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Omega-3 fatty acids suppress inflammatory cytokines production by macrophages and hepatocytes

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Abstract

Objective: Long-term total parenteral nutrition (TPN) in children is often complicated by parental nutrition associated liver disease (PNALD), and may even lead to liver failure. Recently, the addition of omega-3 fatty acids into TPN has been shown to reduce the risk of PNALD. The purpose of this study was to explore the anti-inflammatory effects of omega-3 fatty acids (EPA) in order to demonstrate the protection of the liver against hepatic steatosis and damage.

Materials and Methods: Lipopolysaccharide (LPS) and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) were used to stimulate human peripheral blood mononuclear cells and human liver cell line (THLE-3) to induce an in-vitro inflammatory condition. The cells were then incubated with either omega-3 (EPA) or omega-6 (AA) fatty acids. Supernatants were collected at different time points for the measurement of tumor necrosis factor-\textalpha{} (TNF-\textalpha{}), interleukin 6 (IL-6) and interleukin 10 (IL-10) using ELISA. Furthermore, pre-treated macrophages by LPS stimulation and following incubation with EPA were added to pre-stimulated hepatocytes for the subsequent measurement of cytokine response. Data were analyzed using paired t-test and a p value of <0.05 was taken as statistically significant.

Results: EPA at 100uM concentrations effectively reduced LPS-induced and PGE\textsubscript{2}-induced TNF-\textalpha{}, IL-6 expression and increased IL-10 expression significantly when compared with AA, peaking at 24h time point. Furthermore, supernatant collected after co-culturing EPA with macrophages also suppressed the levels of TNF-\textalpha{}, and IL-6 in hepatocytes. This would suggest that EPA not only had
anti-inflammatory effect on macrophages and hepatocytes directly, but could indirectly reduce inflammations in hepatocytes through activated macrophages.

**Conclusions:** The addition of omega 3 fatty acids in TPN preserves immune function and suppresses the inflammatory response. The findings may help explain the clinical benefits of EPA in pediatric patients receiving long term TPN.

**Keywords**

omega-3 fatty acids (EPA), omega-6 fatty acids (AA), PNALD, TPN, inflammation
Introduction

Total parenteral nutrition (TPN) can provide an effective method for supplying energy and nutrients for children with intestinal failure (1,2). However, long-term application of TPN can cause parental nutrition associated liver disease (PNALD), which was recognized early in the experience almost 30 years ago. This incidence of PNALD is higher in neonates and infants, owing to physiological immaturity (3,4). Indeed, PNALD remains the leading cause of neonatal cholestasis and liver failure and the primary indication for combined liver and intestinal transplantation in children (5-8). As the long-term outcome of liver-intestinal transplantation remains poor, with a 5-year graft survival of only around 50%, the prevention and treatment of PNALD is critical. Thus far, the etiology of TPN-induced liver disease remains unknown but is thought to be related to the direct toxicity of the parenteral nutrition solutions and of the underlying digestive disease (8-13). Recent evidence has indicated the possibility of intravenous emulsified lipid intake with fatal liver disease. The effect may reflect direct hepatotoxicity of some components in lipid emulsion. Additionally, lipid may also provide substrates to fuel the systemic inflammatory response and its deleterious effect on the liver (14-16).

Clinically, excessive inflammation is a response to surgery, trauma, injury, and infection. Patients with short bowel syndrome are especially prone to inflammation and parenteral nutrition may exacerbate the systemic inflammatory response. Excessive inflammation is characterized by the production of inflammatory cytokines and arachidonic acid-derived eicosanoids. Thus, it has been proposed that the use of
omega-6 fatty acid in PN may actually contribute towards hepatic damage, as it is broken down in the body to \( \gamma \)-linolenic acid, then to dihomo-\( \gamma \)-linolenic acid, and finally to arachidonic acid. As a result, some centers in the world recently proposed the use of omega-3 fatty acids instead of omega-6 in the PN formulation. Eicosapentaenoic acid (EPA) is an omega-3 fatty acid, which is a breakdown product of \( \alpha \)-linolenic acid, found in fish oils and breast milk. It suppresses the production of arachidonic acid-derived eicosanoids and is also a substrate for the synthesis of an alternative family of eicosanoids, which have many anti-inflammatory effects. The protective benefits of omega-3 fatty acids may be related to its ability to decrease the production of prostaglandins and subsequently, the release of other inflammatory cytokines. These reductions will inevitably lead to the decrease in the magnitude of inflammation and the severity of insult to the liver (17-20). Indeed, there have been a few case series published recently suggesting the advantages of omega-3 fatty acids in the formulation of parenteral nutrition in the rescue of babies with PNALD (21-24). Despite all these significant and encouraging clinical findings, the exact mechanism of action of omega-3 in preventing PNALD is still not clear.

As the change from pro-inflammatory to anti-inflammatory state has implications for the status and progression of PNALD in response to the initial cholestatic and steatotic insult, it is likely that both pro-inflammatory and anti-inflammatory cytokines could play a role. The pro-inflammatory state is mediated by macrophages and Kupffer cells in liver through the release of cytokines such as tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and interleukin-6 (IL-6), and the plasma levels of TNF-\( \alpha \) and IL-6
correlate positively with the degree of underlying liver damage. On the other hand, interleukin-10 (IL-10), an anti-inflammatory cytokine, has pleiotropic effects in regulating exaggerated immune response and the eventual termination of inflammation.

We therefore hypothesize that omega-3 fatty acid (EPA) could exert its action on cells which produce these pro-inflammatory and anti-inflammatory cytokines. In this study, we aim to explore the anti-inflammatory effects of omega-3 fatty acids on human macrophages and Kupffer cells in order to demonstrate the protective action of the liver against hepatic steatosis and damage.

**Materials and Methods**

**Reagents**

Lipopolysaccharide (LPS) and Prostaglandin E\(_2\) (PGE\(_2\)) were purchased from Sigma-Aldrich (USA). LPS was diluted to 1mg/mL concentration of working solution and stored at 4°C. PGE\(_2\) diluted with PBS to 0.1 ug/ml concentration for subsequent experiments. The format of omega 3 fatty acid and the omega 6 fatty acid (Sigma-Aldrich, USA) used in this study was respectively cis-5, 8, 11, 14, 17 – eicosapentaenoic acid (EPA) and arachidonic acid (AA) derived from porcine liver. Both of them were re-suspended in absolute ethanol and stored at -20°C for experiments.

**Cell culture**
(a) Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells were obtained from donated blood (Hong Kong Red Cross Blood Transfusion Service) by Ficoll Paque gradient method. Briefly, ACK buffer and Ficoll were added to blood and left in room temperature for 15 minutes. The samples were then diluted with phosphate buffered solution (PBS) and centrifuged at 2000rpm for 25 minutes. Cells at the interphase (lymphocytes, monocytes, and thrombocytes) were transferred to a new conical tube filled with PBS and centrifuged at 1200 rpm for 7 minutes at room temperature. The supernatant was carefully removed completely. For removal of platelets, the cell pellet was re-suspended in PBS and centrifuged at 800 rpm for 7 minutes. The supernatant was removed and repeated twice. 35mL of RPMI 1640 medium was added and mixed, then centrifuged at 1200 rpm for 7 minutes. The pellet was re-suspended in 50mL macrophage SFM medium, supplemented with L-glutamine. 2 mL of the solution was added to 6-well plates and cultured at 37 °C and 5% CO₂. After 7 days of incubation, mononuclear cells would change into macrophages, which could be used in further experiments.

(b) Liver THLE-3 Cells

The human liver cell line, THLE-3, was purchased from the American Type Culture Collection (ATCC, USA). The cells were maintained in precoated flasks with a mixture of fibronectin (0.01mg/mL), bovine collagen type 1 (0.03 mg/mL), and bovine serum albumin (0.01mg/mL) dissolved in BEGM medium and incubated at
37°C and 5% CO₂. Medium was changed every 2 to 3 days.

**Experimental Design**

Macrophages and THLE-3 cells were used when 80% confluent. 1x10⁶ of macrophages or THLE-3 was seeded in each well of a 6-well plate. The cells were subjected to four conditions: (1) stimulated with LPS (0.1 µg/mL) or PGE₂ (0.1 µg/ml) alone for 24 h; (2) pre-incubation with 100 µM EPA, 100 µM AA or EPA+AA (ratio, 1:1) for 24 h before LPS or PGE₂ stimulation; (3) co-incubation of EPA, AA or EPA+AA with LPS or PGE₂; (4) post-incubation with EPA, AA or EPA+AA, 24 h after stimulation with LPS or PGE₂. Optimal concentration of EPA and AA was determined to be 100µM. The production of IL-6 and TNF-α were measured using ELISA.

For cell interaction study, pre-stimulated macrophages using LPS were post-incubated with EPA for 24 hours before harvested. The cells were then added to LPS pre-stimulated THLE-3 cells. The subsequent production of IL-6, TNF-α and IL-10 were measured using ELISA.

**Measurements of TNF-α, IL-6 and IL-10 using ELISA**

Human TNF-α, IL-6 and IL-10 ELISA kits were obtained from R & D Systems, USA. The concentration of IL-6, IL-10 and TNF-α in supernatants was measured according to the manufacturer’s instruction. Briefly, ELISA was performed in 96-well plate with 100µl of samples for IL-6 and 200µl for IL-10 and TNF-α at room temperature for 2
h. Following washing for 4 times, 200 µl of conjugate was added to each well at 2 h for IL-6 and 1 h for IL-10 and TNF-α. The concentration of each cytokine was determined by the absorbance at 450nm with reference filter at 540nm.

**Statistics**

All values are measured as means ± SD in experiments. ANOVA and the Student’s t-test were used for statistical analysis. Differences were considered significant at P < 0.05 (*) or P < 0.01 (**).

**Results**

*LPS-induced IL-6 and TNF-α expression in macrophages and THLE-3 cell line*

In the presence of LPS stimulation, there was an increase in IL-6 (2598.1 ± 196.49 pg/ml) and TNF-α (3205.1 ± 123.31 pg/ml) production in macrophages when compared to the medium alone group. However, when EPA was added, either before or after LPS stimulation, there was significant suppression of IL-6 production. This effect was found to be most dramatic in the post-incubation group (decreased by 95.7% to 110.72 ± 12.94 pg/ml). On the other hand, although the addition of AA to the cell culture did seem to suppress the production of IL-6 (1798.3 ± 74.25 pg/ml), this was indeed not statistically significant when compared with control. In addition, the mixture of EPA and AA did not seem to have the same effect as when EPA was added alone (1541.7 ± 43.32 pg/ml) [Figure 1A].

A similar trend could be observed for TNF-α, where EPA was found to decrease its
production most significantly in the post-incubation group by 94.6% (102.3 ± 74.11 pg/ml) [Figure 1B].

These findings suggested that EPA is effective in decreasing LPS-induced IL-6 and TNF-α expression and that EPA addition after LPS stimulation for 24 h is the most effective compared with other groups.

We next used LPS to stimulate THLE-3 cells, a liver cell line. Here, although the effects of EPA on IL-6 and TNF-α production were similar to those seen in macrophages, the overall response of THLE-3 to LPS stimulation was much lower, suggesting that LPS might not be the ideal choice for stimulation [Figure 2A&B].

**PGE2-induced IL-6 expression in macrophages and THLE-3 cell line**

We next used PGE₂ for stimulation to mimic an inflammatory condition in liver. In macrophages, the IL-6 response after stimulation was weak (20.41 ± 0.56 pg/ml) when compared with THLE-3 (1340.6 ± 53.75 pg/ml). However, the same trend could be observed in both cell types.

As with LPS stimulation, the addition of EPA to PGE-2 stimulated cells showed the best suppression in the post-incubation group. For macrophages, IL-6 levels decreased by 74.1% (5.27 ± 0.36 pg/ml) in the EPA treatment group and by 48.7% (10.46 ± 1.02 pg/ml), 52.3% (9.73 ± 0.68 pg/ml) in AA and EPA+AA treatment groups respectively [Figure 3A]. In THLE-3 cell line, IL-6 levels decreased by 80.1% (265.97 ± 11.54 pg/ml) in the EPA treatment group and by 40.4% (798.02 ± 11.75 pg/ml) and 47.9% (698.44 ± 14.57 pg/ml) in AA and EPA+AA treatment groups respectively [Figure
Temporal events of EPA suppression on LPS-stimulated macrophages

Since EPA could effectively suppress the production of pro-inflammatory cytokines, we next asked if it could also alter the production of an anti-inflammatory cytokine, IL-10. Here, a time chase experiment was carried out after EPA had been added to LPS-stimulated macrophages. IL-10 secretion was found to be significantly increased after treatment with EPA. At 24-h time point, its level reached the peak at 280.32 ± 11.86 pg/ml [Figure 4A]. Interestingly, at the same time point, the levels of IL-6 (110.72 ± 12.23 pg/ml) and TNF-α (170.75 ± 15.76 pg/ml) were at the lowest correspondingly [Figure 4B&C].

Co-culture of EPA-treated macrophages with pre-stimulated THLE-3

The above data showed that EPA could significantly decrease the production of IL-6 and TNF-α when cells were either stimulated by LPS or PGE$_2$, with a corresponding increase in IL-10 secretion. So we next asked whether this effect could be mediated indirectly through macrophages on liver cells. We co-cultured EPA-treated, LPS pre-stimulated macrophages with LPS pre-stimulated THLE-3 for 24 h. Results showed that when compared with untreated group, the production of IL-6 and TNF-α decreased by 75.9% (1606.2 ± 77.71 pg/ml to 387.00 ± 5.94 pg/ml) and by 85.2% (136.66 ± 11.33 pg/ml to 20.09 ± 1.16 pg/ml) respectively [Figure 5A&B]. On the other hand, the levels of IL-10 appropriately increased by 12.5 times in the
EPA-treated group (108.23 ± 7.51 pg/ml vs. 8.00 ± 0.81 pg/ml) [Figure 5C]. This would suggest that EPA not only had anti-inflammatory effect on macrophages and THLE-3 directly, but could also indirectly reduce inflammation in hepatocytes through activated macrophages.

Discussion

Hepatobiliary dysfunction, liver cirrhosis and subsequent liver failure are well-known complications of long-time TPN, as the duration of total parenteral nutrition increases, so does the incidence of parental nutrition associated liver disease (PNALD). Throughout these years, the incidence of PNALD and mortality from PNALD has decreased. Many factors have contributed to the decline of incidence and severity of PNALD, including restriction of total energy intake, improvements in total parenteral nutrition composition and catheters and their management (25). Recently, evidence suggested that one major contributing factor could be the composition of the intravenous lipid emulsions that predisposed patients to PNALD. Furthermore, attention has been directed to the inflammatory aspects of PNALD and the role of omega-3 fatty acid supplementation in modifying the hepatic biochemical environment (17,26). Omega-3 polyunsaturated fatty acids have been evidenced to reduce not only the activity of inflammatory processes but also lower inflammatory susceptibility in other disease conditions. Hence, they might be able also to dampen the inflammatory response in liver by regulating Kupffer cell activation and suppressing cytokine production (27). In animal models, it has been shown that
parenteral fish oils did not impair biliary secretion and might prevent hepatic steatosis (17,28,29). Furthermore, the reversal of cholestasis and fatal liver disease has been shown clinically by the use of fish-oil–based emulsion in infants who depended on TPN (22-24,26).

Given the promising findings of inhibited immune response by omega fatty acid in numerous studies, the current study is to investigate the possible mechanism of omega-3 fatty acids (EPA) in the treatment of PNALD. Our study demonstrated that EPA at 100µM concentration effectively suppressed the production of pro-inflammatory cytokines IL-6 and TNF-α in LPS and PGE₂ stimulated macrophages and THLE-3 cells. However, this effect was not as dramatic when EPA was used as a mixture with AA (EPA to AA: ratio: 1:1). This finding may help answer the question of whether to use omega-3 fatty acid alone or as a combination in TPN in the clinical setting.

Although some studies already suggested that fish oils could reduce TNF-α and IL-6 production in LPS-stimulated mononuclear cells, these were done on macrophage cell lines (30-33). Indeed, our study was the first to use stimulated primary human peripheral blood mononuclear cells and to use these to compare the effects of omega-3 fatty acids in different treatment stages. Furthermore, we designed our experiment to mimic the in-vivo condition of the liver, where both hepatocytes and Kupffer cells were present. Our data showed that the anti-inflammatory effects of EPA not only could be exerted directly on these cells, but also indirectly via the secretion of IL-10 by macrophages on hepatocytes. Furthermore, the indirect
anti-inflammatory effect of EPA was more powerful than the direct anti-inflammatory effect (Figure 5B&C). The suppression of inflammation on both fronts could thus explain the significant benefits of omega-3 fatty acids in reversing PNALD in clinical patients.

In future work, we plan to block the action of IL-10 to further explore indirect anti-inflammatory effect of EPA through macrophages, as there are many aspects of the potential anti-inflammatory effects of omega-3 fatty acids metabolism that remain unclear.

On the mechanism level, the inhibitory effect on the secretion of inflammatory factors is associated with lower mRNA levels of these inflammatory factors, suggesting that it might be possible for EPA to alter cytokine protein expression at the transcriptional level (34,35). Since it has been shown that omega-3 fatty acids and some of the metabolites could regulate NF-κB activity, we speculate that the altered expression of these inflammatory factors may have been mediated through NF-κB, which plays key a role in regulating cytokine gene transcription (36-38). In addition, some studies also suggested that the anti-inflammatory effects omega-3 fatty acids were due to the down-regulation of NF-κB activity (38,39). Inflammatory responses are a dynamic reflection of the NF-κB transcriptional complex activation, which may be differentially regulated by EPA. We further speculate that duration of EPA treatment could affect the activation of macrophages by LPS and closely related to the transcription complex activation of NF-κB. The mechanisms underlying will be addressed in future study.
In conclusion, our study has shown that EPA provides potent protection for the liver against steatosis and damage through not only effect on macrophages and hepatocytes directly, but also effect on stimulated hepatocytes through anti-inflammatory macrophages indirectly. The findings may help explain the clinical benefits of EPA in pediatric patients receiving long term TPN. In the future, we hope that omega-3 fatty acids will become a standard for both the rescue as well as prevention of PNALD.

References


**Figure legends**

**Figure 1** - IL-6 (A) and TNF-α (B) expression levels produced by macrophages under three different treatments: pure EPA (100µM), pure AA (100µM) and EPA+AA (1:1) (100µM) added before (pre-incubate), simultaneously with (co-incubate), or after (post-incubate) the addition of LPS simulation (24 h) to cell culture wells. Results represent the mean ± SD of 5–6 independent experiments. *p≤0.05; **p≤0.01 relative to no-treatment. +p≤0.05; ++ p≤0.01 between different treatment groups relative to EPA of the same LPS conditions.

**Figure 2** - IL-10 expression levels measured by ELISA for macrophages activated by 0.1ug/ml LPS for 24 h, then cells were treated with pure EPA (100µM). Supernatants were collected at different time points. Data represent the mean ± SD of 5–6 independent experiments. *p≤0.05; **p≤0.01 between different time points relative to 24 h.

**Figure 3** - IL-6 (A) and TNF-α (B) expression levels measured by ELISA for THLE-3 cell activated by 0.1ug/ml LPS for 24 h. One group was treated with pure EPA (100µM), in the other group, EPA pre-stimulated macrophages were added and co-cultured for 24 hrs. Supernatants were collected. Data represent the mean ± SD of 5–6 independent experiments. *p≤0.05; **p≤0.01 relative to no-treatment. +p≤0.05; ++p≤0.01 between EPA treatment group and co-culture group.