Supplementation of parenteral nutrition with fish oil attenuates acute lung injury in a rat model

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Fish oil rich in n-3 polyunsaturated fatty acids has diverse immunomodulatory properties and attenuates acute lung injury when administered in enteral nutrition. However, enteral nutrition is not always feasible. Therefore, we investigated the ability of parenteral nutrition supplemented with fish oil to ameliorate acute lung injury. Rats were infused with parenteral nutrition solutions (without lipids, with soybean oil, or with soybean oil and fish oil) for three days. Lipopolysaccharide (15 mg/kg) was then administered intratracheally to induce acute lung injury, characterized by impaired lung function, polymorphonuclear leukocyte recruitment, parenchymal tissue damage, and upregulation of mRNAs for inflammatory mediators. Administration of parenteral nutrition supplemented with fish oil prior to lung insult improved gas exchange and inhibited neutrophil recruitment and upregulation of mRNAs for inflammatory mediators. Parenteral nutrition supplemented with fish oil also prolonged survival. To investigate the underlying mechanisms, leukotriene B4 and leukotriene B5 secretion was measured in neutrophils from the peritoneal cavity. The neutrophils from rats treated with fish oil-rich parenteral nutrition released significantly more leukotriene B4, an anti-inflammatory eicosanoid, than neutrophils isolated from rats given standard parenteral nutrition. Parenteral nutrition with fish oil significantly reduced lipopolysaccharide-induced lung injury in rats in part by promoting the synthesis of anti-inflammatory eicosanoids.

Key Words: omega-3 fatty acids, nutritional support, acute lung injury, rat model, fish oil

Parenteral nutrition (PN) is important to supply adequate calories to critically ill patients and is particularly important in patients with intestinal failure. 1,2 Lipid emulsions containing soybean oil-based n-6 polyunsaturated fatty acids (PUFAs) have been traditionally used as a standard intravenous PN supplement. This practice may not be optimal because it may cause an excess of linoleic acid. Meta-analyses have suggested that inclusion of lipids in PN might be detrimental, at least very ill patients; however, most of the studies included in the meta-analysis used soybean oil-based lipid emulsions. 3,4 Alternatives to soybean oil include its partial replacement by fish oil, which is rich in n-3 PUFAs, in particular eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

Acute lung injury (ALI) can lead to morbidity and mortality in the intensive care unit (ICU) and is characterized by neutrophilic lung inflammation, permeability, and intravascular and alveolar fibrin deposition. 5 Clinical and experimental studies have demonstrated that enteral nutrition containing fish oil has diverse immunomodulatory properties and attenuates inflammatory events, including ALI, resulting in improved clinical outcomes of severely ill patients. 6

Lipid emulsions containing fish oil are well established and already commercially available, however, the emulsions are still undergoing trials for use in different patient populations. Additionally, the clinical efficacies of fish oil administered intravenously (such as in PN) and the mechanism by which fish oil elicits protective effects have not been fully elucidated. 7 Herein, we investigated the efficacy of PN emulsions supplemented with fish oil and determined if supplementing PN with fish oil can ameliorate ALI using an established ALI model.

Materials and Methods

Animals. Five weeks old male Sprague-Dawley rats were kept in individual stainless steel cages in a temperature, humidity and light-controlled room (23 ± 3°C, 55 ± 15%, 12-h light-dark cycle) for 3 weeks before the experiments. During this period, all animals were provided standard food (AIN-93G diet; Oriental Kobo Corporation, Tokyo, Japan) and free access to water. All procedures involving rats were conducted in accordance with the guidelines of the Animal Care and Use Committees of the Hyogo College of Medicine and Otsuka Pharmaceutical Factory, Inc. and complied with the National Research Council’s Guide for the Humane Care and Use of Laboratory Animals.

Animal model. Under general anesthesia, catheters for PN were placed into the rats’ external jugular veins. Thereafter, rats were then infused with PN solution (380 ml/kg/day; 270 kcal/kg/day) for 3 days. Basic PN solutions consisted of 11% (w/v) glucose, 2.9% (w/v) amino acids, electrolytes and vitamins; lipid emulsions were supplemented according to the experimental protocol. During PN administration, the animals were fasted and deprived of water. Because the most common cause of ALI in humans is sepsis, intratracheal administration of gram-negative bacterial endotoxin lipopolysaccharide (LPS) was used as an animal model of sepsis-related lung injury in this study. To create a lung injury, 15 mg/kg of lipopolysaccharide (LPS, Escherichia coli, O111: B4, Sigma, St. Louis, MO) was administered intratracheally after 3 days of PN. Preliminary studies revealed that topical instillation of 30 μg LPS into the trachea induced consistent and maximal alveolar cell injury in the instilled lung causing mortality within 24 h. Sham control rats were administered saline instead of LPS. Two hours before the administration of LPS, the infusion of PN was ceased and the lactate ringer’s solution (LR) was infused at 10 ml/kg/h. LR infusion was continued after LPS administration. Rats were sacrificed 3 h or 24 h after LPS administration, and lung tissue and blood samples were harvested. Separate rats were used for analysis of survival. At sampling, the lungs were excised.

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and divided into two sections. The right lobe was snap frozen immediately to liquid nitrogen for further analysis. The left lobe of the lungs was used for histologic examination and immunostaining.

Experimental groups. The rats were assigned to four experimental groups. One group (nonfat) was given PN solution without lipids (lipids calories were compensated by the increase of glucose). The second group (soybean oil) was given PN containing 2.7% (w/v) soybean oil (Intralipos®, Otsuka, Tokyo, Japan). The third group (soy/fish) was given PN containing 1.6% (w/v) soybean oil and 1.1% (w/v) fish oil emulsion (Omegaven®, Fresenius-Kabi, Bad Homburg, Germany) (mixed n-6 PUFAs and n-3 PUFAs; ratio 1.4:1). The control group (control/sham) was given PN supplemented with soy/fish oil but was given sham treatment (saline administration) instead of LPS administration. The PN solutions were prepared to contain the same calories.

Assessment of gas exchange function and blood lactate levels. Partial pressure of oxygen (PO₂) and blood lactate levels were assessed by analysis of blood gases (Rapidpoint 500, Siemens Healthcare Diagnostics Inc., Frimley, Camberley, UK) on a fraction of inspired oxygen of 1.0 in blood drawn from the abdominal aorta 5 min after oxygen inspiration was initiated.

Histopathology. Recruitment maneuvers were performed immediately after thoracotomy to insure that the lungs were free of macroscopic atelectasis. After preparation, the lungs were fixed by inflation with buffered 4% paraformaldehyde for 6 h. After embedding in paraffin, the sections were prepared and stained with hematoxylin and eosin (H&E). Polymorphonuclear neutrophils (PMNs) were stained using a naphthol AS-D chloroacetate esterase staining kit (Sigma Diagnostics, St. Louis, MO) and identified by nuclear morphology stained in bright red. Acute lung injury was blindly scored according to previously described criteria,(13) specifically 1) thickness of the alveolar wall, 2) infiltration or aggregation of neutrophils in air space, alveolar wall, or vessel wall, and 3) alveolar congestion. Each item was graded on a four-point scale (0 to 3), with higher scores indicating more severe damage. ALI scores were assessed in 10 high-power fields per sample.

SYBR green real-time RT-PCR. Rat mRNAs were quantified in duplicate using SYBR Green two-step, real-time RT-PCR, as previously described.(14–16) The following mRNAs were quantitated: rat tumor necrosis factor (TNF)-α, intercellular adhesion molecule (ICAM)-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Optimized and validated primer sets were obtained from realtimeprimers.com (Elkins Park, PA).

Culture of peritoneal exudative neutrophils and measurement of leukotrienes. To measure the leukotriene (LT)-synthesizing capacity of the PMNs, neutrophils from the peritoneal cavity (>90%) were isolated from rats that received each type of PN (without fat emulsion, soybean oil, and soybean and fish oil) for 3 days.(17) PMNs were resuspended in Hanks’ balanced salt solution (HBSS, ~4 × 10⁷ cells/ml) and kept for 10 min at 37°C. The calcium ionophore A23187 was added to a final concentration of 12.5 μM, and the mixture was incubated for 10 min. The sample was then immediately centrifuged to remove the cells, and LTs in the supernatants were quantified by using a previously
reported method. Briefly, 13,14-dihydro-15-keto-PGE₂ (100 ng/sample) was added to the supernatant as an internal standard and citrate buffer was added to adjust the pH to 3.0. The solution was applied to a C18-silica column (GL Sciences Inc., Tokyo), and the LTs were eluted with ethyl acetate:methanol (9:1, vol/vol). The eluent was concentrated under nitrogen, and an aliquot of the residual mixture was used for the quantitation of LTs by high-performance liquid chromatography (HPLC).

Statistical analysis. Results are expressed as mean ± SEM. Parametric data were analyzed with one-way analysis of variance followed by post hoc analysis with the Bonferroni correction. The lung injury score was analyzed with the Mann-Whitney U test with the Bonferroni correction. The cell count was analyzed using Tukey-Kramer methods. JMP version 8 (SAS Institute, Cary, NC) was used for all statistical analyses.

Results

Fish oil supplementation alleviated acute lung injury as determined by blood gas analysis. Exposure to LPS impaired lung function, as evident by a remarkable decrease in PO₂: 24 h after LPS administration in rats given PN (with or without soybean oil) as compared with sham control rats. Supplementation of PN with fish oil significantly increased PO₂ levels 24 after LPS injection (Fig. 1A). There was no significant difference in the partial pressure of carbon dioxide (PCO₂) or blood pH between the groups (data not shown).

Fish oil supplementation reduced blood lactate concentration. Blood lactate levels of the rats given PN without lipids notably increased 24 h after LPS treatment. While supplementation of the PN with soybean fat emulsion did not change blood lactate levels elevated by LPS administration, blood lactate levels did not increase in LPS-treated rats receiving PN supplemented with soy and fish oils (Fig. 1B).

Fish oil supplementation enhanced survival. Supplementation of the PN with fish oil was remarkably effective in prolonging animal survival. Overall survival 24 h after LPS administration was 45.5% (5/11) in rats that received PN without lipids. While soybean oil supplementation did not significantly change survival (63.6% after 24 h, 7/11), supplementation with soy and fish oil significantly prolonged animal survival with 91% (10/11) surviving 24 h after LPS treatment (Fig. 1C).

Fish oil supplementation reduced ALI as determined by histologic analysis. In histologic analysis, the lungs of rats treated with intratracheal LPS showed marked cellular infiltration, edema, and thickening of the alveolar septum. Supplementation of PN with fish oil/ω-3 PUFA reduced both edema and inflammatory cell infiltration (Fig. 2A). The mean ALI score of the rats given PN without lipids was 7.8 ± 1.0, and mean ALI score of the rats given PN with soybean oil was 7.7 ± 0.6. In contrast, the ALI score of the rats given PN supplemented with soybean oil and fish oil was 4.7 ± 0.6, and was significantly lower than in the other experimental groups (p < 0.05 vs without lipids and soybean).
Fish oil inhibited upregulation of proinflammatory mediators in the lungs. Next, we evaluated the effects supplementing PN with fish oil on proinflammatory cytokine expression in the lung. Three hours after LPS inoculation, the mRNAs for TNFα and ICAM-1 were significantly upregulated. PN supplemented with soybean oil did not alter the expression of these genes; however, supplementation with fish oil significantly reduced the peak expression of the transcripts for these inflammatory mediators (Fig. 2B).

Fish oil supplemented PN reduced PMN infiltration after ALI. PMNs are the predominant infiltrating cells in the lung during LPS-induced ALI. While there were scarce PMNs in the sham-treated lungs, intratracheal administration of LPS increased PMN infiltration in the lung within 24 h. Massive neutrophil accumulation in the alveolar space, alveolar capillary congestion, and exudation in the lungs was also seen in the rats given PN with soybean oil emulsion and then treated with LPS. In contrast, PN supplemented with fish oil reduced neutrophil infiltration into the alveolar space after LPS administration (Fig. 3).

Fish oil increased neutrophil LTB₅ production. To further investigate the mechanisms underlying the protective effects of supplementing PN with fish oil, the concentrations of LTB₄ and LTB₅ secreted by the migrated neutrophils were measured using neutrophils isolated from the peritoneal cavity of rats given PN. The neutrophils isolated from rats treated with fish oil-rich PN released significantly more LTB₅, which is a known anti-inflammatory eicosanoid, when exposed to the calcium ionophore A23187 than neutrophils isolated from rats given PN with soybean oil or PN without lipids (Fig. 4). In contrast, the production of LTB₄, a proinflammatory product of n-6 PUFAs, in neutrophils isolated from rats treated with PN supplemented with fish oil was similar to that seen in rats given PN without lipids or PN supplemented with soybean oil.

Discussion

Our study demonstrated that supplementing PN with fish oil significantly reduced LPS-induced ALI in a rat model, as determined by reduction of lung inflammation and improvement of gas exchange, at least in part by increasing the ability of PMNs to secrete anti-inflammatory eicosanoids. Because unbalanced host defense mechanisms result in complications, such as sepsis, the development of immunomodulatory supportive strategies is required despite considerable progress in prophylaxis with modern antibiotics. An “immune-enhancing” enteral diet is a promising option. In fact, clinical and experimental studies have demon-
strated that oral intake of fatty acids had beneficial effects for ICU patients and was associated with attenuation of ALI. However, enteral nutrition is sometimes not feasible, particularly in patients with intestinal failure or impaired gut absorption function. Intravenously injected fatty acids (such as those used to supplement PN) may directly reach their site of action without loss during digestion and may be clinically advantageous for patients in the ICU.

Several recent clinical studies provided evidence of the beneficial effects of supplementation of fish oil-based, long-chain n-3 PUFAs in PN for critically ill patients. Archad et al. reported beneficial effects of an intravenous, omega-3-rich infusion for patients receiving chemotherapy with advanced pancreatic cancer. However, there are also conflicting reports demonstrating that enteral feeding of fish oil does not improve the clinical outcomes of acute lung disease in humans. Some studies even suggest that fish oil may lead to serious complications such as cholestasis, occurring particularly in infants and children with intestinal failure. Thus, further studies are warranted to determine the efficacy of fish oil, the optimal ratio of soybean/fish oil, and molecular mechanisms involved in the beneficial effects conferred by fish oil supplemented into either PN or enteral nutrition.

PUFAs are classified into n-3 and n-6 systems based on the position of the first double bond from the methyl group end and become the base of various bioactive lipid mediators. PUFAs are taken in by the cell membrane, and are metabolized to LTs that exert dual functions. LTs are known potent inflammatory lipid mediators, are involved in hypersensitivity and respiratory disorders, and exert immunoregulatory activities. n-6 PUFAs products, including LTB₄, generally promote inflammation, function as potent chemotactic factors for PMN leukocytes, and induce superoxide generation and degranulation of neutrophils. On the other hand, n-3 PUFAs products, including LTB₅, which compete with n-6 PUFAs products for access to the cyclooxygenase and lipoxygenase enzymes, cause biological responses opposite to those caused by the n-6 PUFAs. The proinflammatory biological activities of LTs are very minor, less than 100-fold in comparison to LTB₄. Fish oil decreases production of n-6 PUFAs-derived mediators, such as PGE₂ and LTB₄, as a result of the reduced n-6 PUFAs content of cell membranes, and increases production of anti-inflammatory eicosanoid mediators such as LTB₅ and LTB₆ from EPA. We previously demonstrated that n-3 PUFA-rich PN regulated neutrophil apoptosis and prevented synthesis of pro-inflammatory eicosanoids, partially explaining the protective effects seen in the clinical setting. Our results are also consistent with previous studies suggesting that utilizing fish oil to partly replace soybean oil in PN may both decrease the amount of linoleic acid and increase the amount of biologically-active n-3 PUFAs. In clinical studies of patients with sepsis who were intolerant of EN and received PN with either standard soybean oil-based emulsion or an emulsion containing fish oil, blood leukocyte counts and serum C-reactive protein concentrations were lower and production of LTB₄ by stimulated neutrophils was much higher in patients receiving fish oil.

Acute inflammation is often noted during ALI and acute respiratory distress syndrome. The pathophysiology of ALI involves inflammation with diffuse alveolar damage, increased capillary permeability, interstitial and alveolar edema, structural damage of the type II alveolar epithelial cells, and influx of circulating inflammatory cells. Reduction of the lamellar structure in the osmiophilic bodies may impact the release of pulmonary surfactant and, thus, affect lung function. The pathogenesis of ALI may result from massive activation of the proinflammatory response, including release of inflammatory mediators, such as eicosanoids. LPS, the main component of the cell wall of gram-negative bacteria, can induce marked acute pulmonary inflammation and lead to further systemic inflammatory response syndrome or sepsis. Low-dose exposure to LPS activates macrophages and PMN cells, presumably to eliminate the toxic agents, whereas higher doses on LPS cause tissue injury. Experimental administration of LPS, often through intratracheal injection, is used to create acute lung inflammation in animal models. Although TNFα and ICAM-1 are not in the final common pathway leading to shock and tissue injury, both ICAM-1 and TNFα are activated in LPS-induced ALI. Our data clearly demonstrated that supplementation of PN with fish oil significantly inhibited the upregulation of mRNAs for ICAM-1 and TNFα in the lung with LPS-induced ALI.

In conclusion, using an LPS-induced rat lung inflammation model, this study demonstrated that fish oil supplemented PN reduces ALI. Fish oil may act as an anti-inflammatory agent, at least in part, by reducing the expression of pro-inflammatory cytokines, such as TNFα and ICAM-1, and altering the balance of LTB₅ and LTB₄.

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**Abbreviations**

- ALI: acute lung injury
- DHA: docosahexaenoic acid
- EPA: eicosapentaenoic acid
- GAPDH: glyceraldehyde 3-phosphate dehydrogenase
- H&E: hematoxylin and eosin
- ICAM: intercellular adhesion molecule
- ICU: intensive care unit
- LPS: lipopolysaccharide
- LT: leukotriene
- PMNs: polymorphonuclear neutrophils
- PN: parental nutrition
- PO₂: partial pressure of oxygen
- PUFAs: polyunsaturated fatty acids
- TNF: tumor necrosis factor

**Conflict of Interest**

No potential conflicts of interest were disclosed.
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