

Nutritional Effect of Oral Supplement Enriched in ω -3 Fatty Acids, Arginine, RNA on Immune Response and Leukocyte–platelet Aggregate Formation in Patients Undergoing Cardiac Surgery

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ABSTRACT: The aim of the present study was to investigate the influence of a supplement enriched in ω -3 fatty acids on immune responses and platelet–leukocyte complex formation in patients undergoing cardiac surgery. Patients in the supplement group ($n = 7$) took a supplement enriched in ω -3 fatty acids (Impact®) in addition to a hospital diet for five successive days before surgery; those in the control group ($n = 7$) took only hospital diet and did not take Impact®. Blood samples in both groups were collected at same time points. Before surgery, samples were collected five days before surgery, at the start of supplementation (baseline), and the end of supplementation (postoperative day (POD)-0). After surgery, samples were collected on POD-1 and POD-7. The expression of human leukocyte antigen (HLA)-DR, the ratio of CD4-/CD8-positive cells, the production of interferon (IFN)- γ by CD4-positive cells, plasma levels of cytokines, and leukocyte–platelet aggregates were measured. Before surgery (POD-0), the supplement caused significant increases in HLA-DR expression, CD4/CD8 ratio, and plasma levels of IFN- γ ; these levels were significantly higher compared to those in the control group ($P < 0.05$, respectively). After surgery (POD-1), all values dramatically decreased in comparison with those of POD-0; however, the values in the supplement group were significantly higher compared to their respective markers in the control group ($P < 0.05$, respectively). Significant differences of HLA-DR expression and CD4/CD8 ratio persisted through POD-7. Before surgery (POD-0), plasma levels of interleukin (IL)-10 in the supplement group decreased significantly compared with those in the control group ($P < 0.05$). After surgery (POD-1), plasma levels of IL-10 in both the control and supplement groups increased; these levels in the supplement group were significantly lower than those in the control group ($P < 0.05$). Significant decreases in the percentage of leukocyte–platelet aggregates were found after supplementation; the difference between the supplement and the control groups was found on POD-0 and POD-1 ($P < 0.05$, respectively). In conclusion, the dietary supplement increased HLA-DR expression, the CD4/CD8 ratio, and the production of IFN- γ by CD4-positive cells; conversely, the levels of IL-10 and the formation of leukocyte–platelet aggregates before and after surgery were suppressed. These beneficial effects may decrease the incidence of complications after surgery.

KEYWORDS: dietary supplement, HLA-DR; CD4/CD8 ratio, platelet–leukocyte aggregates, interferon- γ

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Introduction

Major surgery,^{1,2} including cardiac surgery,^{3,4} is associated with a depression of cellular immunity. Tissue injury caused by major surgery is related to decreased expression of human leukocyte antigen (HLA)-DR on monocytes,^{5,6} which is

correlated with increased postoperative complications and mortality.⁷ Diminished monocytic HLA-DR expression may identify patients with temporary immunodepression, and patients who are therefore at risk for infectious complications.^{8,9} For example, Allen et al reported that decreased



expression of HLA-DR on monocytes was correlated with clinical outcome in pediatric patients undergoing cardiac surgery.¹⁰

The injury to host defense after surgery may be related to a shift in the cell ratio of peripheral blood mononuclear cells, in which there is an increase in prostaglandin E₂-synthesizing monocytes and a simultaneous decrease in functionally competent CD3- and CD4-positive lymphocytes.¹¹ CD4-positive T cells can be divided into T-helper type 1 (Th1) and type 2 (Th2) cells according to distinct profiles of cytokine production. Th1 cells produce interferon (IFN)- γ , interleukin (IL)-2, and IL-12, favoring cell-mediated immunity, whereas Th2 cells produce IL-4, IL-6, IL-10, and IL-13, assisting humoral immune system.¹² CD8-positive cells are cytotoxic T lymphocytes (CTLs), and they secrete molecules that destroy the cell to which they have bound. The role of the CD8-positive T cells is to monitor all the cells of the body, ready to destroy any that express foreign antigen fragments in their class I molecules.^{13,14} Markewitz et al found that IL-2 and IFN- γ secretion are diminished after cardiac surgery;¹⁵ conversely, Muret et al demonstrated that IL-4 and IL-10 production are elevated.¹⁶ Thus, the Th1 and Th2 responses are decreased and increased, respectively, after cardiac surgery.¹⁷ Fatty acids are classified into two main families of polyunsaturated fatty acids; ω -3 fatty acids, which include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and the other is ω -6 fatty acids, which include linoleic acid (LA), arachidonic acid (AA), and γ -linolenic acid. Several studies have reported beneficial effects of preoperative supplementation with specialized diets enriched with ω -3 fatty acids, arginine, and RNA.^{18–20} Furthermore, we have shown that preoperative administration of nutritional supplements improves preoperative nutritional status, and preoperative and postoperative inflammatory and immune responses in cancer patients.²¹

In the present study, we investigated the effects of a dietary supplement enriched in ω -3 fatty acids on the expression of HLA-DR on monocytes, the ratio of CD4-/CD8-positive cells, the production of IFN- γ by CD4-positive cells, plasma levels of cytokines, and the formation of leukocyte-platelet aggregates in patients undergoing cardiac surgery.

Materials and Methods

Reagents. Lysing solution was obtained from BD Biosciences (CA, USA). Ficoll-Hypaque (Histopaque[®] 1077) gradient, 2-mercaptoethanol (ME), concanavalin A, and glutamine were obtained from Sigma-Aldrich Co. (Tokyo, Japan). Acid-citrate-dextrose solution (ACD) was obtained from TERUMO Co. (Tokyo, Japan). IMag buffer (PBS including 5% bovine serum albumin, 20 mM EDTA, and 0.09% sodium azide) was obtained from Becton Dickinson Japan (Tokyo, Japan). RPMI-1640 and heat-inactivated fetal calf serum (FCS) were obtained from Life Technologies, Invitrogen (CA, USA). Penicillin and streptomycin were obtained from Meiji Seika Pharma Co. (Tokyo, Japan).

Antibodies. Fluorescein isothiocyanate (FITC)-labeled antibodies against the monocyte marker CD14 (anti-CD14-FITC), and against the platelet GPIb marker CD42b (anti-CD42b-FITC), phycoerythrin (PE)-labeled antibodies against the monocyte marker HLA-DR (anti-HLA-DR-PE) and against the platelet P-selectin marker CD62 (anti-CD62-PE), and peridinin chlorophyll protein (PerCP)-conjugated antibody against the leukocyte marker CD45 (anti-CD45-PerCP) were purchased from Becton Dickinson (CA, USA). A cocktail of CD3-, CD4-, and CD8-conjugated antibodies (BD[™] Phosflow Human T Cell (CD4/CD8) Antibody Cocktail: anti-Human CD3-PerCP, anti-Human CD4-FITC, anti-Human CD8-PE), FITC-labeled mouse IgG1 or IgG2b antibody, and PE-labeled mouse IgG2a or IgG2b were purchased from BD Biosciences (CA, USA).

Subjects and study design. The study was carried out between May 2005 and September 2005 in patients who underwent cardiac surgery. Patients (seven males and seven females; age, 67 \pm 8 years) were randomized in equal numbers into two groups using computer-generated block randomization (<http://www.randomization.cm>). Exclusion criteria included: pregnancy or lactation; renal or liver insufficiency; use of investigational drugs, steroids, or immunosuppressive medication; malignancy; genetic disorder; human immunodeficiency virus (HIV) infection; splenectomy before hospitalization; inflammatory bowel disease; and insulin-dependent diabetes. Patients were randomly assigned to receive a preoperative oral nutritional supplement (Impact[®], Japan) or not. The composition of omega-3 fatty acids, arginine, and RNA in this supplement is similar to Impact sold in other countries. This supplement contains n-3 fatty acids (14.9%) such as EPA, DHA; n-6 fatty acids (11.9%) such as linolenic acid in fatty acid composition (document of Ajimonoto Co., Ltd.); carbohydrates; minerals; and vitamins. The supplement group ($n = 7$) received Impact[®] for five successive days before surgery other than a conventional hospital diet. The control group ($n = 7$) received only conventional hospital diet and did not take in Impact[®]. Before and after surgery, total caloric intake of both groups was approximately 1100 kcal/day by postoperative day (POD)-1. Subjects were requested to fill in a food diary according to instructions from the principal investigator, where they recorded all food consumption during study. Compliance with consumption of the study product during the intervention period was ensured and checked by doctors or nurses. No postoperative hemorrhage was found in any patient. All subjects gave written informed consent and the study was approved by the Committees on Biomedical Research Ethics for Kagoshima University Hospital.

Blood sampling. Peripheral blood was drawn into vials containing 3.8% sodium citrate. Blood samples in both groups were collected at same time points. Before surgery, samples were collected five days before surgery at the start of supplementation and the end of supplementation (POD-0). After surgery, samples were collected on POD-1 and POD-7.

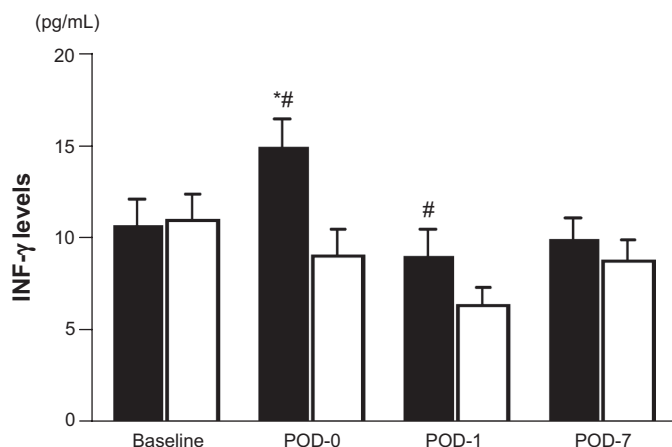


Figure 1. Perioperative changes in plasma levels of IL-6 and IL-10 INF- γ secreted by CD4-positive cells. The supplement group (filled bars, $n = 7$) received Impact[®] for five days preceding surgery. The control group (open bars, $n = 7$) did not receive Impact[®] before surgery. Baseline: the start of supplementation five days before surgery. POD-0: the day after the end of supplementation. POD-1: one day after surgery. Data represent mean \pm SD. ^{*} $P < 0.05$; comparing the levels of INF- γ on POD-0 with those of baseline in the supplement group, [#] $P < 0.05$; comparing the levels of INF- γ in the supplement group with those in the control group.

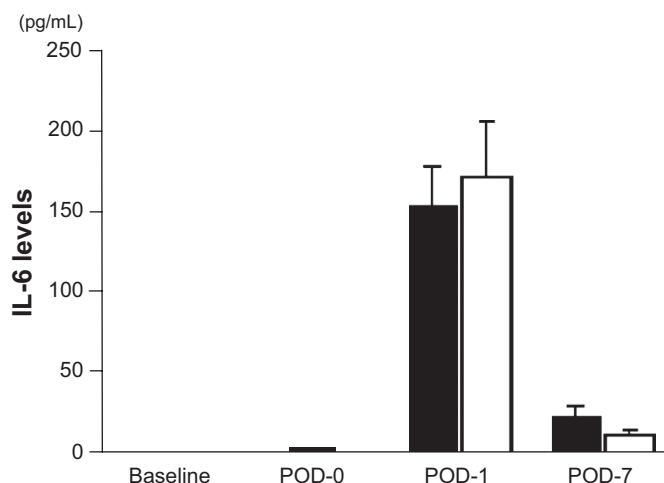


Figure 2. Perioperative changes in plasma levels of IL-6. The supplement group (filled bars, $n = 7$) received Impact[®] for five days preceding surgery. The control group (open bars, $n = 7$) did not receive Impact[®] before surgery. Baseline: the start of supplementation five days before surgery. POD-0: the day after the end of supplementation. POD-1: one day after surgery. Data represent mean \pm SD.

Data represent mean \pm SD.

Blood samples were centrifuged at $1710 \times g$ for 10 minutes; plasma for measuring cytokines was removed, and the samples were stored at -80°C until analysis.

Flow cytometry analysis. Three or four tubes each filled with 100 μL of fresh, anticoagulated blood were incubated with monoclonal antibodies for 15 minutes in the dark at 20°C . Red blood cells were lysed by 1 mL lysing solution. Remaining white blood cells were mixed, incubated in the dark for another 10 minutes at 20°C , and centrifuged at $1400 \times g$ for 15 minutes. Cells were then washed using 1.5 mL PBS, centrifuged at $1400 \times g$ for 10 minutes, re-suspended for fixation in 0.3 mL of 1% paraformaldehyde in PBS, and stored at 4°C in the dark.²² The samples were analyzed within 24 hours of fixation. A minimum of 1×10^4 events for each sample were acquired with a flow cytometer (FACSCalibur, Becton Dickinson, San Jose, CA, USA) using CellQuest software (Becton Dickinson). The appropriate isotype controls were used.

Detection of HLA-DR expression. Anticoagulated whole blood samples (100 μL) were incubated with anti-CD14-FITC (4 μL) and anti-HLA-DR-PE (20 μL) monoclonal antibodies at room temperature for 15 minutes. In the dual staining method, parameter settings of forward light scatter and right-angled side scatter were selected to separate the monocyte populations.²³ Percentage of monocytes co-expressing CD14 and HLA-DR was considered to represent HLA-DR monocytes. The appropriate isotype controls were used. Monocyte HLA-DR measurement was expressed as percentages of HLA-DR-positive monocytes and means of fluorescence intensities (MFIs) related to the HLA-DR

density per cell. Results were expressed as percentages of HLA-DR-positive monocytes and as arbitrary units (MFI). The percentage of HLA-DR-positive monocytes was calculated by the co-expression of CD14 and HLA-DR antigens in the total CD14-positive cells. The arbitrary units were calculated as follows: the MFI of the isotype control was subtracted from the MFI of the sample and the difference was divided by the MFI of the isotype control.

T cell immunophenotyping. The percentage and absolute numbers of the peripheral blood lymphocyte subpopulations were determined by a dual-color direct immunofluorescence technique applied in anticoagulated blood using anti-CD3-FITC, anti-CD4-FITC, and anti-CD8-PE monoclonal antibodies. As a control, Simultest was used. Fluorescence channels were set at logarithmic gain. Lymphocytes were gated according to their forward versus side light-scatter properties. The T cell subpopulation was identified by gating for CD3-positive events. Flow cytometry analysis was carried out by the above mentioned method.

Isolation CD-positive cells from lymphocytes. Purification of platelet-depleted peripheral mononuclear cells (PBMCs) from the platelet donor was based on the method of Pawlowski et al.²⁴ PBMCs were isolated from blood by the standard method of density gradient centrifugation using a Ficoll-Hypaque gradient.²⁵ PBMCs were centrifuged at $120 \times g$ for 15 minutes. The pellets were re-suspended in IMag buffer and centrifuged at $120 \times g$ for 15 minutes. The number of lymphocytes was adjusted to 1×10^6 cells/mL with IMag buffer. CD4-positive cell fraction was obtained from the pellets using BDTM IMag Cell Separation System (Becton Dickinson Japan; Tokyo, Japan) in accordance with



the manufacturer's instructions. Flow cytometry analysis was carried out by the above mentioned method.

Determination of cytokine production by CD-positive cells. CD4-positive cells were re-suspended in culture medium (RPMI-1640 containing inactivated FCS, 2-ME, 3% glutamine solution, penicillin, and streptomycin), and centrifuged at $120 \times g$ for 15 minutes. Pellets were adjusted to 1×10^6 cells/mL with culture medium. CD4-positive cells were stimulated with concanavalin A, and incubated at 37°C for 24 hours. After centrifugation, the supernatant was removed; cytokine levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit. IFN- γ (Human IFN- γ ELISA Kit II, BD Biosciences; San Diego, CA, USA), and IL-4 (IL-4 ELISA kits, R&D Systems, Inc., Minneapolis, MN, USA) were used. Flow cytometry analysis was carried out by the above mentioned method.

Measurement of plasma levels of cytokines. Plasma levels of IL-6 and IL-10 were measured using commercially available ELISA kits (IL-6 and IL-10; R&D Systems, Inc., Minneapolis, MN, USA).

Leukocyte-platelet aggregates. Two-color analysis enabled discrimination of platelet-coupled and platelet-free leukocytes, and calculation of the percentage of platelet-coupled leukocytes in the leukocyte population. Whole blood was incubated with saturating concentrations of anti-CD42b-FITC and anti-CD45-PerCP; background fluorescence was determined using saturating concentrations of anti-CD45-PerCP alone. Fixed samples were analyzed using the FACScan flow cytometer. The double fluorescent particles positive for both the leukocyte CD45 and platelet CD42b were counted as leukocyte-platelet aggregates.

Statistical analysis. All results are expressed as mean \pm standard deviation (SD). Patient's characteristics at baseline were analyzed using Fisher's exact test and two independent samples t test as appropriate. The paired t test was used to compare preoperative values to one-day and seven-day postoperative results. To detect the effect of this nutrient on the supplement group, independent t test was used to determine whether the

mean changes in pre- and postoperative parameters were significant between the supplement and control group. All data did blind and analyzed it. Statistically significant differences were defined as $P < 0.05$. All analyses were performed using SPSS software, version 17.0 (SPSS, Japan, Tokyo, Japan).

Results

Clinical characteristics of patients. Patients' demographic characteristics, including age, gender, operation duration, and surgical procedures are summarized in Table 1. There were no significant differences between groups at entry. Furthermore, no significant differences in the postoperative hospitalization between groups were found. No episodes of bleeding, failure of sutures, or death were found during the one-day observation period after surgery.

Perioperative changes in HLA-DR expression on monocytes. The percentage of peripheral blood monocytes expressing the HLA-DR antigen in healthy donors was $\geq 82\%$. In the same way, the mean level of HLA-DR per monocyte, expressed as MFI, was 50.4 ± 12 in the healthy control. As shown in Table 2, after supplementation (POD-0), the percentage of HLA-DR expression on monocytes in the supplement group increased significantly compared with that of baseline (before supplementation) ($P < 0.05$); the percentage was significantly higher than that in the control group ($P < 0.05$). After surgery (POD-1), percentages of HLA-DR expression in both the supplement and control groups decreased clearly; however, the percentage in the supplement group was significantly higher than that in the control group ($P < 0.05$). Significant difference of this marker persisted through POD-7.

Perioperative changes in the percentage of CD4-positive cells, CD8-positive cells, and ratio of CD4-/CD8-positive cells. As shown in Table 3, after supplementation (POD-0), the percentage of CD4-positive cells and the ratio of CD4-/CD8-positive cells in the supplement group were significantly higher than those at baseline ($P < 0.05$); the percentage and the ratio were significantly higher than those

Table 1. Patients' demographic characteristics.

CHARACTERISTICS	CONTROL GROUP (n = 7)	SUPPLEMENT GROUP (n = 7)	P VALUE
Gender (Male/Female)	3/4	4/3	NS
Mean age (S.D., years)	69 ± 11	65 ± 12	NS
Duration of surgery (S.D., min)	342 ± 10	425 ± 129	NS
Diseases			
Valve diseases	3	2	
Aneurysm	3	4	
Angina pectoris	1	1	
Postoperative hospitalization (S.D., days)	10.5 ± 5.0	9.2 ± 5.2	NS

Data represent mean \pm SD.

Abbreviation: NS, no significant difference between control and nutrient groups.



Table 2. Expression of HLA-DR on CD14+ monocytes in patients undergoing cardiac surgery. The supplement group ($n = 7$) received Impact® for five days preceding surgery. The control group ($n = 7$) did not receive Impact® before surgery. Baseline: the start of supplementation five days before surgery. Postoperative day (POD)-0: the day after the end of supplementation. POD-1: one day after surgery. MFI: mean fluorescent intensity.

DAY	HLA-DR EXPRESSION IN:			
	CONTROL GROUP		SUPPLEMENT GROUP	
	%	MFI	%	MFI
Baseline	68.4 ± 25.9	33.4 ± 12.5	69.1 ± 26.1	33.6 ± 12.7
POD-0	68.7 ± 30.7	34.0 ± 15.3	79.4 ± 27.4*#	46.1 ± 17.3*#
POD-1	41.2 ± 18.6	19.5 ± 8.1	58.1 ± 20.3#	28.6 ± 10.6#
POD-7	57.2 ± 20.4	26.3 ± 9.5	65.1 ± 22.3#	31.4 ± 12.1#

The percentage of HLA-DR-positive monocytes was calculated by the co-expression of CD14 and HLA-DR antigens in the total CD14+ population. * $P < 0.05$; comparing the percentage of HLA-DR expression on monocytes on POD-0 with those of baseline in the supplement group, # $P < 0.05$; comparing the percentage of HLA-DR expression on monocytes in the supplement group with that in the control group.

in the control group ($P < 0.05$, respectively). In contrast, the percentage of CD8-positive cells in the supplement group was significantly lower than that in the control group ($P < 0.05$). After surgery (POD-1), the percentages of CD4- and CD8-positive cells as well as the ratio of CD4-/CD8-positive cells decreased; however, the percentage of CD4-positive cells as well as the ratio of CD4-/CD8-positive cells in the supplement group were significantly higher than those in the control group ($P < 0.05$). Significant difference of these markers persisted through POD-7.

Perioperative changes in cytokine production in CD4-positive cells. As shown in Figure 3, after supplementation, levels of IFN- γ secreted by CD4-positive cells increased significantly compared with those at baseline ($P < 0.05$); these levels in the supplement group were significantly higher compared to those in the control group ($P < 0.05$). On POD-1, levels of IFN- γ in both the supplement and control groups obviously decreased; however, these levels in the supplement group were significantly higher compared to those in the control group ($P < 0.05$). On the other hand, levels of IL-4 in CD4-positive cells were 5 pg/mL or less in all periods, and the meaning change was not found.

Perioperative changes in plasma levels of IL-6 and IL-10. Plasma levels of IL-6 on POD-1 were dramatically

elevated in both the supplement and control groups, though no significant difference between the supplement and control groups was found (Fig. 3). As shown in Figure 3, before surgery (POD-0), plasma levels of IL-10 in the supplement group decreased significantly compared with those in the control group ($P < 0.05$). After surgery (POD-1), plasma levels of IL-10 in both the control and supplement groups increased; however, these levels in the supplement group were significantly lower than those in the control group ($P < 0.05$).

Perioperative changes in leukocyte-platelet aggregates. As shown in Figure 4, after supplementation (POD-0), the percentage of cells co-expressing CD45 and CD42b reflective of leukocyte-platelet aggregates decreased significantly compared with that at baseline ($P < 0.05$); the percentage in the supplement group were significantly lower than that in the control group ($P < 0.05$). On POD-1, the percentage of cells co-expressing CD45 and CD42b in both the supplement and control groups increased clearly; the percentage in the supplement group were significantly lower compared to that in the control group ($P < 0.05$).

Discussion

We paid our attention to Impact®, which contains, in comparison with ω -6 fatty acids, more ω -3 fatty acids and investigated

Table 3. Perioperative changes in CD4/CD8 ratio. The supplement group ($n = 7$) received Impact (Japan) for five days preceding surgery. The control group ($n = 7$) did not receive Impact® before surgery. Baseline: the start of supplementation five days before surgery. POD-0: the day after the end of supplementation. POD-1: one day after surgery.

DAY	CONTROL GROUP			SUPPLEMENT GROUP		
	CD4	CD8	CD4/CD8	CD4	CD8	CD4/CD8
Baseline	68 ± 25	42 ± 19	1.62 ± 0.42	66 ± 27	44 ± 18	1.63 ± 0.41
POD-0	67 ± 25	42 ± 18	1.59 ± 0.41	77 ± 32*#	32 ± 16#	2.26 ± 0.48*#
POD-1	44 ± 15	28 ± 12	1.57 ± 0.36	53 ± 17#	26 ± 11	2.04 ± 0.38#
POD-7	56 ± 19	35 ± 14	1.60 ± 0.37	61 ± 24#	34 ± 15	1.79 ± 0.42#

Data represent mean ± SD. * $P < 0.05$; comparing CD4-positive cells as well as the ratio of CD4/CD8 on POD-0 with each that of baseline in the supplement group, # $P < 0.05$; comparing CD4-positive cells as well as the ratio of CD4/CD8 in the supplement group with that in the control group.

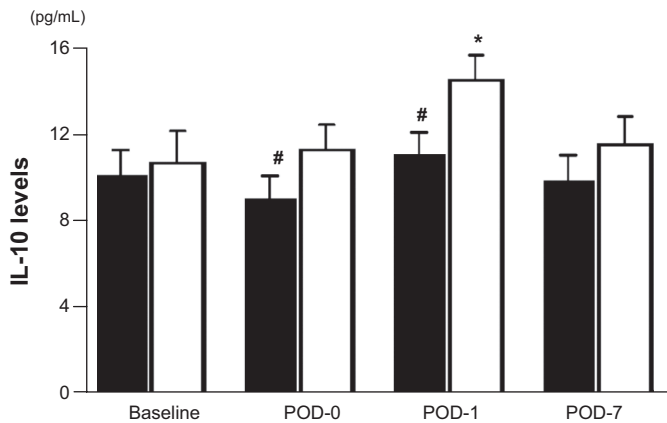


Figure 3. Perioperative changes in plasma levels of IL-10. The supplement group (filled bars, $n = 7$) received Impact[®] for five days preceding surgery. The control group (open bars, $n = 7$) did not receive Impact[®] before surgery. Baseline: the start of supplementation five days before surgery. POD-0: the day after the end of supplementation. POD-1: one day after surgery.

Data represent mean \pm SD. * $P < 0.05$; comparing plasma levels of IL-10 on POD-0 with those of baseline in the supplement group, # $P < 0.05$; comparing plasma levels of IL-10 in the supplement group with those in the control group.

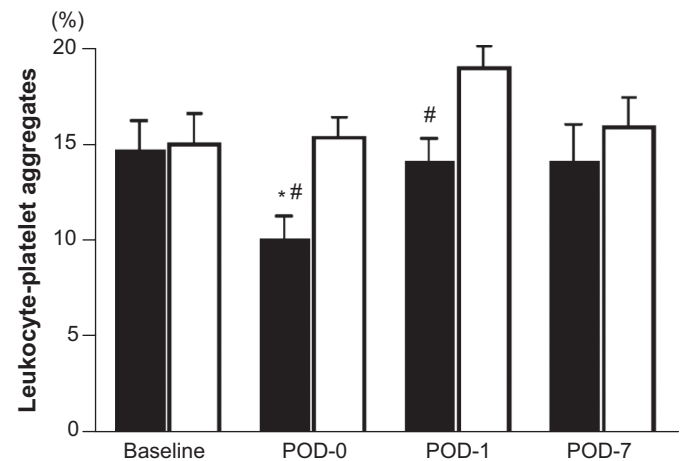


Figure 4. Perioperative changes in leukocyte–platelet aggregates.

The supplement group (filled bars, $n = 7$) received Impact[®] for five days preceding surgery. The control group (open bars, $n = 7$) did not receive Impact[®] before surgery. Baseline: the start of supplementation five days before surgery. POD-0: the day after the end of supplementation. POD-1: one day after surgery.

Data represent mean \pm SD. * $P < 0.05$; comparing leukocyte–platelet aggregates on POD-0 with those of baseline in the supplement group, # $P < 0.05$; comparing leukocyte–platelet aggregates in the supplement group with those in the control group.

the effects of this supplement on immune responses and leukocyte–platelet aggregate formation in patients undergoing cardiac surgery. Previously, we have reported that preoperative supplementation with Impact[®] increased circulating levels of total ω -3 fatty acids including EPA and DHA and decreased the ratio of ω -6/ ω -3 fatty acids in cancer patients. In the present study, Impact[®] increased HLA-DR expression on monocytes, CD4/CD8 ratio, and IFN- γ production by CD4-positive cells. After surgery (POD-1), all values dramatically decreased in comparison with those from POD-0; the values in the supplement group were higher significantly compared to their respective values in the control group. Significant differences of these markers persisted through POD-7. Several reports have shown that surgical intervention induces a reduction of monocyte HLA-DR expression.^{3,4,26} Our results suggest that preoperative administration of Impact[®] is beneficial in protecting from complications caused by infection in patients undergoing cardiac surgery.

Major surgery has been shown to induce a shift in the Th1/Th2 balance toward a Th2-mediated immune response in the early postoperative stage.^{27,28} Such a shift reflects a down-regulation of cell-mediated and an up-regulation of antibody-mediated immunity commensurate with surgical trauma.²⁸ Ishikawa et al²⁷ noted that major postoperative complications were always preceded by a significant decline in the Th1/Th2 balance after major surgeries, and that the increased percentage of Th2 cells may predict the occurrence of postoperative complications. In the present study, we found that the supplement caused an increase in the level of IFN- γ (a Th1 cytokine) prior to surgery, and this effect was maintained until POD-7. In contrast, on POD-0 and POD-1, plasma levels of IL-10

(a Th2 cytokine) in the supplement group were significantly lower than those in the control group (Fig. 3). These results may not express direct effects of this supplement on the inflammatory response. We considered that these phenomena may be supported by the results that the ratio of CD4/CD8 and the percentage of CD8-positive cells on POD-0 in the supplement group were significantly lower than those in the control group. Furthermore, we have reported previously that this supplement reduced plasma levels of inflammatory markers such as high-sensitivity C-reactive protein, α -acid glycoprotein, polymorphonuclear elastase, and soluble tumor necrosis factor receptor I before and after surgery. Therefore, oral administration of this supplement may be useful for the improvement of inflammatory response in patients undergoing cardiac surgery.

Platelet activation not only promotes aggregation and adhesion to the endothelium, but also plays an important role in up-regulating inflammatory processes by interacting with leukocytes, especially monocytes and neutrophils.²⁹ Binding of platelets *via* P-selectin expressed on the surface of activated platelets to the leukocyte counterreceptor P-selectin GP ligand-1 may alter leukocyte recruitment and activation patterns.³⁰ Several reports have shown that the formation of leukocyte–platelet aggregates occurring after coronary ischemia and reperfusion contributes to amplification of local inflammation and tissue damage by up-regulating leukocyte integrin expression and their adhesion to endothelium.^{31,32} In the present study, we found that the supplement caused a decrease in leukocyte–platelet aggregation before and after surgery (POD-1). Thus, perioperative administration of the dietary supplement may be important to avoid the risk



of complications because of increased leukocyte–platelet aggregates.

Previously, we have reported that preoperative supplementation with Impact[®] increased circulating levels of total ω -3 fatty acids including EPA and DHA and decreased the ratio of ω -6/ ω -3 fatty acids in cancer patients.²¹ Based on this report,²¹ we judged that oral administration of supplement enriched in ω -3 fatty acids increased the percentage of positive cells for expression of HLA-DR on monocytes, the percentage of CD4-positive cells, the ratio of CD4/CD8, and the levels of IFN- γ secreted by CD4-positive cells in patients undergoing cardiac surgery. Furthermore, we judged that the supplementation suppressed not only an increase in IL-10 after surgery but also the formation of leukocyte–platelet aggregates before and after surgery. As Impact[®] is rich in arginine, RNA, and ω -3 fatty acids, it is necessary to consider the effects of arginine and RNA on immune responses and leukocyte–platelet aggregate formation after surgery. Several reports have demonstrated that supplementation of arginine may be of clinical benefit in improving wound healing and immune responses.³³ On the other hand, some reports have found no positive effects of arginine supplementation.³⁴ Other experimental reports have suggested that dietary sources of purines and pyrimidines are important for function of the cellular immune response.³⁵ Furthermore, several investigators reported that preoperative oral supplementation of arginine and ω -3 fatty acids leads to beneficial effects such as improved immune metabolic responses, fewer infectious complications, and shorter hospital stays.^{36,37} Therefore, the influence of Impact[®] on immune responses after surgery and formation of leukocyte–platelet aggregates before and after surgery may be concerned with the synergistic effects of these specific substrates and ω -3 fatty acids; however, further study using an isocaloric placebo is required to establish the usefulness of the postoperative supplement enriched in ω -3 fatty acids. Our study group consisted of 14 patients. These numbers were small for a power analysis of the effects of this nutrient on clinical trial. Therefore, more investigations are required to clarify the benefit of this nutrient.

In conclusion, administration of the dietary supplement enriched in ω -3 fatty acids, arginine, and RNA may prevent inflammatory and thrombotic responses in the early stage after cardiac surgery, and thereby decrease the incidence of complications.

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Author Contributions

HI and KN conceived and designed the experiments. HK, JA, and KN performed the experiments. HI, HK, JA, HY, and KN analyzed the data and wrote the paper. HI, HK, JA, HY, and KN agree with manuscript results and conclusions. All authors reviewed and approved of the final manuscript.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

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