Highly-purified eicosapentaenoic acid treatment improves nonalcoholic steatohepatitis

Naoki Tanaka, MD, PhD, [1,2], Kenji Sano, MD, PhD [3], Akira Horiuchi, MD, PhD [4], Eiji Tanaka, MD, PhD [2], Kendo Kiyosawa, MD, PhD [2], and Toshifumi Aoyama, PhD[1]

[1]Department of Metabolic Regulation, Shinshu University Graduate School of Medicine, [2]Department of Gastroenterology, Shinshu University School of Medicine, [3]Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, and [4]Department of Gastroenterology, Showa Inan General Hospital, Komagane, Nagano, Japan.

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Abbreviation: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; FFA, free fatty acid; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment for insulin resistance; NAFLD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; PUFA, polyunsaturated fatty acids; TNF-α, tumor necrosis factor alpha; sTNF-R, soluble tumor necrosis factor receptor.

Correspondence to: Naoki Tanaka, M.D., Ph.D, Department of Metabolic Regulation, Shinshu University Graduate School of Medicine, Asahi 3-1-1, Matsumoto 390-8621, Japan.
E-mail: naopi@hsp.md.shinshu-u.ac.jp
Fax: +81-263-32-9412
Phone: +81-263-37-2634

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Abstract

Recent studies have demonstrated that n-3 polyunsaturated fatty acids (n-3 PUFA) ameliorate nonalcoholic fatty liver disease. Although eicosapentaenoic acid (EPA), one of the major components of n-3 PUFA, is widely used as an anti-lipidemic agent, its single efficacy for nonalcoholic steatohepatitis (NASH) remains unclear. As such, we aimed to evaluate the efficacy and safety of EPA on 23 biopsy-proven NASH patients in a pilot trial. Highly-purified EPA (2,700 mg/day) was administered for 12 months and efficacy was assessed by biochemical parameters and liver histology. All patients completed the treatment with no adverse events, indicating acceptable tolerance to the treatment. After 12 months, serum alanine aminotransferase levels were significantly improved (from 79 $\pm$ 36 to 50 $\pm$ 20 U/L), and serum free fatty acids, plasma soluble tumor necrosis factor receptor 1 and 2 levels, and serum ferritin and thioredoxin levels, which may reflect hepatic oxidative stress, were significantly decreased. Body weight, blood glucose, insulin, and adiponectin concentrations remained unchanged. Seven of the 23 patients consented to undergo post-treatment liver biopsy, which showed improvement of hepatic steatosis and fibrosis, hepatocyte ballooning, and lobular inflammation in 6 patients. In conclusion, EPA treatment seems to be safe and efficacious for patients with NASH, largely due to its anti-inflammatory and anti-oxidative properties. To confirm these results, appropriately powered, controlled trials are needed.

Introduction

Nonalcoholic steatohepatitis (NASH) is characterized by hepatic steatosis with necroinflammation, hepatocyte ballooning, and perisinusoidal/perivenular fibrosis. Similarly to obesity and related metabolic disorders, such as impaired glucose tolerance and dyslipidemia, NASH is becoming a common type of chronic hepatitis both in Japan and worldwide[1,2] and may progress to cirrhosis and ultimately develop into hepatocellular carcinoma or hepatic failure.[3-6] In addition, NASH patients have been reported to experience a higher mortality compared with patients with simple steatosis because of a higher incidence of cardiovascular events and liver-related death.[6] Thus, NASH should be recognized as a serious disease and appropriate therapeutic interventions are needed. Various pharmacological treatments for NASH have been attempted with variable success, including insulin sensitizers,[7-10] vitamin E,[11] ursodeoxycholic acid,[12] and lipid-lowering agents, such as probucol[13] (reviewed by Ref. 1). However, specific and definitive therapeutic strategies against NASH have not yet been fully established.

There is evidence from epidemiologic studies and randomized controlled trials that supplementation of n-3 polyunsaturated fatty acids (n-3 PUFA), abundantly contained in fish oil, lowers blood triglyceride levels and reduces the risk of coronary heart disease, sudden death, and mortality.[14-17] Eicosapentaenoic acid (EPA, C20:5 n-3) is one of the principal components of n-3 PUFA, and highly-purified EPA is widely used for treatment of hyperlipidemia and atherosclerosis. The Japan EPA Lipid Intervention Study has demonstrated that increases in plasma EPA levels by the addition of EPA to statin has beneficial effects in preventing coronary heart diseases in hypercholesterolemic patients.[18] Recently, it has also been reported that treatment with n-3 PUFA, a mixture of EPA and docosahexaenoic acid, ameliorates hepatic steatosis and necroinflammation in humans[19] and rats,[20] probably due to a reduction of hepatic tumor necrosis factor (TNF)-α expression and improvement of insulin sensitivity. However, the efficacy of single treatment of EPA for NASH patients has not yet been elucidated.

In the present study, we conducted a pilot trial to evaluate whether highly-purified EPA treatment improves the biochemical and histological abnormalities found in patients with NASH.

Patients and Methods

Patients

Twenty-three patients with NASH were enrolled in this study. All patients demonstrated hepatic steatosis by abdominal ultrasonography and persistent abnormal serum alanine aminotransferase (ALT) levels ($\geq$45 U/L) for more than six months, regardless of repeated instructions to reduce calorie intake by dietitians, and were confirmed as having NASH by liver biopsy. Histological diagnosis of NASH was made according to the following criteria: macrovesicular steatosis mainly present in zone 3, hepatocellular ballooning and/or perisinusoidal/perivenular fibrosis. Exclusion criteria of this study were positive results for hepatitis B surface antigen or anti-hepatitis C virus antibody, other causes of chronic liver disease (e.g. drug-induced liver injury, autoimmune liver disease, Wilson's disease, hereditary hemochromatosis and α1-antitrypsin deficiency), secondary causes of NASH (e.g. tamoxifen treatment, surgical procedures), chronic alcohol consumption of more than 40 grams of ethanol per week, and persistent intake of any lipid-lowering drugs, such as fibrates, statins, or probucol.

After an overnight fast, patients were admitted to our hospital and underwent blood tests and percutaneous liver biopsy. At the time of admission, body mass index (BMI) was calculated, and a BMI of more than 25 kg/m^2 was considered as obese according to criteria for the Japanese population. Patients were considered to be hypertensive if their systolic/diastolic pressure was greater than 140/90 mmHg, or if they were taking anti-hypertensive drugs. Patients were considered to be diabetic if they had a fasting glucose level equal to or higher than 126 mg/dL, or if they were taking insulin or oral hypoglycemic drugs. Patients were diagnosed as having hyperlipidemia if their fasting serum levels of cholesterol and triglycerides were equal to or higher than 220 mg/dL and 150 mg/dL, respectively.

Intervention

Patients were told of the study following a liver biopsy and histological confirmation of NASH. In total, 23 patients with NASH (14 men and 9 women), aged 27-74 years (mean 56), underwent this trial between January 2004 and December 2005. Highly (>98%) purified EPA ethyl ester (ethyl all-cis-5, 8, 11, 14, 17-icosapentaenoate) (Epadel^R, Mochida Pharmaceutical Co, Ltd, Tokyo, Japan) was administered at a dose of 2,700 milligrams per day, a maximum dose certified by Japanese medical insurance system, for 12 months. All patients had been under dietary restrictions and some had previously been taking other agents, such as calcium channel antagonists, angiotensin II

receptor antagonists, or allopurinol for at least 12 months, though these interventions remained unchanged during the study. The presence of any adverse effects of EPA was monitored once a month verbally with patients and with physical and blood examinations. Compliance of EPA intake was assessed by repeated interviews and measurement of plasma EPA concentrations. Therapeutic efficacy was evaluated mainly by clinical and laboratory data. Afterwards, seven patients agreed to undergo repeated liver biopsy at the end of the trial and assessment of any changes in histological findings. Informed consent, in writing, was obtained from all patients in this study.

Laboratory examination

All data were obtained in a fasting state. Routine examinations were performed by standard methods. The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the following equation: [fasting plasma glucose level (mg/dL) x fasting plasma insulin level (μU/mL)]/405. Serum fatty acid composition was examined by gas chromatography. Plasma concentrations of tumor necrosis factor-α (TNF-α), soluble TNF-α receptor-1 (sTNF-R1), and sTNF-R2 were measured using enzyme immunoassay kits (R&D systems Inc., Minneapolis, MN). Serum adiponectin concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). Serum thioredoxin levels were measured using a sandwich ELISA kit (Redox Bioscience Inc., Kyoto, Japan).

Evaluation of hepatic steatosis using ultrasonography

B-mode ultrasonography was performed by the same operator who had not known about clinical data of the patients using a Hitachi model EUB-525 ultrasound device equipped with a 3.5 MHz convex-type transducer (Hitachi Medical Corp., Tokyo, Japan). The patients fasted for at least 12 hours before ultrasonography, and were examined at the beginning and end of the study. Liver echogenicity was scored in a blinded manner by an independent ultrasonographer on a four-grade scale as follows: Grade 0, no steatosis; Grade 1, mild steatosis (lightly and homogeneously increased liver echogenicity; obvious intra-hepatic vascular pattern; no profound attenuation); Grade 2, moderate steatosis (moderate increase in liver echogenicity; partial dimming of the intra-hepatic vessels; mild profound attenuation); and Grade 3, severe steatosis (diffuse increase in liver echogenicity; no longer visible intra-hepatic vessels; heavy profound attenuation).

Histological evaluation

Liver biopsy specimens were obtained using 14-G needles and immediately fixed in 10% neutral formalin. Sections were cut at 4-μm thicknesses and stained using hematoxylin and eosin and Azan-Mallory methods. Histological findings were assessed in a blinded fashion by an independent pathologist (KS), and were scored according to the staging/grading system proposed by Kleiner et al.[21] The nonalcoholic fatty liver disease (NAFLD) activity score (NAS) was defined as the unweighted sum of steatosis (0-3), lobular inflammation (0-3), and hepatocyte ballooning (0-2), which ranged from 0 to 8. In accordance with the report of the NASH Clinical Research Network, a NAS of $\geq$5 corresponded to a diagnosis of "definitive NASH", a score of 3-4 corresponded to "borderline NASH", and a score of <3 corresponded to "not NASH".

Ethics

This study was carried out in accordance with the World Medical Association Helsinki Declaration, and was approved by the ethics committee of Showa Inan General Hospital.

Statistics

Statistical analyses were performed using SPSS software 11.0J for Windows (SPSS Inc., Chicago, IL). Qualitative variables were expressed as numbers (percentages in parentheses) and were compared using the χ^2 test. Quantitative data were expressed as mean $\pm$ standard deviation and were compared using the paired two-tailed t test. A probability value of less than 0.05 was considered statistically significant.

Results

Baseline characteristics of the patients

The baseline characteristics of all 23 patients are shown in Table 1. Nineteen (83%) patients were obese, and mean BMI was 28.1 ± 3.2 kg/m^2. Thirteen (57%) and four (17%) patients had hypertension and diabetes, respectively. All patients had hyperlipidemia, and eleven (48%) and sixteen (70%) demonstrated elevation of serum cholesterol and triglyceride levels, respectively. Eleven (48%) patients showed grade 3 severe steatosis by abdominal ultrasonography, and all patients had a NAS of more than 5. The fibrosis stage of many patients was 1 or 2. Five (22%) patients had bridging fibrosis, and none had cirrhosis.

Effect of EPA on Biochemical Parameters

All patients completed the 12-month treatment. There were no differences in mean body weight before and after the treatment (73.7 ± 11.0 vs. 73.4 ± 11.2 kg, P=0.846). Compared to baseline, serum ALT (79 ± 36 vs. 50 ± 20 U/L, 26% decrease from baseline, P=0.002) and serum aspartate aminotransferase (AST) levels (50 ± 17 vs. 36 ± 15 U/L, 24% decrease, P=0.001) levels were both decreased after treatment (Figure 1). These improvements occurred progressively to month 6 of treatment, then leveled off after that. In five (22%) patients, serum ALT levels normalized (< 30 U/L) using EPA treatment; on the contrary, three (13%) showed no significant improvement in serum ALT levels (Figure 1). Platelet counts and serum levels of albumin, alkaline phosphatase, γ-glutamyltransferase, hyaluronic acid, and type 4 collagen 7S did not change.

Total cholesterol and free fatty acid (FFA) levels were improved after treatment (6% and 32% decrease from baseline, P=0.039 and 0.012, respectively), but triglyceride and high-density lipoprotein (HDL) cholesterol levels remained unchanged (Table 2). Serum fatty acid profile assays revealed that both EPA content and the proportion of EPA to arachidonic acid (C20:5, n-3 / C20:4, n-6) were markedly increased. Serum arachidonic acid concentrations were reduced with treatment (10% decrease, P=0.041). On the other hand, there were no significant changes in fasting glucose, insulin and adiponectin levels, HOMA-IR, or glycohemoglobin values after treatment, suggesting that the decrease in serum AST and ALT levels by EPA did not correlate with amelioration of insulin resistance and hypoadiponectinemia.

Serum proinflammatory cytokine levels, which may reflect the necroinflammatory activity of NASH,[22,23] were then examined. Mean plasma TNFα levels tended to be lower after treatment, but this decrease was not statistically significant. Plasma

sTNF-R1 and sTNF-R2 levels were found to be significantly decreased (10% and 15% decrease from baseline, P<0.001, respectively). Although serum iron concentrations and transferrin saturation ratios remained unchanged, serum ferritin concentrations were significantly reduced after 12 months (46% decrease, P<0.001). Serum thioredoxin levels, which is known to be elevated with an increase in hepatic oxidative stress,[24] were also decreased (27% decrease, P=0.036).

Effect of EPA on Ultrasonographic Findings

Twelve (52%) patients showed an improvement in steatosis grade: eleven (48%) patients had one level of reduction, and one (4%) patient had two levels of steatosis reduction. In five (22%) patients, steatosis disappeared completely after taking EPA for 12 months. Overall, the mean steatosis grade was significantly improved by the treatment (from 2.1 $\pm$ 0.9 to 1.6 $\pm$ 1.1, P=0.004).

Effects of EPA on Histological Findings

Seven (30%) patients consented to undergo repeated liver biopsy following treatment. The remainders refused post-treatment biopsy because of busyness of works and domestic duties (9 and 4 patients, respectively), and pain or discomfort experienced at the liver biopsy (3 patients). Changes in histological findings are shown in Table 3. The mean steatosis score decreased from 2.4 $\pm$ 0.5 to 1.7 $\pm$ 0.5, and the mean fibrosis score markedly improved from 1.7 $\pm$ 1.1 to 0.7 $\pm$ 0.5 after treatment. Four (57%) of the patients who underwent serial biopsy had at least one level of fibrosis score reduction, and two (29%) had two levels of reduction. No patients experienced worsened fibrosis. Mean scores of lobular inflammation and hepatocyte ballooning were also improved.

Overall, the mean NAS decreased from 6.1 $\pm$ 1.3 to 3.7 $\pm$ 1.4, and ameliorated in six (86%) patients. A histological response was defined as a reduction in NAS by 3 points or more, with an improvement of at least 1 point in each of steatosis, lobular inflammation, and hepatocyte ballooning. According to this strict definition, three (43%) patients underwent a histological response. Five (71%) patients had a NAS of less than 5, resulting in a downgrading from “definitive NASH” to “borderline” or “not NASH” at the end of the study.

Safety of EPA

No dropout was recorded in the treatment group, and no adverse events occurred during EPA treatment. Bleeding tendency was not observed, even in patients having NASH with advanced fibrosis.

Discussion

In this 12-month pilot study, 87% of the patients who enrolled experienced a decrease in serum ALT levels, which became normal in 22% of cases. Similarly, an improvement of hepatic steatosis was documented by hepatic ultrasonography in 74% of patients. Eighty-six percent of 7 patients who underwent serial biopsy showed an improvement in several key features of NASH, including hepatic steatosis, fibrosis, lobular inflammation, and hepatocyte ballooning, and 43% demonstrated a significant improvement in NAS by more than 3 points. Moreover, no adverse reactions were reported by patients or found in tests. Although there is a previous report concerning the efficacy of n-3 PUFA, a mixture of EPA and docosahexaenoic acid, for ultrasonography-confirmed NAFLD, this is the first pilot trial to seek to evaluate the pharmacological effect of EPA alone in patients with biopsy-proven NASH.

EPA treatment reduced hepatic steatosis in patients with NASH, as evidenced by ultrasonographic and histological examinations. In steatotic livers, most hepatic triglycerides are derived from circulating FFA released mainly from adipose tissue and from de novo lipogenesis in hepatocytes.[25] It has been reported that EPA administration leads to a reduction of fatty acid synthase expression in white adipose tissues and a resultant decrease in plasma FFA concentrations in *ob/ob* mice.[26] Accordingly, a significant decrease in serum FFA concentrations by EPA treatment was found in the present study. Additionally, in livers, EPA suppresses de novo lipogenesis through down-regulation of sterol regulatory element-binding protein 1 (26). PUFA is also known to decrease lipogenic gene expression by reducing the DNA binding activities of nuclear factor Y, stimulatory protein 1, and possibly hepatic nuclear factor 4 (27). These molecular mechanisms may account for some of the findings obtained in this study.

Serum ferritin and thioredoxin levels, the surrogate markers reflecting hepatic oxidative stress at least in part,[24,28] significantly dropped after EPA treatment. EPA administration to mice has been found to markedly reduce hepatic malondialdehyde and 4-hydroxynonenal contents through enhanced expression of copper, zinc, and manganese-superoxide dismutases (Tanaka N and Aoyama T, unpublished results). Similarly, a decrease in hepatic triglyceride/FFA accumulation may also contribute to a reduction of hepatic lipid peroxides, and our results may indicate an unanticipated beneficial antioxidant effect.

Evaluation of histological findings and plasma sTNF-R levels confirmed that EPA treatment significantly ameliorated NASH activity. The EPA-induced anti-inflammatory effects are suspected to result from mechanisms such as decreased FFA-derived lipotoxicity in hepatocytes,[26,29] elimination of oxidative stress, antagonism of

arachidonic acid metabolism which produces proinflammatory eicosanoid derivatives such as 5-hydroxyeicosatetraenoic acid,[30] and suppression of nuclear factor-kappa B activation due to decreased degradation of its inhibitory subunit.[31,32] In addition, EPA supplementation might alter cell/organella membrane lipid composition and improve its fluidity,[33] probably leading to a decline in hepatocyte damage.

In this study, EPA did not increase serum adiponectin concentrations or improve insulin resistance. Recently, there have been several reports demonstrating the efficacy of pioglitazone for NASH.[7-9] In contrast with EPA, the chief pharmacological effect of pioglitazone is an adiponectin-mediated improvement in insulin sensitivity and enhancement of hepatic fatty acid metabolism rather than modulation of proinflammatory cytokines and oxidative stress.[34] Therefore, combination therapy of EPA and pioglitazone may compensate for the individual imperfections of these agents and become more efficacious for NASH treatment than each agent alone.

This study provided useful information about the safety of EPA administration at a dose of 2,700 milligrams per day for NASH patients. The most commonly reported serious adverse effect of EPA is bleeding tendency. Although bleeding was not observed in this study, careful monitoring is mandatory, especially in patients with advanced hepatic fibrosis.

Although Capanni et al. have previously clarified the steatosis-reducing effects of n-3 PUFA in NAFLD patients,[19] the other effects of PUFA have not yet been investigated. The present study preliminarily demonstrated an EPA-induced improvement not only of steatosis, but also of hepatic inflammation, fibrosis, and ballooning degeneration, all major elements of NASH. This is also the first study to demonstrate pre- and post-histological findings of EPA on NASH. Therefore, these results may advance our understanding of EPA treatment as a possible therapeutic option for NASH.

The major limitation of this study is lack of control group. As evidenced by recent placebo-controlled trials,[9] the probability of spontaneous hepatic fibrosis and ballooning necrosis regression is considered to be very low. As such, the improvements seen in this study should be regarded as EPA's absolute effects on NASH. Moreover, many participants were unwilling to be allocated to a control group after being explained the progressiveness of NASH and necessity of liver biopsy and adequate therapeutic interventions. To overcome the shortcomings of this study design and to further confirm our results, appropriately powered, large-scale trials are needed.

In conclusion, EPA treatment seemed to be safe and efficacious for patients with NASH. This beneficial effect was mainly attributed to its anti-inflammatory and

anti-oxidative properties. EPA might become one of the promising agents for treatment of NASH, and deserves further evaluation in a randomized, placebo-controlled study.

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Figure Legend

Figure 1. Changes in serum AST and ALT levels during treatment with EPA.
***, $P<0.01$ compared with baseline.