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Effect of immune-enhancing enteral nutrition formula enriched with plant-derived *n*-3 fatty acids on natural killer cell activity in rehabilitation patients

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BACKGROUND/OBJECTIVES: Enteral nutrition formulas with immune-enhancing nutrients, such as *n*-3 fatty acids, may manage patients' nutritional status and pathophysiological processes. The aim of our study was to investigate natural killer (NK) cell activity alterations and related cytokine changes resulting from feeding with soybean oil-containing enteral nutrition formula (control group) and plant-derived *n*-3 fatty acid-enriched enteral nutrition formula.
SUBJECTS/METHODS: Subjects participated for 14 consecutive days and consumed enteral formula containing canola and flaxseed oil (n3EN, test group) in nonsurgical patients hospitalized for rehabilitation. Blood samples were collected on the first day and 14 days after the consumption of each formula daily, and anthropometric parameters were collected. Hematology and biochemical values were analyzed, and NK cell activities and serum cytokine concentration were measured. A total of sixty subjects were included in the analysis, excluding dropouts.
RESULTS: No significant differences were found in biochemical parameters. The n3EN group's NK cell activities at effector:tumor cell ratios of 10:1, 5:1, 2.5:1 and 0.625:1 were significantly higher than those of the control group after two weeks ($P < 0.05$). However, there were no statistically significant differences in serum cytokine interleukin (IL)-12, interferon-γ, IL-1β, IL-6 and tumor necrosis factor-α values between the two groups.
CONCLUSIONS: In conclusion, this study elucidates the beneficial effects of plant-derived *n*-3 fatty acid supplementation in enteral formula on NK cell activity.

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INTRODUCTION

Enteral nutrition is preferred over parenteral nutrition for hospitalized patients who are unable to meet their nutritional requirements with an oral diet [1,2]. Immune-enhancing enteral nutrition formulas containing *n*-3 fatty acids, selenium, and antioxidants may manage patients' nutritional status and pathophysiological processes [3,4]. The fat sources traditionally used in enteral nutrition are based on *n*-6 fatty acid-rich oils, such as soybean oil; however, such sources may not be ideal because they may provide excess linoleic acid [5], which is a precursor for pro-inflammatory arachidonic acid synthesis [6]. Clinical trials regarding the immunomodulatory effect of *n*-3 fatty acids have been reported in previous studies [7,8]. Meta-analyses of controlled, randomized clinical trials using *n*-3 fatty acids or similar formulas to enhance immune functions have shown marked decreases in the length of hospital stay and infection events; however, these effects are more significant in critically ill [9-11] or postoperative patients [12].

Immune-enhancing enteral formulas, also known as immune-enhancing formulas, typically include arginine, glutamine, nucleic acids, and *n*-3 fatty acids [13]. The aim of these formulas is to reduce complications associated with infection and protect and stimulate the immune system [11,14-16]. Perioperative immunomodulating enteral nutrition has been recommended for surgical patients by the European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines [17]. Although many immune-enhancing enteral nutrition guidelines strive to

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achieve better patient care outcomes, a precise definition of the desirable properties of immune-strengthening fatty acid components in enteral nutrition formulas for chronic nonsurgical hospitalized inpatients is still lacking.

Natural killer (NK) cells regulate immune and inflammatory reactions by presenting multiple cytokines, particularly interferon (IFN)-γ, a potent immune-activating cytokine [11]. NK cell activity and cytokines can serve as parameters of a patient's immune status. Moreover, rehabilitation inpatients are vulnerable to infectious complications because of their prolonged hospital stays [18]. Although no clear evidence regarding the immune-enhancing effect of *n*-3 fatty acids derived from plants on NK cell activity is available, plant-derived *n*-3 fatty acids can be effective for regulating the immune system [19,20] because they are rich in α-linolenic acid. However, few studies have compared the effect of enteral formulas enriched with plant-derived *n*-3 fatty acids in nonsurgical rehabilitation patients with that of a soybean oil formula as a control.

Malnutrition is commonly encountered in long-term hospitalized patients, such as rehabilitation patients, and these patients require specific nutritional strategies [21]. Therefore, the goal of our research was to compare NK cell activity alterations and related cytokine changes associated with a soybean oil formula (control) and a plant-derived *n*-3 fatty acid-enriched formula (test) in nonsurgical patients hospitalized for rehabilitation.

SUBJECTS AND METHODS

Participants

From August 2015 to March 2018, ninety-four patients were enrolled in this study after admission to the department of rehabilitation at Yonsei University Severance Hospital (Seoul, Republic of Korea). The eligibility criteria included adult male and female patients aged 19 years and older who received enteral nutrition through tube feeding. The exclusion criteria were hepatic impairment, renal impairment, or diabetic problems, active chemotherapy, a life expectancy of less than 1 month, pregnancy, and the presence of other acute diseases that could affect a patient's pathophysiological condition.

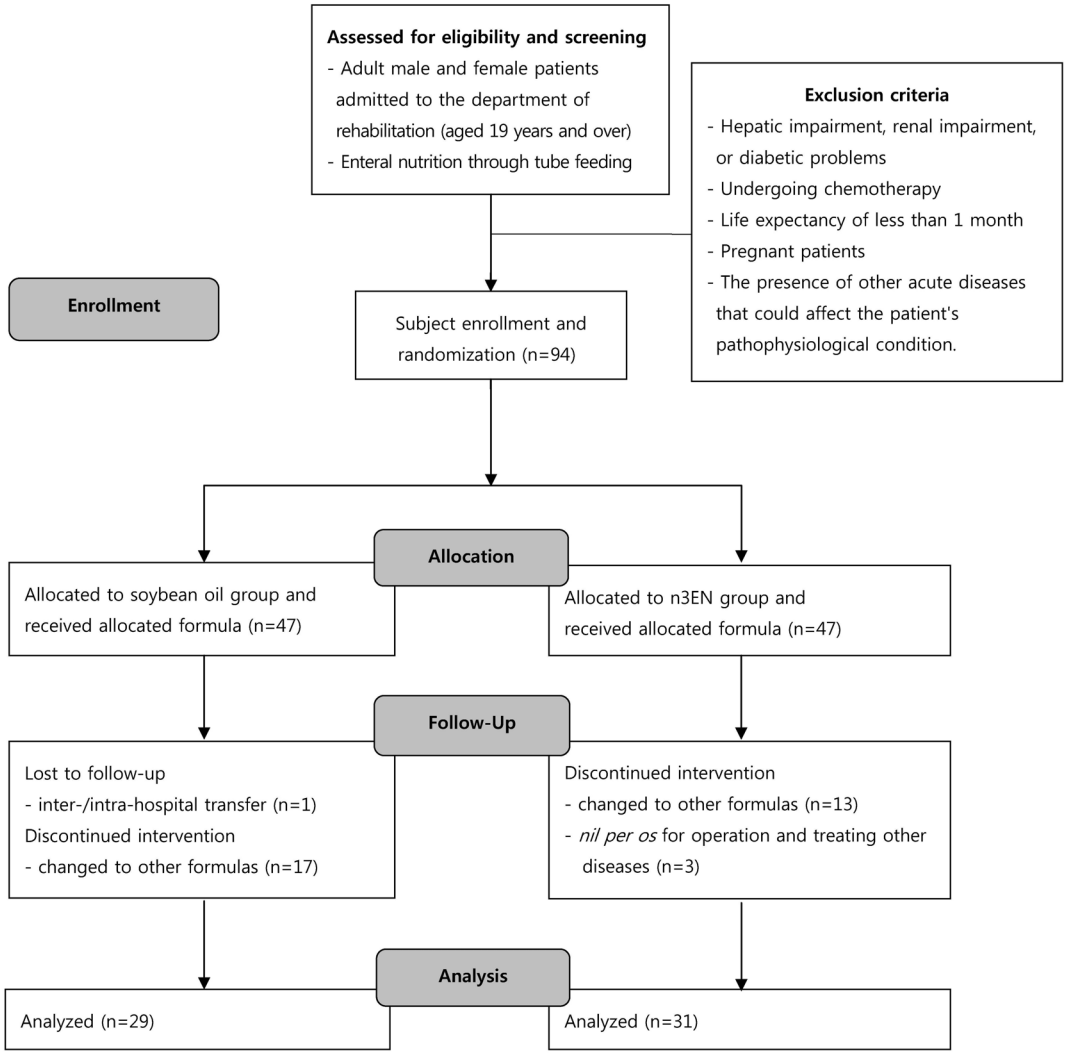

Fig. 1. Flow diagram of randomized participants

However, thirty- four subjects discontinued the study for personal reasons, including a change to another formula or withdrawal, inter/ intrahospital transfer, or *nil per os* for the treatment of other diseases. None of the withdrawals were due to serious adverse effects. The results of this study are based on an analysis of sixty subjects after excluding the dropouts. In total, forty-three quadriplegic patients, five patients with hemiplegia of a cerebellar pathophysiological origin, eleven patients with ataxic quadriparesis, and one patient with lateral medullary syndrome trauma were enrolled. No differences were identified between the test and control groups regarding the diagnostic distribution ($P = 0.100$, chi-square test). Informed consent was provided by the patients or a close family member. This research was approved by the Institutional Review Board of Yonsei University Severance Hospital, Seoul, Korea (Identification Approval number: 2015-0907). All compliance measures and medical histories of the study subjects were tracked under the IRB tracking number (ClinicalTrials.gov: NCT03638661; http://www.clinicaltrials.gov).

Implementation of the randomized clinical trial

The principal indication for enteral nutrition is a patient with an intact and functional gastrointestinal tract but an impaired ability to swallow and/or difficulty with oral intake [22]. Because of severe metabolic changes and unstable vital signs, critically ill or postoperative patients are not suitable for enteral nutrition for more than 2 weeks and were therefore ineligible for this clinical trial. Additionally, an appropriate intestinal approach cannot be carried out in pre-/peri-/postoperative patients, and these subjects may have several contraindications to enteral nutrition. Therefore, we decided that rehabilitation patients were suitable for the current clinical study using standard nutrients with an *n*-3 fatty acid formula.

The first patient was enrolled in August 2015, and the last (94^{th}) patient's 2-week follow-up ended in March 2018. A flow diagram of the randomization of the subjects is presented in Fig. 1. The present study was designed as a parallel, randomized, human clinical trial. Using computer-generated simple sampling randomization lists (the SAS randomization program), ninety-four patients were randomized to receive either a soybean oil-containing enteral nutrition formula (control) or an *n*-3 fatty acid-enriched enteral nutrition formula containing canola and flaxseed oil (n3EN, test). Yonsei Dairy Co. generated the random allocation sequence and concealed the allocation table. The attending physicians of the Department of Rehabilitation at Yonsei University Severance Hospital enrolled the participants and allocated the subjects using the given sequence. The patients assigned to the control group received a soybean oil formula by tube feeding. Those assigned to the n3EN group received the *n*-3 fatty acid-enriched formula by tube feeding (product; Yonsei Dairy Co., Seoul, Republic of Korea). The ready-to-use soybean oil formula and n3EN formula had an identical appearance, with no dissimilarity in packing, texture, or smell. All of the participants, health care providers, outcome assessors and data analysts were blinded after assignment to the intervention groups. The concealed allocation tables were not uncovered before all the participants' data had been clarified.

Enteral nutrition formula intervention

The nutritional compositions of both the control and test enteral formulas are shown in Table 1. The nutrient (fatty acids) compositions of canola, flaxseed and soybean oils according to the *United States Department of Agriculture database* [23] are attached as supplemental data (Supplemental Table 1). The subjects participated for 14 consecutive days and consumed the enteral formula by either naso-gastric enteral tube feeding or a percutaneous endoscopic gastrostomy (PEG) enteral tube. Enteral nutrition was started within 24 h of hospital admission and supplied at a continuous rate to achieve a minimum of 50% basal energy expenditure (BEE; determined using the Harris-Benedict equation) × 1.2 within the first 12 h. If well tolerated, enteral nutrition was increased to achieve BEE × 1.2 within 48 h. Complementary delivery with parenteral nutrition was allowed for the initial 48 h. From the third day, the subjects received a minimum of 75% of BEE × 1.2.

Table 1. Composition of the enteral nutrition product of the control and test groups

	Soybean oil formula (control group)	*n*-3 fatty acid-enriched formula (n3EN, test group)
Fat ingredients/source (percentage ratio)	Soybean, medium chain triglycerides (MCT) (55:45)	Canola, flaxseed, MCT (44.4:11.1:44.5)
Calories (kcal)	200	200
Total fat (g)	4.5	6.0
Saturated fat (g)	2.0	2.4
Trans fat (g)	0.0	0.0
Total carbohydrate (g)	34.0	30.0
Protein (g)	8.0	8.0
Fiber (g)	3.0	2.5
Cholesterol (mg)	0.00	0.00
Vitamin A (μgRE)	150.00	150.00
Vitamin B1 (mg)	0.24	0.24
Vitamin B2 (mg)	0.30	0.30
Vitamin B6 (mg)	0.30	0.30
Vitamin B12 (μg)	0.48	0.48
Vitamin C (mg)	20.00	20.00
Vitamin D (μg)	1.00	2.00
Vitamin E (mgα-TE)	2.00	2.00
Vitamin K (μg)	15.00	15.00
Niacin (mg NE)	3.20	3.20
Folic acid (μg)	80.00	80.00
Pantothenic acid (mg)	1.00	1.00
Biotin (μg)	6.00	6.00
Calcium (mg)	140.00	140.00
Phosphorus (mg)	140.00	140.00
Potassium (mg)	200.00	260.00
Magnesium (mg)	44.00	44.00
Iron (mg)	2.00	2.00
Zinc (mg)	2.00	2.00
Sodium (mg)	120.00	120.00
Copper (mg)	0.10	0.16
Manganese (mg)	0.46	0.70

Anthropometric parameters and blood collection

The subjects' gender, age, height (cm), weight (kg), and weight change were tracked. Body weight during the hospital stay was derived from the medical records on the first and 14th days after consuming each formula and was used to calculate the body mass index (BMI = body weight (kg) / height (m^2)) [24]. Venous blood samples were collected in EDTA-treated and plain tubes and centrifuged to acquire plasma and serum. The collected blood samples were preserved at -70°C until analysis.

Hematology and biochemical analysis

Serum glucose levels were measured according to the hexokinase method on a Hitachi 7600 autoanalyzer. Serum albumin concentrations were analyzed according to the BCG method with an ALB kit (Siemens, Tarrytown, NY, USA) using an ADVIA 2400 autoanalyzer (Siemens, Tarrytown, NY, USA). The leukocyte count was acquired using the HORIBA ABX diagnostic analyzer (HORIBA ABX SAS, ParcEuromedicine, Montpellier, France). Serum high-sensitivity C-reactive protein (hs-CRP) levels were determined with a kit from the N-Assay LA CRP-S D-TYPE (Nittobo, Tokyo, Japan) with a Hitachi 7600 autoanalyzer.

Natural killer (NK) cell activity

NK cell activity was the primary outcome measure of this study. Isolated PBMCs from the whole-blood specimens were incubated with K562 cells to measure the cytotoxicity of NK cells. The blood sample was mixed with the same amount of RPMI medium 1640 (Gibco, Thermo Fisher Scientific, Waltham, MA) and then carefully overlaid on Histopaque®1077 (Sigma-Aldrich, Irvine, UK) and centrifuged for 20 min at 1,800 rpm at 15°C. After separation, the buffer coat layer was isolated, washed once with RPMI 1640 medium, and then re-suspended in 1 mL of 10% fetal bovine serum. The isolated PBMCs (E, effector cells) were seeded onto 96-well plates at ratios of 10:1, 5:1, 2.5:1, 1.25:1 and 0.625:1 with the K562 cells (T, target cell) and then incubated at 37°C under 5% CO2 for more than 4 h. The cytolytic activities of the NK cells were measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega Co., Fitchburg, WI, USA) according to the manufacturer's instructions. The optical density was read at 490 nm using a Victor × 5 2030 multi-label plate reader (PerkinElmer, Hopkinton, MA, USA), and the results were calculated by the following formula:

% Cytotoxicity = (Experimental-Effector Spontaneous-Target Spontaneous)/
(Target Maximum-Target Spontaneous) × 100

Cytokine concentrations in serum

Serum cytokines were secondary outcome measures of this study. Interferon gamma (IFN-γ) was measured with an IFN-γ High-Sensitivity Human ELISA Kit (Abcam plc-Cambridge Science Park, Cambridge, UK) following to the manufacturer's instructions. Interleukin (IL)-12 concentrations were analyzed with a High-Sensitivity Human IL-12 (P70) ELISA kit (Genway Biotech, Inc., San Diego, CA, USA) using a Victor × 5 2,030 multi-label reader (PerkinElmer, Hopkinton, MA, USA) at 450 nm. IL-6, IL-1β, and tumor necrosis factor (TNF)-α concentrations in serum were measured using the Bio-Plex™ Reagent Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Noncontinuous variables are presented as *n* values and percentages, whereas continuous variables are shown as the averages and the means ± standard errors (SE). All data were analyzed using SPSS version 24.0 (IBM/SPSS Corp., Chicago, IL, USA). Independent *t*-tests were used to compare parameters between the control and test (n3EN) groups. Chi-square tests were used to examine categorical values. For descriptive purposes, the mean values are presented using untransformed values. A two-tailed *P*-value less than 0.05 was considered statistically significant.

RESULTS

Basic characteristics and anthropometric values

Table 2 outlines the basic characteristics at baseline and after two weeks for the control group and the n3EN group. At baseline, no significant differences were noted between the two groups in gender, age, weight, BMI, waist circumference, and the estimated average calorie intake (Table 2). After 2 weeks of consuming the enteral nutrition formulas, no statistically significant changes were observed in these clinical characteristics in the control and n3EN groups. Additionally, no noticeable differences were observed in the changes (differences from baseline) in these clinical characteristics between the two groups (Table 2).

Table 2. Basic characteristics and anthropometric values in the control and test groups at baseline and at the 2-week follow up

	Control group (n = 29)		Test group (n = 31)		*P*-value[1]	*P*-value[2]
	Baseline	Follow-up	Baseline	Follow-up		
Age (yrs)	58.66 ± 2.90		57.26 ± 2.99		0.739	
Male/female, n (%)	17 (58.6) / 12 (41.4)		21 (67.7) / 10 (32.3)		0.464	
Weight (kg)	55.79 ± 1.71	56.33 ± 1.68	57.71 ± 1.81	57.20 ± 1.84	0.444	0.730
Body mass index (kg/m^2)	20.27 ± 0.56	20.27 ± 0.58	21.17 ± 0.56	20.93 ± 0.53	0.262	0.410
Waist circumference (cm)	81.19 ± 2.11	82.45 ± 2.32	83.75 ± 1.40	83.02 ± 1.57	0.316	0.839
Calorie needs (kcal)	1,413.58 ± 34.27		1,380.44 ± 37.53		0.517	
Average calorie intake (kcal)	1,367.97 ± 43.24		1,437.12 ± 46.57		0.282	

Mean ± SE (standard error).
[1] *P*-values were derived from the chi-square test and independent *t*-test at baseline.
[2] *P*-values were derived from independent *t*-tests at the 2-week follow up

Biochemical parameters

No significant differences were found in the concentrations of white blood cells, red blood cells, hemoglobin, platelets, serum albumin, prealbumin, transferrin, glucose, HDL cholesterol, LDL cholesterol, GOT, GPT, hs-CRP, and free fatty acids at baseline between the control and n3EN groups (Table 3).

Natural killer (NK) cell activity and cytokine concentrations

NK cell activities (%) were investigated based on effector: tumor (E:T) ratios of 10:1, 5:1, 2.5:1, 1:25:1 and 0.625:1. As outlined in Table 4 and Fig. 2, no significant differences were found in the NK cell activities measured at baseline between the two groups. The n3EN group's NK cell activities at the 10:1, 5:1, 2.5:1 and 0.625:1 E:T ratios were significantly higher than those of the control group at week 2 ($P < 0.05$) compared with the baseline values (Fig. 2). The n3EN group had larger increases in NK cell activity at ratios of E:T = 10:1, 5:1, and 0.625:1 than the control group ($P < 0.05$). No statistically significant differences were found in cytokine concentrations (IL-12, IFN-γ, IL-6, IL-1β and TNF-α) between the two groups (Table 4).

Table 3. Biochemical parameters of the control and test groups at baseline and at the 2-week follow up

	Control group (n = 29)		Test group (n = 31)		*P*-value[1]	*P*-value[2]
	Baseline	Follow-up	Baseline	Follow-up		
White blood cells ($\times 10^3/\mu L$)	7.51 ± 0.57	7.28 ± 0.41	6.33 ± 0.39	6.84 ± 0.51	0.095	0.499
Red blood cells ($\times 10^6/mm^3$)	4.03 ± 0.11	4.05 ± 0.08	3.98 ± 0.12	3.85 ± 0.11	0.748	0.131
Hemoglobin (g/dL)	12.01 ± 0.32	12.20 ± 0.26	12.13 ± 0.38	11.69 ± 0.32	0.808	0.220
Platelets ($\times 10^3/mm^3$)	269.71 ± 16.27	282.17 ± 15.97	261.68 ± 13.61	275.69 ± 13.19	0.706	0.756
Serum albumin (g/dL)	3.93 ± 0.07	3.99 ± 0.06	4.02 ± 0.08	3.94 ± 0.08	0.426	0.586
Prealbumin (mg/dL)	24.38 ± 1.15	28.86 ± 1.44	27.26 ± 1.47	26.61 ± 1.42	0.129	0.270
Transferrin (mg/dL)	223.55 ± 9.77	229.69 ± 8.50	242.94 ± 7.89	239.87 ± 8.41	0.128	0.398
Glucose (mg/dL)	104.83 ± 5.81	98.31 ± 3.86	97.36 ± 5.03	105.48 ± 5.11	0.335	0.268
HDL cholesterol (mg/dL)	42.21 ± 1.84	44.00 ± 2.11	41.71 ± 2.26	46.03 ± 2.40	0.865	0.527
LDL cholesterol (mg/dL)	96.34 ± 5.98	108.34 ± 5.96	83.63 ± 5.78	89.28 ± 7.57	0.131	0.053
GOT (IU/L)	29.90 ± 3.92	29.55 ± 3.46	25.58 ± 2.05	27.10 ± 2.32	0.335	0.558
GPT (IU/L)	25.10 ± 3.01	30.34 ± 4.21	23.16 ± 2.09	26.39 ± 4.23	0.599	0.510
hs-CRP (mg/L)	12.77 ± 3.86	9.78 ± 2.52	7.05 ± 1.37	9.30 ± 2.56	0.171	0.894
Free fatty acids (uEq/L)	344.96 ± 25.69	380.55 ± 42.51	315.19 ± 29.93	372.19 ± 48.50	0.454	0.898

Mean ± SE.
[1] *P*-values were derived from independent *t*-tests at baseline.
[2] *P*-values were derived from independent *t*-tests at the 2-week follow up.

Table 4. Natural Killer (NK) cell activity and cytokine values in the control and test groups at baseline and at the 2-week follow up

	Control group (n = 29)		Test group (n = 31)		*P*-value[1]	*P*-value[2]	*P*-value[3]
	Baseline	Follow-up	Baseline	Follow-up			
NK cell activity E:T = 10:1 (%)	22.30 ± 2.94	16.74 ± 3.41**	19.33 ± 2.19	34.87 ± 5.06**	0.423	0.005	
Change	-4.94 ± 4.28		14.86 ± 5.09				0.005
NK cell activity E:T = 5:1 (%)	17.78 ± 2.27	15.02 ± 3.22*	15.11 ± 1.96	26.40 ± 3.49*	0.377	0.020	
Change	-3.01 ± 3.53		11.07 ± 3.85				0.009
NK cell activity E:T = 2.5:1 (%)	14.85 ± 2.03	12.80 ± 3.13*	13.46 ± 1.48	22.65 ± 3.56*	0.582	0.043	
Change	-0.98 ± 3.90		7.99 ± 4.26				0.127
NK cell activity E:T = 1.25:1 (%)	12.13 ± 1.62	12.91 ± 2.11	12.98 ± 1.63	19.51 ± 2.83	0.713	0.067	
Change	2.18 ± 2.99		6.39 ± 3.12				0.335
NK cell activity E:T = 0.625:1 (%)	14.20 ± 2.96	11.80 ± 2.29**	14.50 ± 2.16	25.41 ± 4.13**	0.934	0.006	
Change	-2.66 ± 4.06		8.25 ± 5.01				0.097
Serum cytokines							
IL-12 (pg/mL)	121.16 ± 42.81	54.83 ± 14.00	101.53 ± 36.56	70.26 ± 23.10	0.729	0.571	
IFN-γ (pg/mL)	6.19 ± 1.22	6.73 ± 0.99	20.32 ± 12.80	18.35 ± 6.93	0.280	0.107	
IL-1β (pg/mL)	2.10 ± 0.80	3.28 ± 2.23	1.64 ± 0.31	1.68 ± 0.30	0.590	0.482	
IL-6 (pg/mL)	8.03 ± 1.69	10.17 ± 3.62	9.99 ± 1.96	7.13 ± 1.14	0.452	0.430	
TNF-α (pg/mL)	18.32 ± 7.08	24.65 ± 12.83	19.81 ± 4.49	18.32 ± 3.83	0.860	0.639	

Mean ± SE.
[1] *P*-values were derived from independent *t*-tests at baseline.
[2] *P*-values were derived from independent *t*-tests at the 2-week follow up.
[3] *P*-values were derived from independent *t*-tests for changed values.
* $P < 0.05$ and ** $P < 0.01$ derived from paired *t*-test.

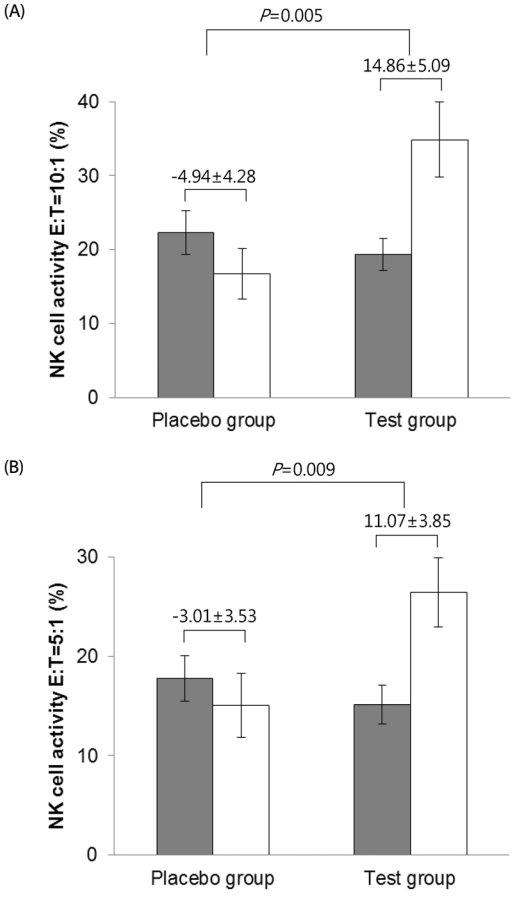

Fig. 2. Increment of NK cell activities between the two groups at baseline (0 week, ■) and follow up (2 week, □). Values are Mean ± SE. *P*-values were derived from independent *t*-tests.

DISCUSSION

Fatty acids can alter cell signaling, membrane properties, gene expression and the stimulation of bioactive regulators [25-27]. The lipid mediator system is important for mediating the inflammatory response [28]. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in fish oil and α-linolenic acid in plant oil are well known for promoting immune competence through promotion of anti-inflammatory eicosanoid synthesis [29]. Plant oils, especially flaxseed and canola oils, are good sources of *n*-3 fatty acids (C18:3). In a practical context, an enteral formula is generally used in large amounts on a daily basis for the long term. Therefore, a patient's tolerance and preference and the stability of an enteral formula are considered important factors. The main reason that plant-derived *n*-3 fatty acid was incorporated into the test products used in our clinical trial rather than *n*-3 fatty acid from fish oil is because fish oil introduces stability problems and promotes an unacceptable taste and odor, and oxidation and hydrolysis quickly deteriorate the quality of fish oil [30]. Another issue is that fish oil's functional substance may vary due to the variety of fish species and seasonal and geographical differences. When exposed to oxygen, fish oil can undergo oxidation and deterioration of its functional efficacy [31]. The stability and consistent quality of effective ingredients are crucial for enteral formulas given to patients. In addition, research showing that enteral formulas containing fish oil-derived *n*-3 PUFA are superior to plant-derived *n*-3 fatty acid formulas in hospitalized patients is lacking. Thus, we hypothesized that plant-derived *n*-3 oils may be desirable as fat components of an immune-enhancing enteral nutrition formula for rehabilitation patients.

In this randomized, placebo-controlled study, the main findings indicated that the use of canola and flaxseed oils for enteral nutrition can be effective for activating the immune system by enhancing NK cell activities. Compared with the soybean oil formula (control) group, the plant-derived *n*-3 fatty acid-enriched formula group showed significantly greater increases in changes in NK cell activities at 10:1, 5:1, 2.5:1 and 0.625:1 E:T ratios. This immune-modulating finding in our study shows that plant *n*-3 fatty acids had beneficial effects on NK cell activity, a marker of immune competence [28,32,33].

This result is in accordance with those of a previous reports in which *n*-3 fatty acids were shown to have immunomodulatory effects in humans [7,34]. Generally, the evidence of an immune modulation effect of *n*-3 fatty acids is strongly supported by numerous studies. P.C. Calder summarized beneficial immune and inflammatory effects of *n*-3 fatty acids on monocytes in terms of phagocytosis, lymphocyte proliferation, cytokine production and human leucocyte antigen-D-related expression [34]. Recently, *n*-3 fatty acids have been reported to stimulate the immune system, inhibiting the cyclooxygenase (COX)-2 pathway of eicosanoid synthesis; therefore, a marked decrease occurs in pro-inflammatory prostaglandins such as PGE2, which favorably influences immune system modulation [35]. A recent report showing that NK cells have beneficial anti-infection and anti-inflammatory activities [36] supports the idea that therapeutic immune interventions applied to patients can stimulate the function of NK cells [37].

NK cells are a crucial factor in the innate immune system, representing 10% of the cells in the total peripheral blood mononuclear cell (PBMC) population of circulating human lymphocytes [38]. Additionally, previous studies have demonstrated that cytokine production by PBMCs and several circulating cytokines also play vital roles in critically ill patients [18-20]. The stimulated levels of NK cell activity and blood cytokine concentrations are good indicators of host immunity because they reflect actual cell function, such as their ability to generate the important cytokines involved in immune defense [39]. Critically ill patients are known to exhibit hyporesponsive NK cell activity [40]. In this previous study, NK cell expression was significantly lower in septic subjects than that in non-septic subjects and a healthy control group.

Decreases and increases in NK cell activity levels are related to the production of cytokines. A decrease in the expression level of NK cells results in altered production of cytokines. In a clinical prospective cohort study, the number of $CD14^+$ monocytes yielding IL-12, TNF-α, and IL-6 was 40% to 70% lower in trauma patients than that in healthy control subjects [8]. In another study, critically ill pediatric patients with low stimulated TNF-α production were vulnerable to developing life-threatening complications [41,42].

Although the canola and flaxseed oil formula group had higher NK cell activity levels in our study, no significant changes were observed in the five measured cytokines: IL-12, IFN-γ, IL-1β, TNF-α and IL-6. Serum cytokine changes between the two groups and within the groups after two weeks of consuming each formula were not remarkable. These results regarding serum cytokines are inconsistent with the finding of Heyland DK *et al*. [11], which indicated that NK cells contribute to the immune reaction by presenting various potent immune-stimulating cytokines. On the other hand, our previously published study showed that despite NK cell activity upregulation, no corresponding trends for serum cytokine activation were noted in critically ill patients [43]. In addition, Lee *et al*. [44] demonstrated serum cytokine and NK cell activity alterations according to age, and no positive or negative associations were found between NK cell activity and serum cytokines. These observations closely concurred with our findings. IL-6 and TNF-α have a short half-life [45], which may explain why we failed to find an association between hyper-NK cell activity and altered serum cytokine concentrations.

Furthermore, the wide discrepancy in cytokine levels in our study may be another factor underlying the lack of association found among immune markers, and serum cytokine homeostasis is unstable in hospitalized-rehabilitation patients [46]. In addition, Agarwal *et al*. [47] reiterated that the bioavailability and/or action of proinflammatory cytokines may depend on circulating cytokine receptors. Additionally, since cytokines are released in a paracrine manner, the levels may vary widely depending on when a subject's blood sample is collected. Serum and plasma cytokine levels can be affected by receptor binding, temperature-induced degradation, urinary excretion, and cytokine breakdown within reacting cells [48]. Thus, these properties of cytokines may be another factor underlying the lack of association with NK cell activities and other immune parameters in our study. Additionally, this contradictory finding may be due to dissimilarities in the patients' pathophysiological conditions. Our subjects were in the general inpatient ward, while most previous studies were conducted in critically ill patient care settings or in healthy subjects. Despite the small sample of sixty rehabilitation participants, this randomized placebo-controlled clinical study clearly showed that a combination of canola and flaxseed oils as a source of immune-modulating *n*-3 fatty acids in an enteral nutrition formula had beneficial effects without causing significant changes in serum cytokine levels, including IL-12, IFN-γ, IL-1β, IL-6 and TNF-α.

Several points should be considered when interpreting the present results. First, the small sample size of sixty enteral tube-fed patients warrants attention. The results should be confirmed by larger randomized controlled trials with sub-analyses by diagnosis, disease severity and age. Second, although the fatty acid profile of lipids (the fatty acid fraction) is less likely to be a practical parameter in our clinical setting because the fatty acid profile can be easily altered by total parenteral nutrition usage [49], we could not report the plasma fatty acid profiles of these patients because of limited blood (serum) volumes; therefore, NK cell activity, cytokine, and hematologic assessments were prioritized. Third, our results do not directly show that the increase in NK cell activity was correlated with cytokine changes as the circulating levels of measured cytokines may not reflect their true biological activity. Finally, as we focused on immune-specific parameters during two weeks of the patients' hospitalizations, this study could not elucidate practical values such as the involuntary readmission rate, mortality, patients' subjective tolerance or satisfaction scores, administrative costs, hospital stay durations, and follow-up clinical data after discharge regarding immune functions. Further investigation is required to evaluate the effects of plant-derived *n*-3 fatty acids on the immune system and clinical outcomes in larger long-term larger trials.

Despite these limitations, we demonstrated a greater increase in NK cell activity in the n3EN group. Immune-enhancing enteral nutrition enriched with canola and flaxseed oils yielded significant elevations in NK cell activities compared with the baseline levels, and a greater increase was observed in the n3EN group than that in the control group. In conclusion, this study shows the beneficial effects of plant-derived *n*-3 fatty acid supplementation in an enteral formula on NK cell activity.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

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