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Eicosapentaenoic acid and docosahexaenoic acid in fish oil capsule supplementation in obese children decreases serum interleukin-6 and hepcidin and improves iron status



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ABSTRACT

Background: Chronic low-grade inflammation in obese children causes iron restriction, resulting in hypoferrremia. Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), active metabolites of long chain n-3 polyunsaturated fatty acid have an anti-inflammatory effect.

Objective: We investigated the effect EPA and DHA in fish oil capsule supplementation on inflammation and iron status in obese children.

Methods: A double-blind randomized controlled trial was conducted among 70 obese children aged six to ten years old. Thirty-five obese children received a fish oil capsule containing 45 mg EPA and 225 mg DHA (the supplemented group) and the others a virgin coconut oil capsule (the control group) for eight weeks, respectively. The

changes in IL-6 and Hepcidin levels and iron status before and after supplementation were analyzed using MANCOVA test ($\alpha=0.05$).

Results: The study indicates a significant decrease of serum IL-6 by 1.8 pg/ml (95%CI 0.5-3.1 $p=0.005$), Hepcidin 9.6 nmol/l (95%CI 5.1-14.0 $p=0.0001$), and ferritin 10.2 ng/dl (95%CI 0.9-19.6 $p=0.032$). In addition, after the supplementation, we found an increase in serum iron by 13.6 $\mu\text{g/dl}$ (95%CI 4.1-23.2 $p=0.006$) and transferrin saturation by 4.6% (95%CI 1.7-7.6 $p=0.002$).

Conclusion: Supplementation with fish oil capsule containing EPA and DHA in obese children improved the iron status through decreasing serum IL-6 and Hepcidin.

Keywords: iron, obesity, EPA, DHA

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INTRODUCTION

Recently, obesity has been recognized as a risk of iron metabolism disorder from triggering hypoferrremia.¹⁻³ It is known that hypoferrremia in children associates with low physical performance and low cognitive score.^{4,5} Therefore, it is important to study the iron metabolism disorder in obese children.

We know that obesity is an accumulation of fat in the adipose tissue. The function of the adipose tissue is not only as an energy reservoir. But, it also functions as an endocrine organ that produces many adipokines and cytokines with a broad biological activity.⁶ Several inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) are produced by the adipose tissue. Adipose cells contribute 15%-30% of the circulating IL-6 in the absence of an acute inflammation. The inflammatory cytokine directly stimulates hepcidin synthesis through induction and subsequent promoter binding of signal transducer and activator of transcription 3 (STAT-3).⁷

Hepcidin is mainly produced by hepatocytes. The increased hepcidin production in obesity

causes an internalization and a degradation of ferroportin—the sole known cellular exporter of iron. It blocks the pathway for transferring iron from enterocytes to the plasma, and blocks the iron release from macrophage to the plasma, causing a hypoferrremia.⁸ Obesity-related hypoferrremia is associated with a diminished response to oral iron therapy.⁹ In consequence, anti-inflammatory agents are needed to suppress the obesity-related inflammation to improve the iron status in obese children.

Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) are active metabolites of long chain n-3 polyunsaturated fatty acid (LC-PUFA). They are known to have three mechanisms which have anti-inflammatory effects: (1) they decrease the production of eicosanoid mediators from arachidonic acid (AA), (2) they increase the production of inflammatory resolving agents such as resolvin and protectin, and (3) hinder the production of pro-inflammatory cytokines such as IL-6.¹⁰ Up until we conducted our study, there had not been a study to investigate the effects of EPA and DHA supplementation on improving the

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iron status in obese children. Thus, we conducted this study to reveal the effects of EPA and DHA supplementation on serum IL-6, hepcidin and iron status in obese children.

METHODS

Study design and randomization

The study was a double-blind randomized clinical controlled trial. The children were randomized to receive either 500 mg fish oil capsule (contain 45 mg EPA and 225 mg DHA) or 500 mg coconut oil capsule to be consumed twice per day for 8 weeks. The capsules were taken monthly at our clinic and the parents reported the compliance weekly. There was not any other intervention in the children's diet or any other aspect of their daily routine. The dietary intake was estimated by three-day food record. We recorded the child one day of the weekend dietary intake and two days of the weekdays. The dietary intake data were collected and analyzed using the Nutrition Data System for Research software by the Nutrition Center, Sanglah General Hospital, Bali, Indonesia.

Blood samples were drawn on the study day 0 (baseline) and on day 56 (end intervention), both after 12-hours fasting. Plasma and sera were prepared and stored for the IL-6, hepcidin and iron status studies. The samples were examined for the concentrations of serum IL-6, hepcidin, serum iron, serum ferritin, and transferrin saturation.

A written informed consent was obtained before each child participation. An ethical approval for the study was given by The Ethical Committee of Udayana University and the university teaching hospital Sanglah General Hospital, Denpasar, Bali, Indonesia.

Study population

Obese children (with body mass index or BMI \geq P-95) aged 6-10 years old were recruited for the study. The children were excluded if they have any condition or illness that could affect study outcomes including acute/chronic infection, malignancy, liver function disorder, renal function disorder, current adherence to a weight-loss program, and current use of iron or omega-3 fatty acid supplementation.

Anthropometric measurements

Subjects were weighed in minimal clothing to the nearest 01 kg using a digital scale (Seca® 881). The height was measured to the nearest 01 cm using a fixed stadiometer (Seca® 206). The BMI was calculated as weight in kilogram divided by height in meters squared. Obesity was established if BMI \geq

percentile-95 according to BMI chart based on the age and gender.

IL-6, Hepcidin and iron status measurements

IL-6 was measured using dHuman IL-6 – hs immunoassay (Quantikine®). The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. The standards and the samples are pipetted into the wells and the immobilized antibody binds any IL-6 present. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and a color develops in proportion to the amount of IL-6 bound in the initial step. The color development is stopped and the intensity of the color is measured.

Hepcidin was measured using DRG® Hepcidin ELISA method. The DRG Hepcidin ELISA is an enzyme immunoassay for the quantitative in vitro measurement of Hepcidin in serum. The kit is a solid phase enzyme-linked immunosorbent assay, based on the principle of competitive binding. Endogenous Hepcidin of a patient sample competes with the added Hepcidin-biotin conjugate for binding to the coated antibody. The intensity of the color developed after an addition of substrate solution is reverse proportionally to the concentration of Hepcidin in the patient sample.

Serum iron was determined using ferrozine methods on the ADVIA Chemistry System. Ferric ion is dissociated from its carrier protein, transferrin, in an acid medium and simultaneously reduced to the ferrous form. The ferrous iron is then a complex with ferrozine, a sensitive iron indicator, to produce a colored chromophore. Serum ferritin was determined using COBAS®, the electrochemiluminescence immunoassay (ECLIA) with sandwich principle. The ADVIA Chemistry System determined serum TIBC. The ADVIA TIBC method uses 2 reagents in a sequential process that is monitored spectrophotometrically. The transferrin saturation in percent was calculated with serum iron divided by TIBC \times 100.

Sample size and statistical analysis

Differences in serum iron between groups were our main-endpoint and therefore used to determine the sample size. The number of subjects required for detecting 20 μ g/dl difference with 80% power, $\alpha=0.05$, was 33 subjects per group.

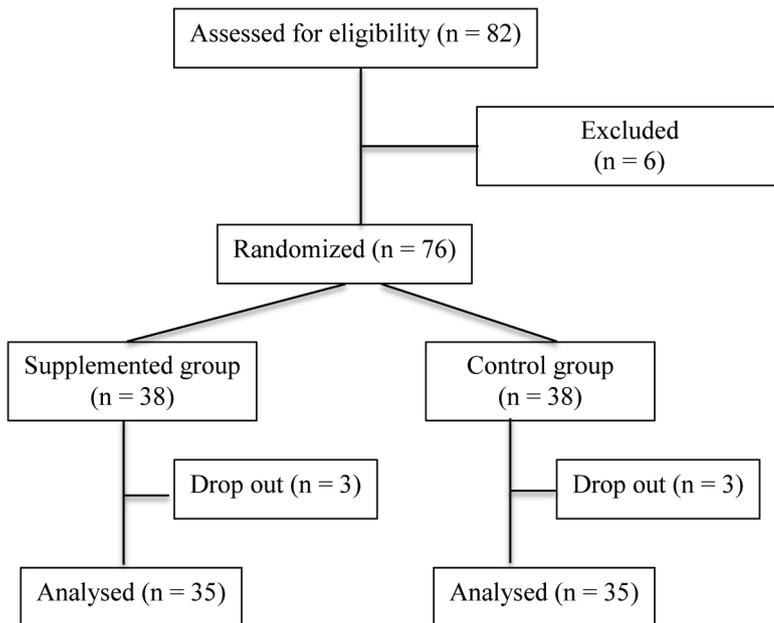


Figure Consort Flow Diagram

Table 1 Baseline Characteristics

	Supplemented (n = 35) f(%)	Control (n = 35) f(%)
	mean (SD)	mean (SD)
Gender, male	18 (48.6)	19 (51.4)
Age (year)	9.9 (0.6)	9.7 (0.7)
Weight (kg)	53.8 (9.8)	53.1 (9.8)
Height (cm)	140.7 (8.7)	138.5 (7.1)
BMI (kg/m ²)	27.1 (3.4)	27.4 (3.5)
Energy (kcal)	1730 (294)	1774 (353)
Protein (g)	67.0 (18.7)	72.2 (20.4)
Fat (g)	55.1 (20.7)	62.0 (29.0)
Pufa (g)	12.0 (7.4)	13.1 (11.1)
Iron (mg)	6.3 (3.1)	7.2 (3.8)
Serum IL-6 (pg/ml)	4.1 (4.0)	2.8 (2.1)
Serum Hcpidin (nmol/l) *	22.7 (14.9)	17.0 (5.5)
Serum Iron (µg/dl)	61.2 (29.1)	62.6 (21.7)
Serum ferritin (ng/dl)	93.0 (68.3)	76.7 (33.1)
Transferrin saturation (%)	18.1 (8.6)	18.5 (5.9)

*) P < 0.05; other variables P > 0.05.

Table 2 Independent T-test for BMI Percentile and Nutrient Intake Mean Differences After Supplementation

	Supplemented (n = 35) mean (SD)	Control (n = 35) mean (SD)	P
BMI (kg/m ²)	27 (3.2)	27.2 (3.8)	0.768
Energy (kcal)	1555 (296)	1603 (311)	0.510
Protein (g)	63.7 (17.6)	64.7 (18.2)	0.830
Fat (g)	48.7 (15.4)	51.7 (20.2)	0.492
Pufa (g)	9.3 (4.5)	11.2 (8.3)	0.229
Iron (mg)	6.3 (2.4)	6.0 (2.6)	0.663

The descriptive statistics include mean \pm standard deviation (SD) for continuous variable, and frequency for categorical variables. The variable distributions were tested using Kolmogorov-Smirnov test. The groups' mean differences were compared using the student's t-test. Dichotomous categorical variables were compared between groups using X^2 . We analyzed the effect of the supplementation on serum IL-6, hepcidin, iron, ferritin, and transferrin saturation levels using ANCOVA test and then MANCOVA test for multivariate analysis.

RESULTS

There were 82 subjects recruited and enrolled in the trial from January to August 2014 (Fig.). Seventy-six subjects met the inclusion criteria and were randomized into an equal number of 38 for the supplementation and the control group. During the 8 weeks intervention, 6 subjects were drop out, they consist of 3 subjects from each group. In the end of the study, 70 subjects were analyzed (on treat analysis). The baseline characteristics of the subjects are shown in Table 1. Most of the characteristics were comparable between groups, except the serum hepcidin which was significantly higher in the supplemented group than control group. After the supplementation, the BMI percentile BMI and nutrient intake were not statistically different between groups (Table 2). The final analysis using ANCOVA and MANCOVA showed that the effects of EPA and DHA supplementation significantly decreased the serum levels of IL-6, hepcidin, and ferritin and significantly increased serum levels of iron and transferrin saturation (Table 3 and Table 4).

DISCUSSION

The major function of the adipocyte is to store energy during an excess food consumption and to release energy during a starvation. Recent studies indicate that adipose tissue is an endocrine organ producing several adipokines and cytokines with broad biological activity.⁶ The most important of cytokine mediators of inflammation produced by adipose tissue are IL-6 and TNF- α . In obese children, an increase of the cytokines, especially IL-6, directly stimulates hepcidin production by the liver.

Hepcidin is a 25-amino acid peptide. It is not only an iron-regulatory hormone but also an important link between host defense and iron metabolism. During an inflammation, hepcidin synthesis is markedly increased by a mechanism

Table 3 ANCOVA Comparing EPA and DHA Supplementation Effects on Serum IL-6, Hepcidin, Ferritin, Iron and Transferrin Saturation

Variable	Point estimate	95% CI	p
Serum IL-6 (pg/ml)	-1.9	-3.2; -0.6	0.004
Serum Hepcidin (nmol/l)	-8.8	-13.1; -4.6	0.0001
Serum Iron (μ g/dl)	13.3	4.7; 21.9	0.003
Serum Ferritin (ng/dl)	-11.7	-20.6; -2.8	0.011
Transferrin saturation (%)	4.6	1.9; 7.2	0.001

Table 4 MANCOVA of EPA and DHA Supplementation on Serum IL-6, Hepcidin, Ferritin, Iron and Transferrin Saturation

Variable	Point estimate	95% CI	p
Serum IL-6 (pg/ml)	-1.8	-3.1; -0.5	0.005
Serum Hepcidin (nmol/l)	-9.6	-14.0; -5.1	0.0001
Serum Iron (μ g/dl)	13.6	4.1; 23.2	0.006
Serum Ferritin (ng/dl)	-10.2	-19.6; -0.9	0.032
Transferrin saturation (%)	4.6	1.7; 7.6	0.002

independent of iron status or erythrocyte activity.¹¹ The cytokine IL-6 is an important inducer of hepcidin synthesis during an inflammation such as in obese children.

Hepcidin regulates systemic iron flow by binding itself to ferroportin and inducing its internalization and degradation.¹¹ Ferroportin is a transmembrane protein known as the sole cellular exporter of iron. Ferroportin is important for iron transfer from the mother to the fetus, for iron absorption in the intestine, and for iron export from macrophages. When iron stores are adequate, the liver produces hepcidin, which circulates to the small intestine. In the small intestine, hepcidin causes ferroportin to be internalized, blocking the pathway for the transfer of iron from enterocytes to plasma. When iron stores are low, hepcidin production is suppressed, ferroportin molecules are displayed on basolateral membranes of enterocytes, and there they transport iron from the enterocyte to plasma. When serum hepcidin concentrations are high such as in obese children, hepcidin binds to ferroportin, ferroportin is internalized, and iron export is blocked, resulting a low serum iron, a low transferrin saturation with normal or high serum ferritin concentration. The development of anti-inflammatory agents should be useful in the treatment of these conditions.

In our study, 45 mg EPA and 225 mg DHA (fish oil capsule) supplementation twice per day in six to ten years old obese children decreased serum IL-6 level by 1.9 pg/ml, decreased serum hepcidin level by 8.8 nmol/l, decreased serum ferritin level by 11.7 ng/dl. In contrast, it

increased serum iron concentration by 13.3 μ g/dl and increased transferrin saturation by 4.6%. The decreased serum IL-6, hepcidin, and ferritin and the increased of both serum iron and transferrin saturation were still significantly higher after adjusted of others confounding variable such as age, gender, BMI and nutrition intake using multivariate analysis.

We know that EPA (20:5n-3) and DHA (22:6n-3) are essential polyunsaturated fatty acids with anti-inflammatory effects. They decrease eicosanoid mediators production from arachidonic acid; increase anti-inflammatory and inflammation resolving resolvins production; and hinder pro-inflammatory cytokines and other pro-inflammatory proteins production induced via the NFkB system.¹⁰ Therefore, EPA and DHA supplementation improve inflammatory conditions, resulting decrease serum IL-6 and hepcidin levels and eventually improves the iron status of obese children. This study supports an argument claiming that the iron dysregulation status obese children was caused by inflammation, not by inadequate dietary iron intake. The data supports the hypothesis because dietary iron intake in both groups before and after supplementation was similar.

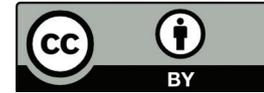
There had been no data about the benefit of EPA and DHA to improve iron status in obese children through decreasing serum IL-6 and hepcidin levels. The results of this study can be used as an alternative treatment of iron dysregulation caused by inflammation related to obesity in children.

It can be concluded that fish oil capsule supplementation (45 mg EPA and 225 mg DHA) twice a day for 8 weeks in six to ten-year-old obese children could improve the iron status through decreasing serum IL-6 and hepcidin.

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