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Participation of the anti-inflammatory and antioxidative activity of docosahexaenoic acid on indomethacin-induced gastric injury model

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ABSTRACT

Adverse gastrointestinal (GI) effects caused by nonsteroidal anti-inflammatory drugs (NSAIDs), including indomethacin, are recognized as the major limitation to their clinical use. NSAID-induced gastric damage is generated by cyclooxygenase inhibition, activation of inflammatory processes, and oxidative stress. Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid, has shown gastroprotective effects; however, the molecular mechanisms underlying these effects have not been fully explained. As a result, the aim of this study was to examine DHA's anti-inflammatory and antioxidative actions in a mouse model of indomethacin-induced gastric injury. Oral administration of DHA (3, 10, 30, and 100 mg/kg) caused a reduction in indomethacin-induced gastric hemorrhagic lesions. We found that the gastroprotective effects of DHA treatment (100 mg/kg) were accompanied by decreases in several parameters: in leukocyte recruitment; gastric levels of myeloperoxidase; leukotriene B_4; intercellular adhesion molecule-1; tumor necrosis factor alpha; and nuclear translocation of nuclear factor-κB. Concurrently, we observed an improvement in antioxidant defenses produced by the increase in superoxide dismutase and glutathione activities but not catalase; in addition, a decrease in some oxidative damage markers such as malondialdehyde and carbonyl proteins in lipids and proteins was observed. Furthermore, resolvin D1 production and expression of free fatty acid receptor 4 were stimulated by DHA. Therefore, this study identified the antioxidant and anti-inflammatory actions of DHA as the main mechanisms involved in DHA's gastroprotective effects against indomethacin-induced gastric damage.

1. Introduction

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is limited by the extensive damage induced in the gastrointestinal (GI) tract (Bindu et al., 2013; Whittle, 2003). Inhibition of cyclooxygenase (COX) enzymes and subsequent suppression of gastric prostaglandin (PG) production has been considered the major reason for NSAID-induced gastric pathogenesis (Sinha et al., 2015; Yadav et al., 2012). In addition, during the past few decades, several studies have demonstrated that NSAIDs (such as indomethacin) generate gastric injury due to the production of reactive oxygen species (Bastaki and Wallace, 1999; Bindu et al., 2013); reactive oxygen species are associated with inflammatory process activation in damaged gastrointestinal tissue (Suleyman et al., 2010; Wallace, 2008).

The current prevention strategies for NSAID-associated gastropathy have not been completely effective (Wallace, 2013), as was shown in the case of proton pump inhibitors (PPIs). Despite the capability of these inhibitors to reduce gastric acid secretion, they have been reported to induce adverse effects (such as dysbiosis) as a consequence of chronic, long-term use (Wallace et al., 2011a).

Docosahexaenoic acid (DHA, C22:6), an omega-3 polyunsaturated fatty acid, which is abundant in fish oil (Holub, 2002), has shown

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neuroprotective, antinociceptive, antioxidative, and anti-inflammatory effects in experimental murine models such as those for hypoxia-ischemia brain and liver injury, formalin testing, and experimental colitis (Bento et al., 2011; Mayurasakorn et al., 2011; Nakamoto et al., 2010; Türkez et al., 2012). Recently, we reported that DHA, as a pure compound, has gastroprotective effect in the indomethacin-induced gastric injury model (Pineda-Peña et al., 2012). In this study, DHA did not reverse gastric prostaglandin E_2 (PGE_2) levels but partially prevented the increase in indomethacin-induced gastric leukotriene B_4 (LTB_4) levels (Pineda-Peña et al., 2012). Furthermore, DHA's protective effect has been associated with reduction of oxidative stress in brain (Hossain et al., 1999), liver (González-Périz et al., 2006), kidney (Ajami et al., 2013) and cardiac (Jahangiri et al., 2006) tissues by increasing the expression of superoxide dismutase (SOD) (Ajami et al., 2013), catalase (CAT) (Jahangiri et al., 2006), and glutathione levels (GSH) (Hossain et al., 1999). In addition, a decrease in malondialdehyde (MDA) was also observed (González-Périz et al., 2006). Several anti-inflammatory activities of DHA have been demonstrated: reduction of myeloperoxidase (MPO) in a murine ear inflammation model (Raederstorff et al., 1996); reduction of tumor necrosis factor alpha (TNF-α) in LPS- stimulated macrophages (Honda et al., 2015); reduction of intercellular adhesion molecule-1 (ICAM-1) expression in atherosclerosis (Huang et al., 2015); and prevention of nuclear factor-κB (NF-κB) activation in THP1-macrophages (Harvey et al., 2015; Yang et al., 2013). In addition, in a *fat*-1 transgenic mice model, eicosapentaenoic acid (EPA), another omega-3 polyunsaturated fatty acid, appeared to exert protective effects on the GI tract via activation of free fatty acid receptor 4 (FFA4 receptor) after NSAID-induced GI damage had occurred (Han et al., 2016).

However, the anti-inflammatory and antioxidative pathways have not been studied with respect to DHA's gastroprotective actions. Thus, the aim of this study was to evaluate the anti-inflammatory and antioxidative mechanisms of DHA in the indomethacin-induced gastric injury model.

2. Material and methods

2.1. Drugs and reagents

DHA (D2534), indomethacin (I7378), omeprazole (O104), and olive oil (1514), were purchased from Sigma Aldrich (Toluca, Mexico). Olive oil was utilized as the vehicle for DHA, indomethacin was dissolved in 5% $NaHCO_3$, and omeprazole was dissolved in 0.9% saline solution. All reagents were prepared prior to use.

2.2. Animals

Male Balb-c mice, weighing 20–25 g, were obtained from Centro de Investigación y de Estudios Avanzados (CINVESTAV) del Instituto Politécnico Nacional (Mexico City, Mexico) (Protocol number: 0184-03). All treatments for the animals, their care, and surgical procedures were performed in accordance with the Mexican Official Norm for Animal Care and Handling (NOM-062-ZOO-1999) and the Bioethics Committee of ENMyH-IPN (registry number: ENMH-CB-139–2015) and were in compliance with international rules and standards on the care and use of laboratory animals. Sample size per group consisted of five to seven animals. Animals were fed with standard laboratory chow and tap water *ad libitum*. Mice were placed in cages with wire-net floors to minimize coprophagy and fasted 12 h prior to experimentation but were allowed free access to tap water while fasting.

2.3. Induction of gastric ulceration and assessment of gastric mucosal lesions

Mice were randomly divided into equal groups and treated via oral gavage as follows: 1.) Group 1, Control (olive oil); 2.) Groups 2, 3, 4, and 5, received a single administration of DHA (at doses of 3, 10, 30, and 100 mg/kg, respectively); 3.) Group 6, vehicle for DHA (olive oil) that was administered 2 h prior to indomethacin; 4.) Group 7, received omeprazole (30 mg/kg); and 5.) Group 8, vehicle for omeprazole (0.9% saline solution) 30 min prior to administration of the ulcerogenic agent. Five h after oral administration by gavage of the ulcerogenic agent (indomethacin 30 mg/kg) or the same volume of vehicle (5% $NaHCO_3$ for control), mice were anesthetized with ketamine (100 mg/kg) and xylazine (7.5 mg/kg) and killed. Stomachs were removed, opened along the greater curvature, and thoroughly rinsed with saline solution. The extent of the gastric-damaged area was scored blindly. For this, a picture of the fully extended stomach was taken; the length and width of each lesion was measured using ImageJ software (Version 1.45), and the total lesion area of the stomach (mm^2) was obtained for each mouse (Navarrete et al., 2005; Pineda-Peña et al., 2012; Wallace et al., 2011b, 2000). Based on the dose-response curve performed, we selected 100 mg/kg, p.o. of DHA for further analysis.

2.4. Histological study

For histological assessment, gastric tissue was excised and fixed with 10% formaldehyde in phosphate buffered saline (PBS) for 24 h. These tissues were then washed with tap water, dehydrated in alcohol, and embedded in paraffin. Sections of 4–5 μm were mounted on glass slides covered with silane. Hematoxylin and eosin staining was performed on each slide (Reyes-Gordillo et al., 2007), and slides were then examined under an optical microscope (Nikon Eclipse Slog) equipped with a high-resolution digital camera (Nikon Digital Sight DS-2mv).

2.5. Measurement of gastric MPO levels

Myeloperoxidase (MPO) tissue concentrations were determined employing a modified version of previously described methods (Jung et al., 2012; Seo et al., 2012; Yan et al., 2011). The MPO value was calculated by measuring the absorbance of samples at 620 nm and comparing them to a MPO standard (Yan et al., 2011).

2.6. Determination of gastric mucosal LTB_4, TNF-α, ICAM-1 and RvD1 levels

A sample of the corpus region of the stomach was excised, weighed, and added to a tube containing 1 ml of PBS (10 mmol/l; pH 7.4). The tissue sample was minced with scissors for 30 s and then placed in a shaking water bath (37 °C) for 20 min. The samples were centrifuged (9000*g*) for 1 min, and the supernatant was snap-frozen and then stored at −70 °C (Díaz-Triste et al., 2014; Wallace et al., 2000). The supernatant was used for determination of LTB_4, TNF-α, ICAM-1, and RvD1 levels by enzyme-linked immunosorbent assay (ELISA) using commercially available ELISA kits from Cayman Chemical Co. (Ann Arbor, MI, USA) and Thermo Fisher Scientific, Inc. (Waltham, MA, USA), according to the manufacturer's instructions. Values obtained were expressed per mg of tissue (Wallace et al., 2000).

2.7. Nuclear protein isolation and assay for NF-κB nuclear translocation

To obtain nuclear extracts from the corpus region of the gastric tissue to assay for nuclear translocation of NF-κB, samples from control and experimental sets of mice were utilized as previously described (Dimauro et al., 2012). Protein contents of the cytosolic and nuclear extracts were determined using the bicinchoninic acid protein assay, and the purity of the nuclear extract was determined by Western Blot for lamin-β1. The nuclear translocation of NF-κB was estimated using a commercial NF-κB (p65) Transcription Factor Assay Kit, which combines the principles of the electrophoretic mobility shift assay and ELISA as indicated by the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI, USA).

2.8. Assessment of gastric mucosal SOD, CAT, activity and GSH levels

Samples were prepared by homogenizing gastric tissues on cold phosphate buffered saline solution and centrifuged at 900*g* for 5 min at 4 °C. The resulting supernatant was used for SOD and CAT assays. SOD activity was determined using the method described by Sun et al. (1988) with minor modifications. The SOD value was determined by measuring the absorbance at 560 nm. SOD activity was calculated as Ung^{-1} protein (López-López et al., 2011). Hydrogen peroxide (H_2O_2) consumption was measured at 480 nm as a marker for gastric CAT activity (Cohen et al., 1970). CAT activity was calculated as the first-order reaction rate constant of H_2O_2 decomposition ($K \times 10^2\ min^{-1}$) (López-López et al., 2011).

A modified version of the method previously described by Galicia-Moreno et al. (2013) was performed for gastric GSH determinations. Briefly, gastric tissue samples were homogenized on a solution consisted of 5 mM EDTA, pH 8% and 5% of trichloroacetic acid, which was used as a protein precipitant. The total homogenate was centrifuged at 4 °C at 100,000*g* for 20 min to obtain a supernatant for the assay. GSH values were determined spectrophotometrically at 412 nm and compared with GSH standards. The results are expressed in nmol GSH/*g* tissue (Galicia-Moreno et al., 2016).

2.9. Assessment of lipid peroxidation

The extent of lipid peroxidation was determined in gastric homogenates through the quantification of malondialdehyde (MDA) formation using the thiobarbituric acid method (Galicia-Moreno et al., 2016; Ohkawa et al., 1979). The MDA content was calculated from the absorbance measurement at 532 nm and an absorption coefficient = $1.56 \times 10^5\ cm^{-1}\ M^{-1}$. The content of total protein was measured using the Bradford method with bovine serum albumin as the standard (Bradford, 1976; Galicia-Moreno et al., 2016).

2.10. Serum carbonyl protein determination

2,4- Dinitrophenylhydrazine was used for determining the carbonyl content in proteins using a previously described method (Galicia-Moreno et al., 2016). Serum carbonyl content was calculated from the absorbance measurement at 360, 370, and 390 nm and an absorption coefficient = $22{,}000\ M^{-1}\ cm^{-1}$.

2.11. Western blot analysis for FFA4 receptor expression

The extracts were prepared in ice-cold lysis buffer composed of 20 mmol/l Tris-HCl, (pH 7.4), 1% of Triton X-100, and protease and phosphatase inhibitor cocktail employing a modified version of previously described method (Martin et al., 2008). The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories Hercules, CA). After blocking with 15% nonfat dry milk and 2% albumin bovine in Tris-buffered saline containing 0.5% Tween-20 for 1 h, the membranes were incubated with primary antibody for the FFA4 receptor (SC-390752 at 1:1000 dilution, Santa Cruz Biotechnology, Santacruz, CA) for 3 h at room temperature. After incubation with the appropriate peroxidase-conjugated secondary antibodies, the signals were visualized using a commercial kit (ECL by Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's recommendations and then exposed to T-mat G/RA film (Kodak, Rochester, NY, USA). The membranes were reblotted with anti-β-actin antibody (SC- 1615 at 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) to verify equal loading of the protein in each lane.

2.12. Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Comparisons among controls were performed utilizing one-way analysis of variance (ANOVA) followed by the Newman–Keuls test. $P < 0.05$ was considered as a statistically significant difference between means.

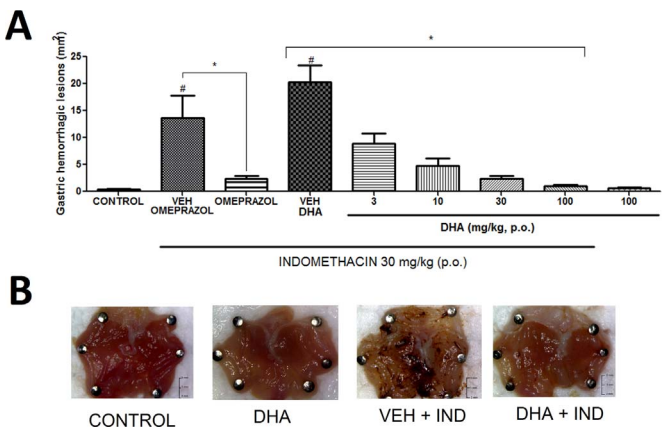

Fig. 1. (A) Gastroprotective effects of DHA (docosahexaenoic acid 3, 10, 30, and 100 mg/kg, p.o.) compared to gastroprotective effect of omeprazole in the indomethacin-induced gastric injury model in mice. DHA was gavaged 120 min before indomethacin administration. Omeprazole (30 mg/kg, p.o.) was gavaged 30 min before indomethacin administration. Control = olive oil + 5% $NaHCO_3$, VEH = 0.9% saline solution + indomethacin for omeprazole, olive oil + indomethacin for DHA. Data are presented as mean ± S.E.M. (n = 5–7) $^{*}P < 0.05$ versus respective vehicle (VEH), $^{\#}P < 0.05$ versus control group. (B) Representative images of gastric lesions in the corpus of the stomach following different treatments.

3. Results

3.1. Gastroprotective effects of DHA on indomethacin-induced gastric injury

Oral administration of DHA (3, 10, 30, and 100 mg/kg per os [p.o.]) caused a reduction in indomethacin-induced gastric hemorrhagic lesions when compared with the indomethacin-treated group (Fig. 1A; $P < 0.05$). Histological examination showed that indomethacin administration caused disruption (black arrow) and neutrophil infiltration (green arrow) into the gastric mucosa (Fig. 2C), while neutrophils were not observed in the gastric mucosa of control group (Fig. 2A and B). Interestingly, DHA pre-treatment (100 mg/kg, p.o.) of indomethacin-treated animals protected stomach mucosa, thus preserving both the well-defined gastric folds and pits without congestion, cellular infiltrate, or damage (Fig. 2D). In addition, DHA pre-treatment (100 mg/kg, p.o.; 1.04 ± 0.18 mm^2) was as effective as a proton pump inhibitor (such as omeprazole, 30 mg/kg, p.o.; 2.38 ± 0.54 mm^2) in protecting gastric tissues against indomethacin-induced damage (18.37 ± 3.18 mm^2) as shown in Fig. 1A.

3.2. Effects of DHA in the anti-inflammatory pathway on indomethacin-induced gastric injury

Neutrophil infiltration in the indomethacin-treated group was followed by a significant increase in gastric MPO, LTB_4, TNF-α, and ICAM-1 levels and NF-κB activation when compared with the control group (Table 1; $P < 0.05$). DHA pretreatment (100 mg/kg, p.o.) remarkably attenuated neutrophil infiltration (Fig. 2D) with a significant consequent reduction in MPO, LTB_4, TNF-α, and ICAM-1 levels and NF-κB translocation when compared with the non-protected indomethacin-treated group (Table 1). In summary, pretreatment with DHA of indomethacin-treated mice was successful in maintaining basal gastric mucosal biochemical parameters that are closely related to neutrophil infiltration.

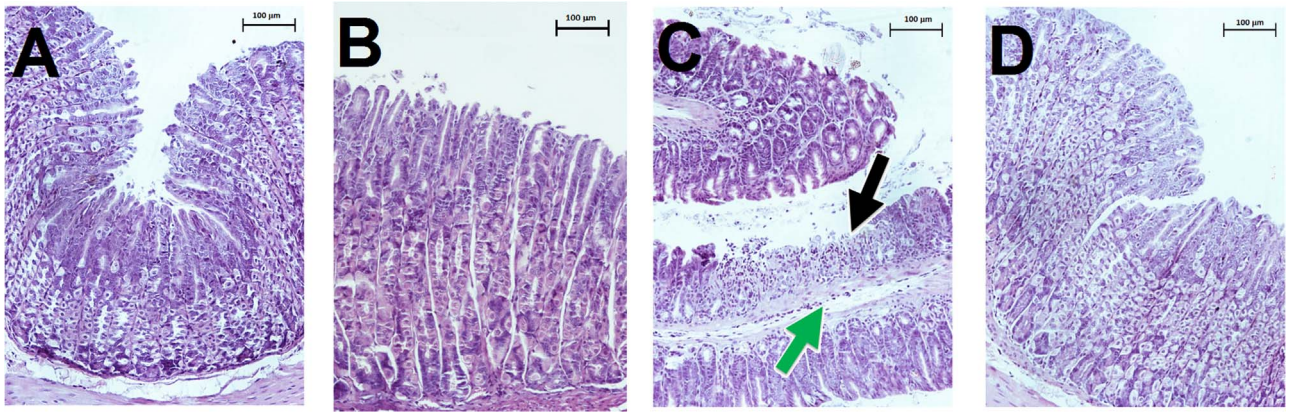

Fig. 2. Representative histopathological sections of gastric mucosa following different treatments. Control group (Panel A), DHA 100 mg/kg + 5% $NaHCO_3$ (Panel B), Olive oil + indomethacin 30 mg/kg (Panel C) and DHA 100 mg/kg + indomethacin 30 mg/kg (Panel D). Disruption in the surface region of the glands of the gastric mucosa (black arrows), congestion, and cell infiltration (green arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Effects of DHA on antioxidants molecules in indomethacin-induced gastric injury

Indomethacin administration significantly decreased gastric mucosal SOD and CAT activities and GSH levels in comparison to the control group (Table 2; $P < 0.05$). On the contrary, DHA pretreatment improved the antioxidant status of the gastric mucosa by causing a significant increase in SOD activity and GSH levels when compared with the indomethacin-treated group; however, CAT activity was not restored in DHA-treated mice (Table 2).

3.4. Effect of DHA in oxidative damage on lipids and proteins induced by indomethacin administration

MDA and carbonylated proteins content of the gastric mucosa were significantly higher after indomethacin-induced acute gastric injury compared to the control group (Table 2; $P < 0.05$). DHA pretreatment (100 mg/kg, p.o.) reversed the effect of indomethacin on gastric mucosa by causing a significant reduction in the formation of MDA and carbonylated proteins when compared with the indomethacin-treated group. Moreover, DHA pretreatment was successful in maintaining basal levels of lipid peroxidation even in the presence of indomethacin (Table 2).

3.5. Effects of DHA on RvD1 levels in indomethacin-induced gastric injury

Our results showed that oral administration of indomethacin caused a decrease in RvD1 gastric levels when compared with those of the control group. Pre-treatment with DHA (100 mg/kg, p.o.) prevented the indomethacin-induced decrease of RvD1 gastric levels in a significant manner (Fig. 3; $P < 0.05$).

3.6. FFA4 receptor expression

Western blot analysis showed that the full length FFA4 receptor (52 kDa) was expressed in the GI tract (Fig. 4B). The expression of the FFA4 receptor was significantly decreased by indomethacin administration when compared with the control group. Nonetheless, DHA pretreatment (100 mg/kg, p.o.) restored the basal expression of the FFA4 receptor when compared with the indomethacin-treated group (Fig. 4A; $P < 0.05$).

4. Discussion

The adverse effects of NSAIDs and the limited effectiveness in treating these effects has driven the search for novel treatments of NSAID-induced gastric damage. Administration of indomethacin, a representative of this group of drugs, has long been used as a reproducible and clinically relevant experimental model for the induction of acute gastric ulcers in experimental animals (Chanudom and Tangpong, 2015; Souza et al., 2004; Wallace et al., 2011a). The present study showed that a single administration of DHA provided significant gastroprotective effects in mice that had undergone indomethacin-induced gastric ulceration. Our findings are consistent with our previous report in which DHA pre-treatment generated gastroprotective effects against indomethacin-induced gastric injury in another murine model, and the effects correlated with a decrease in LTB_4 levels in gastric tissue (Pineda-Peña et al., 2012) although the complete mechanism had not been fully investigated. A novel finding from this study was the occurrence of a decrease in reactive oxygen species production and consequent modulation of inflammatory pathways; these appear to be the main mechanisms involved in DHA's gastroprotective effects; additionally, these effects were associated with an increase in RvD1, a pro-resolutive fatty acid-derived, and the DHA-stimulated expression of the FFA4 receptor. Furthermore, DHA could be employed as an

Table 1
Effect of docosahexanoic acid (DHA) in the anti-inflammatory pathway on the indomethacin-induced gastric damage in mice.

Group/treatment	MPO level (nmol/g tissue)	LTB_4 level (ng/g tissue)	TNF-α level (ng/g tissue)	ICAM-1 level (µg/g tissue)	Nuclear NF-κB (O.D. NF-κB /ng of protein)
Control	2.17 ± 0.34	2.53 ± 0.52	12.65 ± 1.90	7.32 ± 1.50	9.44 ± 0.82
DHA + $NaHCO_3$ 5%	2.29 ± 0.50	1.37 ± 0.25	3.11 ± 0.43[a,b]	8.07 ± 1.61	9.80 ± 0.24
Vehicle + indomethacin	4.56 ± 0.12[b]	6.25 ± 0.96[b]	22.38 ± 2.45[b]	19.32 ± 1.97[b]	19.21 ± 0.50[b]
Indomethacin + DHA	0.35 ± 0.11[a,b]	1.10 ± 0.12[a]	8.30 ± 1.53[a]	8.61 ± 1.54[a]	11.72 ± 0.47[a]

Vehicle = olive oil + indomethacin, IND = indomethacin (30 mg/kg, p.o.), DHA = docosahexanoic acid (100 mg/kg, p.o.). Results are expressed as relative optical density (O.D.) values obtained using spectrophotometric analysis. Values expressed as mean ± S.E.M. (n = 5–7), statistical analysis was performed using ANOVA followed by Newman – Keuls test.

[a] $P < 0.05$ vs. vehicle + indomethacin.

[b] $P < 0.05$ vs. control.

Table 2
Effect of docosahexanoic acid (DHA) on oxidative stress in the indomethacin-induced gastric damage in mice.

Group/treatment	GSH (μmol/mg tissue)	SOD (U/ng protein)	CAT (K × 10^2 min^{-1})	MDA (nmol/ng protein)	Carbonyl (nmol/ng protein)
Control	1.37 ± 0.18	35.17 ± 1.84	76.47 ± 5.6	29.14 ± 9.21	1.58 ± 0.24
DHA + $NaHCO_3$ 5%	2.30 ± 0.05[a,b]	32.89 ± 2.26	88.48 ± 4.0[a]	31.62 ± 7.28[a]	1.51 ± 0.35[a]
Vehicle + indomethacin	1.04 ± 0.08[b]	31.13 ± 4.38[b]	45.44 ± 10.08[b]	99.86 ± 11.98[b]	2.76 ± 0.10[b]
Indomethacin + DHA	2.84 ± 0.09[a]	32.74 ± 1.69	41.32 ± 7.94[b]	36.78 ± 7.85[a]	1.51 ± 0.08[a]

Vehicle = olive oil + indomethacin, IND = indomethacin (30 mg/kg, p.o.), DHA = docosahexanoic acid (100 mg/kg, p.o.). Values expressed as mean ± S.E.M. (n = 5–7), statistical analysis was performed using ANOVA followed by Newman – Keuls test.

[a] $P < 0.05$ vs. vehicle + indomethacin.

[b] $P < 0.05$ vs. control.

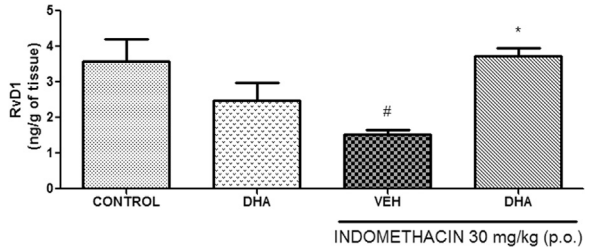

Fig. 3. Effect of DHA (100 mg/kg, p.o.) on RvD1 levels in gastric mucosa in the indomethacin-induced gastric injury model in mice (VEH = olive oil + indomethacin). Data are presented as mean ± S.E.M. (n = 5–7) $^{*}P < 0.05$ versus vehicle (VEH), $^{\#}P < 0.05$ versus control group.

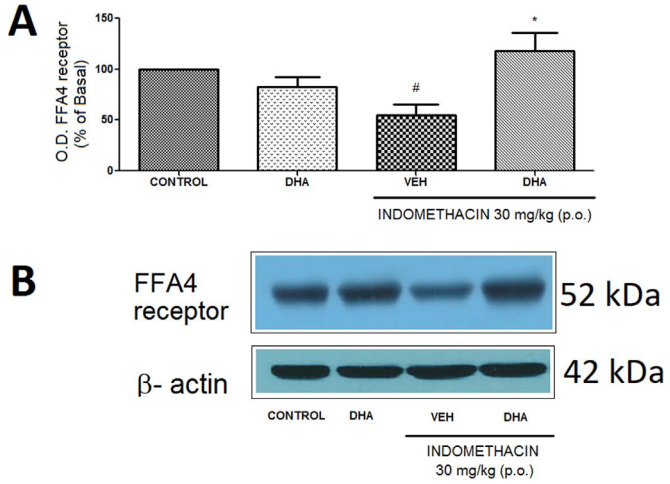

Fig. 4. (A) Assessment of DHA's effects (100 mg/kg, p.o.) on FFA4 receptor expression in gastric mucosa in the indomethacin-induced gastric injury model in mice determined by densitometric analyses of the Western immunoblot data. β-actin was used as an internal control. VEH = olive oil + indomethacin. Values calculated as the radio of FFA4 receptor/β-action. Results are expressed as relative optical density (O.D.) values obtained using spectrophotometric analysis. Each bar represents the mean value ± S.E.M. (n = 4) $^{*}P < 0.05$ versus vehicle (VEH), $^{\#}P < 0.05$ versus control group. (B) Representative Western immunoblot of FFA4 receptor of DHA pre-treatment (100 mg/kg, p.o.) in gastric mucosa of indomethacin-treated mice.

alternative therapeutic approach for the treatment of NSAID-induced gastric ulcers without compromising NSAID therapeutic actions since DHA has demonstrated synergistic interactions and gastric safety in the antinociceptive effect of indomethacin (Arroyo-Lira et al., 2014). In addition to PG inhibition (Martin and Wallace, 2006), it has been reported that indomethacin generates gastric ulcers through several processes, including an increase in reactive oxygen species production (Bastaki and Wallace, 1999; Wallace, 2013), initiation of lipid peroxidation (Beck et al., 2000), and neutrophil infiltration associated with the activation of inflammatory responses (Bindu et al., 2013; Laine et al., 2008). Our results suggest that the ability of DHA to modulate leukocyte recruitment or activity is reflected by the decrease of MPO, ICAM-1, LTB_4, and TNF-α levels; thus, DHA pre-treatment can be associated with a minimal inflammatory response leading to gastric protection, thus supporting the idea of inflammatory cytokine modulation exerted by omega-3 polyunsaturated fatty acids (Calder, 2013; Renier et al., 1993). The central role for oxidative stress in NF-κB activation has been well established (Bowie and O'Neill, 2000), and the nuclear translocation of NF-κB has been shown to stimulate the expression of proinflammatory cytokines (Bindu et al., 2013). As a consequence, we wanted to establish whether NF-κB comprises the key steps between the decrease in oxidative stress, inhibition of pro-inflammatory cytokine increase, and neutrophil-recruitment inhibition by DHA pre-treatment. In this study, we demonstrated that DHA-exerted gastroprotective effects involved the regulation of the inflammatory pathway via inhibition of TNF-α-mediated activation of NF-κB. Our results are consistent with previous studies in in vitro models in which THP-1 macrophages were stimulated (Harvey et al., 2015) and high glucose-treated neurons (Gao et al., 2015), in which DHA inhibited NF-κB gene expression and promoted inactivation of NF-κB through the decreased nuclear factor kappa B kinase subunit beta (IKKB) inhibitor phosphorylation (Yang et al., 2014).

Neutrophils play a key role in inflammation by releasing large amounts of reactive oxygen species in response to TNF-α stimulation and lipid peroxidation (Chanudom and Tangpong, 2015; Handa et al., 2014; Liu et al., 2014). The acute indomethacin-induced gastric damage model is associated with gastric lipid peroxidation and inflammatory reaction producing reactive oxygen species, thus leading to severe ulceration of gastric tissues (Chanudom and Tangpong, 2015). Our results show a DHA pretreatment-associated decrease in the formation of MDA and carbonyl proteins indicating the capacity of DHA to prevent oxidative damage in lipids and proteins necessary to maintain cellular homeostasis even in the presence of indomethacin. In addition, our data suggest that DHA pretreatment might preserve the normal redox potential by maintaining basal GSH and SOD levels, which, when acting in conjunction, improve the endogenous antioxidant defense that counteracts free radicals and cytotoxicity. The CAT experimental results showed that it was expressed abundantly in the cytoplasm, and its activity was coordinated by their protein abundance and enzymatic activity (Chen et al., 2016; Zhan et al., 2004). Some authors have indicated that cellular antioxidant responses to DHA-associated reactive oxygen species production could have occurred in the mitochondria in in vitro studies; the amount of CAT is less than that found in the cytoplasm, but was greater for GSH (Garrel et al., 2012; Weydert and Cullen, 2010). This could explain the lack of increase in CAT in gastric tissues. The data obtained in this study demonstrated the antioxidant capacity of DHA pretreatment in indomethacin-induced gastric damage. Furthermore, DHA-associated reduction of oxidative stress is consistent with the decrease in NF-κB activation and consequent attenuation of the pro-inflammatory response in the gastric mucosa after the indomethacin insult; as a result, gastric damage is prevented.

Despite excellent documentation of DHA-associated regulation of oxidative stress and inflammatory responses in a variety of neuropathological models (Ajami et al., 2013; Harvey et al., 2015; Mayurasakorn et al., 2011), there is only one report dealing with the

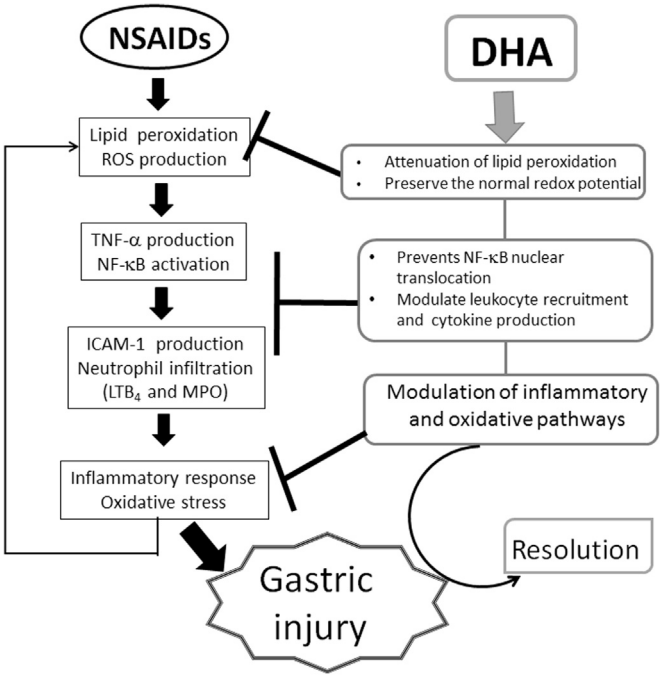

Fig. 5. Proposed mechanism of gastroprotective effect of DHA pretreatment against indomethacin-induced gastric damage in mice.

effects of omega-3 polyunsaturated fatty acids on indomethacin-gastric injury in *fat-1* transgenic mice (Han et al., 2016). Nonetheless, only the therapeutic mechanism of eicosapentaenoic acid (EPA) was examined. Since it has been documented that DHA and EPA may have independent effects when they are tested separately (Anderson and Ma, 2009; Dyall, 2015; Mayurasakorn et al., 2011; Mozaffarian and Wu, 2012; Sublette et al., 2011), we consider that our investigation provides a better understanding of the individual role of DHA in indomethacin-induced gastric injury.

Recently, the mechanisms underlying the beneficial effects of fatty acids have been partially explained by the discovery of omega-3 fatty acid-derived specialized pro-resolving mediators (SPMs) (Serhan et al., 2015), including resolvins of the D series (RvD 1–5) that are synthesized from DHA (Calder, 2009; Serhan et al., 2015). Transcellular resolvin synthesis involves both the cyclooxygenase and lipooxygenase pathways (Calder, 2013), and it has been reported that nanomolar doses of resolvins are enough to trigger their actions (Liu et al., 2014; Serhan et al., 2015). To our knowledge, there is no information of the possible implication of resolvins in DHA's gastroprotective effects. Furthermore, results in this study suggest for the first time that indomethacin administration could significantly reduce gastric RvD1 levels, and DHA was able to prevent the indomethacin-induced decrease of RvD1 gastric levels by enhancing the production and release of RvD1 in gastric tissues. Our results support several reports that indicate the ability of RvD1 to reduce LTB_4 expression (Norling et al., 2012), TNF-α release (Abdelmoaty et al., 2013), and ICAM-1 expression (Bento et al., 2011), and prevent nuclear translocation of NF-κB (Eickmeier et al., 2013) in inflammatory models of peritonitis (Norling et al., 2012), colitis (Bento et al., 2011), and inflammation-induced by mechanical hypersensitivity (Abdelmoaty et al., 2013). Besides its anti-inflammatory actions, RvD1 has been involved in the attenuation of oxidative stress (Cox et al., 2015; Lee and Surh, 2013) and prevention of lipid peroxidation (Spite et al., 2009) in murine models of peritonitis (Spite et al., 2009), hyperoxic injury (Cox et al., 2015), and oxidative stress-induced apoptosis in murine macrophage-like RAW264.7 cells (Lee et al., 2013). Nevertheless, in contrast to our study, few studies have made direct connections between the anti-inflammatory and antioxidative effects of DHA-derived resolvins such as RvD1.

It is important to note that certain molecular targets for omega-3 fatty acids include FFA4 receptors (Calder, 2015, 2013; Im, 2012). The FFA4 receptor is expressed in the GI tract (Mobraten et al., 2013) and abundantly expressed in inflammatory macrophages (Calder, 2015). In our study, FFA4 receptor expression decreased after indomethacin-induced gastric injury, and this effect was prevented by DHA administration. Recent studies have reported that the inhibitory effects of DHA on NF-κB occurs via the FFA4 receptor (Calder, 2015; Oh et al., 2010), this might explain the inhibition of NF-κB translocation to the nucleus and the decrease of pro-inflammatory cytokines observed herein. In addition, in vitro studies have demonstrated that DHA inhibits neutrophil recruitment and reactive oxygen species accumulation during inflammation through its incorporation into cellular membranes (Calder, 2013; Chattopadhyay et al., 2017; Lin et al., 2015; Nordgren et al., 2014), which consequently alters the fluidity of the membrane in leukocyte–endothelial cell interactions, reduces lipid peroxidation of membrane's lipids, and probably modifies other cell effectors (Liu et al., 2016; Rodrigues et al., 2016). More importantly, it has been suggested that some fatty acids, including DHA, might regulate the activity of intracellular signaling pathways and restore the imbalance of oxidative damage through different mechanisms (Martins de Lima et al., 2007). Considering the results obtained in the present study and the extensive search of relevant information available in the literature, we have proposed a scheme to give a logical explanation regarding to the gastroprotective effect of DHA, in which the previously reported FFA4 receptor and DHA interactions and resolvins could fully explain our results (Fig. 5).

5. Conclusions

In conclusion, this investigation clearly identifies the mechanism by which DHA affords an important gastroprotective effect against indomethacin- induced gastropathy, demonstrating that attenuation of pro-inflammatory cascade and activation of antioxidative pathway are the main mechanisms. In addition, our findings strongly suggest that the modulation of these pathways could cause expression of the FFA4 receptor. However, further studies need to be done to investigate the participation of RvD1 and the FFA4 receptor in the gastroprotective effects of DHA. Our results indicate that DHA might be considered a promising potential candidate for the treatment of indomethacin-induced gastropathy.

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Conflict of interest statement

The authors declare no conflict of interest.

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