this rather nonselective model, such as the release of biogenic amines (as histamine and serotonin) and inhibitions of COXs and their metabolites (as PGE_2 and $PGF_{2\alpha}$) as well as of the opioid system [61,62]. It is also established that the nociceptive response caused by acetic acid is dependent upon the release of cytokines, such as TNF-α, interleukin-1β, and interleukin-8, via modulation of macrophages and mast cells in the peritoneal cavity [63].

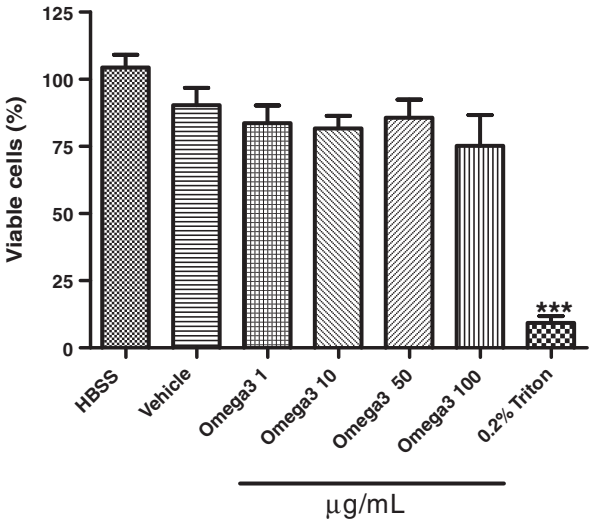

Fig. 10 – Omega-3 PUFA (1-100 µg/mL) effects evaluated by the MTT assay in human neutrophils in vitro. HBSS and 0.2% Triton were used as negative and positive controls, respectively. Values are expressed as means ± SEM. ***P < .001 vs all other samples (1-way ANOVA followed by the Student-Newman-Keuls as the post hoc test).

Although omega-3 PUFA significantly inhibited both phases of the formalin test, its effect occurred predominantly at the second phase of the test. The first phase of the formalin test corresponded to acute neurogenic pain, whereas the second phase corresponded to inflammatory pain. They are believed to reflect the excitation of peripheral afferent nociceptors and central sensitization [64,65]. Substance P and bradykinin participated in the first phase, whereas serotonin, histamine, nitric oxide, and PGs were involved in the second phase [66]. The first and second phases were attenuated by opioids, whereas COX inhibitors were known to attenuate only the second phase. Interestingly, we found that the naloxone pretreatment partially reversed the omega-3 PUFA effects in both phases of the formalin test, suggesting that the opioid system participated in that action. A recent work [20] also showed that antinociceptive effects of DHA were abolished after the naloxone pretreatment, which confirms our results. Thus, we could assume that omega-3 PUFA acts by both peripheral and central mechanisms because the formalin test encompasses inflammatory, neurogenic, and central mechanisms of nociception [24].

Carrageenan-induced inflammation is a classical model of edema formation and hyperalgesia, extensively used in studies of NSAIDs. It is known that peripheral inflammation involves an increase in COX-2–mediated PG synthesis in the central nervous system, contributing to allodynia and

hyperalgesia [25]. We observed that omega-3 PUFA reduced the carrageenan-induced edema that leads us to assume that these PUFA inhibit COX-2, thus decreasing PG concentrations.

We also found that the omega-3 PUFA decreased MPO release to the peritoneal cavity, as evaluated by the carrageenan-induced peritonitis. In addition, the number of neutrophils was significantly reduced. This is a model of acute inflammation [28] characterized by a rapid influx of polymorphonuclear neutrophils, followed by mononuclear cell infiltration. It is often used to assess the anti-inflammatory effects of drugs and as an in vivo model to study inflammatory mediators [67,68].

The Hargreaves method used mild radiant heat to measure thermal nociception in cutaneous hyperalgesia, and the time for foot withdrawal characterized the pain response [28]. It can be used as an in vivo model where inflammation is produced by a chemical agent as carrageenan. This test enabled one to discern the drug effect on a peripherally mediated thermal stimulation, in the unrestrained rat, and is sensitive to COX inhibitors. We demonstrated that the omega-3 PUFA significantly increased the withdrawal threshold, not only in the plantar test, but also in the hot plate test. This last test is considered to be suitable for measuring the effects of opioid analgesics and is not sensitive to analgesic effects of NSAIDs [69]. Like the Hargreaves method, the hot plate is a common test that measures the response to thermal nociception of drugs acting by a central mechanism. Thus, as far as the antinociceptive effects are concerned, our data from both the Hargreaves method and the hot plate test confirm that these omega-3 PUFAs act by both peripheral and central mechanisms.

It is assumed that omega-3 PUFA may have antinociceptive properties, in part by inhibiting microglial release of matrix metalloproteinases [70]. Microglial cells appear to play a vital role in the initiation of processes promoting persistent pain states [71]. Glial activation can be induced by C-fiber nociceptive input from the sciatic nerve, and thus, the nociceptive-induced glial activation appears to be crucial in contributing to acute and inflammatory pain in rodent models [72]. The pretreatment of RAW 264.7 cells with omega-3 was shown to significantly attenuate TNF-α production in lipopolysaccharide-stimulated macrophages [73].

Clinical trials show that omega-3 PUFA may be of benefit in the management of patients with neuropathic pain [74]. A significant factor in neuropathic pain is the activation of spinal cord glial cells [75]. Activated glial cells are characterized by the proliferation, hypertrophy, and increased production of inflammatory cytokines, such as interleukin-1β, interleukin-6, and TNF-α. Eicosapentaenoic acid and DHA could possibly reduce the production of these cytokines. In the present work, we demonstrated that the omega-3 treatment reduced immunostaining for TNF-α in the inflamed rat paw, as evaluated by the carrageenan-induced edema.

Our in vitro studies showed that the omega-3 supplement significantly decreased MPO release from PMA-stimulated human neutrophils, confirming the anti-inflammatory action of EPA and DHA. In addition, it did not present any cytotoxic effect on those cells at the concentration range used as assessed by the LDH and MTT assays. However, EPA and DHA increased lipid peroxidation at higher concentrations, as demonstrated by the TBARS assay.

Although we did not explore some other molecular/cellular targets for EPA and DHA actions, these may involve inhibition of COXs and microglial activation, leading to a reduced release of proinflammatory cytokines, such as TNF-α. The EPA and DHA could be combined to reduce the amount of NSAIDs for the management of inflammatory diseases and pain. However, some additional translational studies are needed to confirm the beneficial effects of EPA and DHA and/or their active metabolites, as previously reported [76], alone or in conjunction with NSAIDs, for their potential in the treatment of inflammation and pain-related processes.

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