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Effects of fish oils on *ex vivo* B-cell responses of obese subjects upon BCR/TLR stimulation: a pilot study☆

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Abstract

The long-chain n-3 polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) in fish oil have immunomodulatory properties. B cells are a poorly studied target of EPA/DHA in humans. Therefore, in this pilot study, we tested how n-3 LC-PUFAs influence B-cell responses of obese humans. Obese men and women were assigned to consume four 1-g capsules per day of olive oil (OO, n=12), fish oil (FO, n=12) concentrate or high-DHA-FO concentrate (n=10) for 12 weeks in a parallel design. Relative to baseline, FO (n=9) lowered the percentage of circulating memory and plasma B cells, whereas the other supplements had no effect. There were no postintervention differences between the three supplements. Next, *ex vivo* B-cell cytokines were assayed after stimulation of Toll-like receptors (TLRs) and/or the B-cell receptor (BCR) to determine if the effects of n-3 LC-PUFAs were pathway-dependent. B-cell IL-10 and TNFα secretion was respectively increased with high DHA-FO (n=10), relative to baseline, with respective TLR9 and TLR9 + BCR stimulation. OO (n=12) and FO (n=12) had no influence on B-cell cytokines compared to baseline, and there were no differences in postintervention cytokine levels between treatment groups. Finally, *ex vivo* antibody levels were assayed with FO (n=7) after TLR9 + BCR stimulation. Compared to baseline, FO lowered IgM but not IgG levels accompanied by select modifications to the plasma lipidome. Altogether, the results suggest that n-3 LC-PUFAs could modulate B-cell activity in humans, which will require further testing in a larger cohort.

Keywords: Fish oil; B cells; Cytokines; Antibody levels; Lipidomics; Toll-like receptors; B-cell receptor

1. Introduction

The long-chain n-3 polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) are consumed in low levels in the Western Diet [1,2]. There is evidence that increasing the consumption of these fatty acids has potential health benefits for a range of inflammatory and autoimmune diseases [3]. Animal studies across model systems show that n-3 LC-PUFAs robustly improve inflammatory outcomes and aid in the resolution of inflammation [3–5]. However, studies in humans have generally provided mixed results about the effectiveness of n-3 LC-PUFAs for innate or adaptive immunity [3]. One major limitation in developing n-3 LC-PUFAs for clinical applications related to immunity is that their cellular targets and underlying mechanisms are not well delineated, particularly within the human population [6].

B cells are not well studied in response to n-3 LC-PUFA intervention at the human level. B cells are associated with antibody production but also play a role in cytokine secretion and antigen presentation to T cells. A series of rodent studies shows that n-3 LC-PUFAs, administered as fish oil or as purified ethyl esters, enhance B-cell cytokine secretion and/or antibody production in lean and obese mice [7–13]. Furthermore, rodent studies suggest that DHA is more effective than EPA in

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enhancing B-cell cytokine secretion and antibody production, presumably through a lipid-raft-mediated mechanism [9–12]. The enhancement in B-cell activity with n-3 LC-PUFAs is of biological relevance given that a range of metabolic diseases are associated with impaired B-cell responses. To exemplify, subjects recently diagnosed with diabetes have diminished production of B-cell cytokines [14]. Similarly, obese mice and humans, compared to lean controls, display lower antibody production in parallel with chronic inflammation upon influenza infection or vaccination [13,15–18]. Thus, n-3 LC-PUFAs may have potential health applications for select clinical populations that have impaired B-cell activity.

The overall goal of this study was to determine if administration of supplements containing n-3 LC PUFAs to obese subjects would influence select B-cell responses. The primary objectives of this double-blind study were to determine if administration of one of two fish oil (FO) supplements or olive oil (OO) in a parallel design would change the frequency of circulating B-cell subsets relative other immune cell populations and *ex vivo* B-cell cytokine secretion after stimulation with agonists targeting TLR1/2, TLR9, BCR + TLR9. Cytokine secretion was assessed in response to stimulation of the B-cell receptor (BCR) and Toll-like receptors (TLRs) to determine if the biochemical effects of n-3 PUFAs were mechanistically pathway dependent. Finally, *ex vivo* B-cell antibody production was assayed with one of the FO supplements upon BCR + TLR9 stimulation accompanied by a plasma lipidomic analysis. The rationale for the lipidomic analyses was to determine if known lipid meditators such as lipoxin A4 that modulate antibody production were modified by the FO supplement [19–22].

2. Methods

2.1. Subjects and inclusion/exclusion criteria

Obese men and women with a body mass index (BMI; kg/m^2) >30 were recruited from the general population (Table 1). Approval for the study was obtained by the East Carolina University Institutional Review Board. The recruitment strategy was the following. First, potential participants completed a phone screen for initial eligibility based on age, body weight, absence of pregnancy, and low consumption of fatty fish and/or fish oil supplements. After passing the initial phone screen, written informed consent was obtained before participation, and each subject received signed approval from a physician. Exclusion criteria for males and females were the following: blood clotting, taking aspirin, taking more than one fish meal per week, consuming short-chain or long-chain n-3 PUFA supplements in the last 3 months prior to enrollment, history of autoimmune diseases, allergies to fish or shellfish, atrial fibrillation or flutter, high LDL cholesterol, liver dysfunction, problems with blood clotting, underactive thyroid, or taking estrogen or thiazide diuretics. In addition, those females that were pregnant, breastfeeding or lactating were also excluded.

A total of 36 subjects were enrolled with 12 subjects randomly assigned per treatment group. Subjects were age-matched between the three supplement groups described below. One subject withdrew from the study due to mild discomfort from burping. Another subject started the use of an anti-inflammatory drug during the study and was therefore removed.

2.2. Intervention and study design

Enrolled participants were assigned to receive four identical 1-g capsules per day of either olive oil (OO), concentrated fish oil (FO) (~400 mg EPA/300 mg DHA ethyl esters per capsule) or a high-DHA concentrated FO (~100 mg EPA/500 mg DHA ethyl esters per capsule). The OO supplement provided 2.8 g of oleic acid per day, the FO concentrate supplement provided 1.7 g of EPA and 1.2 g of DHA per day, and the high-DHA concentrated FO provided 0.5 g EPA and 2.0 g DHA per day. The minor differences in dosing arose from the need to maintain the concentration of total oil constant at 4 g between the three arms of the study. The FO concentrate, the high-DHA-FO concentrate and olive oil were encapsulated and provided by AlaskOmega, Organic Technologies (Coshocton, OH, USA). The olive oil was not extra virgin olive oil. The fatty acid analyses of the three dietary supplements are provided in Table 2.

Supplements were provided for 12 weeks and blood was obtained prior to and after intervention. Subjects were instructed to consume two capsules with breakfast and two with dinner on a daily basis. The study was double-blinded and all samples were collected and stored with a subject number. Results were unblinded after all analyses were completed.

Subjects completed surveys at the initial and final blood draws to assess work behavior as a factor that can influence stress and food questionnaires to confirm that n-3 PUFA intake was low [23,24]. An additional survey was also administered to account for potential differences in physical activity [25]. Subjects were provided 6 weeks of supplements after which they were brought in to receive another round of supplementation for 6 weeks. Compliance was assessed based on pill count, as provided by the subjects at each visit, and was measured to be 100%. Compliance was also confirmed by measuring EPA and DHA levels in circulation. This pilot study was not registered at clinicaltrials.gov since the study did not have any clinical outcomes.

Table 1
Patient characteristics

Parameters	OO	FO	High DHA-FO
Age (mean and range)	42.50 (30–55)	45.33 (31–56)	39.10 (29–55)
BMI (mean and range)	39.39 (30.19–65.57)	40.15 (29.17–52.67)	35.72 (30.96–41.33)
Sex *n (%)*			
Female	9 (75.00%)	6 (50.00%)	3 (30.00%)
Male	3 (25.00%)	6 (50.00%)	7 (70.00%)
Race *n (%)*			
Caucasian	10 (83.33%)	10 (83.33%)	6 (60.00%)
African American	2 (16.67%)	1 (8.33%)	3 (30.00%)
Hispanic	0 (0%)	1 (8.33%)	0 (0%)
Asian	0 (0%)	0 (0%)	1 (10.00%)
Medications *n (%)*			
Biguanide	1 (8.33%)		2 (20.00%)
Antidepressant	1 (8.33%)	1 (8.33%)	2 (20.00%)
SSRI	1 (8.33%)	1 (8.33%)	1 (10.00%)
Statin	2 (16.67%)		
Insulin			2 (20.00%)
ACE inhibitor	2 (16.67%)	2 (16.67%)	2 (20.00%)
Benzodiazepine		1 (8.33%)	2 (20.00%)
GABA analog			1 (10.00%)
Xanthine oxidase reducer	1 (8.33%)		
Calcium channel blocker	1 (8.33%)		1 (10.00%)
Antihyperglycemic			
Antiepileptic		1 (8.33%)	
Proton pump inhibitor	2 (16.67%)		
Antihistamine			2 (20.00%)
Beta-blocker		1 (8.33%)	
Diuretic		1 (8.33%)	1 (10.00%)
Birth control		1 (8.33%)	1 (10.00%)

Subjects consumed OO, FO, or high-DHA-FO supplements for 12 weeks.

2.3. Peripheral blood mononuclear cell (PBMC) analyses

Subjects were fasted overnight before obtaining blood. Peripheral blood taken in vacutainer tubes (Franklin Lakes, NJ, USA) was diluted 1:1 in PBS followed by separation of PBMCs using Ficoll Paque (GE Healthcare, Washington, NC) gradient centrifugation. The following subsets were analyzed using a BDLSRII flow cytometer: $CD45^+CD3^+CD4^+$ (CD4 helper T cells), $CD45^+CD3^+CD8^+$ (CD8 cytotoxic T cells), $CD45^+CD3^-CD14^+$ (monocytes) and $CD45^+CD14^-CD19^+$ (B cells) [13]. All fluorophore antibody markers were obtained from Biolegend (San Diego, CA,USA) or Miltenyi Biotech (San Diego CA, USA) and consisted of: CD45 (PE), CD3 (Pacific Blue), CD4 (FITC), CD8 (PE-Cy5), CD14 (FITC) and CD19 (APC).

2.4. B-cell purification and analyses of subsets

B cells were isolated from PBMCs using a B-cell isolation kit II (Miltenyi Biotec) with a resulting purity of>99%. Given that the number of B cells was limited, B-cell subsets were analyzed from a fraction of the total number of subjects, as indicated in the results. The following subsets were analyzed using a BDLSRII flow cytometer: $CD19^+CD27^+IgD^+$ (memory B cells), $CD19^+CD27^-IgD^+$ (naïve B cells) and $CD19^+CD38^+CD27^+IgD^-$ (plasma cells). All fluorophore-conjugated antibodies were obtained from Biolegend or Miltenyi Biotech and consisted of CD19 (APC), CD27(Pacific Blue), CD38 (FITC) and IgD (PE-Cy7).

2.5. B-cell stimulation and proliferation

Purified human B cells were cultured in RPMI 1640 with 5% FBS, 2 mM L-glutamine, 50 μM 2-betamercaptoethanol, 10 mM HEPES and 50μg/ml gentamicin at a concentration of $2.9–3.1\times10^6$ cells/ml. B cells were stimulated with: (1) CpG oligodeoxynucleotides (ODN) 2395 (a TLR9 agonist; Hycult Biotech, Plymouth Meeting, PA, USA) and 1μg/ml plus BCR stimulation using rabbit anti-human IgM Ab fragment (Jackson ImmunoResearch, West Baltimore Pike, PA, USA) at a concentration of 2μg/ml; (2) PAM3CSK4 (a TLR1/2 agonist; Invivogen, San Diego, CA, USA) at a concentration of 10μg/ml; (3) CpG-ODN targeting TLR9 at a concentration of 10μg/ml. B cells were plated