Effect of immune-enhancing enteral nutrition formula enriched with vegetable derived *n*-3 fatty acids on NK cell activity in hospitalized patients for rehabilitation

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Materials 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. Yet, thirty-four subjects discontinued the study for personal reasons (withdrawn consent for clinical trial, move to another department or hospital, other disease treatment, Nil Per Os, etc.). There were no serious adverse effects for the withdrawal reasons. Results of this study were analyzed with sixty subjects, except for the dropouts. Majority of patients were forty-three quadriplegia patients and five hemiplegia patients with cerebellar pathophysiological origin. Eleven patients with ataxic quadriparesis and one patient with lateral medullary syndrome trauma were also enrolled. There was no difference between two groups regarding diagnostic distribution (p=0.100, chi-square test). Informed consent was provided by patients or their close family member. This investigation was approved by the Institutional Review Board at Yonsei University Severance Hospital, Seoul, Korea (Approval number: 2015-0907). All comorbidities and histories of the study participants were recorded under IRB tracking number.

Randomization and Intervention

Using computer-generated randomization lists, ninety-four patients were randomized to receive one of two types of enteral nutrition: soybean enteral nutrition (control), vegetable (canola, flaxseed) derived *n*-3 fatty acid enriched enteral nutrition (n3EN). The ready-to-use soybean used formula and n3EN formula had identical packaging with no differences in appearance, texture, or smell. Patients assigned to the control group received a soybean used formula by tube feeding. Those assigned to the n3EN group received vegetable *n*-3 fatty acid

enriched formula by tube feeding (product; Yonsei Dairy Co., Seoul, South Korea). The composition of both control and test group enteral formula shown in Table 1. The nutrient (fatty acids) composition of Canola, Flaxseed and Soybean oil by *United States Department of Agriculture database* (25) attached as supplemental data (Supplemental table 1).

Enteral nutrition was initiated within 24 h of admission. Enteral feeding was delivered at a constant 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 advanced to achieve a BEE × 1.2 within 48 h. Complementary feeding with enteral or parenteral nutrition was allowed for an initial 48 h. From the third day, the patient received a minimum of 75% of BEE × 1.2. The daily enteral intake was recorded to obtain the total volume and calories delivered to the patients. The data on daily total calorie intake were abstracted from the medical records.

Anthropometric Parameters and Blood Collection

The subjects' gender, age, height (cm), weight (kg), weight change were tracked. Body weight during hospital stay was derived from the medical records on the first day and 14 days after consumption of each formula and was used to calculate BMI (Body Mass Index = body weight (kg) / height (m^2)) (26). Venous blood specimens were collected in EDTA-treated and plain tubes and centrifuged to obtain plasma and serum. The collected blood samples were stored at −70 °C until analysis.

Hematology and biochemical analysis

Serum glucose concentrations were measured according to the hexokinase method on

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

Cytokine Assay in Serum

Interferon (IFN)-γ was measured with a kit from an IFN gamma High-Sensitivity Human ELISA Kit (Abcam plc-Cambridge Science Park, Cambridge, UK) according to the manufacturer's instructions. Interleukin (IL)-12 levels were analyzed by a High-Sensitivity Human IL-12 (P70) ELISA kit (Genway Biotech Inc., San Diego, CA, USA) using a Victor ×5 2030 multi label plate reader (PerkinElmer, Hopkinton, MA, USA) at 450 nm. IL-6, IL-1β, and tumor necrosis factor (TNF)-α levels in serum were measured using the Bio-Plex™Reagent Kit (Bio-Rad Laboratories, Hercules, CA, USA).

NK Cell Activity

Isolated PBMCs (peripheral blood mononuclear cells) from the whole blood samples were incubated with K562 cells to analyze the cytotoxic activity of NK cells. A whole blood sample was mixed with the same volume of RPMI medium 1640 (Gibco, Thermo Fisher Scientific, Waltham, MA), then gently overlaid on Histopaque®1077 (Sigma-Aldrich, Irvine, UK) and centrifuged for 20 min at 1800 rpm at 15 °C. After separation, a buffer coat layer was isolated, washed once with RPMI 1640 medium, and then resuspended in 1 mL of 10% fetal

bovine serum. The isolated PBMCs (effector cell, E) were seeded into 96-well plates at ratios of 10:1, 5:1, 2.5:1, 1.25:1 and 0.625:1 with the K562 cells (target cell, T) and then incubated at 37 °C under 5% CO2 for more than 4 h. The cytolytic activities of NK cells were analyzed via the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega Co., Fitchburg, WI, USA) according to the manufacturer's instructions. The color reactions were 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