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Evidence of anti-inflammatory effect and percutaneous penetration of a topically applied fish oil preparation: a photoacoustic spectroscopy study

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Abstract. This paper investigates the topical anti-inflammatory effect of a fish oil preparation (FOP) in a croton oil (CO) model of skin inflammation. The photoacoustic spectroscopy (PAS) was applied to estimate the percutaneous penetration of the FOP and as a model to evaluate the topical inflammatory response. After applying CO, the groups of mice received a topical application of a FOP on the left ear. The right ear received the vehicle that was used to dilute the CO. After 6 h, ear tissue was collected to determine the percent inhibition of edema, myeloperoxidase (MPO) activity, and cytokine levels and to perform PAS measurements. Treatment with FOP reduced edema and MPO activity, which was at least partially attributed to a decrease in the levels of tumor necrosis factor, interleukin-1β, interleukin-6, keratinocyte-derived chemokine, and monocyte chemoattractant protein-1. The topically applied FOP penetrated into the tissue and decreased the area of the bands that characterize inflamed tissue. The present results demonstrated the topical anti-inflammatory effect of the FOP. PAS suggests that FOP anti-inflammatory activity is linked with its ability to penetrate through the skin. [DOI: 10.1117/1.JBO.22.5.055003]

Keywords: Fish oil; inflammation; ear edema; croton oil; photoacoustic spectroscopy.

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1 Introduction

Fish oil (FO) is a rich natural source of omega-3 polyunsaturated fatty acids, including eicosapentaenoic acid (EPA; $20:5n-3$) and docosahexaenoic acid (DHA; $22:6n-3$), with various beneficial effects in many autoimmune and inflammatory diseases when supplemented in the diet.[1–3] The anti-inflammatory activity of FO in these studies may be at least partially attributable to the insertion of EPA and DHA, which partially substitute for arachidonic acid (ARA; $20:4n-6$) in phospholipids of the cell membrane.[4] This substitution changes membrane fluidity, decreases the production of eicosanoids that are derived from ARA metabolism, and increases the synthesis of lipid mediators that are derived from EPA that are less potent than those that are derived from ARA. It also induces the synthesis of compounds that stimulate the resolution of inflammation (e.g., resolvins that are derived from DHA and EPA), and induces the synthesis of protectins and maresins that are derived from DHA.[1,5]

The anti-inflammatory activity of FO also involves other mechanisms, including actions on signaling pathways that reduce the expression of proinflammatory genes by binding to G protein-coupled receptor 120, activate peroxisome proliferator-activated receptor-γ, and inhibit the activation of factor kappa B (NF-κB).[3,6]

Our group recently reported that a single oral dose of FO had anti-inflammatory activity in two acute models of inflammation (paw edema and pleurisy) that was induced by carrageenan in rats. Treatment with a fish oil preparation (FOP) reduced the formation of edema and leukocyte recruitment at the site of injury. Such effects were at least partially attributed to an inhibitory effect of the FOP on the production or release of cytokines and nitric oxide.[7]

These reports indicate beneficial effects of FO when orally administered, either in a single dose or when FO is supplemented in the diet. Still unknown, however, is whether FO has anti-inflammatory activity when it is topically applied to the skin. The use of topically applied drugs has the advantage of minimizing adverse effects that can be caused by systemic administration.[8] Few studies[9,10] have evaluated the anti-inflammatory activity of topically applied FO.

We used a model of croton oil (CO)-induced ear edema, which has been widely used to evaluate the topical anti-inflammatory activity of compounds that are able to effectively be used for the treatment of cutaneous inflammatory diseases.[11,12]

The adequate penetration of topical anti-inflammatory agents to reach regions of the skin where the inflammatory process occurs is crucial for their efficacy. Thus, the efficacy of topically applied compounds depends on their penetration characteristics, which are related to the composition of the formulation and conditions of the skin, such as moisture content and structure.[13] Photoacoustic spectroscopy (PAS) has been shown to be an effective method in dermatological research to characterize tissue and lesions[14] and evaluate the distribution and penetration of substances through the skin where the formulations are applied.[13,15] Furthermore, PAS is relatively easy to perform, does not require the use of reagents, and is nondestructive. These features are important and desirable for preclinical studies

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in pharmaceutical research because of the need for a small number of animals that are used and of the possibility of reusing samples in additional tests.

PAS is a method that instead of measuring the radiation transmitted or reflected by the sample, as happens for conventional spectroscopic techniques operating in the UV–Vis region, determines the fraction of the absorbed energy that is transformed into heat.[16] The result is a spectrum similar to optical absorption, enabling studying solid or semisolid materials in bulk or powder and amorphous or crystalline substances, gel, and others.[17] Furthermore, scattered light from the sample generally is a problem for conventional spectroscopic techniques, for example, to analyze biological tissues. However, these features present no difficulties in PAS justifying its potential as an inspection method to study healthy and lesioned tissues.

In fact, there is nowadays a great effort to establish photoacoustic and photothermal methods for clinical diagnosis. The literature survey reveals some papers in this area using since UV–Vis conventional PAS until those more sophisticated related with photoacoustic imaging.[18,19] There are even works using these methods for diagnosis of inflammation in systemic diseases.[20,21] This was our motivation to apply a photoacoustic method in this work. We explored the simplicity of the conventional photoacoustic method in an important area related to the search of new formulations envisaging anti-inflammatory effects.

This study had two purposes: (1) to investigate the topical anti-inflammatory effect of an FOP in a CO model of skin inflammation in mice and (2) to evaluate the percutaneous penetration of an FOP by PAS.

2 Materials and Methods

2.1 *Animals*

Male Swiss mice, weighing 25 to 30 g, were kept in an environment under controlled temperature (22°C ± 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 Maringá (ECAE/UEM 045/2012).

2.2 *Fish Oil Preparation*

The FO was commercially obtained from Naturalis®. According to the manufacturer, each 500-mg capsule contains 250 mg DHA and 50 mg EPA. A previous study[7] quantified the fatty acids that were present in the capsule, validating the aforementioned information from the manufacturer (data not shown).

The contents of the FO capsule were diluted in olive oil (OO) or in a solution of 70% acetone to obtain the fish oil preparations (FOP_{OO} and FOP_{AC}) in amounts of (i) 0.25 mg DHA and 0.05 mg EPA, (ii) 0.5 mg DHA and 0.1 mg EPA, (iii) 1 mg DHA and 0.2 mg EPA, (iv) 2 mg DHA and 0.4 mg EPA, and (v) 4 mg DHA and 0.8 mg EPA. These preparations were topically applied to the ears of different groups of animals. For simplicity, we refer to the preparations below according only to the amount of DHA in each preparation.

2.3 *Croton Oil-Induced Ear Edema*

Ear edema was induced on the ventral side of the left ear by applying 20 μl of CO (200 μg) diluted in 70% acetone (vehicle), adapted from the technique of Schiantarelli et al.[22] The right ear received only topical application of 20 μl of vehicle (70% acetone) as a noninflamed control or negative control. Immediately after CO application, the animals received a topical application of 20 μl of 70% acetone (inflamed control or positive control), FOP_{OO} (0.25, 0.5, 1, 2, or 4 mg DHA/ear) or FOP_{AC} (0.25, 0.5, 1, 2, or 4 mg DHA/ear). The other groups of animals received a topical application of 20 μl of OO, dexamethasone (Dexa; 0.1 mg/ear, used as anti-inflammatory reference drug), or indomethacin (Indo; 1 mg/ear, used as anti-inflammatory reference drug) immediately after CO application. After 6 h, the animals were anesthetized and euthanized. The ears were sectioned into 6.0-mm diameter discs and weighed (in milligrams) using an analytical balance. Ear edema was evaluated as an increase in ear weight ($n = 7$ animals/group). The percent inhibition of edema was determined according to the following equation:

$$\%\text{inhibition} = \frac{(\text{left ear weight}_{\text{positive control}} - \text{left ear weight}_{\text{treated}})}{\text{left ear weight}_{\text{positive control}} - \text{right ear weight}_{\text{negative control}}} \times 100. \quad (1)$$

2.4 *Myeloperoxidase Activity*

Myeloperoxidase (MPO) activity was assessed in the supernatants of homogenates of ear sections ($n = 7$/group) in the control groups and mice that received the aforementioned treatments (CO-induced ear edema) according to the technique of Bradley et al.[23] The ear tissue was placed in a solution that contained 0.5% hexadecyltrimethyl ammonium bromide in potassium phosphate buffer (50 mM) and processed in a homogenizer (60 s at 0°C). The homogenate was centrifuged at 2500 rotations per minute (rpm) for 5 min. Afterward, 10 μl of the supernatant (in triplicate) was added to a 96-well microplate, followed by the addition of 200 μl of a mixture that contained *O*-dianisidine dihydrochloride (0.167 g/ml), hydrogen peroxide (0.0005%), and potassium phosphate buffer (50 mM). The reaction was stopped by the addition of 1.46 M sodium acetate solution. Enzyme activity was determined by the end-point technique by measuring absorbance at a wavelength of 460 nm and expressed in optical density (OD). The percent inhibition of MPO activity was determined according to the following equation:

$$\%\text{inhibition} = \frac{(\text{left ear OD}_{\text{positive control}} - \text{left ear OD}_{\text{treated}})}{(\text{left ear OD}_{\text{positive control}} - \text{right ear OD}_{\text{negative control}})} \times 100. \quad (2)$$

2.5 *Determination of Cytokine Levels*

Cytokine levels were determined in the supernatants of homogenates from ear sections in the control groups and mice that were treated with FOP_{OO} (4 mg DHA/ear) or OO alone 6 h after topical CO application ($n = 7$/group). The ear tissue was placed in phosphate-buffered saline and processed in a homogenizer (60 s at 0°C). The homogenate was centrifuged at 2500 rpm for 10 min, and the supernatant was separated and stored at −70°C until the assay was performed. The analyses were performed using a magnetic bead reader (Luminex 100TM/200TM) and Cytokine Mouse Magnetic 20-Plex Kit (Invitrogen, Waltham, Massachusetts, catalog no. LMC006M, lot no. 1000793) according to the manufacturer's recommendations.

2.6 *Determination of Percutaneous Penetration and Topical Inflammatory Response by Photoacoustic Spectroscopy*

The measurements were performed on sections of right ear tissue (negative control) and 6 h after applying CO in the positive control group, FOP_{OO} (4 mg DHA/ear) and OO alone, using the experimental setup shown in Fig. 1 ($n = 5$/group). The PAS homemade experimental setup is composed of a 1000-W Xenon arc lamp (Oriel, model 68820) used as a light source, which is diffracted when passing through the monochromator (Oriel, model 77250) with 3.16-mm input and output slits. The monochromator was equipped with a diffraction grade (Oriel, model 77296) for UV–Vis spectral range between 200 up to 800 nm. The nominal power of the light source was 800 W. The mechanical chopper (Stanford Research Systems, model SR 540) was tuned at 15 Hz modulating the light that impinges the sample. Higher order diffractions were eliminated by band-pass filters. Thus, the monochromatic beam was focused in the sample placed inside the photoacoustic cell, which was sealed with a transparent quartz window (8-mm diameter and 2-mm thickness). A Brüel and Kjaer model 2669 capacitive microphone was coupled to collect the photoacoustic signal that was generated from the pressure variation resulting from the periodic heating of the sample. The used lock-in amplifier was a EG&G Instruments, model 5110. The depth of the skin sample contributing to the photoacoustic signal was estimated using the thermal diffusion length (μs): $\mu s = (d/\pi f)1/2$, in which d is the sample thermal diffusivity and f is the light modulation frequency. Taking the thermal diffusivity of the skin (ventral and dorsal) as $d = 4.0 \times 10^{-4}$ cm^2 s^{-1} (Ref. 24) and $f = 15$ Hz, the estimated μs value of our measurements was 30 μm. The final photoacoustic signal is proportional to

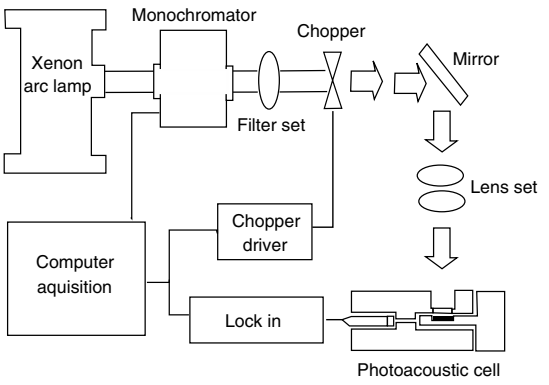

Fig. 1 Experimental setup of the used PAS.

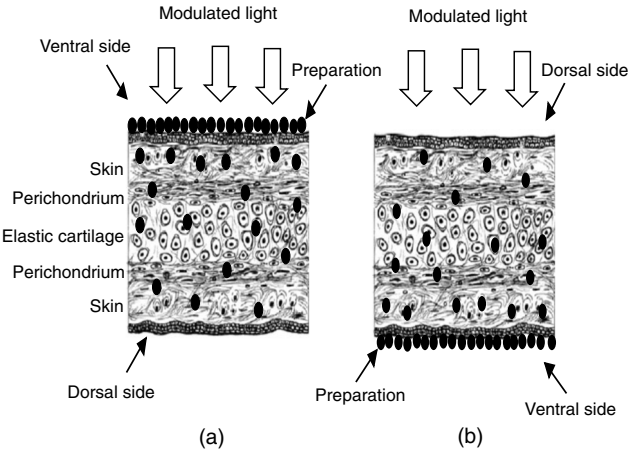

Fig. 2 Schematic of spectral readings for illumination of the (a) ventral surface and (b) dorsal surface of ear tissue in mice (modified from Ref. 15).

the sample absorption coefficient, then the photoacoustic spectra can be interpreted by means of absorption bands.[17] To correct the source emission intensity in each wavelength, all spectra were normalized with respect to a carbon black sample signal.

The spectra of each ear were obtained positioning the sample into the photoacoustic cell, illuminating the face to be measured. Afterwards, the ear was turned upside down to illuminate the opposite side, as shown in Figs. 2(a) and 2(b). Considering the chosen light modulation frequency at 15 Hz (μs of about 30 μm) and that the total sample thicknesses were always around 445 μm, in all cases the measurements were performed near the surface of the samples exposed to the incident light. Then, detecting the presence of FOP_{OO} band on the dorsal side (opposite side of FOP_{OO} application) indicates that the topically applied formulation permeated through the ear tissue. In addition, the quantification of the bands associated with the inflammatory response in the inflamed and treated ears may provide information regarding the inflammatory processes and the treatment effect.

2.7 *Statistical Analysis*

Edema values, MPO activity, and cytokine levels are presented as mean ± standard error of the mean (SEM). The data were analyzed using analysis of variance (ANOVA) followed by Tukey's test. Values of $p < 0.05$ were considered statistically significant.

3 Results

3.1 *Effect of Fish Oil Preparations on Croton Oil-Induced Ear Edema and Myeloperoxidase Activity*

The application of CO on the left ear (inflamed control or positive control) caused an evident inflammatory response at 6 h compared with the right ear (noninflamed control or negative control). Topical application of FOP_{OO} at 0.25, 0.5, 1, 2, and 4 mg DHA/ear and OO alone significantly reduced the formation of ear edema [93.3%, 81.4%, 85.5%, 72%, 83.7%, and 85.5%, respectively; Fig. 3(A)]. Because these reductions of ear edema were similar, independent of treatment, we conducted a further experiment using 70% acetone as the diluent to discard the possible action of OO on the FO response.

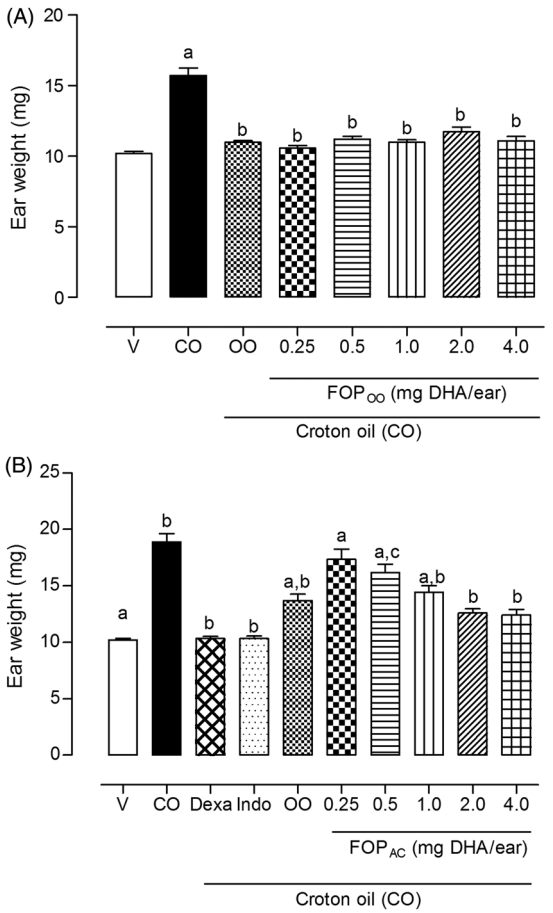

Fig. 3 Effect of FOP on ear edema induced by CO (200 μg) in mice. (A) FOP_{OO} at the amounts indicated and OO alone were topically applied immediately after the application of CO on the left ear. (B) FOP_{AC} at the amounts indicated and OO diluted in 70% acetone were topically applied immediately after the application of CO on the left ear. Dexa and Indo were used as anti-inflammatory reference drugs and diluted in 70% acetone and topically applied at doses of 0.1 and 1 mg/ear, respectively. V = right ears that received only application of the vehicle (70% acetone) as a negative control. The data are expressed as the mean ± SEM ear weight for each group ($n = 7$) 6 h after the application of CO. [a]$p < 0.001$, compared with V; [b]$p < 0.001$, compared with positive control group (CO); [c]$p < 0.05$, compared with positive control group (CO; ANOVA followed by Tukey's test).

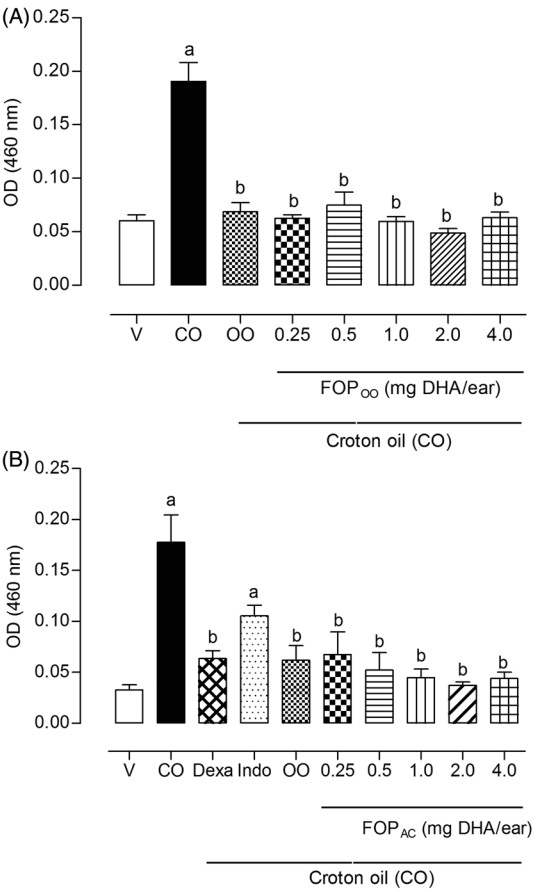

Fig. 4 Effect of FOP on MPO activity in supernatants of homogenates of ear tissue in mice. (A) FOP_{OO} at the amounts indicated and OO alone were topically applied immediately after the application of CO on the left ear. (B) FOP_{AC} at the amounts indicated and OO diluted in 70% acetone were topically applied immediately after the application of CO on the left ear. Dexa and Indo were used as anti-inflammatory reference drugs and diluted in 70% acetone and topically applied at doses of 0.1 and 1 mg/ear, respectively. V = right ears that received only application of the vehicle (70% acetone) as a negative control. The data are expressed as the mean ± SEM OD for each group ($n = 7$) 6 h after the application of CO. [a]$p < 0.001$, compared with positive control group (CO; ANOVA followed by Tukey's test).

FOP_{AC} at 0.5, 1, 2, and 4 mg DHA/ear and OO diluted in 70% acetone significantly reduced the intensity of ear edema (31.4%, 51.3%, 72.4%, 74.4%, and 59.9%, respectively). These reductions were less than those of Dexa (0.1 mg/ear) and Indo [1 mg/ear; 98.5% and 98.3%, respectively; Fig. 3(B)].

MPO activity increased after CO application. The topical application of FOP_{OO} at 0.25, 0.5, 1, 2, and 4 mg DHA/ear and OO significantly inhibited MPO activity [98.3%, 89%, 99%, 99%, 97.8%, and 93.6%, respectively; Fig. 4(A)].

FOP_{AC} at 0.25, 0.5, 1, 2, and 4 mg DHA/ear and OO diluted in 70% acetone significantly reduced MPO activity (76.1%, 86.6%, 91.6%, 96.9%, 92.3%, and 80.1%, respectively). These reductions of MPO activity were greater than the effect of Indo (1 mg/ear; 48%), which did not significantly inhibit MPO activity. Dexa (0.1 mg/ear) also inhibited MPO activity [80.6%; Fig. 4(B)].

3.2 *Effect of Fish Oil Preparation on Cytokine and Chemokine Levels in Supernatants of Homogenates from Ear Sections*

The levels of the proinflammatory cytokines tumor necrosis factor (TNF), interleukin-1β (IL-1β), and IL-6 as well as chemokines keratinocyte-derived chemokine (KC/CXCL-1) and monocyte chemoattractant protein-1 (MCP-1/CCL2) increased in the supernatants of homogenates from the ear sections 6 h after the induction of the inflammatory response by topical application of CO. Topical treatment with FOP_{OO} at 4 mg DHA/ear reduced TNF, IL-1β, IL-6, KC, and MCP-1 levels in the supernatants of homogenates from the ear sections [Fig. 5(A)–5(E), respectively]. Topical application of OO reduced IL-1β, IL-6, and MCP-1 levels [Fig. 5(B), 5(C), and 5(E), respectively] but did not reduce TNF or KC levels [Fig. 5(A) and 5(D), respectively]. Topical treatment with FOP_{OO} at 4 mg DHA/ear and OO alone did not alter the levels of the anti-inflammatory cytokines IL-10 and IL-4 (data not shown).

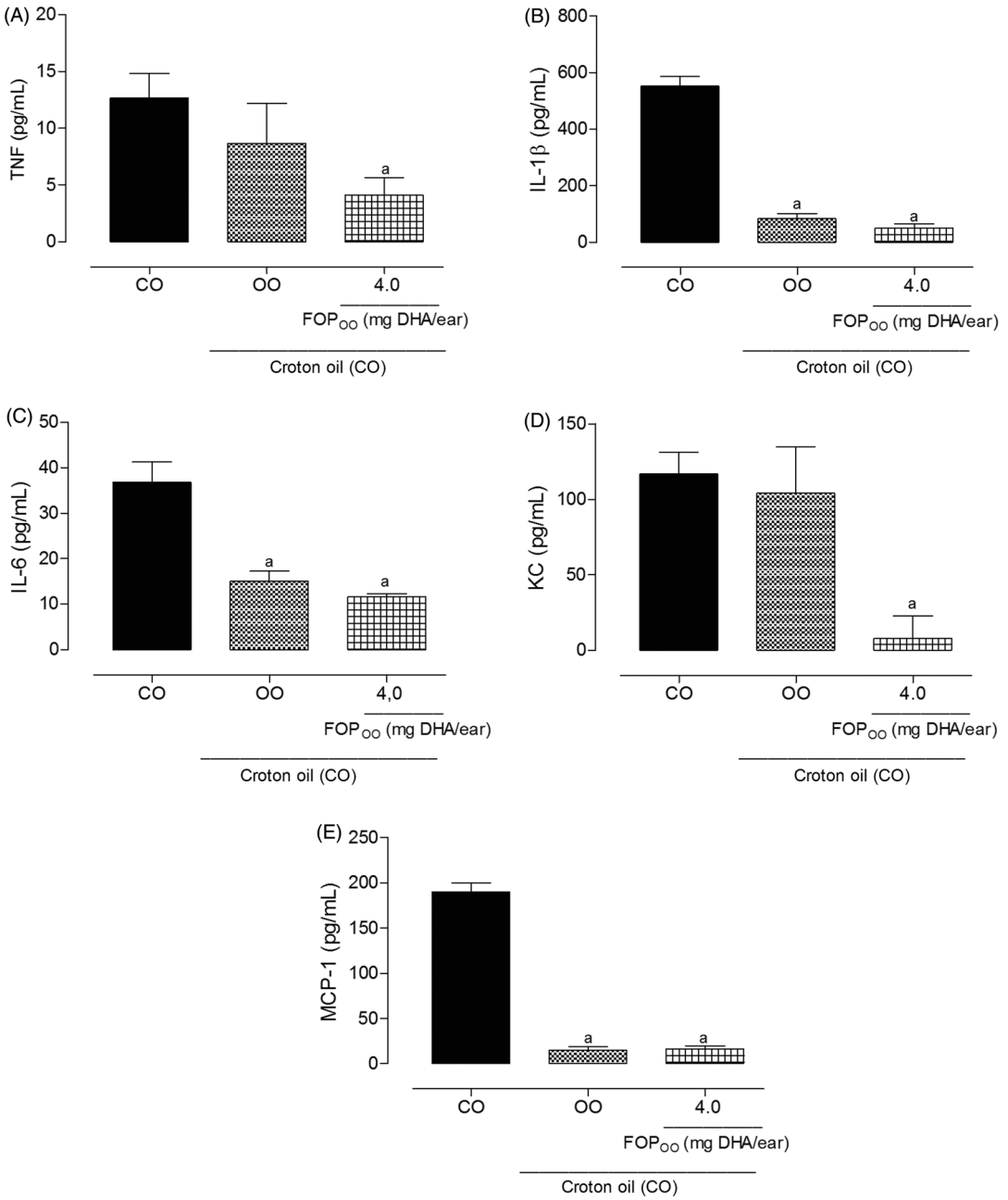

Fig. 5 Effect of FOP on the levels of the cytokines (A) TNF, (B) IL-1β, and (C) IL-6 and chemokines, (D) KC, and (E) MCP-1 in supernatants of homogenates of ear tissue in mice. FOP_{OO} (4 mg DHA/ear) and OO alone were topically applied immediately after the application of CO on the left ear. Cytokine and chemokine levels were calculated by subtracting the value obtained for the right ear (V, negative control) from the value obtained for the left ear in the different groups. The data are expressed as the mean $\pm$ SEM for each group ($n = 7$) 6 h after the application of CO. $^{a}p < 0.05$, compared with positive control group (CO; ANOVA followed by Tukey's test).

3.3 *Determination of Percutaneous Penetration of Fish Oil Preparation and Its Effect on the Topical Inflammatory Response by Photoacoustic Spectroscopy*

The photoacoustic spectra for CO, FO, OO, and FOP_{OO} at 4 mg DHA/ear (Fig. 6) indicated that these substances featured absorbing chromophores in the region from 200 to 350 nm. The CO spectrum was broadband, without the presence of well-defined bands compared with the FO, OO, and FOP_{OO} spectra. The FO spectrum had two absorption bands with peaks around 246 and 280 nm. The OO spectrum had a band centered around 234 nm. The FOP_{OO} spectrum had two absorption bands with peaks around 246 and 273 nm. The detection of these characteristic bands along the thickness of the ear tissue indicated the presence of FOP_{OO} and OO.

The readings from the ventral and dorsal sides of the right (negative control) and the left (positive control) ear tissue 6 h after the application of CO showed the presence of two bands in the left ear tissue spectrum centered around 225 and

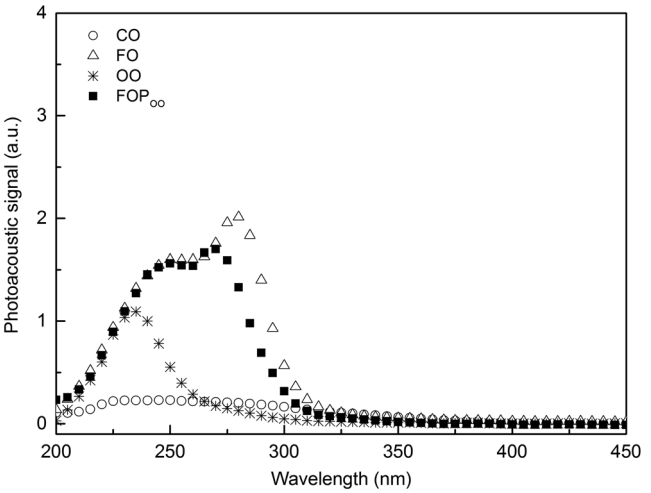

Fig. 6 Photoacoustic spectra of CO, FO, OO, and FOP_{OO} (4 mg DHA/20 μl).

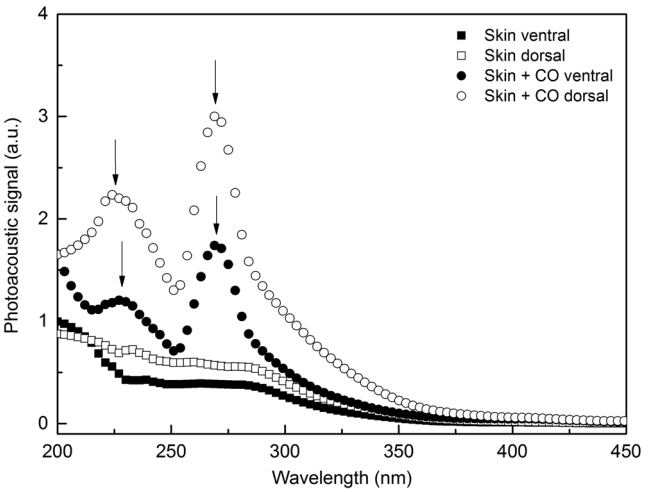

Fig. 7 Photoacoustic spectra for the ventral and dorsal sides of right ear tissue (negative control, skin ventral, skin dorsal) and left ear tissue (positive control, skin + CO ventral, skin + CO dorsal) 6 h after the application of CO on the ventral side of the left ear ($n = 5$).

270 nm (Fig. 7, arrows). These bands were absent in the right ear tissue spectrum. These results show that PAS detected the inflammatory response in ear tissue 6 h after the CO-induced inflammatory response.

Fig. 8(a) shows the spectra that were obtained from readings on the ventral side of the right and left ears tissue (negative and positive controls, respectively) and ear tissue that was treated with FOP_{OO} (skin + CO + FOP_{OO}) and OO (skin + CO + OO). The spectra for ear tissue that was treated with OO have spectral characteristics that were similar to the positive control ear tissue spectrum, with bands at 225 and 270 nm. The ear tissue that was treated with FOP_{OO} presented a band at 270 nm, but at a lower intensity compared with this same band from the positive control and no band at 225 nm. This strong reduction of the 225 nm band appears to be a fingerprint showing that the FOP_{OO} presented an anti-inflammatory effect on the ventral side of the ear.

Because of the overlapping of the 210 to 250 nm bands from FOP_{OO} and OO with that characteristic of inflamed tissue, we

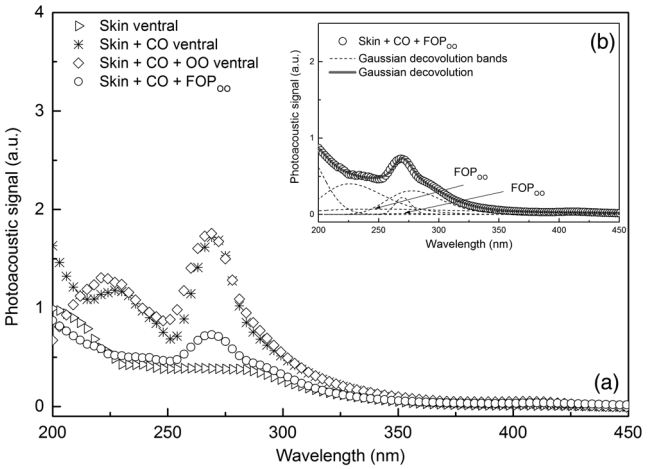

Fig. 8 (a) Photoacoustic spectra obtained from readings of the ventral side of right ear tissue (negative control, skin ventral), left ear tissue (positive control, skin + CO ventral), ear tissue treated with FOP_{OO} (4 mg DHA/ear; skin + CO + FOP_{OO} ventral) and ear tissue treated with olive oil (skin + CO + OO ventral), 6 h after topical application of CO on the ventral side of the left ear ($n = 5$). (b) Gaussian adjustments of spectra for skin + CO + FOP_{OO} ventral. The dashed lines show the bands that make up the spectrum, and the solid line indicates the sum of all bands.

could not directly observe the presence of substances that were topically applied on the ears. Therefore, fittings were made using Gaussian functions [Fig. 8(b)] to decompose the photoacoustic spectra into constituent bands, thus allowing the identification of characteristic bands for FOP_{OO} and OO in the ear tissue. This procedure was performed for all of the photoacoustic spectra for the treated ear tissue and pure substances. In Fig. 8(b), the dashed lines show the bands in the photoacoustic spectrum for the ventral side of the ear tissue that was treated with FOP_{OO}. The solid line is the Gaussian functions sum, which represents the best fitting of the sample spectrum.

With Gaussian fitting, the separation of the spectrum of the positive control ear tissue (skin + CO) showed peaks centered at 200, 225, 270, and 280 nm. The separation of the spectrum of the ear tissue that was treated with FOP_{OO} (skin + CO + FOP_{OO}) had bands centered at 200, 246, 270, 273, and 280 nm. The separation of the spectrum of ear tissue that was treated with OO (skin + CO + OO) exhibited Gaussian peaks centered at 200, 225, 234, 270, and 280 nm. Thus, the bands of the substances that were topically applied that differed from the characteristic bands of inflamed tissue (225 and 270 nm) were centered at 246 and 273 nm for FOP_{OO} and 234 nm for OO, indicating the presence of such substances on the ventral surface of the treated ears. These same bands were obtained with the Gaussian fittings of the pure substances.

The spectra that were obtained from reading the dorsal side of the right and left ears tissue (negative and positive controls, respectively) and ear tissue that was treated with FOP_{OO} (skin + CO + FOP_{OO}) and OO (skin + CO + OO) are shown in Fig. 9. Figure 9(a) shows the presence of the optical absorption bands that characterize inflamed tissue in the photoacoustic spectra of the ear tissue that was treated with FOP_{OO} and OO, but at a lower intensity compared with the positive control ear, indicating an inhibitory effect. Similar to the ventral side, measurements of the dorsal side were also performed using Gaussian fittings [Fig. 9(b)]. In the photoacoustic spectra of the samples that were treated with FOP_{OO} and OO, decomposed by Gaussian

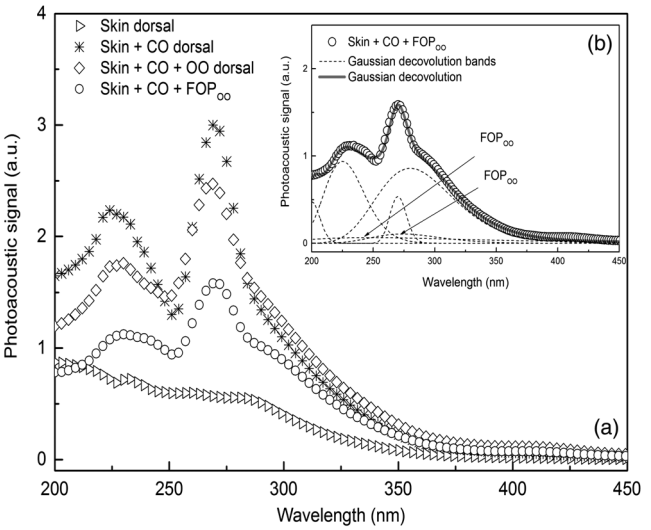

Fig. 9 (a) Photoacoustic spectra obtained from readings of the dorsal side of right ear tissue (negative control, skin dorsal), left ear tissue (positive control, skin + CO dorsal), ear tissue treated with FOP_{OO} (4 mg DHA/ear; skin + CO + FOP_{OO} dorsal) and ear tissue treated with olive oil (skin + CO + OO dorsal), and 6 h after topical application of CO on the ventral side of the left ear ($n = 5$). (b) Gaussian adjustments of spectra for skin + CO + FOP_{OO} dorsal. The dashed lines show the bands that make up the spectrum, and the solid line indicates the sum of all bands.

functions, bands were centered at 246 and 273 nm for FOP_{OO} and 234 nm for OO, indicating the presence of these substances on the dorsal side of the ear tissue. This shows that both FOP_{OO} and OO permeated the ear tissue to the dorsal side. The topical application of both substances occurred on the ventral side.

Fig. 10 shows the average areas of the optical absorption bands that characterize inflamed tissue that was induced by CO, centered at 225 and 270 nm, obtained from readings from the ventral and dorsal sides of inflamed control ear tissue (skin + CO) and ear tissue that was treated with FOP_{OO} (skin + CO + FOP_{OO}) and OO (skin + CO + OO). The areas of each photoacoustic spectra were obtained using Gaussian fittings. On the ventral side [Fig. 10(a)], there was a reduction of the areas of the bands that characterize inflamed tissue for ear

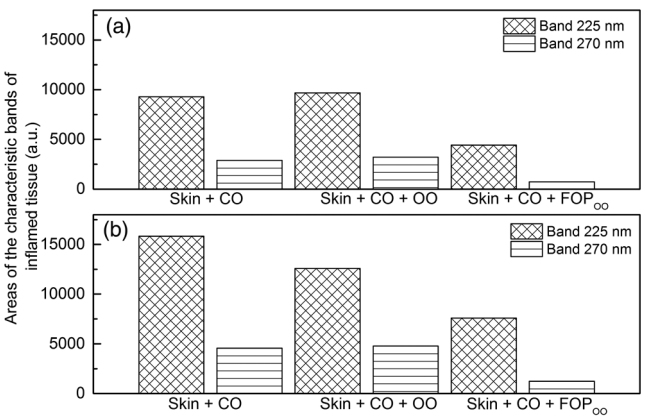

Fig. 10 Average areas of optical absorption bands that characterize inflamed tissue (225 and 270 nm) in photoacoustic spectra after the CO-induced inflammatory response in positive control ear tissue (skin + CO), ear tissue treated with FOP_{OO} [4 mg DHA/ear (skin + CO + FOP_{OO})], and ear tissue treated with olive oil (skin + CO + OO) ($n = 5$). (a) Ventral side. (b) Dorsal side.

tissue that was treated with FOP_{OO} compared with the inflamed control ear. Ear tissue that was treated with OO presented areas of the bands that characterize inflamed tissue that were similar to the control inflamed ear. Figure 10(b) shows a reduction of the areas of the optical absorption bands that characterize inflamed tissue on the dorsal side for both FOP_{OO} and OO treatment compared with the control inflamed ear. Note that in the treated ear with FOP_{OO}, the areas of the bands related to the inflamed tissue were smaller than those treated with OO.

4 Discussion

This study demonstrated an anti-inflammatory effect of topical application of FOP_{OO} on CO-induced ear edema in mice. We also found evidence of the percutaneous penetration of FOP_{OO} after topical application.

CO is a complex mixture of lipids that are extracted from *Croton tiglium* L. (Euphorbiacea), which contains 12-*O*-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters with irritating properties. The topical application of CO induces a local inflammatory response that is characterized by edema formation and leukocyte infiltration through the direct activation of protein kinase C and subsequent activation of other enzymatic systems, including the production of prostaglandins, leukotrienes, and proinflammatory cytokines.[11,25,26] In this model of ear edema, topical treatment with FOP_{OO}, regardless of the amount tested, prevented the formation of edema and reduced MPO activity in homogenates of ear tissue. MPO activity was used as an indirect marker of the recruitment or activation of neutrophils in the inflamed tissue.[23]

The levels of the proinflammatory cytokines TNF, IL-1β, and IL-6 and chemokines KC and MCP-1 increased in the inflamed ear tissue, which is consistent with previous reports.[27] TNF plays a role in the cutaneous inflammatory response by inducing the expression of leukocyte adhesion molecules, which stimulates the release of other cytokines, such as IL-1β and IL-6, and increases vascular permeability.[25,28,29] The actions of IL-1β involve the activation of leukocytes and induction of the expression of adhesion molecules, chemokines, and other cytokines.[30,31] IL-6, in turn, induces the expression of chemokines and adhesion molecules and consequently influences the recruitment of leukocytes to the inflammatory site.[31,32] Chemokines, such as KC and MCP-1, are low-molecular-weight cytokines that stimulate leukocyte migration and actively participate in the inflammatory response after injury.[33]

Thus, the inhibitory effect of topically applied FOP_{OO} on the CO-induced inflammatory response was at least partially attributed to a decrease in the levels of these cytokines. Our data corroborate other studies that reported inhibitory effects of FO on the inflammatory response and cytokine production in other experimental models.[34,35]

We also investigated the percutaneous penetration of topically applied FOP_{OO} using PAS. The photoacoustic signals that are obtained by this technique depend on the optical and thermal properties of the sample. When samples undergo changes in their composition and/or structure, such as in cases of inflammatory responses associated with the formation of edema, recruitment of leukocytes, and production of inflammatory mediators, the heat propagation and the optical absorption of the sample can change, thus altering the photoacoustic signal.[13] Thus, PAS can also be used to evaluate changes that occur in the photoacoustic signal of tissue after establishment of the inflammatory process.

As observed, the photoacoustic signal of inflamed ear tissue had two characteristic bands with peaks around 225 and 270 nm. It is important to mention that these bands were not present in the negative control. Thus, these bands can be attributed to the presence of components that participate in inflammatory events, demonstrating the applicability of PAS for studies on skin inflammation. Previous studies have shown that leukocytes exhibit characteristic fluorescence patterns with an intense excitation peak in the region between 250 and 265 nm.[36,37] More recently were demonstrated absorption bands of leukocytes between 200 to 220 nm and 240 to 290 nm.[38] These bands of leukocytes are similar to the absorption bands that were observed centered at 225 and 270 nm in the inflamed ear tissue. Therefore, based on that, we hypothesize that the bands deteced in this study are due to the leukocyte infiltration in the ear tissue that occurs after the CO-induced inflammatory response. In addition, the reduction of the 225 and 270 nm bands of the FOP_{OO} treated ear may be associated with a reduction of the leukocytes population in the inflamed area, as demonstrated by the evaluation of the MPO activity, occurring as a consequence of the FOP_{OO} effect.

The analysis of the photoacoustic signals clearly demonstrated the percutaneous permeation of FOP_{OO} after topical application, which also evidenced that PAS is an effective technique that may be used safely for determining the optical absorption characteristics of inflamed ear tissue. It was possible to detect the inhibitory effect of the topically applied FOP by decreases on the area of the optical absorption bands that characterize inflamed tissue 6 h after the CO-induced inflammatory response. The anti-inflammatory effect that was observed after topical treatment with FOP_{OO} and OO was reflected by decreases in edema intensity, MPO activity, and cytokine levels. These decreases were strongly correlated with the inhibitory effect that was observed in the PAS analysis. We have previously used PAS to evaluate the penetration of a topically applied plant extract immediately after the CO-induced inflammatory response in ear tissue in mice.[15] However, to our knowledge, for the first time this technique was used in order to exploit some features of the inflammatory response in this experimental model.

The different intensities that were observed between the photoacoustic spectra of the ventral and dorsal sides may be attributable to the different time intervals that the sample was placed in the photoacoustic cell. Both sides of the same sample were measured. The time of light exposure during the reading of the first side may have caused dehydration of the tissue, thus making it slightly thinner. Therefore, when the other side was read, the tissue may have filled the photoacoustic cell differently, thus causing differences in the signal intensity between readings.

The choice of OO as the diluent to obtain the FOP_{OO} was based on studies that evaluated the benefits of FO in arthritis and administered OO in control animals.[39,40] Topically applied OO exerted an inhibitory effect on ear edema that was induced by CO. de la Puerta et al.[41] showed that specific minor compounds of OO [phenolic compounds (e.g., tyrosol, hydroxytyrosol, caffeic acid, and oleuropein) and compounds of the nonsaponified fraction (e.g., β sitosterol and erythrodiol)] exert anti-inflammatory effects in models of ear edema that is induced by ARA and TPA. One explanation for these effects is that these compounds inhibit prostanoid production and consequently reduce the influx of neutrophils, which may contribute to the anti-inflammatory properties of OO.

To discard the possible effect of OO on the FO response (FOP_{OO}) in ear edema, an additional experiment was performed using 70% acetone as the diluent. We found an inhibitory effect of FOP_{AC} on both the development of edema and MPO activity, demonstrating that FO, even in the absence of OO, exerts an anti-inflammatory effect. When equal amounts of FOP_{OO} and FOP_{AC} were applied, the first preparation produced more intense inhibitory effects, suggesting that OO can contribute to the anti-inflammatory effect that is observed in this experimental model of ear edema. The PAS data may confirm this theory, in which the topical application of FOP_{OO} more effectively reduced the optical absorption bands that characterize inflamed tissue compared with topical administration of OO alone.

5 Conclusion

The results of this study showed evidence that the topical application of FOP_{OO} inhibited the inflammatory response in an experimental model of CO-induced ear edema. The mechanism of action of FOP_{OO} was at least partially attributable to inhibition of the production of proinflammatory cytokines. Furthermore, PAS showed that this anti-inflammatory activity was associated with percutaneous penetration of FOP_{OO}.

Disclosures

The authors declare that there are no conflicts of interest.

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