

The effect of fish oil supplementation on cytokine production in children

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ABSTRACT. The *ex vivo* production of inflammatory cytokines during fish oil supplementation (n-3 polyunsaturated fatty acids, n-3 PUFA) is a matter of considerable controversy. Studies on human subjects have generally reported decreased lymphocyte proliferation and decreased production of IL-2, interferon- γ , IL-1 β , IL-6 and TNF- α , but other studies showed no effect or even increased production. There are no published reports on *ex vivo* cytokine production in children on long-term, n-3 PUFA supplementation. The current double-blind study explored cytokine production by peripheral blood mononuclear cells (PBMCs), with and without lipopolysaccharide (LPS) stimulation in children on 12 weeks' supplementation with 300 mg/day of n-3 PUFA. Twenty-one children (aged 8-12 years) were randomized to receive 1 g canola oil (control) or 300 mg n-3 PUFA + 700 mg canola oil in a chocolate spread. Blood was then drawn and PBMCs were separated and cultured for 24 h in a culture medium with or without 10 μ g/mL LPS for 5×10^6 PBMCs. The pro-inflammatory cytokines, IL-1 β , TNF- α and IL-6, and the anti-inflammatory cytokines, IL-10 and IL-1RA, were evaluated by ELISA. The levels of all the cytokines were higher in non-stimulated and LPS-stimulated cultures, from n-3 PUFA-treated subjects as compared to controls. There was no difference in the IL-1 β /IL-1RA ratio between the two groups, with and without LPS stimulation. Nevertheless, the ratio tended to be lower in the treated subjects on both occasions. In conclusion, our results indicate an increased production of both pro-inflammatory and anti-inflammatory cytokines, with and without LPS stimulation, in children on 12 weeks' n-3 PUFA supplementation.

Keywords: fish oil, DHA, cytokines

Different studies have suggested that unsaturated fatty acids can modulate immune functions [1, 2]. Most of the studies were performed in animal models and showed that omega-3 polyunsaturated fatty acids (n-3 PUFAs) are especially potent, while other fatty acids had various effects too. Cytokines are key mediators of immune function and can be either pro-inflammatory or anti-inflammatory. The *ex vivo* production of inflammatory cytokines during fish oil supplementation is a matter of considerable controversy [1]. Studies in animal models and in human subjects generally reported a decrease in lymphocyte proliferation

[3] and a decreased production of IL-2, interferon γ , IL-1 β , IL-6 and TNF- α [4-6]. However, other studies have shown no effect [7-10] and even an increased production [11, 12]. There are no published reports on *ex vivo* cytokine production in children who receive long-term fish oil supplementation. The current study demonstrates changes in *ex vivo* production of pro-inflammatory and anti-inflammatory cytokines after 3 months of fish oil supplementation in children aged 8-12 years.

METHODS

Study design

The effect of n-3 PUFA dietary supplementation was investigated using a double-blind, placebo-controlled study design. Children were randomized to receive either 300 mg n-3 PUFA (180 mg EPA, 120 mg DHA) + 700 mg canola oil or 1 g of canola oil blended in chocolate spread. The chocolate mixture was delivered weekly to the children's home and compliance was ensured by receiving the empty containers on the spot. There were no other interventions in the children's diet or any other aspect of their

Abbreviations:

PUFA	polyunsaturated fatty acids
IL	interleukin
TNF	tumor necrosis factor
LPS	lipopolysaccharide
PBMCs	peripheral blood mononuclear cells
EPA	eicosapentaenoic acid
DHA	docosapentaenoic acid
ELISA	enzyme-linked immunosorbent assay
n-3	omega-3

daily routine. Ten mL of whole blood were drawn at the end of three months for the cytokine studies. The study was approved by the local Human Subject Committee.

Study population

Twenty-one (7 controls) subjects aged 8-12 years were recruited for the study. The male/female ratio was 5/2 and 9/5 in the in control and treatment group, respectively. Exclusion criteria included routine supplementation of n-3 fatty acids, chronic disease or intestinal impairment that could have affected the absorption of nutrients.

Preparation of peripheral blood mononuclear cells (PBMCs)

Ten mL of whole blood were drawn into heparin-containing tubes between 08.00-10.00 a.m.

The blood was layered onto Ficoll-Hypaque gradients (density 1.077 g/mL; ratio of blood to Ficoll 3:2) (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged for 30 min at 1500 rpm and 20°C. The PBMCs were collected from the interface and washed twice with RPMI medium containing 0.75 mmol glutamine/L and antibiotics (penicillin and streptomycin) for 10 min at 1200 rpm. After resuspension in 2.5 mL of culture medium, the cells were counted on a Coulter Z1 Cell Counter (Beckman Coulter Ltd., Bucks, United Kingdom), and diluted to a concentration of 5×10^6 cells/mL. PBMCs were cultured for 24 h in a culture medium with or without 10 µg/mL lipopolysaccharide (LPS) (Sigma, St Louis, MO, USA) [13] for 5×10^6 PBMCs. At the end of the incubation, the plates were centrifuged for 7 min at 3000 rpm, at room temperature, and the culture medium was collected and frozen in aliquots.

Cytokine measurements

The levels (pg/mL) of the pro-inflammatory cytokines, IL-1β, TNF-α and IL-6, and the anti-inflammatory cytokines, IL-10 and IL-1RA, were measured by solid phase enzyme-linked, immunosorbent assay (ELISA, R&D Systems Inc., Minneapolis, USA) in supernatants of PBMCs,

with and without LPS stimulation. A quantitative “sandwich” enzyme immunoassay technique was used as previously described [13].

Statistical analysis

Comparisons between the cytokine levels of supplemented (n-3 PUFA-treated) and non-supplemented (control) subjects were performed with the Mann-Whitney, non-parametric test. The statistical significance level was set at 0.05 and the SPSS for Windows software version 12.0 was used for the analysis.

RESULTS

No differences in plasma TNF-α or IL-6 concentrations were found between the two groups before fish oil supplementation. TNF-α concentrations were 141.7 ± 81.9 versus 194.0 ± 68.6 pg/mL in the controls and the supplementation group respectively, and the IL-6 levels were 2.07 ± 1.18 pg/mL in the controls and 1.66 ± 0.85 in the supplementation group. After treatment, IL-1β, TNF-α and IL-6 concentrations were significantly higher in the supernatants of the n-3 PUFA-treated subjects as compared to the controls ($p = 0.003$, $p = 0.052$ and $p = 0.004$, respectively) (figure 1). The concentrations of the anti-inflammatory cytokines, IL-10 and IL-1RA, in the supernatants were also higher in n-3 PUFA-treated subjects as compared to controls ($p = 0.004$ and $p = 0.02$ respectively) (figure 2). After stimulation of PBMCs by LPS, the concentrations of the pro-inflammatory cytokines, IL-β, TNF-α and IL-6 were higher in n-3 PUFA-treated subjects as compared to controls ($p = 0.002$, $p = 0.011$ and $p = 0.006$, respectively) (figure 3). The concentrations of the anti-inflammatory cytokines, IL-10 and IL-1RA after stimulation, were also higher in n-3 PUFA-treated subjects as compared to the controls ($p = 0.073$ and $p = 0.008$ respectively) (figure 4). The ratio of IL-1β/IL-1RA between the groups did not change significantly with or without LPS stimulation ($p = 0.76$ versus $p = 0.20$ respectively).

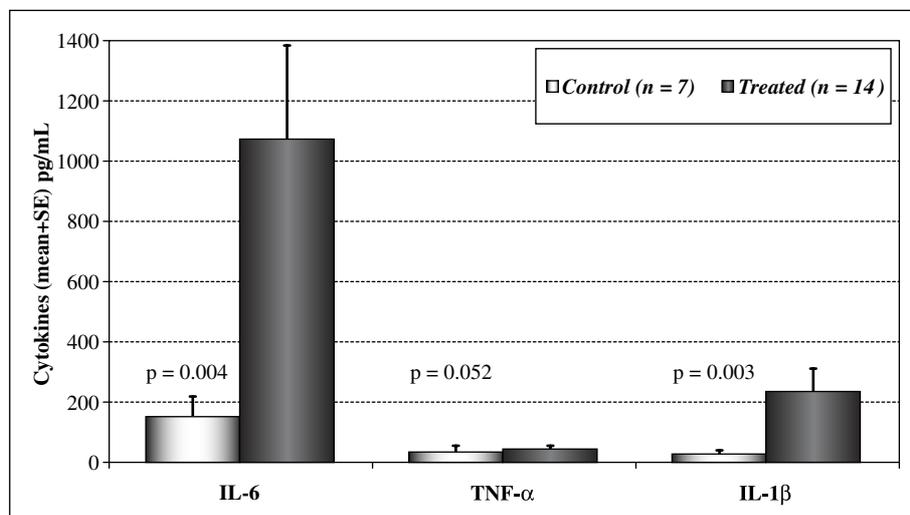


Figure 1

Pro-inflammatory cytokine production in non-stimulated culture.

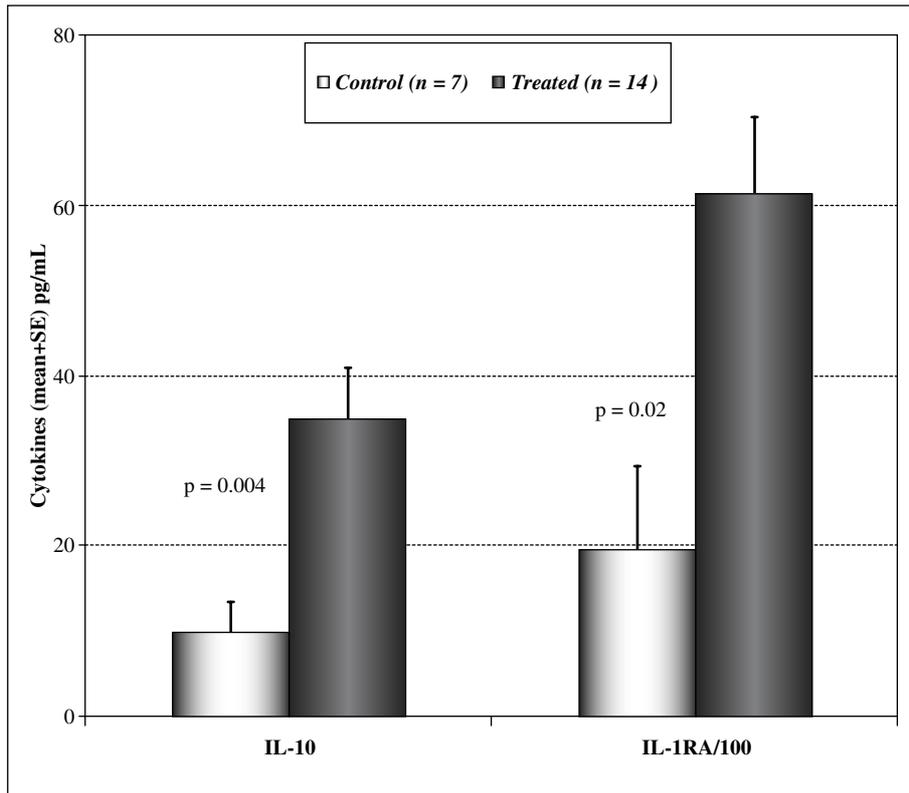


Figure 2

Anti-inflammatory cytokine production in non-stimulated culture.

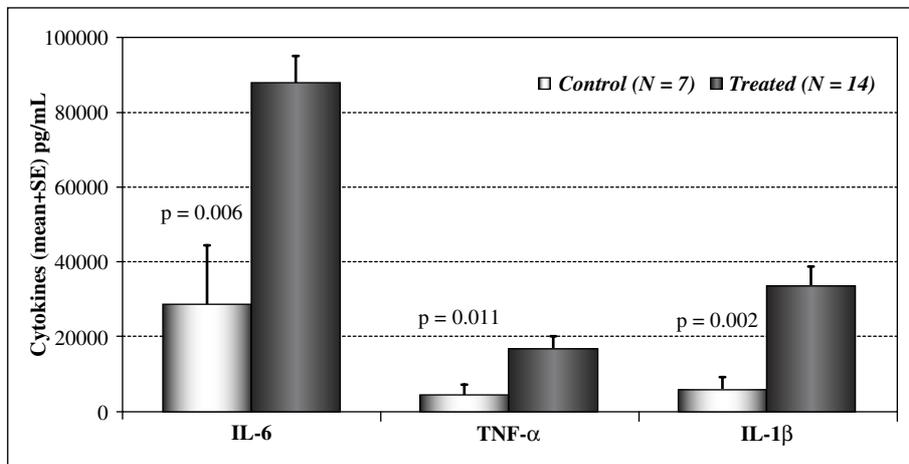


Figure 3

Pro-inflammatory cytokine production in LPS stimulated culture.

DISCUSSION

Our results indicate that long-term supplementation of moderate amounts of n-3 fatty acids to the diets of 8-12 year-old children increases both *ex vivo* production of non-stimulated and LPS-stimulated pro- and anti-inflammatory cytokines. These are the first results to be reported in children, and are different from some of the results previously reported in adult subjects. Most of the studies on adults reported only the concentrations of pro-inflammatory cytokines and showed inconsistencies in the results. In an attempt to explain this discrepancy, Calder

[2] looked at certain aspects that may affect cytokine production. These key factors included: the dose of n-3 PUFA supplementation, the medium that was sampled (whole blood, supernatants or cell lysate), and the dose of stimulation. Calder's general conclusion was that studies using higher doses (> 5.2 g eicosapentaenoic acid [EPA] + docosahexaenoic acid [DHA] per day) reported inhibition in pro-inflammatory cytokine production, while those using the lowest doses (< 1 g EPA + DHA per day) reported no effect. This does not however, entirely account for the inconsistency, because some studies employing high doses of n-3 PUFA showed no effect on cytokine

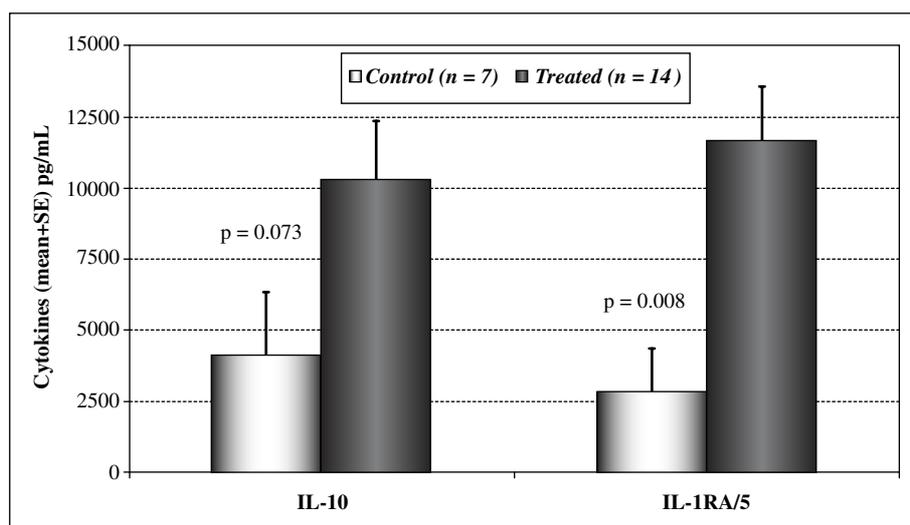


Figure 4

Anti-inflammatory cytokine production in LPS stimulated culture.

production, while others using low doses reported inhibition [1]. Healy *et al.* [8] conducted a dose-response study using different concentrations of tuna oil and found no effects on neutrophil chemotaxis or superoxide production. Blok *et al.* [11] found no consistent differences in the response to different doses, suggesting an increase in both pro- and anti-inflammatory cytokine production (IL-1 β and IL-1RA) in response to long-term fish oil supplementation. Since no comparable studies have been done in children, there are no data for comparison, but, in terms of the effect of dose on our findings, the absolute amounts used in our study seem to be on the lower side (a total of 300 mg) as compared to studies in adults and so the results may indicate a different effect of n-3 PUFA supplementation in children as compared to adults.

The second factor which may affect our results is the nature of the sample in which cytokines are assayed. Calder [2] concluded that the effects of “moderate” levels of EPA + DHA might be exerted at the level of intracellular cytokine concentrations rather than at the level of extracellular cytokine concentrations. Molvig *et al.* [14] reported a decrease in cellular IL-1 β content, while no difference was found in the medium. In our current study, we collected only culture medium and not cell lysates. The increase in cytokine production that we found may, therefore, point to a different response on the part of children as compared to adults.

The third of Calder’s [2] three key factors is the cell culture conditions, i.e. the amount of LPS used to stimulate the PBMCs. We used 10 μ g LPS for stimulation of 5×10^6 PBMCs, which is higher than the concentrations used in most of the previous studies. This was done in order to simulate sepsis in an attempt to clarify some of the inconsistencies found in the literature. Our results do not support the hypothesis that n-3 PUFA supplementation attenuates cytokine production in severe sepsis conditions, but on the other hand, they may indicate a different threshold of sensitization of children’s PBMCs as compared to adults. They may also indicate that the decreased production reported in previous studies may not apply to severe infections.

Another key factor which may affect the results is the length of time over which the supernatants were harvested.

In a comparative analysis of stimulated PBMCs, Reddy *et al.* [15] showed differences in the excretion of cytokines over time. The cytokine levels continued to increase with time and peaked during the first 24-48 h in most of the assays. This emphasizes the importance of comparing results of the same duration of stimulation. Our samples were cultured for 24 h, and differences in time collection cannot explain the differences between the non-supplemented and supplemented subjects.

In addition to the above, different studies have suggested that a difference in the prostaglandin and oxidative status, caused by the various fatty acids, may also affect cytokine production [16].

The potentially different immuno-modulatory properties of the different n-3 PUFAs (EPA and DHA) are also major issues. Most of the published studies used a combination of both fatty acids in different ratios, ranging between 1-4.8 (EPA/DHA) [2]. The ratio we used in our preparation was 3:2, well within the range of the other studies. The intracellular concentrations of the different fatty acids was also suggested to play a role in cytokine production. For example, Caughey *et al.* [4] suggested that the EPA content of mononuclear cells is strongly associated with the *ex vivo* production of IL-1 β and TNF- α , and that a concentration of 1.5% EPA results in maximum suppression of cytokine synthesis.

Recent studies have suggested that the capacity to produce cytokines is genetically determined. Polymorphism in the promoter regions of the genome was reported to be responsible for the differences, and such polymorphisms have been described for different cytokines [17]. Polymorphism may explain the large variation in our results, but it did not have an effect on the significant statistical differences. Moreover, there was no difference in spontaneous cytokine production between groups as indicated by the concentrations of TNF- α and IL-6 in the plasma, prior to study intervention.

The influence of aging on cytokine production has not yet been studied in the pediatric population. Different studies on adults, however, suggest alterations in cytokine production with age, and a tendency towards a decline in production in older subjects [18].

The results of our study indicate that there is an increased level of stimulation of PBMCs, expressed as a higher level of cytokine production, with and without LPS stimulation. Since this increase involves both pro- and anti-inflammatory cytokines, it may indicate a higher level of response in children with higher n-3 PUFA levels. The fact that there is increased production of both pro- and anti-inflammatory cytokines indicates a stronger activation of the immune cells involved (B cells, T cells, NK cells and monocytes) that may, nevertheless, lead to a better clinical response in inflammatory conditions. Moreover, the ratio of IL-1 β to IL-1RA is smaller for supplemented subjects on both occasions (with and without LPS stimulation), suggesting a relative increase in the anti-inflammatory response in these subjects as compared to the controls. This concomitant increase in anti-inflammatory cytokine production may suggest a mechanism of attenuation of the inflammatory response, especially as the timing of increase of the different cytokines may differ [19].

In conclusion, we have demonstrated that the production of both pro-inflammatory and anti-inflammatory cytokines increases significantly after 3 months of fish oil supplementation in children aged 8-12 years. The importance of the changes in cytokine production and excretion can only be interpreted in clinical settings.

Additional studies using different doses of supplementation and different concentrations of stimulants are needed to verify the clinical significance of these findings.

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