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Effects of Soybean Oil Emulsion and Eicosapentaenoic Acid on Stress Response and Immune Function After a Severely Stressful Operation

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Objective
To investigate the effects of soybean oil emulsion and oral or enteral administration of eicosapentaenoic acid (EPA) on stress response, cytokine production, protein metabolism, and immune function after surgery for esophageal cancer.

Summary Background Data
It has been reported that safflower oil, rich in n-6 polyunsaturated fatty acid (n-6 PUFA), affects the survival rate of septic animals and decreases the immune function. It has also been reported that the administration of fish oil, in contrast, reduces these stress responses and stress-induced immunosuppression. In humans, the effects of soybean oil emulsion and the administration of EPA on stress response and immune function after surgery have not been established.

Methods
Patients who underwent esophagectomy with thoracotomy were divided into three groups. Seven patients were fed by total parenteral nutrition (TPN) with soybean oil emulsion, which accounted for 20% of total calories. Seven patients were given oral or enteral administration of 1.8 g/day EPA, in addition to TPN with soybean oil emulsion. Nine patients served as the control group; these patients received fat-free TPN. Serum interleukin-6 (IL-6), C-reactive protein, concanavalin A (con A)- or phytohemagglutinin (PHA)-stimulated lymphocyte proliferation, natural killer cell activity, and stress hormones were measured.

Results
The postoperative level of serum IL-6 was significantly higher in the group receiving soybean oil emulsion than in the fat-free group. Oral or enteral supplementation of EPA with soybean oil emulsion significantly reduced the level of serum IL-6 compared with the patients receiving soybean oil emulsion. Con A- or PHA-stimulated lymphocyte proliferation decreased significantly on postoperative day 7 in all groups of patients. The supplementation of EPA with soybean oil emulsion significantly improved the lymphocyte proliferation and natural killer cell activity on postoperative day 21 compared with the group receiving soybean oil emulsion.

Conclusions
Soybean oil emulsion amplifies, and the supplementation of EPA reduces, the stress response and stress-induced immunosuppression.

Linoleic acid, one of the n-6 polyunsaturated fatty acids (PUFA), is the precursor of arachidonic acid, which in turn gives rise to the dienoic prostaglandins and leukotrienes.[1–6] It has been shown that levels of prostaglandin (PG) E_2, derived from arachidonic acid, increase in a stressed state and suppress immune function. Therefore, n-6 PUFA may adversely affect inflammatory and immunologic responses in critically ill patients. Animal experiments have indicated that safflower oil, rich in n-6 PUFA, enhances the stress response and stress-induced immunosuppression.[7–12] The fat emulsion currently used in clinical practice is limited to

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Table 1. FATTY ACID COMPOSITION OF SOYBEAN OIL EMULSION

Myristic acid	(C14:0)	NR
Palmitic acid	(C16:0)	12.4%
Palmitoleic acid	(C16:1)	NR
Stearic acid	(C18:0)	4.4%
Oleic acid	(C18:1)	23.9%
Linoleic acid	(C18:2)	51.6%
Linolenic acid	(C18:3)	7.7%
Arachidonic acid	(C20:4)	1.6%
EPA	(C20:5)	<0.1%

NR, not reported; EPA, eicosapentaenoic acid

soybean oil emulsion containing >50% of linoleic acid (Table 1). The perioperative use of soybean oil emulsion may amplify the stress response and stress-induced immunosuppression in critically ill patients. However, n-3 PUFA, the other type of essential fatty acid, decreases the synthesis of PGE_2 and generates the biologically less active metabolite PGE_3 from eicosapentaenoic acid (EPA).[1,4–6] Administration of n-3 PUFA may reduce the adverse effects of n-6 PUFA.

In this study, we investigated the effects of soybean oil emulsion and EPA on stress response and immune function after surgery for esophageal cancer.

PATIENTS AND METHODS

Patients and Clinical Protocol

This prospective, randomized, double-blind protocol was approved by the Ethics Committee of Chiba University School of Medicine. Twenty-three patients were enrolled into the study. Informed written consent was obtained from all patients. They underwent esophagectomy with thoracotomy and three-field lymph node dissection. This was followed by reconstructive surgery using a gastric tube or colon replacement by the retrosternal route. According to our previous study,[13] they were fed exclusively by total parenteral nutrition (TPN), which provided 1.5 g of protein/kg/day and 40 kcal/kg/day from the seventh day before surgery to postoperative day (POD) 14. The rate of parenteral nutrition started at 20 kcal/kg/day and was gradually increased according to patient tolerance to a target rate that would deliver 40 kcal/kg/day.

The patients were divided into three groups. Nine control patients received fat-free TPN (group I). Seven patients received TPN with a soybean oil emulsion (Intralipid), which accounted for 20% of the total calories (group II). Seven patients were given orally or enterally 1.8 g/day of EPA in addition to TPN with soybean oil emulsion (group III). After POD 14, TPN was gradually switched to enteral feeding. This consisted of 17.7% medium-chain triglyceride and 4% linoleic acid. Soybean oil emulsion continued to be administered to groups II and III and EPA to group III until POD 21. The soybean oil emulsion contained 51.6% linoleic acid and 7.7% linolenic acid (see Table 1).

All patients had normal hepatic and renal function and were not diabetic. They did not receive preoperative chemoradiation therapy.

Blood and Urine Sampling

Interleukin-6 (IL-6), C-reactive protein (CRP), and glucagon can be used to gauge the intensity of the injury stress response. Concanavalin A (con A)- and phytohemagglutinin (PHA)-stimulated lymphocyte proliferation and natural killer (NK) cell activity are indices of cell-mediated immunity, and the rapid turnover proteins and nitrogen studies give some index of the amount of acute protein breakdown that was occurring.

The serum concentration of IL-6 was determined before surgery; 1, 2, and 12 hours after surgery; and 3 and 10 days after surgery. Glucagon and CRP were determined before surgery and on POD 1, 3, and 10. Con A- or PHA-stimulated lymphocyte proliferation and NK cell activity were determined before surgery and on POD 7 and 21. Rapid turnover proteins were measured before surgery and on POD 1, 3, 10, and 21. Urine was collected daily, and the daily nitrogen losses were calculated by multiplying the nitrogen concentration measured in urine by the total amount of ultradiafiltrate produced over a 24-hour period and by adding to this the standard estimate for insensible nitrogen losses (15 mg/kg/day). Daily nitrogen balance was estimated as the difference between intake and losses. The daily nitrogen balance was assessed through POD 7.

Laboratory Analyses

The concentration of serum IL-6 was assayed using a commercial human cytokine enzyme-linked immunosorbent assay kit (Amersham, UK). The cytokine assay was standardized by inclusion of a titration of the appropriate purified recombinant cytokine of known concentration. The absorbance of the sample was determined with 450 nm as the primary wave length.

Fasting glucagon was measured in plasma from venous blood that was mixed with Trasyrol immediately after collection in EDTA tubes and centrifuged at 4°C. Glucagon concentrations were assayed using a double-antibody ^{125}I radioimmunoassay kit (Daiich RI, Tokyo, Japan).

Mononuclear cells were separated from venous blood by density gradient centrifugation on Conray/Ficoll. Mononuclear cells removed from the interface were washed two times in phosphate-buffered saline. 1×10^5 cells in a final volume of 200 μl of RPMI 1640 with 10% fetal calf serum were plated in microtiter wells. The cells were incubated with the T-cell mitogens, Con A and PHA, in final concentrations of 10 μg/ml. Assays were performed in duplicate, and unstimulated background control cultures were incubated with every assay. Cells were incubated at 37°C for 64

Table 2. DEMOGRAPHIC, PREOPERATIVE, AND SURGICAL DATA

	Group I	Group II	Group III
Number of patients	9	7	7
Age (years)	64 ± 5	58 ± 4	61 ± 3
Sex (men/women)	8/1	6/1	6/1
Albumin (g/dl)	3.8 ± 0.1	4.0 ± 0.1	3.9 ± 0.2
Prealbumin (mg/dl)	29 ± 1	26 ± 1	29 ± 2
Transferrin (mg/dl)	224 ± 12	260 ± 8	245 ± 23
Con A (SI)	142 ± 24	130 ± 43	182 ± 44
PHA (SI)	175 ± 37	149 ± 44	214 ± 45
NK (%)	29 ± 5	34 ± 7	39 ± 9
Operation time (minutes)	592 ± 42	595 ± 45	642 ± 48
Blood loss (g)	979 ± 213	961 ± 444	810 ± 336

Data are expressed as means ± SEM.
p = not significant among all categories
Con A = con A-stimulated lymphocyte proliferation; PHA = PHA-stimulated lymphocyte proliferation; NK = natural killer cell activity; SI = stimulation index.

hours with an 8-hour pulse labeling with ^{3}H-thymidine, 0.25 μCi/well. Cells were harvested onto glass-fiber filters, and ^{3}H-thymidine content and hence proliferation were determined by liquid scintillation counting. Stimulation indices were calculated by dividing the counts per minute (cpm) of ^{3}H-thymidine in mitogen-stimulated cells by the cpm in cells cultured without mitogens.

Natural killer cell activity was measured by a standard 4-hour ^{51}Cr-release assay, using K 562 as target cells at effector/target cell (E/T) ratios of 20:1. NK cell activity, expressed as percentage cytotoxicity, was calculated by the following formula: % cytotoxicity = ([experimental release − spontaneous release]/[total release − spontaneous release]) × 100.

C-reactive protein and rapid turnover proteins such as transferrin, retinol binding protein, and prealbumin were measured by radial immunodiffusion assay. This technique allows quantitative determination of human plasma proteins after 24 hours of diffusion. Serum containing the protein to be tested is placed in a well on the test plate. The area of the resulting antibody–antigen precipitin zone is directly related to the concentration of the substance placed in the plate well.[14]

Urinary concentrations of nitrogen were measured using a chemiluminescence method. Briefly, nitrogen in biologic samples is oxidized at 1100°C, yielding nitric oxide. On contact with ozone, a metastable nitrogen dioxide is generated that emits photons on decay. The intensity of the emitted light is proportional to the nitrogen content of the sample.[15]

Statistical Analyses

All values are expressed as mean ± standard error of the mean. Statistical analyses were performed using Fisher's protected least significant difference when the overall analysis of variance was significant. $P < 0.05$ was considered significant.

RESULTS

The clinical details of all patients studied are shown in Table 2. There were no statistical differences among these three groups in age, sex ratio, baseline nutritional date, preoperative cell-mediated immunity, blood loss, and operating time.

The profiles of IL-6 production are illustrated in Figure 1. In group II, serum IL-6 levels peaked 2 hours after surgery (569 ± 111 pg/ml). Serum IL-6 levels in group II were statistically higher than in group I (321 ± 21 pg/ml) at 2 hours after surgery ($p < 0.05$). Serum IL-6 levels were significantly lower in group 3 (195 ± 54 pg/ml) than in group II at 2 hours after surgery ($p < 0.01$). A statistically significant difference between the two groups was maintained for the duration of the study ($p < 0.01$ at 10 days after surgery, $p < 0.05$ at 1 and 12 hours and 3 days after surgery). Serum IL-6 levels in group III returned to preoperative levels on POD 10.

The profiles of CRP are illustrated in Figure 2. CRP levels peaked on POD 3 in all groups. On POD 3, the CRP levels in group III (13 ± 2.1 mg/dl) were significantly lower ($p < 0.05$) than that in group II (22.3 ± 2.1 mg/dl).

The profiles of glucagon are illustrated in Figure 3. In all groups, glucagon peaked on POD 1 and declined in the subsequent days. On POD 1, the glucagon levels in group III (188 ± 16 pg/ml) were significantly lower ($p < 0.05$) than those in group II (298 ± 52 pg/ml). A statistically significant difference between the two groups was maintained throughout the study ($p < 0.05$).

Con A-stimulated lymphocyte proliferation (stimulation index) decreased significantly on POD 7 compared with the preoperative value in all groups ($p < 0.05$) (Fig. 4). On POD 21, Con A-stimulated lymphocyte proliferation in

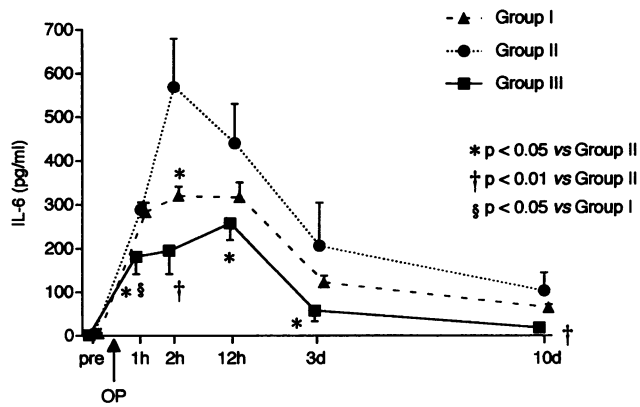

Figure 1. Serum concentration of interleukin-6 (IL-6). Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group 2; † $p < 0.01$ *vs.* group 2; § $p < 0.05$ *vs.* group 1. pre, before surgery; OP, operation; h, postoperative hours; d, postoperative days.

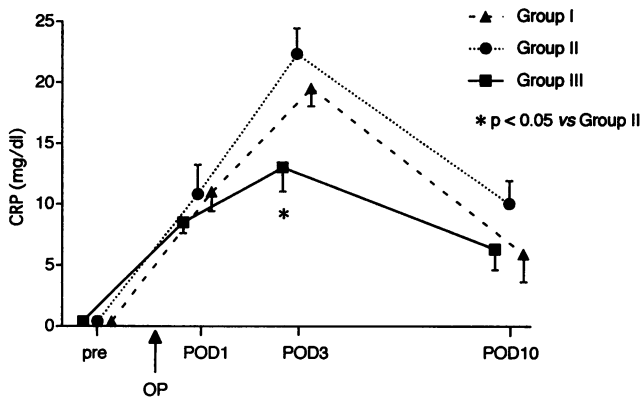

Figure 2. Serum concentration of C-reactive protein. Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group II. pre, before surgery; OP, operation; POD, postoperative days.

group III (200 ± 33) was statistically higher ($p < 0.05$) than on POD 7 (65 ± 26), and returned to preoperative levels (182 ± 44). It was also significantly higher than in the other groups ($p < 0.05$).

The stimulation index of PHA-stimulated lymphocyte proliferation also decreased significantly on POD 7 compared with the preoperative value in groups II and group III ($p < 0.05$). On POD 21, PHA-stimulated lymphocyte proliferation in group III (242 ± 36) was statistically higher ($p < 0.05$) than on POD 7 (81 ± 40), and returned to preoperative levels (214 ± 45). It was also statistically significantly higher than that in group I ($p < 0.05$).

On POD 21, NK cell activity in group III (49 ± 8%) was significantly higher ($p < 0.05$) than in group II (28 ± 7%) (Fig. 5).

Transferrin, retinol binding protein, and prealbumin levels decreased significantly after surgery, but there were no significant differences among the three groups (Fig. 6). Although a gradual reduction in cumulative nitrogen balance was observed after surgery, there were no statistically significant differences among the three groups (Fig. 7).

DISCUSSION

It has been generally accepted that supplementation of adequate amounts of lipid is useful in critically ill patients. Lipids are inexpensive, provide essential fatty acids, and reduce the glucose load. Critically ill patients have been shown to metabolize lipid normally or even at an accelerated rate.[16]

Commercially available fat emulsion is limited to soybean oil emulsion, which contains >50% linoleic acid. Overactivation of the arachidonic acid pathway through the provision of excessive amounts of the precursor linoleic acid by soybean oil emulsion has been suggested to have deleterious effects in critically ill patients.

We have investigated the effect of intravenous n-6 fat emulsion using safflower oil on nitrogen retention, protein kinetics, cytokine production, and cell-mediated immune function in burned rats. We concluded that the administration of n-6 PUFA-enriched fat emulsion increased levels of serum cytokines such as tumor necrosis factor-alpha and IL-6, IL-8, and IL-10 in burned rats. We also demonstrated that nitrogen retention was affected and delayed-type hypersensitivity was suppressed by the administration of safflower oil emulsion in burned rats.[7–10]

Mochizuki et al[11] have reported adverse effects when safflower oil emulsion accounted for approximately 30% to 50% of nonprotein calories. These effects negatively impacted muscle mass, nitrogen balance, and serum protein in burned guinea pigs. Alexander et al[12] have found that enteral administration of safflower oil increased the release of PGE_2 from splenic macrophage and decreased delayed-type hypersensitivity in burned guinea pigs.

According to our clinical experiment, soybean oil emulsion increased the level of serum IL-6. Nitrogen balance tended to be aggravated when compared with the patients given soybean oil emulsion. Cell-mediated immune function was suppressed after surgery for esophageal surgery, which is one of the most severe surgical procedures. The administration of soybean oil emulsion did not result in significant differences in cell-mediated immune function and NK cell activity when compared with the group given fat-free TPN.

The other type of essential fatty acid, n-3 PUFA, has been reported to modulate inflammatory and immune responses in animals and humans. We performed an animal experiment to investigate the effect of fish oil emulsion on protein metabolism, inflammatory response, cytokine production, and immune function. We observed that the administration of fish oil emulsion decreased the serum levels of IL-6, IL-8, IL-10, and tumor necrosis factor-alpha when compared with the burned rats fed safflower oil emulsion. Protein metabolism was also improved by the administration of fish oil emulsion.[7–10]

Alexander et al[12] have reported that the administration of fish oil significantly improved weight loss, promoted skeletal muscle mass, decreased energy expenditure, and in-

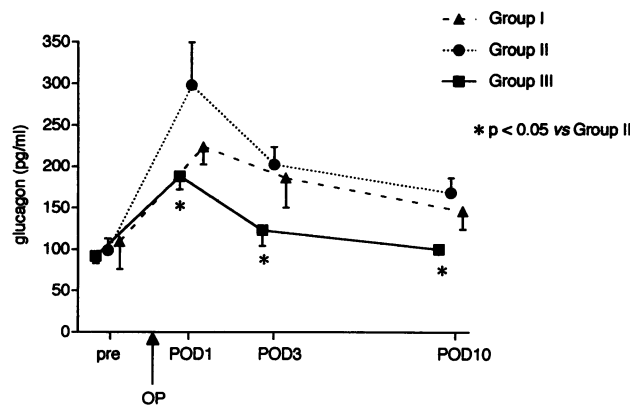

Figure 3. Serum concentration of glucagon. Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group II. pre, before surgery; OP, operation; POD, postoperative days.

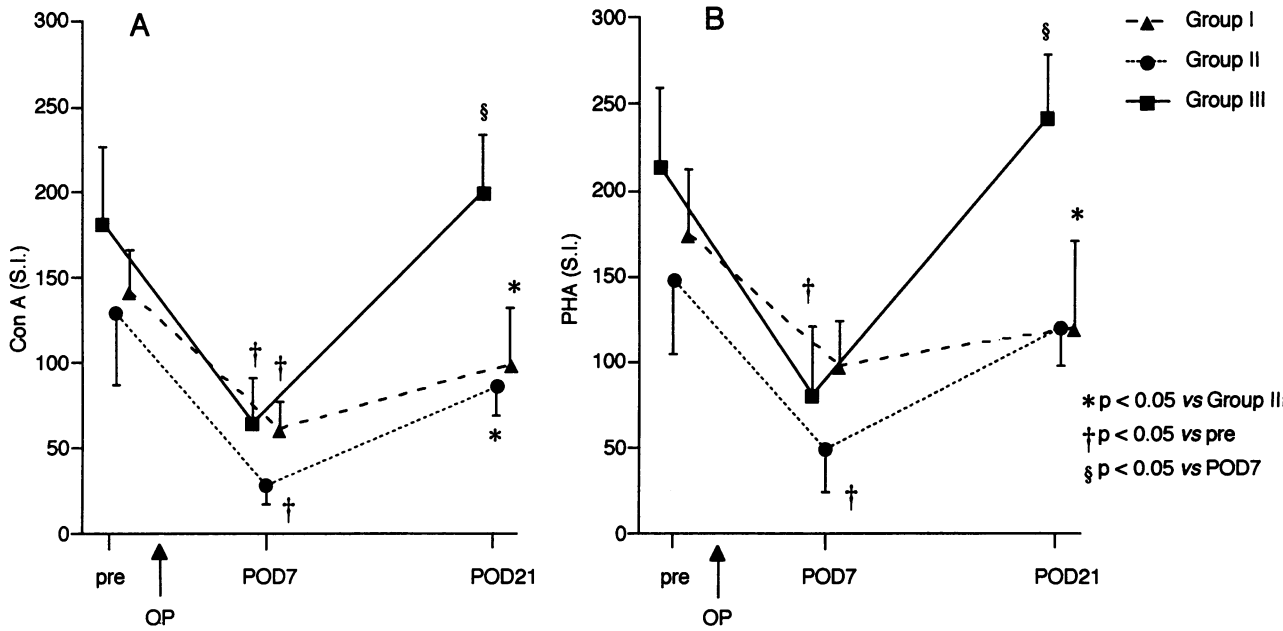

Figure 4. (A) Concanavalin A-stimulated lymphocyte proliferation. (B) Phytohemagglutinin-stimulated lymphocyte proliferation. Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group III; † $p < 0.05$ *vs.* pre; § $p < 0.05$ *vs.* POD 7. pre, before surgery; OP, operation; POD, postoperative days; S.I., stimulation index.

creased delayed-type hypersensitivity. Saito et al[17] have reported that resting metabolic expenditures and immunologic responses were improved when fish oil was substituted for linoleic acid or when indomethacin was given in conjunction with a safflower oil-based lipid diet.

In humans, the effects of n-3 PUFA have been investigated in patients with chronic inflammatory disease, such as rheumatoid arthritis, psoriasis, multiple sclerosis, or ulcerative colitis.[18–22] Meydani et al[23] found that n-3 fatty acid supplementation in both young and older women suppressed cytokine production. Endres et al[24] found that n-3 fatty acid supplementation reduced the synthesis of IL-1 and tumor necrosis factor by mononuclear cells. However, the effects of n-3 PUFA have not been clarified in critically ill patients with multiple trauma, extensive burn injuries, or sepsis.

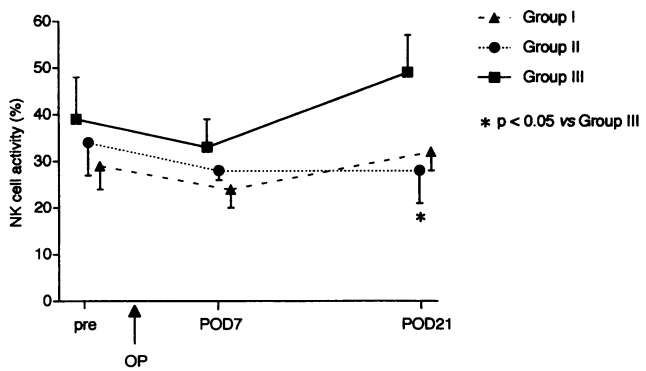

Figure 5. Natural killer cell activity. Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group III. pre, before surgery; OP, operation; POD, postoperative days.

In this study, oral or enteral administration of EPA significantly reduced the postoperative serum IL-6 level compared with the group given fat-free TPN with soybean oil emulsion. Postoperative stress-induced immunosuppression was significantly reduced by the addition of EPA, which accounted for only 1% of total caloric intake, even though the same amount of soybean oil emulsion was infused in this study group.

The immunologic effects of n-3 PUFA are not uniform. Yoshino and Ellis[25] have found that fish oil-derived fatty acids modulated chronic inflammation and cell-mediated immunologic reactions by reducing the synthesis of arachidonic acid metabolites in rats. Calder[26] found that supplementation of the diet of healthy humans with fish oil capsules suppressed lymphocyte proliferation and IL-2 production. Endres et al[27] found that dietary n-3 fatty acids decreased the production of IL-2 and reduced mononuclear cell proliferation in healthy humans. All of these studies were performed in nonstressed patients. The improvements in immune function in a stressed patients obtained from both our experimental and clinical studies demonstrate the possibility that the effect of n-3 PUFA on immune function in a stressed state may be different from that in a nonstressed state, as reported before.[7–10] It is widely known that cell-mediated immune functions are affected by severe stress. The data from our studies indicate that n-3 PUFA reduced rather than increased stress-induced immunosuppression.

Several reports exist regarding the mechanism underlying the effects of n-3 PUFA. Alterations in the type of arachidonic acid metabolites produced during stimulation of the

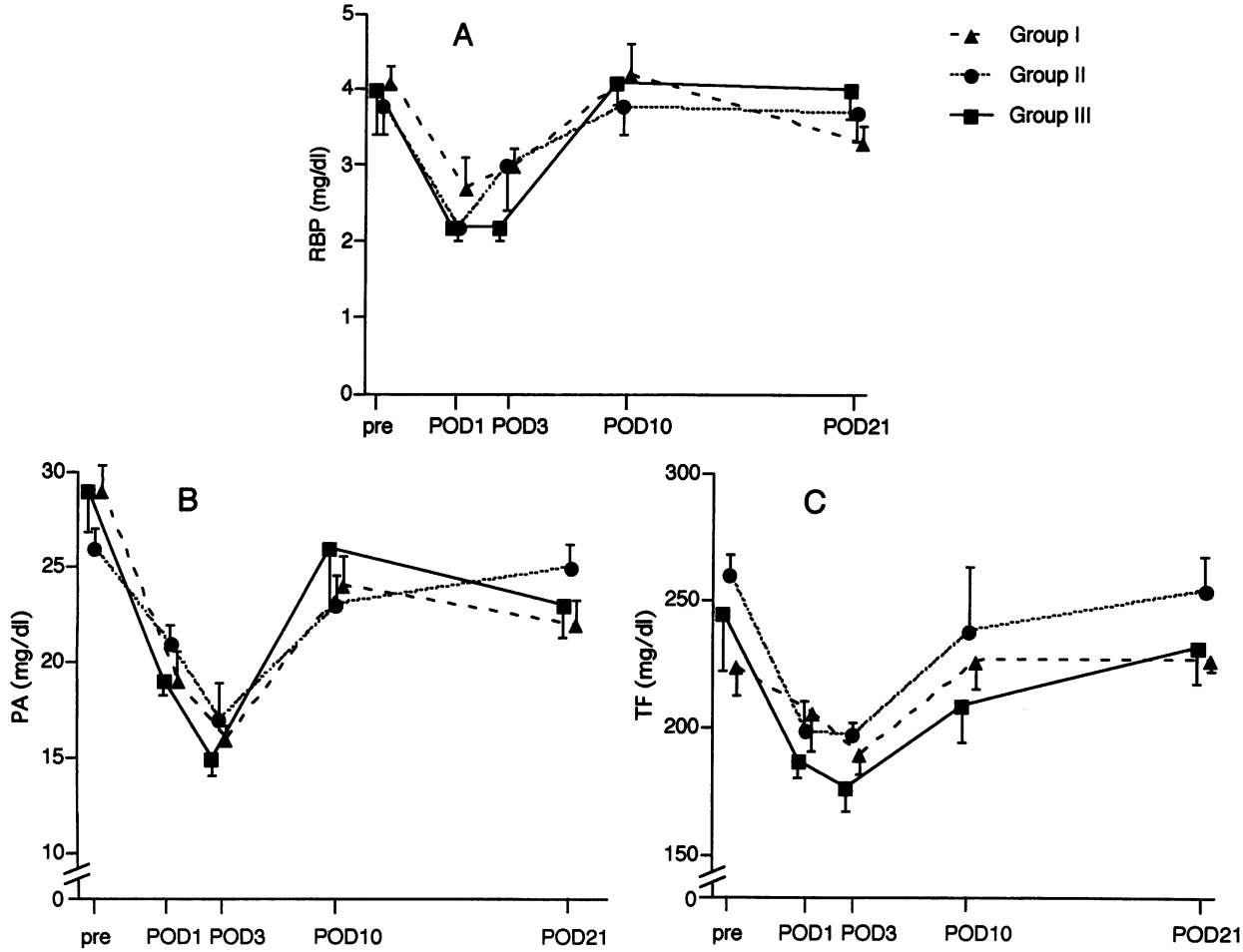

Figure 6. (A) Serum concentration of retinol binding protein. (B) Serum concentration of prealbumin. (C) Serum concentration of transferrin. Data are expressed as means ± SEM. pre, before surgery; POD, postoperative days.

mononuclear cells may explain, in part, the changed production of IL-6 in the cytokine network. A possible mechanism for the changed IL-6 production due to n-6 and n-3 PUFA is altered synthesis of leukotriene B4 and generation of the biologically less active metabolite leukotriene B5 from EPA.[1-6,24] It is well known that levels of PGE_2, derived from arachidonic acid, increase in a stressed state and suppress immune function. Thus, a possible mechanism for reduced immunosuppression by the administration of n-3 PUFA in a stressed state is the decreased synthesis of PGE_2 and the generation of the biologically less active metabolite PGE_3 from EPA.[1,4-6] Another possible mechanism for the effects of n-6 and n-3 PUFA is the result of changes in membrane fluidity,[28] as well as the changes in the release of membrane-associated intracellular messengers, such as phosphate inositol and diacylglycerol.[29,30] Chang et al[31] have suggested that fish oil enhances tumor necrosis factor-alpha mRNA expression of macrophage at the transcriptional level. Details of the exact mechanism remain to be worked out, however.

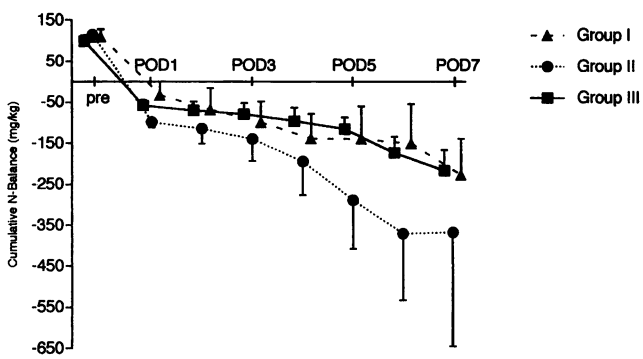

Figure 7. Cumulative nitrogen balance. Data are expressed as means ± SEM. pre, before surgery; POD, postoperative days.

Recent studies, as well as the experimental data from our laboratory and from others, suggest that n-3 PUFA has beneficial effects in critically ill patients in both stress response and immune function. However, n-6 PUFA is still an essential component of cell membrane phospholipids and is critical to the maintenance of cellular functions, particularly in critically ill patients. Excessive levels of n-6 PUFA

without n-3 PUFA have been shown to increase the production of cytokines and promote immunosuppression. It was also found that the addition of EPA to soybean oil emulsion reduced the stress response, improved protein metabolism, and prevented stress-induced immunosuppression.

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