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α-Linolenic acid (ALA) is an anti-inflammatory agent in inflammatory bowel disease

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Abstract

Studies suggest that consumption of omega-3 (n-3) polyunsaturated fatty acids (PUFA) plays a protective role in inflammatory bowel disease; however, the use of plant-derived oils rich in α-linolenic acid (ALA) has not been widely investigated. The aims of this study were to test the effects of two different sources of (n-3) PUFA, fish and plant-derived oils, in two animal models of experimental colitis and to determine whether the (n-3) PUFA-enriched diets could ameliorate the inflammatory status. Rats were fed diets rich in corn, fish or sage oil with or without vitamin A supplementation for 3 weeks then colitis was induced by adding dextran sodium sulfate to the drinking water or by injecting 2,4,6-trinitrobenzene sulfonic acid. We show that colitic rats fed the sage oil diets had a lower inflammatory response, improved histological repair and had less necrotic damage in the mucosa when compared to the corn and fish oil groups. Colonic damage and myeloperoxidase activity were significantly lower. Colonic mRNA levels of pro-inflammatory genes including interleukin IL-6, cyclooxygenase 2 and tumor necrosis factor α were markedly down-regulated in rats fed fish and sage oils compared to control. These results were supported by experiments in the human colonic epithelial cell line Caco-2, where ALA supplementation was shown to be effective in inhibiting inflammation induced by IL-1β by down-regulating mRNA levels of pro-inflammatory genes including IL-8, COX2 and inducible nitric oxide synthase. Taken together, these results suggest that plant-derived oil rich in ALA could ameliorate the inflammatory damage in colitis.

Keywords: Omega-3; Alpha-linolenic acid; Inflammatory bowel disease; Vitamin A; Colitis

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract that includes ulcerative colitis and Crohn's disease. Worldwide, the prevalence of IBD is on the rise, particularly in the industrialized countries of the Western World [1,2]. Over 1 million people in America suffer from IBD and its symptoms, which include diarrhea, loss of appetite, joint pains, sores in the anal area, rectal bleeding and fistulas. The etiology of the disease is poorly understood; however, it is assumed that the disease is controlled by multiple environmental, genetic and immune-regulatory factors [3–5].

The current drug regimens available for IBD patients include a combination of steroids, immunosuppressants and antibiotics. These drugs cause side effects, and in many cases, complications develop [3,6]. In an attempt to overcome these challenges, a large number of studies have been carried out in order to discover a more effective and safe treatment for IBD, frequently by examining different anti-inflammatory agents.

Omega-3 (n-3) polyunsaturated fatty acids (PUFA) exhibit anti-inflammatory properties due to the activity of eicosanoids derived from eicosapentaenoic acid [EPA, 20:5(n-3)], which can be synthesized after ingestion from α-linolenic acid [ALA, 18:3(n-3)]. These eicosanoids exhibit properties opposite to those derived from arachidonic acid [AA, 20:4(n-6)]. The eicosanoids that are derived from AA, including prostaglandin PGE_2, thromboxane TXA_2 and leukotriene LTB_4, are potent vessel constrictors, vasodilatation agents and platelet aggregators. Alternatively, eicosanoids that are formed from EPA, including prostaglandin PGE_3, thromboxane TXA_3 and leukotriene LTB_5, are weaker platelet aggregators; they exhibit a lower chemotactic activity and are less pro-inflammatory mediators [7–10]. Numerous studies suggested a protective role of the dietary intake of (n-3) PUFA in IBD due to their anti-inflammatory properties [7,9,11–15].

Recent experimental studies in rats with experimental colitis, induced by intrarectal injection of 2,4,6-trinitrobenzene sulfonic acid (TNBS model), have documented that treatment with (n-3) PUFA reduces mucosal damage as assessed by biochemical and histological markers of inflammation [11]. Fish oil, rich in EPA and docosahexaenoic acid (DHA) (n-3) PUFA, has been demonstrated as a supplement that reduces colonic damage and inflammatory markers in numerous animal studies [12,13]. Most of the studies that investigated (n-3) fatty acids have focused on fish oil. Few studies have examined the effect of plant-derived oils rich in ALA, like flaxseed and perilla oils, on various inflammatory markers. The effect of a

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flaxseed oil-based diet on tumor necrosis factor α (TNF-α) and interleukin IL-1β synthesis was examined in healthy volunteers. Use of flaxseed oil in domestic food preparation for 4 weeks inhibited TNF-α and IL-1β production by more than 30% [16]. Another experiment investigated the effect of a perilla oil-enriched diet on mucosal levels of leukotrienes (LTs) in rats with experimental colitis induced by dextran sulfate sodium (DSS). The production of LTs from the colonic mucosa in the experimental group was significantly lower than that of controls [17]. Despite the small number of studies using plant-derived oils rich in ALA, there is a growing body of evidence suggesting that ALA alone might have an anti-inflammatory effect [18–20]. Therefore, the aim of this research was to study the effect of two different sources of (n-3) PUFA, fish and plant-derived oils, in two animal models of colitis and to determine whether the different (n-3) PUFA-enriched diets could ameliorate the inflammatory status in colitis with the same efficacy. In addition, a series of studies have demonstrated that vitamin A deficiency (VAD) alters and broadens inflammatory responses while supplementation of the vitamin activates anti-inflammatory cascades [21]. Vitamin A is a lipid-soluble antioxidant, a regulator of epithelial proliferation and differentiation and is vital for optimal immune function. Studies show that dietary VAD has a profound effect on the membrane fatty acid profile in rat tissues. Moreover, a combined vitamin A and (n-3) fatty acid deficiency dramatically shifts the balance of n-3/n-6 PUFA in rat liver and colon tissues and alters the entire cell membrane PUFA profile [22]. Considering the protective properties of vitamin A and the existing evidence, we aimed to ascertain whether an ALA-enriched diet with vitamin A supplementation had a synergistic and more potent effect on reducing inflammatory markers.

2. Materials and methods

This study received ethical approval from the ethics committee of the Hebrew University for studies involving animals and was carried out in a full compliance with The Hebrew University Policy on Animal Use and Care.

2.1. Reagents and composition of the diets

The different diets used in the present study were prepared according to the American Institute of Nutrition (AIN) diet #D10012G (AIN-93G diet). The diets differed in the source of fat (5% wt/wt) included in each diet. The diets were designated as CO [corn oil rich in (n-6) fatty acids], FO [fish oil rich in long-chained (n-3) PUFA — *fish oil from Menhaden fish (Sigma F-8020)*], SO (sage oil rich in ALA) and SO&A (sage oil along with retinyl palmitate supplement equivalent to 2000 IU vitamin A given twice a week). The diets were composed of 20% protein, 5% oil (as previously described), 5% dietary fiber (in the form of cellulose) and 65% carbohydrates. Other components such as mineral and vitamin mix represented 4% and 1%, respectively. The digestible energy of each diet was 3.9 kcal/g. The fatty acid composition of the diets is shown in Table 1.

2.2. Experimental design

Pathogen-free, male Wistar rats (170–190 g) were obtained from the Harlan Laboratory at The Weizmann Institute of Science, Rehovot, Israel. They were housed in metal cages in a room with controlled temperature (22±2°C), humidity (65±5%) and light (0800–2000 h) where they had free access to tap water and food. The rats were randomly assigned to 8 groups (n=8); 2 groups (DSS and TNBS colitic groups) were fed the CO, FO, SO and SO&A diets each. Three weeks after the beginning of the experiment, the rats colitis was induced as described previously [23], by replacing their normal drinking water with water containing 5% DSS (wt/v, prepared daily, molecular weight 36,000–50,000; MP Biomedicals, USA) for 7 days or by injecting the rats with 0.2 ml of TNBS (100 g/L dissolved in 50% ethanol) solution into the colonic lumen, at a site 8 cm proximal to the anus. At the end of the seventh day (DSS group) or 24 h (TNBS group), the rats were sacrificed by decapitation; plasma and colon tissues were removed for various analyses.

2.3. Assessment of colonic damage

Animal body weight was recorded weekly for each rat. After the rats were sacrificed, their colons were removed and rinsed with PBS, and then each sample length was measured. The colon was opened longitudinally; a section was fixed in 4% formaldehyde and embedded in paraffin for histological analysis. The colon was then subsequently divided into segments for biochemical determinations and the fragments were frozen at −80°C for myeloperoxidase (MPO) activity and for TNF-α, IL-6 and COX2 gene expression. MPO activity was measured according to the technique described by Uritski *et al.* [24]. The results were expressed as MPO units per gram of wet tissue; 1 unit of MPO activity was defined as that degrading 1 μmol H_2O_2 per minute at 20°C.

Table 1
Fatty acid composition of diets

Fatty acid	Diet		
	CO	FO	SO
	g/100 g fatty acids		
16:0	10.1	21.4	7.5
18:0	3.3	4.5	2.7
18:1(n-9)	29.7	13.1	29.7
18:2(n-6)	55.1	1.5	14.3
18:3(n-3)	1.0	1.8	43.9
20:4(n-6)	ND	0.8	0.3
20:5(n-3)	ND	11.7	0.1
22:6(n-3)	ND	15.7	ND
∑SFA	13.4	25.9	10.1
∑MUFA	29.7	13.1	29.7
∑PUFA	56.0	31.6	58.7
∑(n-3)	1.0	29.2	43.9
∑(n-6)	55.1	2.4	14.6
∑(n-9)	29.7	13.1	29.7
18:2(n-6)/18:3(n-3)	55.2	0.9	0.3
20:4(n-6)/20:5(n-3)	-	0.1	3.0
(n-6)/(n-9) ratio	1.9	0.2	0.5
(n-6)/(n-3) ratio	55.2	0.1	0.3

ND, not detected.

2.4. mRNA analysis and RT-PCR

mRNA was extracted from colons of rats using Total RNA Extraction Mini Kit — Tissue (RBC Bioscience) and reverse-transcribed into cDNA using the components of the EZ-first strand cDNA synthesis kit for RT-PCR (Biological Industries Ltd, Beit Ha'Emek, Israel). Colonic TNF-α, IL-6 and COX2 gene expression was determined by real-time PCR. Amplification of the mRNA was performed using the Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen Life Technologies) on a Real-Time PCR System 7300 instrument (Applied Biosystems). All samples were run in triplicate. Quantitation of gene expression by real-time RT-PCR was calculated by the comparative threshold cycle ($\Delta\Delta C_T$) method following the manufacturer's instructions. All quantitations were normalized to an endogenous control, the 18S rRNA.

The primers used were as follows: 18S: forward primer, 5′-CACGGACAGGATTGACAGAT-3′, reverse primer, 5′-CAAATCGCTCCACCAACTAA-3′ [25]; COX2: forward primer, 5′-CCCCAAGGCACAAATATGATG-3′, reverse primer, 5′-CAGGTCCTCGCTTCTGATCTG-3′ (Primer Express, Applied Biosystems); TNF-α: forward primer, 5′-ACAAGGCTGCCCCGACTAT-3′, reverse primer, 5′-CTCCTGGTATGAAGTGGCAAATC-3′ [26]; IL-6: forward primer, 5′-GCCCTTCAGGAACAGCTATGA-3′, reverse primer, 5′-TGTCAACAACATCAGTCCCAAGA-3′ [27].

2.5. Fatty acid profiles in plasma and colonic tissue

A total plasma free fatty acid profile was determined after plasma or colon tissues were homogenized in a mixture of chloroform/methanol (2:1) and then dried under N_2. Methylation solution (5% sulfuric acid in methanol) was then added to each sample and total lipid was extracted by a modified method of Folch and Lebaron [28]. GC analyses were performed in an Agilent Technologies GC7890N gas chromatograph equipped with a flame ionization detector and provided with an N10149 automatic liquid sampler. Hydrogen was used as the carrier gas at a flow rate of 1.0 ml/min. The separation of the compounds was performed using an Agilent DB-23 GC column, 60 m×0.25 mm i.d., 0.25 μm film thickness. The oven temperature was programmed from 130°C (1.0 min) to 170°C at 6.5°C/min, to 215°C at 2.75°C/min (7 min) and to 230°C at 2.5°C/min (5.0 min). The detector temperature was set at 260°C and the injector port temperature was set at 270°C.

2.6. Histological analysis

Colon sections were stained with hematoxylin and eosin and graded by a pathologist unaware of the experimental groups according to the criteria described previously by Reifen *et al.* [29], after which microphotographs were taken.

2.7. Histopathological semiquantitative scoring

Histopathology examination was limited to the colon and rectum. Three transversal samples, each one in the length of approximately 15 mm, were trimmed from each colon and rectum, as follows: First segment (i.e., proximal segment) taken 1 cm distal from the cecum–colon junction; second segment (i.e., middle segment) taken 1 cm

Table 2
Fatty acid composition in plasma from TNBS-induced colitic rats fed the CO, FO, SO or SO&A diet

Fatty acid	Diets			
	CO	FO	SO	SO&A
	g/100 g fatty acids			
16:0	18.6±0.6[a]	21.5±0.9[ab]	18.4±1.4[a]	20.9±0.7[b]
18:0	14.9±0.7[a]	14.5±0.7[a]	17.1±0.2[b]	17.6±0.8[b]
18:1(n-9)	9.0±0.9	10.3±0.4	10.3±0.3	9.7±0.8
18:2(n-6)	21.7±1.3[c]	13.3±0.7[a]	19.5±1.8[b]	18.5±0.5[b]
18:3(n-3)	0.1±0.1[a]	0.1±0.1[a]	4.8±1.6[b]	6.1±0.9[b]
20:4(n-6)	30.9±2.7[b]	14.7±1.0[a]	15.6±3.0[a]	12.2±0.9[a]
20:5(n-3)	0.0±0.0[a]	10.4±1.0[c]	7.0±0.8[b]	5.3±0.5[b]
22:6(n-3)	1.7±0.1[a]	7.8±0.3[c]	3.1±1.2[b]	4.1±0.3[b]
∑SFA	33.5±1.0[a]	36.1±1.3[ab]	35.4±1.4[ab]	38.5±0.9[b]
∑MUFA	9.0±0.9	10.3±0.4	10.3±0.3	9.7±0.8
∑PUFA	54.4±1.4[b]	46.3±1.6[a]	50.0±2.8[ab]	46.2±0.5[a]
∑(n-3)	1.8±0.1[a]	18.3±1.1[b]	14.9±2.0[b]	15.5±0.6[b]
∑(n-6)	52.6±1.4[b]	28.0±1.6[a]	35.1±4.6[a]	30.7±0.6[a]
∑(n-9)	9.0±0.9	10.3±0.4	10.3±0.3	9.7±0.8
18:2(n-6)/18:3(n-3)	210.8±0.1[c]	120.7±0.1[b]	4.1±1.1[a]	3.0±0.5[a]
20:4(n-6)/20:5(n-3)	–	1.4±0.2[a]	2.2±0.2[b]	2.3±0.2[b]
(n-6)/(n-9) ratio	5.9±0.7[b]	2.7±0.3[a]	3.4±0.5[a]	3.2±0.4[a]
(n-6)/(n-3) ratio	28.5±2.2[c]	1.5±0.1[a]	2.4±0.8[b]	2.0±0.1[ab]

Values are means±S.E.M., $n=4$. Means in a row with superscripts without a common letter differ, $P<.05$.

Table 3
Fatty acid composition in colon from TNBS-induced colitic rats fed the CO, FO, SO or SO&A diet

Fatty acid	Diets			
	CO	FO	SO	SO&A
	g/100 g fatty acids			
16:0	21.9±0.3[a]	33.3±1.6[b]	25.1±1.6[a]	22.2±0.8[a]
18:0	3.0±0.2	4.8±0.7	3.5±0.2	4.4±0.5
18:1(n-9)	29.8±0.2	28.1±1.5	30.6±0.4	29.9±0.9
18:2(n-6)	33.9±0.8[c]	12.7±0.5[a]	14.6±1.0[ab]	15.6±0.8[b]
18:3(n-3)	0.9±0.1[a]	1.4±0.1[a]	16.0±1.4[b]	15.0±0.4[b]
20:4(n-6)	0.9±0.2	1.3±0.4	0.8±0.1	1.3±0.2
20:5(n-3)	0.0±0.0[a]	1.3±0.1[c]	0.2±0.1[b]	0.5±0.1[b]
22:6(n-3)	0.0±0.0[a]	3.7±0.5[c]	0.2±0.08[b]	0.4±0.1[b]
∑SFA	24.9±0.5[a]	38.1±2.3[b]	28.5±1.7[a]	26.7±1.2[a]
∑MUFA	29.8±0.2	28.1±1.5	30.6±0.4	29.9±0.9
∑PUFA	35.7±0.5[b]	20.4±2.7[a]	31.7±2.3[b]	32.8±1.1[b]
∑(n-3)	1.2±0.3[a]	5.9±0.3[b]	16.4±1.4[c]	15.9±0.4[c]
∑(n-6)	34.8±0.7[c]	13.9±0.5[a]	15.4±0.9[ab]	16.9±0.8[b]
∑(n-9)	29.8±0.2	28.1±1.5	30.6±0.4	29.9±0.9
18:2(n-6)/18:3(n-3)	37.3±3.6[c]	8.8±0.1[b]	0.9±0.02[a]	1.0±0.05[a]
20:4(n-6)/20:5(n-3)	–	0.9±0.1[a]	4.0±0.7[b]	2.6±0.1[b]
(n-6)/(n-9) ratio	1.2±0.02[b]	0.5±0.03[a]	0.5±0.03[a]	0.6±0.04[a]
(n-6)/(n-3) ratio	38.3±3.7[c]	2.1±0.1[b]	0.9±0.05[a]	1.1±0.05[a]

Values are means±S.E.M., $n\geq4$. Means in a row with superscripts without a common letter differ, $P<.05$.

distal to the previous; third segment (i.e., distal segment) taken 1 cm distal to the previous. Each segment was put in a separate vial, marked, respectively, segments A, B and C. The vials were transferred to the histology laboratory, for further trimming and processing. Each segment was trimmed to five transversal slices (each one in the length of 3 mm). Trimmed tissues were embedded perpendicularly in paraffin, cut at thickness of 5–6 microns, stained by hematoxylin and eosin and "blindly" evaluated by light microscopy.

A Lateral extension — Distribution of colonic changes (inflammation)
Grade

0 None (or 0%) inflammation
1 Focal (or 1–25% of the circumference of the intestinal section) inflammation
2 Multifocal (or 26–50% of the circumference of the intestinal section) inflammation
3 Nearly diffuse (or 51–75% of the circumference of the intestinal section) inflammation
4 Diffuse (or 76–100% of the circumference of the intestinal section) inflammation

B In-depth layers involved (reflecting in-depth extension of inflammation)
Grade

0 No inflammation
1 Half (up to 50%) mucosal inflammation
2 Total (up to 100%) mucosal inflammation
3 Mucosal and submucosal inflammation
4 Transmural inflammation

C Lateral extension — Distribution of colonic changes (necrosis and/or ulceration)
Grade

0 No necrosis and/or ulceration
1 Focal (or 1–25% of the circumference of the intestinal section) inflammation
2 Multifocal (or 26–50% of the circumference of the intestinal section) inflammation
3 Nearly diffuse (or 51–75% of the circumference of the intestinal section) inflammation
4 Diffuse (or 76–100% of the circumference of the intestinal section) inflammation

D In-depth layers involved (reflecting in-depth extension of necrosis and/or ulceration)
Grade

0 No necrosis and/or ulceration
1 Half (up to 50%) mucosal necrosis and/or ulceration
2 Total (up to 100%) mucosal necrosis and/or ulceration
3 Mucosal and submucosal necrosis and/or ulceration
4 Transmural necrosis and/or ulceration

2.8. Cell culture

The human colon adenocarcinoma cell line Caco-2 was used as an experimental model. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamine (Biological Industries Ltd, Beit Ha'Emek, Israel) and were maintained at 37°C in a humidified atmosphere containing 5% CO_2. In order to induce inflammation, Caco-2 cells were incubated for 4 h with 5 ng/ml IL-1β in FBS-free DMEM. To investigate the effect of fatty acids, Caco-2 cells were incubated for 48 h with ALA, oleic acid (OA), fish oil (FO) or sage oil (SO), all in FBS (final concentration, 10 mM).

2.9. mRNA analysis

For cDNA arrays, Caco-2 cells were harvested, and total RNA was prepared from cells by the Tri-Reagent (Sigma-Aldrich, Rehovot, Israel). The quality and quantity of total RNA were determined by spectrophotometer using the absorbency at $A_{260}/A_{280\ nm}$. Reverse transcription of total RNA from Caco-2 cells was performed using the components of the EZ-first strand cDNA synthesis kit for RT-PCR (Biological Industries Ltd, Beit Ha'Emek, Israel).

The primers used were as follows: GAPDH: forward primer, 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, reverse primer, 5′-AGCCTTCTCCAGGTGGTGAAGAC-3′ (306 bp); COX2: forward primer, 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′, reverse primer, 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (305 bp); IL-8: forward primer, 5′-ATGAGTTCCAAGCTGGCCGTGGCT-3′, reverse primer, 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′ (289 bp); inducible nitric oxide synthase (iNOS): forward primer, 5′-CGGTGCTGTATTACGGGCGAAGAAGG-3′, reverse primer, 5′-GGTGCTGCTTGTTAGGAGGTCAAGTAAAGGGC-3′ (259 bp).

PCR amplification consisted of 35 cycles of 2 min denaturation at 95°C, 1 min annealing at 55°C (COX2) or 58°C (GAPDH, IL-8, iNOS) and extension at 72°C. Samples were preheated to 95°C before addition of *Taq* polymerase (Biological Industries Ltd, Beit Ha'Emek, Israel) was used to increase specificity of the amplification. Amplified cDNAs were separated by agarose gel electrophoresis and bands were visualized by ethidium bromide staining. The expression of each gene was reported as the ratio of the value obtained after each treatment relative to control after the normalization of the data.

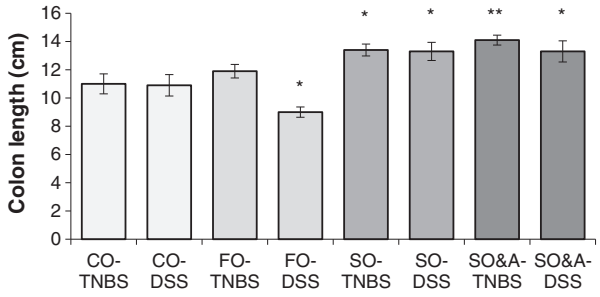

Fig. 1. Colon lengths in DSS- and TNBS-induced colitic rats fed the CO, FO, SO or SO&A diet. */**Different from the DSS or TNBS colitic control group (CO), $P<.05$/$P<.01$, respectively.

2.10. Statistics

All results are expressed as means±S.E.M. The data were analyzed by 1-way ANOVA, using Tukey–Kramer honest significant difference and Student's *t* tests. All statistical analyses were conducted with the JMP 5.1 software, with differences considered significant at $P<.05$.

3. Results

This study examined the effect of fish and plant-derived oils in two animal models of colitis and in *in vitro* Caco-2 model to determine whether the ALA-enriched diets could ameliorate the inflammatory status in colitis and to compare the ALA-rich SO and FO anti-inflammatory effects. The main differences among the three diets were the contents of linoleic acid (LA) [18:2(n-6)], ALA [18:3(n-3)], EPA [20:5(n-3)], and DHA [22:6(n-3)]. Levels of saturated fatty acids (SFA) also differed among the diets. LA concentrations were much lower, in the SO and FO diets than in CO diet, whereas EPA and DHA concentrations were much higher in the FO diet, and ALA concentrations were much higher in the SO diet. Although the (n-6):(n-3) ratio was much lower in SO diet than in CO diet (1:3) due to high ALA concentration, it was even lower in the FO diet (1:10) as a consequence of the concentration of EPA and DHA (Table 1).

Consumption of the different diets affected lipid profiles in the plasma (Table 2) and colon tissues (Table 3). The differences in lipid composition in plasma and colon samples of TNBS-induced colitic rats paralleled the differences in the composition of fatty acids in the different diets, confirming the *in vivo* incorporation of dietary lipids into different body compartments. The same effects were observed in plasma and colon samples of DSS-induced colitic rats (data not shown). Compared with the CO-fed rats, the reduction in the LA content in SO- and SO&A-fed rats due to the substitution by ALA was evident in all of the tissues analyzed and in both TNBS and DSS models. Moreover, EPA and DHA levels were elevated in the tissues of FO-fed rats compared with CO-, SO- and SO&A-fed rats. This increase in the (n-3) EPA and DHA led to a higher level of total (n-3) PUFA and to a shift of the (n-6):(n-3) ratio. Likewise, there was an increase in ALA in the tissues of SO- and SO&A-fed rats compared with both CO- and FO-fed rats, leading to a higher level of total (n-3) PUFA and to a modification of the (n-6):(n-3) ratio. Levels of (n-9) fatty acids did not differ among plasma and colon tissues of the colitic rats fed the CO, FO, SO or SO&A diet in both TNBS and DSS groups.

Exposure to DSS in rats' drinking water as well as TNBS induction led to inflammation in the colon with characteristics similar to those described previously [24,30–32] and several bleeding events, mostly in the FO groups in both TNBS and DSS models. Macroscopic examination of the colonic samples after exposure of 24 h to TNBS induction as well as after 7 days of DSS treatment assessed the intestinal inflammatory status and revealed a significantly greater colon length measurement in SO- and SO&A-fed rats compared with CO- and FO-fed rats (Fig. 1). During the first 3 weeks of the experiment, weight gain did not differ among the groups. However, as a consequence of a progressive inflammatory process, the weight gain in all experiment groups decreased in week 4 after colitis induction. From that time point until the end of the experiment, weight did not change significantly in the CO-, SO- and SO&A-fed colitic rats while significant weight loss was observed in the FO-fed rats.

Histological examination of the colonic sections evaluated the intestinal inflammatory state in both TNBS and DSS models (Figs. 2 and 3, respectively). Microscopically, the samples from both DSS- and TNBS-treated CO-fed colitic rats demonstrated typical acute inflammatory changes in the colonic structure, i.e., changes in the colonic wall involving the mucosa, submucosa and muscle layer, as well as multifocal mucosal necrosis, ulceration, cell depletion and crypt dilation. According to the histological examination of the colonic wall (graded as described in the methods section), the TNBS-treated colitic rats fed the FO and SO&A diets had a comparable level of colonic inflammation and ulceration as the CO-fed group.

There were no differences in the mucosal necrosis evaluation, among the various groups (Fig. 4). Among the DSS-treated colitic rats, the SO&A group showed significantly less wall inflammation than other groups (including the control — CO). Mucosal necrosis scoring indicated that the FO group had significantly greater inflammation than the control group (Fig. 5).

The biochemical determinations showed that TNBS as well as DSS administration led to significantly higher colonic MPO activity in both CO- and FO-fed colitic rats when compared to SO- and SO&A-fed rats, while results from SO and SO&A were similar (Fig. 6). In addition, the

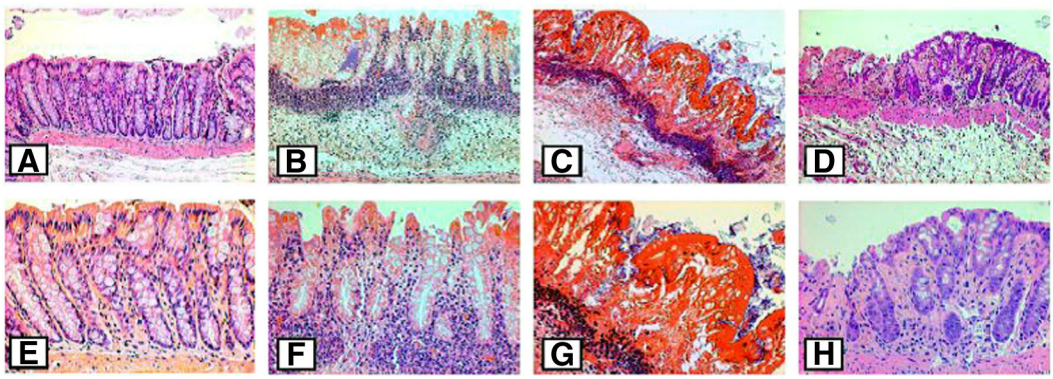

Fig. 2. Histological sections of colonic mucosa from TNBS-induced colitic rats fed the CO, FO, SO or SO&A diet stained with hematoxylin and eosin. (A and E) Colitic rat fed the SO&A, (B and F) CO-fed rat, (C and G) FO-fed rat and (D and H) SO-fed rat (original magnifications ×10 and ×20).

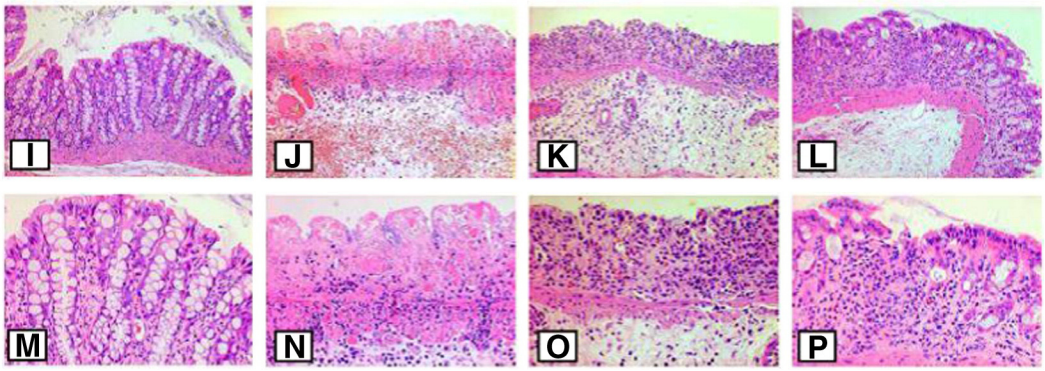

Fig. 3. Histological sections of colonic mucosa from DSS-induced colitic rats fed the CO, FO, SO or SO&A diet stained with hematoxylin and eosin. (I and M) Colitic rat fed the SO&A diet, (J and N) CO-fed rat, (K and O) FO-fed rat and (L and P) SO-fed rat. (original magnifications ×10 and ×20).

elevated inflammatory status was also associated with higher levels of IL-6, TNF-α and COX2 mRNA, as a consequence of the TNBS-induced inflammatory process. A significant up-regulation of IL-6 and COX2 mRNA levels was demonstrated in CO-fed rats compared with FO-, SO- and SO&A-fed rats, while TNF-α mRNA levels did not significantly differ between FO- and CO-fed rats. The lowest mRNA levels were consistently seen in SO- and SO&A-fed rats; however, for the most part, significant differences were not reached when compared to FO-fed rats (Fig. 7).

After exposure of the Caco-2 cells to the cytokine IL-1β during 4 h, a significant up-regulation of COX2, IL-8 and iNOS mRNA levels was observed compared with the control Caco-2 cells from all the experiment groups. In addition, significantly higher levels of COX2 and IL-8 mRNA were observed in IL-1β-treated naïve cells and in cells treated with OA or fish oil (FO) compared with the ALA- and SO-treated cells ($P<.05$ and $P<.001$, respectively, compared to naïve cells). A significantly lower iNOS mRNA level was observed in ALA-treated Caco-2 cells compared to all other treatments (Fig. 8).

4. Discussion

To date, numerous studies suggest a protective role of the dietary intake of (n-3) PUFA in IBD due to their anti-inflammatory properties both in animal models of colitis and in humans [11–13,16,17]. Most of the studies focused on fish oil, rich in EPA and DHA, while only a few have been performed using plant-derived oils rich in ALA. The accumulating findings emphasize the importance of (n-3) PUFA in the diet. However, it is unclear whether the amount of (n-3) PUFA leads to their beneficial anti-inflammatory effect or rather their source (marine vs. plant-derived) or their ability to alter the (n-6):(n-3) ratio. It is currently recommended by healthcare and nutritional authorities that the Western diet alters its current (n-6):(n-3) ratio of 15–30:1 [8,20] toward ~2–5:1, which is believed to be the optimal ratio for various inflammatory conditions [10,15,18,32–34]. As a result of these recommendations and due to the difficulties of increasing consumption of fatty marine fish, fish oil supplements became popular in many of Western countries. However, it is unclear whether the fish oil can be successfully substituted by another cheaper and more accessible source of (n-3) as a potent anti-inflammatory agent.

The primary objective of this work was to compare the effect of two sources of (n-3) PUFA, fish and plant-derived oils, in models of colitis and to determine whether the different (n-3) PUFA-enriched diets could ameliorate the inflammatory status in colitis with the same efficacy. We analyzed the effects of a dietary modification involving the lipid composition in the diet on the progression and the severity of an inflammatory process in two experimental models of colitis in rats. We chose two of the most common and reliable experimental models of colitis in rats that are widely used in IBD research in order to examine the possible differences of the models.

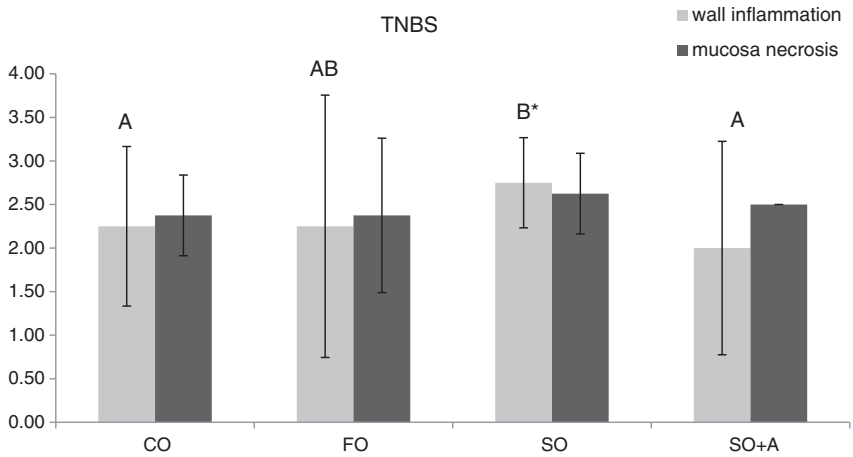

Fig. 4. Histophatological evaluation of the colon — TNBS-induced colitic rats fed the CO, FO, SO or SO&A diet.Values are means±S.D., $n=8$. */**Different from the naive control or colitic groups, $P<.05$/$P<.001$, respectively.

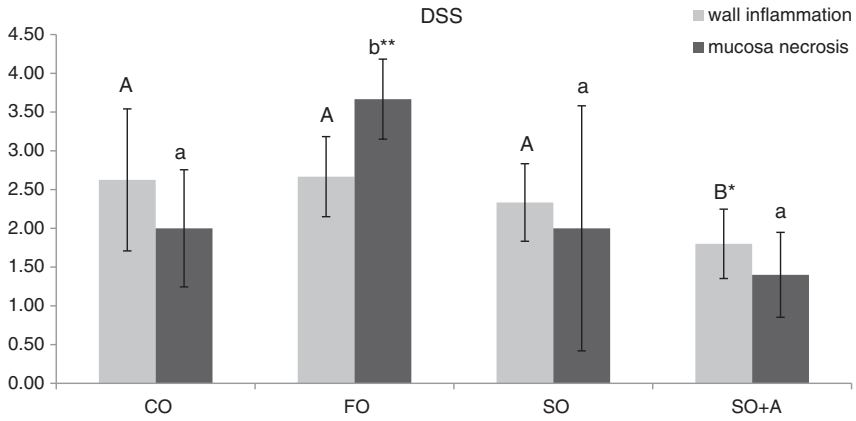

Fig. 5. Histophatological evaluation of the colon — DSS-induced colitic rats fed the CO, FO, SO or SO&A diet.Values are means±S.D., $n=8$. */**Different from the naive control or colitic groups, $P<.05$/$P<.001$, respectively.

In order to evaluate the efficacy of ALA from plant-derived oil compared to EPA- and DHA-rich fish oil, we have accentuated the unique properties of each (n-3) source and determined a single fat source in each of the experimental diets. The corn oil represented a source rich in (n-6) and low in (n-3). This way we were able to compare a control Western-type diet (CO) with a diet rich in EPA and DHA (FO) and with a diet rich in ALA (SO), with or without vitamin A supplementation.

Dietary fatty acid incorporation into tissue membrane phospholipid pools has previously been shown to alter the production of eicosanoids and to increase membrane fluidity. Furthermore, dietary fats can modulate fatty acid-dependent intracellular signaling events taking place in immune cells. This includes generation of second messengers, activation or stabilization of enzymes in the signaling pathways, effecting signal transduction and modifying gene expression, consequently modulating the inflammatory response [12,15]. The preventive, anti-inflammatory effect shown by the lipid dietary intervention in colitic rats in both TNBS and DSS colitis animal models was associated with a significant modification in the lipid profile in both plasma and colonic tissue. These findings show that dietary fats were well absorbed based on the tissue incorporation of the corresponding fatty acids present in the different diets administered.

As expected, macroscopic examination of the colonic samples revealed significantly greater colon length measurements in SO- and SO&A-fed rats compared with CO-fed rats. A reverse trend was discovered in relation to the lesion length in the colon. The observed shortening of the colon and enlargement of the lesions in the control (CO) group indicate an inflammatory state as well as tissue damage. Furthermore, histological examination of the colonic samples from both DSS- and TNBS-treated CO-fed colitic rats demonstrated typical acute inflammatory changes in the colonic structure, as was observed in previous studies [24,35,40–44]. Colitic rats fed SO&A diets had an overall lower level of colonic damage compared with CO-fed group.

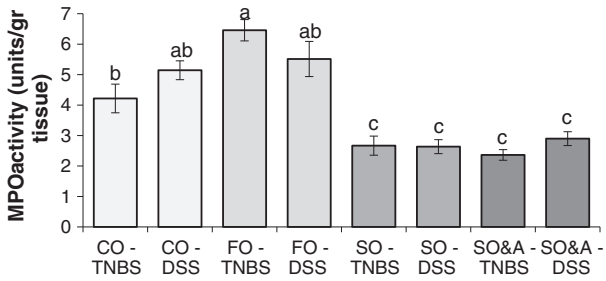

Fig. 6. MPO activity in the colon of DSS- and TNBS-induced colitic rats fed the CO, FO, SO or SO&A diet. One unit of MPO activity was defined as that degrading 1 μmol hydrogen peroxide per minute at 20°C. Values are means±S.E.M., $n=6$–8 rats per group. Values without a common letter differ, $P<.01$.

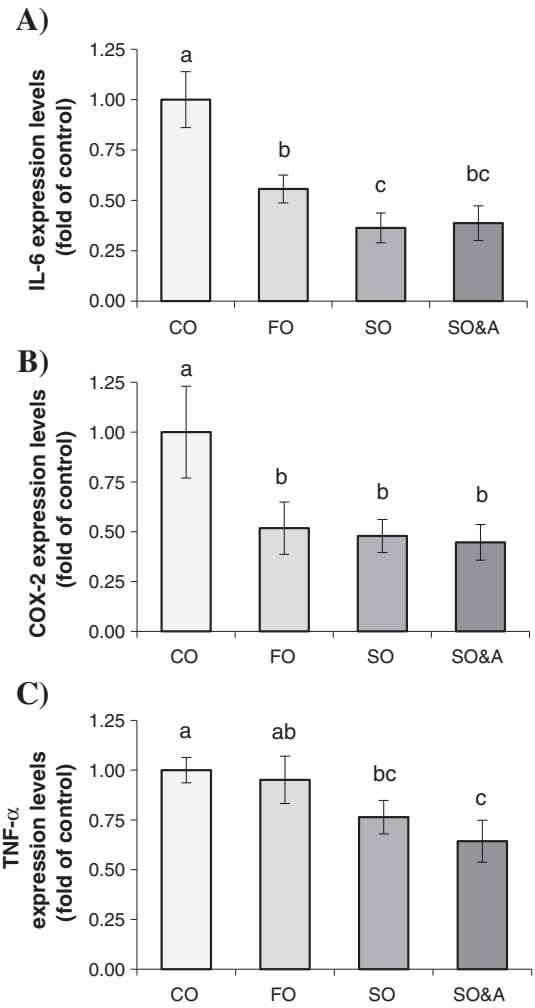

Fig. 7. Gene expression of (A) Interleukin IL-6, (B) cyclooxygenase COX2 and (C) TNF-α in colon tissue of TNBS-induced colitic rats fed the CO, FO, SO or SO&A diet as determined by real-time RT-PCR. The mRNA levels were normalized to 18S. Values are means±S.E.M., $n=6$–8 rats per group. Values without a common letter differ, $P<.05$.

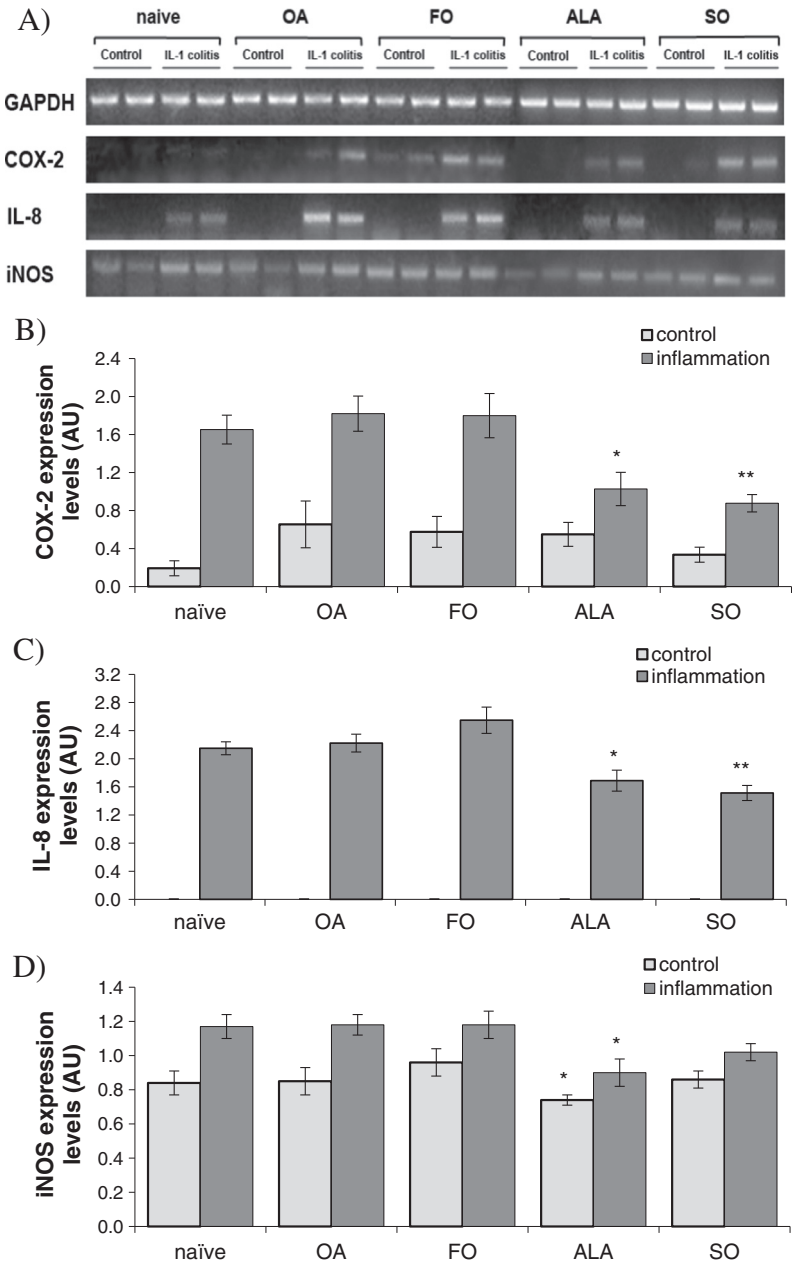

Fig. 8. Gene expression of cyclooxygenase COX2, interleukin IL-8 and iNOS in Caco-2 cells as determined by RT-PCR. Caco-2 cells were incubated during 48 h with ALA, OA, fish oil (FO) or sage oil (SO) (10 μM) with or without inflammation induction by a 4-h incubation with 5 ng/ml IL-1β. (A) Representative autoradiogram for gene expression of COX2, IL-8, iNOS and GAPDH. The mRNA levels of COX2 (B), IL-8 (C) and iNOS (D) were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values are means±S.E.M., $n=8$. */**Different from the naive control or colitic groups, $P<.05$/$P<.001$, respectively.

Additionally, the biochemical determinations revealed that both TNBS and DSS administration significantly higher colonic MPO activity in CO-fed colitic rats when compared to SO-fed rats, and the difference between the CO- and SO&A-fed rats was significantly pronounced as well. MPO activity has been widely used to identify and observe the progression of intestinal inflammation, and a reduction in the activity of this pro-inflammatory enzyme can be interpreted as evidence of the anti-inflammatory activity of a given substance [21].

The anti-inflammatory effects demonstrated by biomarkers (MPO, IL-6, COX2, TNF-α) by SO diets in both models of experimental colitis can be explained by the involvement of several different mechanisms that may interactively act to ameliorate the tissue damage induced by DSS or TNBS. These include antioxidant properties as well as suppression in the production and release of pro-inflammatory mediators such as cytokines, eicosanoids and NO [21]. Other mechanisms might be associated with a considerable decrease in colonic LTB_4 levels, supporting previous observations in animal experimental colitis models and in humans [21,28,35]. The beneficial effects stimulated by SO intake in colitic rats might also be associated with suppression of the production of colonic TNF-α, a potent pro-inflammatory cytokine that is considered to play a key role in inflammatory intestinal conditions [21], as well as other potent pro-inflammatory agents, such as IL-6 and COX2. Gene expression of these active and crucial players of the inflammatory cascade supported the beneficial effects exerted by sage oil and vitamin A dietary supplementation. Fish oil had a similar effect on the pro-inflammatory

agents' mRNA levels. Vitamin A supplementation showed a beneficial effect when added to ALA-rich diet, apparently due to its properties as a regulator of epithelial proliferation and differentiation and as an anti-inflammatory agent and antioxidant. Its effect can be explained by the participation of various mechanisms that may synergistically act to ameliorate the colitic damage. These findings are consistent with previous findings in animals [22,45,46].

Is should be noted that fish oil supplementation in many instances led to severe bleeding events. Opposing to the findings of numerous studies, which showed a decrease in the inflammatory state and in the colonic damage as a result of using dietary fish oil supplementation [16,17,21], our results clearly show the opposite effect. The beneficial effects exerted by SO and SO&A when compared to FO dietary supplementation were evidenced histologically, with a reduction in the colon tissue and the lesion lengths, and also biochemically, by a decrease in colonic MPO activity. Animal studies have shown that fish oil might reduce platelet AA and thromboxane A_2 levels and decrease the level of vitamin K-dependent coagulation factors, leading to a hypocoagulable condition [36]. Moreover, human studies demonstrated that fish oil at doses greater than 2 g/day may cause platelet vascular dysfunction, as evidenced by extension of bleeding times [37,38]. In addition, it was shown that high consumption of fish oil might cause bleeding events in people with a corresponding tendency [47,48] as well as increasing the risk of intracranial hemorrhage [18,49,50]. These findings suggest that a relatively high dose of fish oil can act as an anticoagulant and inhibit platelet function that might cause damage and raise the risk of bleeding. Due to such potent anticoagulant properties of the fish oil, caution should be taken when FO doses are considered, particularly in patients who consume anticoagulant medications and/or suffering from conditions that involve bleeding events, such as active IBD. Other possible problems might have occurred over the 4-week period of consuming a diet with fish oil as the only fat source. A lack of potent antioxidants in the diet might have led to EPA and DHA's oxidation [35]. The FO diet also had reduced levels of the essential (n-6) LA. Increased oxidation of the long chain and highly unsaturated EPA and DHA may have reduced their beneficial effects. Likewise, insufficient LA may have caused an imbalance between the fatty acids in the tissues and disturbed the proper stimulation of wound healing in the colon [51,52]. This might explain the histological findings and the inflammatory status of FO-fed rats in both the TNBS and DSS models observed in this study.

Whether the essentiality of ALA in the diet reflects the activity of ALA itself or of longer-chain PUFA, EPA and DHA synthesized from ALA is a matter for debate among scientists. Studies demonstrate a significant linear relationship between ALA intake and EPA but not DHA incorporation into tissues [53,54]. Our findings also show significantly higher EPA levels as well as a moderate increase in DHA levels in the tissues of SO-fed rats. However, considering the high amount of ALA in the SO diet (44%) compared with CO (1%) and FO diets (1.8%), comparison of the amounts of EPA and especially DHA levels in plasma and colonic tissues of the CO-, FO- and SO-fed rats leads to the conclusion that the extent of ALA conversion to EPA and DHA was rather limited. Therefore, it is unlikely that such modest conversion could be accredited for the beneficial effects stimulated by SO intake in colitic rats rather than the activity of ALA itself.

Since it is impossible in an *in vivo* model to administer a diet containing exclusively ALA, in order to investigate its specific anti-inflammatory properties and efficacy, we used an *in vitro* model for IBD using the human colon adenocarcinoma cell line Caco-2. Caco-2 cells were supplemented with SO as well as with ALA alone in order to isolate it from other multiple components of the sage oil. ALA supplementation was shown to be effective at inhibiting inflammation induced by IL-1β by down-regulating mRNA level of pro-inflammatory genes including IL-8, COX2 and iNOS compared to naïve cell as well as FO- and OA-treated cells. Similar to the *in vivo* models, the results of the *in vitro* Caco-2 model suggest a consistent advantage of SO and ALA supplementations compared to FO. The comparison of ALA [18:3(n-3)] and OA [18:1(n-9)] was designed in order to compare the potential of the two fatty acids as anti-inflammatory agents, mainly due to the concern that ALA is more easily oxidized and therefore might have caused damage to the colonic cells. In the current Caco-2 model, this concern was not supported as ALA showed a significantly higher efficacy as an anti-inflammatory agent when compared with OA. It is plausible that other components present in sage oil may have contributed to the anti-inflammatory effect of the oil; however, this is beyond the scope of this paper and should be investigated in future studies.

In conclusion, our findings from both DSS and TNBS *in vivo* colitic models as well as from a Caco-2 *in vitro* inflammation model support the beneficial role of diet enriched in plant-derived oil rich in ALA in rats with intestinal inflammation. Our results also confirm the anti-inflammatory efficiency of plant-derived (n-3) fatty acid supplementation in colitis, as has been suggested in previous studies [16,17,36,39]. Furthermore, our findings present a possible advantage of plant-derived oil rich in ALA compared with fish oil supplementation, particularly in patients who take anticoagulant medications and/or suffer from conditions that involve bleeding events, such as active IBD. Taken together, the outcomes of this study suggest that ALA alone has potential as an anti-inflammatory agent that is not necessarily dependent on its conversion to the longer (n-3) PUFA, EPA and DHA.

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