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EFFECTS OF PARENTERAL FISH OIL LIPID EMULSIONS ON COLON MORPHOLOGY AND CYTOKINE EXPRESSION AFTER EXPERIMENTAL COLITIS

Nutrición Hospitalaria, vol. 28, núm. 3, mayo-junio, 2013, pp. 849-856
Grupo Aula Médica
Madrid, España

Available in: http://www.redalyc.org/articulo.oa?id=309226242041
Original

Effects of parenteral fish oil lipid emulsions on colon morphology and cytokine expression after experimental colitis

Ricardo Garib, Priscila Garla, Raquel S. Torrinhas, Pedro L. Bertevello, Angela F. Logulllo and Dan L. Waitzberg


Abstract

Aim: To study the effects of different protocols of fish oil lipid emulsion (FOLE) infusion on acute inflammation in a rat model of colitis.

Methods: Adult male Wistar rats (n = 51) were randomized into 5 groups to receive parenteral infusion of saline (SS) or soybean oil lipid emulsion (SO), as controls, and FOLE composed of: fish oil alone (FO); a mixture (9:1 v/v) of SO with FO (SO/FO); or 30% soybean oil, 30% medium-chain triglycerides, 25% olive oil, and 15% fish oil (SMOF). After 72 h of intravenous infusion, experimental colitis was induced with acetic acid. After 24 h, colonic samples were analyzed for histological and cytokine changes.

Results: In relation SS group, macroscopic necrosis was less frequent in the FO group and histological necrosis was more frequent in the SMOF group. There was a direct and inverse relation of colon interleukin (IL)-1 and IL-4 respectively, with histological necrosis. In comparison to the SS group, FO increased IL-4 and IFN-gamma and decreased TNF-alpha, SO/FO decreased TNF-alpha, and SMOF increased IL-1 and decreased IL-4.

Conclusion: In acetic acid-induced colitis, the isolate infusion of FOLE composed of fish oil alone was more advantageous in mitigating inflammation than the infusion of FOLE containing other oils, and this difference may be due the influences of their different fatty acid contents.

(Nutr Hosp. 2013;28:849-856)
DOI:10.3305/nh.2013.28.3.6404

Key words: Lipid emulsion. Fish oil. Soybean oil. Experimental colitis. Inflammatory response. Parenteral nutrition.

EFFECTOS DE LAS EMULSIONES PARENTERALES DE LÍPIDOS DE PECES SOBRE LA MORFOLOGÍA DEL COLON Y DE LA EXPRESIÓN DE CITOQUINAS DESPUÉS DE COLITIS EXPERIMENTAL

Resumen

Objetivo: Estudiar los efectos de los diferentes protocolos de infusión de la emulsión de lípidos de aceite de pescado (Fole) sobre la inflamación aguda en el modelo de colitis en la rata.

Material y métodos: Ratas Wistar macho adultas (n = 51) fueron asignadas al azar en 5 grupos para recibir infusión parenteral de solución salina (SS) o emulsión de lípidos de aceite de soja (SO), como controles, y Fole compone de: aceite de pescado solo (FO), una mezcla (9:1 v/v) de SO con FO (SO/FO), o 30% de aceite de soja, 30% triglicéridos de cadena media, 25% de aceite de oliva, y 15% de aceite de pescado (SMOF). Después de 72 h de infusión intravenosa, colitis experimental fue inducida con ácido acético. Después de 24 h, las muestras de colon se analizaron para determinar cambios histológicos y citoquinas.

Resultados: En relación en el SS grupo, necrosis macroscópica fue menos frecuente en el grupo FO y necrosis histológica fue más frecuente en el grupo SMOF. Existe una relación directa e inversa de colon interleukina (IL)-1 e IL-4, respectivamente, con necrosis histológica. En comparación con el grupo SS, en el FO hubo aumento de IL-4 e IFN-gamma y disminución de TNF-alpha, SO/FO disminuyó TNF-alpha, y en el SMOF hubo aumento de IL-1 y la disminución de IL-4.

Conclusion: En la colitis inducida por ácido acético, la infusión aislada de Fole compuesto de aceite de pescado por sí solo fue más ventajosa en la atenuación de la inflamación que la infusión de Fole conteniendo otros aceites, y esta diferencia puede ser debida las influencias de su diferente contenido de ácido graso.

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Introduction

Commercial parenteral lipid emulsions (LEs) containing fish oil have been designed to provide essential omega-3 polyunsaturated fatty acids (PUFA). They also function to prevent elevated omega-6 to omega-3 PUFA ratios in cell membranes, which can occur after the infusion of standard soybean oil LEs that are rich in potentially inflammatory omega-6 PUFA. In addition, omega-3 PUFA in fish oil LEs can mitigate inflammation by positively affecting the production of eicosanoids, cytokines, and resolvins. Therefore, fish oil LEs are of major interest for use in clinical settings to treat inflammation. Their potential antiinflammatory effects were shown to be influenced by the ratio of omega-3 to omega-6 PUFA infused.

Commercially available fish oil LEs are composed of fish oil alone or of fish oil mixed with other oils. In patients under parenteral nutrition therapy, LE composed of fish oil alone is traditionally infused as a supplement, and physically mixed with standard available LEs [based on soybean oil, based on olive oil, or rich in medium chain triglycerides (MCT)]. Fish oil LEs are infused in the amount of 10-20% of the total fat to supply essential fatty acids and to attain the currently recommended ratio of omega-6 to omega-3 PUFA to modulate a favorable immune response (3:1).

Mixed fish oil LEs may contain different amounts of soybean oil, olive oil, and/or MCT that dilute the fish oil. They are ready to use LE in nutritional therapy by providing essential fatty acids in adequate concentrations and to attain the recommended omega-6 to omega-3 PUFA ratios for a favorable immune response.

Nowadays, the optimal omega-6 to omega-3 fatty acid ratio has been considered as less important than having an adequate intake for both omega-6 and omega-3 PUFA. When testing different omega-6 to omega-3 PUFA ratios, Hagi et al. identified that the release of the anti-inflammatory leukotriene B5 (LTB5) was directly proportional to the total amount of omega-3 PUFA infused. Their results suggest that providing high amounts of omega-3 PUFA could be a better approach to attain their anti-inflammatory properties.

In this field of research, the isolated infusion of LE containing fish oil alone has been considered, and it can represent an alternative to provide larger amounts of omega-3 PUFA than the amounts supplied by supplemental and ready-to-use forms for infusing fish oil LEs. In addition, while LE composed of fish oil alone is a high source of omega-3 PUFA, both the supplemental and ready-to-use forms for infusing parenteral fish oil can also supply high amounts of omega-6 PUFA and omega-9 monounsaturated fatty acids (MUFA) from the distinct oils used in combination with fish oil. These other fatty acids have also been shown to influence immune functions and may counterbalance the inflammatory modulation by omega-3 PUFA from the fish oil.

Our hypothesis considered that the parenteral infusion of fish oil LEs given as supplement and in ready-to-use forms may modulate acute inflammation differently than the isolated infusion of LE containing fish oil alone by providing a lower amount of omega-3 fatty acids and by being influenced by other fatty acids. In order to test this hypothesis, we compared the effects of these different forms for the parenteral infusion of fish oil LEs by studying colon damage and cytokine expression following experimentally induced colitis in rats.

Materials and methods

Fifty-one adult male Wistar rats (250-300 g) were obtained from the Vivarium Center of the School of Medicine, University of Sao Paulo. Prior to the experimental procedures, the animals were adapted for 5 days in metabolic cages at a controlled room temperature (22 ± 2°C) with a 12-h light-dark cycle and with free access to standard rodent chow and water. Two weeks before the experimental procedures, the animals were treated in sequence with vermifuge praziquantel (25 mg/kg body weight) and ivermectin/pyrantel (2.0 g/kg body weight), both from Merck Sharp & Dohme (Germany).

Parenteral access

Animals were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg of body weight) from Parke-Davis (Ache, Sao Paulo, Brazil). Parenteral access was achieved by jugular vein cannulation, according to a standard technique, followed by connection to a swivel apparatus that allowed the animals free mobility. All rats were then housed in metabolic cages. All intravenous treatments were delivered at a rate of 0.5 mL/h with a multichannel peristaltic pump (Rainin Rabbit-Plus, Procter & Gamble, NY, USA) for 72 h.

The animals were randomized for intravenous infusions with one of five parenteral regimens, as follows: 0.9% saline solution (SS); LE composed of 100% soybean oil (SO; Lipovenoes® 20%, Fresenius-Kabi, Bad Homburg, Germany); LE composed of 100% fish oil (FO; Omegavenos® 10%, Fresenius-Kabi, Bad Homburg, Germany); a mixture (9:1 v/v) of Lipovenoes® 20% with Omegavenos® 10% (SO/FO); and LE composed of 30% soybean oil, 30% MCT, 25% olive oil, and 15% fish oil (SMOF; SMOFlipid® 20%, Fresenius-Kabi, Bad Homburg, Germany). All LE regimens were delivered at doses of 8-9 g of fat/kg body weight. The animals in the SS group received a standard oral diet (AIN-93M), and the LE treated groups received isocaloric and isonitrogenated lipid-free oral diets. The
Parenteral fish oil in mitigating inflammation


fatty acid compositions of the studied LEs are described in table I.

Experimental colitis

After 72 h of intravenous infusion, experimental colitis was induced in all of the animals by a 5 mL intrarectal administration of 10% acetic acid solution (Dinâmica, São Paulo, Brazil), as described elsewhere.12-15 The animals were maintained under parenteral infusion treatment and then sacrificed 24 h after the colitis procedure. Laparotomy was performed for complete colon resection and sample collection. The colon specimens were washed with saline solution and dissected longitudinally for macroscopic analysis. Then, the specimens were placed in a 10% formaldehyde buffer (Merck & Co. Inc., NJ, USA). After dehydration and standard processing, the colon samples were embedded in paraffin in individual blocks for further histological and immunohistochemical analyses.

Morphological evaluation

The colon specimens were evaluated macroscopically for the presence of ulceration and tissue necrosis. These evaluations were performed with an optical microscope equipped with 200-400x objectives (standard objectives; Nikon, Tokyo, Japan; and Zeiss, Jena, Germany) by two independent observers who were blinded to the experimental groups. Measurements from five randomized, high-power optical fields were averaged for each rat. Disagreements regarding observations between the two investigators (e.g., presence vs. absence of necrosis) were reviewed simultaneously, and a consensus was reached.

Cytokine evaluation

We determined the expression of the inflammatory cytokines interleukin (IL)-1, IL-4, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ with immunohistochemical methods that were standardized and described previously.12 Briefly, after deparaffinization, the 3.0 μm histological colon sections were incubated overnight with primary cytokine-specific antibodies. Antibodies were diluted with phosphate buffered saline (PBS) to 1:300 for anti-rIL-1β and to 1:30 for anti-rIL-4, anti-rIL-6, anti-rIFN-γ, and anti-rTNF-α (all from R&D Systems, Minneapolis, USA). All of the test reactions (tissue with primary antibody) were run in parallel with negative controls (tissue and reaction buffer with no primary antibody).

Two observers, who were blinded to the experimental groups, counted positive cells in ten different fields (400x magnification) with high concentrations

<table>
<thead>
<tr>
<th>Table I</th>
<th>Fatty acids composition of studied lipid emulsions</th>
</tr>
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<tbody>
<tr>
<td>Oil source (% by weight)</td>
<td>Lipovenous (20%)</td>
</tr>
<tr>
<td>Soybean (100%)</td>
<td>Fish (100%)</td>
</tr>
<tr>
<td>Caproic</td>
<td>–</td>
</tr>
<tr>
<td>Caprylic</td>
<td>–</td>
</tr>
<tr>
<td>Capric</td>
<td>–</td>
</tr>
<tr>
<td>Lauric</td>
<td>–</td>
</tr>
<tr>
<td>Myristic</td>
<td>–</td>
</tr>
<tr>
<td>Palmitic</td>
<td>10</td>
</tr>
<tr>
<td>Stearic</td>
<td>4</td>
</tr>
<tr>
<td>Oleic</td>
<td>24</td>
</tr>
<tr>
<td>Linoleic</td>
<td>54</td>
</tr>
<tr>
<td>α-Linolenic</td>
<td>8</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>–</td>
</tr>
<tr>
<td>Eicosapentaenoic</td>
<td>–</td>
</tr>
<tr>
<td>Docosapentaenoic</td>
<td>–</td>
</tr>
<tr>
<td>Docosahexaenoic</td>
<td>–</td>
</tr>
<tr>
<td>α-Tocopherol (mg/L)</td>
<td>~38 mg/l</td>
</tr>
</tbody>
</table>

Source: Informations provided by the lipid emulsions manufacturer, Fresenius Kabi®. *SO/FO: Physical mixture of Lipovenous® and Omegaven® (9:1).
The mean of the preliminary ten results obtained by each observer for each rat was computed, and then a new mean was calculated from the two obtained means. Cases with severe disagreement were reviewed simultaneously to reach a diagnostic consensus.

**Statistical analysis**

For macroscopic and histological analyses, the Fisher exact test or Chi-square test was used. Post hoc used to assess associations in the groups, was the adjusted standard residuals. For cytokine evaluation, the Kruskal-Wallis test was used, and multiple comparisons between the groups were carried out with the Behrens-Fisher test. We considered p values < 0.05 to be statistically significant. Statistical analyses were carried out with PASW 18.0 for Windows (Chicago, IL, USA).

**Ethics**

The Research Ethical Committee (CAPPesq) of the School of Medicine at the University of Sao Paulo (FMUSP), Sao Paulo, Brazil, approved all of the experimental procedures.

**Results**

**Morphological alterations**

Macrosopic and histological alterations were observed in the colons of all animals submitted to colitis, but significant differences were only observed for necrosis (Table II). Macroscopically, necrosis was less frequent in the FO group in relation to all of the groups (p = 0.003). The SMOF group had the highest number of macroscopic and histological necrosis cases in relation to all of the groups (p < 0.05), respectively. In addition, histological necrosis was less frequent in the SO group in relation to the other LE groups (p = 0.013), but not in relation to control SS.

**Cytokine alterations**

We found a direct and inverse relation of colon IL-1 (p = 0.005) and IL-4 (p = 0.015), respectively, with histological necrosis. We also observed an inverse relation of IL-4 with histological ulceration (p = 0.008). The comparison of colon cytokine expression between the groups is shown in figure 1. The FO group had higher IL-4 (p = 0.027) and IFN-γ (p = 0.001) expression levels compared to the other groups, with the exception of the SO group for IFN-γ. The FO and SO/FO groups had lower TNF-α expression compared to the other groups (p < 0.001). The SMOF group had significantly higher IL-1 expression compared to the other groups (p = 0.07), except for the SO group, and it had lower IL-4 expression than all of the other groups (p < 0.001).

**Discussion**

In our study, different protocols used in clinical practice to infuse parenteral LEs containing fish oil within a nutrition regimen were compared to a more pharmacological protocol by infusion of a LE composed by fish oil alone regarding their capacity to mitigate inflammation in a model of experimental acetic acid-induced colitis. We observed different effects on colon damage and cytokine expression that mainly depended on the amounts of omega-3 PUFA and also on the types of fatty acids provided by the infusion protocol.

Acetic acid–induced colitis adequately reproduces ulcerative colitis by leading to a significant colon mucosa and submucosa inflammatory infiltrate. There is a diffuse lymphocyte response with an

<table>
<thead>
<tr>
<th>Alternations</th>
<th>SS</th>
<th>SO</th>
<th>SO/FO</th>
<th>FO</th>
<th>SMOF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro ulcer</td>
<td>09/10</td>
<td>06/10</td>
<td>10/10</td>
<td>06/10</td>
<td>07/11</td>
</tr>
<tr>
<td>Micro ulcer</td>
<td>08/10</td>
<td>10/10</td>
<td>10/10</td>
<td>08/10</td>
<td>11/11</td>
</tr>
<tr>
<td>Macro necrosis</td>
<td>07/10</td>
<td>02/10</td>
<td>02/10</td>
<td>01/10*</td>
<td>08/11*</td>
</tr>
<tr>
<td>Micro necrosis</td>
<td>04/10</td>
<td>03/10*</td>
<td>06/10</td>
<td>06/10</td>
<td>11/11</td>
</tr>
</tbody>
</table>

SS: Saline control; SO: Animals treated with soybean oil based lipid emulsion; FO: Animals treated with lipid emulsion containing only fish oil; SO/FO: Animals treated with a mixture (9:1 v/v with SO and FO); SMOF: Animals treated with lipid emulsion containing 30% soybean oil, 30% medium-chain triglycerides, 25% olive oil and 15% fish oil.

Data expressed as number of events/number of animals.

*p < 0.05 vs LE groups.

*p < 0.05 vs all groups.
increase in INF-γ and IL-2 and a decrease in IL-4 in the colon mucosa after acid-induced ulcerative colitis. In addition, we have previously shown a significant increase in colon IL-1, IFN-γ, and IL-6 expression and a higher frequency of colonic necrosis after 24 h of acetic acid-induced colitis, compared to the non-colitis controls.

The effects of LEs containing fish oil alone or mixed with soybean oil, MCT, and olive oil on colon inflammation were compared to those of standard LEs based in soybean oil and saline solution. Our infusion protocols were designed to provide 30-40% of non-protein calories as fat, similar to the percentages used by other authors and in accordance with the recommendations of the European Society for Clinical Nutrition and Metabolism (ESPEN).

One limitation of our study was that it did not measure the cell or plasma incorporation of omega-3 PUFA. However, the total period of our infusion protocols should be considered adequate for promoting fatty acid incorporation into cell membranes and for priming the cell membranes with different amounts of omega-3 PUFA. Experimentally, 24 h after infusion of pure fish oil LE, the omega-3 PUFA content in splenocyte cell membranes rose to 70% of the peak value and nearly reached a plateau after 72 h. In addition, we choose to infuse LE before trauma, based on the cytokine kinetics. After injury, cytokine expression may peak at different times prior to 72 h, and reasonable changes should be detectable after 24 h.

Previously, we reported that after acetic acid-induced colitis in rats, a 7-day infusion with parenteral LE containing fish oil (supplement) was associated with fewer inflammatory and morphological consequences and decreased colonic concentrations of pro-inflammatory lipid mediators, including leukotriene B4 (LTB-4), prostaglandin E-2 (PGE-2), and tromboxane (TXA-2), compared to the saline control. In agreement, other authors using different post-trauma oral supplementation protocols for omega-3 PUFA in different colitis models showed favorable modulation of inflammatory mediators, including increased IL-10 levels and decreased TNF-α, inducible nitric oxide synthetase (Nos), cyclooxygenase-2 (COX-2), and myeloperoxidase (MPO) activities. These inflammatory alterations were associated with a beneficial morphological impact and with improvements in histological scores and microscopic colonic damage.

According to our current data, the pre-trauma infusion of fish oil LE in the supplementation form

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**Fig. 1.—Colon cytokines expression in rats treated with different lipid emulsions (LE), previously to the induction of acetic acid-colitis. SS: Saline control; SO: Soybean oil lipid emulsion; FO: Fish oil lipid emulsion; SOFO: Mixture of SO and FO (9:1 v/v); SMOF: Lipid emulsion containing 30% soybean oil, 30% medium-chain triglycerides, 25 olive oil and 15% fish oil. 1A: IL-1 expression (SMOF > all groups, except SO); B: IL-4 expression (FO > all groups); C: TNF-α expression (FO and SO/FO < all groups); D: INF-γ expression (FO > all groups, except SO); E: IL-6 expression (no changes). The symbols "*" and "o" in figure 1A and in figures 1B and 1E, respectively, represent animals which presented very different mean values from the major individuals of the group.**
the mucosa and reduced intestinal atrophy.\textsuperscript{15,35} Induced colitis was also associated with protection of parenteral infusion of MCT-based LE in rats with (SO/FO) also decreased pro-inflammatory TNF-α and IL-1β in a similar way or to a lesser extent than soybean oil parenteral LEs.\textsuperscript{36,37} In a previous experimental study, we observed that a LE treatment composed of a 1:1 mixture of soybean oil and MCT combined with 20% fish oil (MCT/FO) increased the number of liver and lung resident phagocytizing macrophages.\textsuperscript{38} In contrast, SMOF, which differs from MCT/FO because it includes olive oil, did not change phagocytosis.\textsuperscript{36} We concluded that olive oil could have interfered with the immune response by inhibiting the modulation of phagocytizing macrophages by MCT/FO. Furthermore, in 20 healthy volunteers, the infusion of olive oil-based LE decreased lymphocyte proliferation and induced lymphocyte necrosis.\textsuperscript{39,40} Omega-9 MUFA was previously reported to have deleterious effects on inflammatory bowel disease by Gassul et al. in humans.\textsuperscript{41} In their randomized, double-blind study, the remission rate of active Crohn’s disease was significantly lower in patients after four weeks of treatment with enteral diets rich in omega-9 MUFA (27%) compared to those treated with an enteral diet rich in omega-6 PUFA (63%).\textsuperscript{42} It should be emphasized that human inflammatory bowel diseases are physiopathologically different from chemically-induced acetic colitis, which precludes any generalizations to humans based on our results. However, we recently have observed that olive oil-based LE can increase the expression of the pro-inflammatory colon cytokine IL-6 and the frequencies of ulceration and necrosis in rats with acetic acid-induced colitis.\textsuperscript{35} The isolated infusion of fish oil LE (FO group) favorably modulated colon cytokine expression by increasing anti-inflammatory IL-4 and decreasing proinflammatory TNF-α. This infusion produced a positive impact on colon damage and lowered the necrosis frequency when compared with others FOLE. These IL-4 and TNF-α alterations suggest that there is an activation of the regulatory immune response mediated by Th helper (Th) type 2 lymphocytes that counteracts the effects of the Th1 cytokines.\textsuperscript{15,42} Although the FO group also showed increased IFN-γ, this increase was not associated with severe colon

<table>
<thead>
<tr>
<th>Group</th>
<th>Omega-3 (g/L)</th>
<th>Omega-6 (g/L)</th>
<th>Omega-9 (g/L)</th>
<th>Omega-3:Omega-6</th>
<th>Omega-9:Omega-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SO</td>
<td>16</td>
<td>108</td>
<td>48</td>
<td>1:6.7</td>
<td>2:3</td>
</tr>
<tr>
<td>FO</td>
<td>41.1</td>
<td>5.5</td>
<td>10.9</td>
<td>11.7:1</td>
<td>1:4</td>
</tr>
<tr>
<td>SO/FO</td>
<td>21.0</td>
<td>87.5</td>
<td>48.6</td>
<td>1:4.2</td>
<td>2:2</td>
</tr>
<tr>
<td>SMOF</td>
<td>14.6</td>
<td>38.4</td>
<td>55.6</td>
<td>1:2.6</td>
<td>4:1</td>
</tr>
</tbody>
</table>

SS: Saline; SO: 100% soybean oil; FO: 100% fish oil; SO/FO: 90% soybean oil lipid emulsion and 10% fish oil lipid; SMOF: 30% of soybean oil, 30% medium-chain triglycerides, 25% olive oil and 15% fish oil; n: Omega.
damage, probably because the increase was followed by an increase in IL-4. Our data identified a significant inverse association between these cytokine levels and the histological frequencies of ulceration and necrosis.

The isolate infusion of LE containing fish oil alone is not influenced by fatty acids from other oils and is a high source of omega-3 PUFA, providing amounts of these PUFA that are two and tree times higher than the SO/FO and SMOF groups, respectively (table III). At the moment, LE containing fish oil alone has mainly been infused in experimental studies, but it has also been infused in low amounts in some initial clinical trials as a pharmacological agent to modulate the immune response.

In summary, we observed that the type of infusion with parenteral fish oil LEs before the induction of experimental colitis influenced the modulation of the colonic inflammatory response. According to our data, the isolated infusion of LE containing fish oil alone as a high source of omega-3 PUFAs was more effective in showing a favorable modulation of colon cytokine expression and had a positive influence on colon damage than ready-to-use LE containing fish oil mixed with soybean oil, olive oil and medium chain triglycerides. Additional experimental and clinical studies are needed to explore our preliminary findings in the future.

Comments

Parenteral fish oil lipid emulsions (FOLE) are potentially anti-inflammatory by providing omega-3 polyunsaturated fatty acids. We have shown in a rat model of colitis that the isolate infusion of FOLE composed of fish oil alone was more advantageous in mitigating inflammation than the infusion of FOLE containing other oils, by providing a higher amount of omega-3 fatty acids and by being not influenced by other fatty acids. Our findings contribute with scientific data to support the infusion of FOLE composed of fish oil alone as a pharmacological agent in clinical settings enrolling inflammation.

Acknowledgements

This study was supported by CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico do Estado de São Paulo (process number 140925/00-7). The authors thank Fresenius-Kabi for kindly providing the lipid emulsions and Abbott Laboratories for donating the peristaltic pumps and João Italo Dias França and Prof. Julio Cesar Rodrigues Pereira for the statistical discussion.

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