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Research Article

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Usefulness of Beta-Cryptoxanthin for Nonalcoholic Fatty Liver Diseases

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Abstract

We previously reported that in nonalcoholic steatohepatitis (NASH) patients, there was excess intake of carbohydrates and low intake of protein, polyunsaturated fatty acids and zinc. We also reported that a two-year modification of these diets was effective for improving anthropometric and biological parameters in NASH patients. There have been a few reports on NASH patients about the effectiveness of antioxidant carotenoids, especially beta-cryptoxanthin (b-crypt). In this study, we clarified intake and serum levels of b-crypt in NASH patients, and conducted a clinical trial to assess whether b-crypt inhibits the progression of NASH. Intake and serum levels of b-crypt were significantly lower in NASH patients than in nonalcoholic fatty liver (NAFL) patients and healthy controls. We randomly assigned NAFLD patients to receive either a trial beverage containing 3 mg of b-crypt or a placebo beverage for 12 weeks. In both NASH and NAFL patients, serum GGT levels were decreased with b-crypt treatment, as compared with placebo. Serum oxidative LDL and interleukin (IL)-6 levels were decreased and serum superoxide dismutase and IL-10 levels were increased in both NASH and NAFL patients who received b-crypt treatment. We also randomly assigned NAFLD patients to receive either a trial beverage containing 3 mg of b-crypt, 12 mg of Zn and 30 mg of a-tocopherol or a trial beverage containing only 3 mg of b-crypt. Both trial beverages had the same effect. These findings suggest that b-crypt treatment is effective in promoting anti-oxidant and anti-inflammation activities in NAFLD patients.

Keywords

Beta-cryptoxanthin; Antioxidant; Non-alcoholic steatohepatitis; Non-alcoholic fatty liver; Randomized control trial

Introduction

Nonalcoholic fatty liver disease (NAFLD) has become a common cause of chronic liver disease worldwide, and the prevalence of NAFLD is expected to increase. NAFLD affects approximately 30 million people in Japan and 1 billion people in the world [1-7]. NAFLD is a spectrum of clinicopathological conditions characterized by lipid deposition in the liver parenchyma of patients who have no history of excessive alcohol intake. Within this category, simple steatosis (NAFL) is benign, while nonalcoholic steatohepatitis (NASH), which is characterized by ballooning degeneration and sinusoidal/pericellular fibrosis, is a progressive disease to cirrhosis and hepatocellular carcinoma [8].

Although the exact pathogenesis of NASH is not clear, recent studies have shown that NASH is associated with insulin resistance and oxidative stress [9-13]. Insulin resistance causes metabolic syndrome, including glucose intolerance, dyslipidemia, hypertension and obesity, and it increases the risk for cardiovascular disease [14]. Diet is strongly related to insulin resistance [15]. We previously reported that in patients with NASH, there was an excess intake of carbohydrates, especially simple carbohydrates, and low intakes of protein, polyunsaturated fatty acids and zinc [16]. We also reported that an interventional diet, consisting of 30-35 kcal/kg of the patient's ideal body weight, 55% energy from carbohydrate, 20% from protein, 25% from fat, 1.0-2.0 ratio of polyunsaturated to saturated fatty acids, and sufficient vitamin A, C and E, and zinc for two years, was effective in improving anthropometric and biological parameters in patients with NASH [17]. The most common simple carbohydrates are glucose and fructose, which can result in a high glycemic index, leading to insulin resistance [18,19]. Polyunsaturated fatty acids increase insulin sensitivity in the liver, and vitamins and zinc have antioxidant effects in the liver [20,21].

Oxidative stress is thought to play a key role in the pathogenesis of liver injury. Therefore, antioxidant micronutrients, such as a-tocopherol and b-carotene, can be expected to protect against liver injury. Indeed, some recent intervention studies indicate that a diet high in antioxidants, including vitamins C and E and carotenoids, or pharmacological supplements of vitamins C and E, reduced serum liver enzymes and liver pathology [22-24].

An epidemiological approach concerning the association between antioxidant carotenoid levels and liver disease has not been well tested. Sugiura et al. [25] reported that antioxidant carotenoids, especially b-cryptoxanthin, may prevent earlier pathogenesis of nonalcoholic liver disease in Japanese subjects [26,27].

In this study, we first clarified intake and serum levels of b-cryptoxanthin in NASH patients. Intake and serum levels of b-cryptoxanthin were significantly lower in NASH patients than in NAFL patients and healthy controls. Second, we conducted a clinical trial (1) to assess whether b-cryptoxanthin inhibits the progression of NASH. In both NASH and NAFL patients, serum GGT levels were decreased with b-cryptoxanthin treatment, as compared with placebo. Third, we conducted a clinical trial (2) to assess whether additional treatment with a-tocopherol and Zn with b-cryptoxanthin is more effective than treatment with only b-cryptoxanthin. Both trial beverages had the same effect. Our current studies suggest that b-cryptoxanthin treatment is effective for lowering serum liver enzymes in NAFLD patients.

Subjects and Methods

Cross-sectional study

Thirty NASH patients, 30 NAFL patients, and 15 healthy volunteers (HC) were studied in the Department of Gastroenterology and Metabology in Ehime University Hospital from 2010 to 2012. A priori approval for the study was obtained from the Ehime University

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Hospital Research Ethics Board (#1009002). The following causes of liver disease were ruled out by appropriate examination: daily alcohol consumption greater than 30 g in men and 20 g in women, drug-induced hepatotoxicity, infection with Hepatitis B virus or Hepatitis C virus, genetic metabolic diseases, autoimmune liver diseases, biliary diseases, and thyroid diseases.

After providing written informed consent, each patient underwent a liver biopsy using a 16-guage needle. Samples longer than 15 mm were diagnosed. All samples were randomly collected and diagnosed by an experienced liver pathologist twice at a 1-month interval. All samples were diagnosed using the scoring system proposed by Brunt et al. [28].

Dietary habits and intake of nutrients were recorded for 3 consecutive days by each subject. Moreover, these were confirmed by detailed questioning by physicians and dieticians. These were converted to nutrient and food intake data using a computational database (Deltis, Olympus Optical Co., Japan) based on the 6th revised edition of The Standard Table of Food Composition in Japan.

Body mass index (BMI) was calculated as body weight [kg]/(body height [m])^2. Waist (cm) measurement was taken as the circumference between the lowest rib and the top of the pelvis.

Plain computed tomography (CT) was performed in the supine position in all patients within 1 month of diagnosis for evaluating abdominal fat deposition using the Light Speed Ultra 16 (GE Medical System, Milwaukee, WI). Visceral fat area (VFA, (cm^2)) and subcutaneous fat area (SFA, (cm^2)) at the navel level were estimated using NIH image software.

A history of diabetes mellitus was established in patients with fasting glucose ≥ 126 mg/dL or glycated hemoglobin (HbA1c) ≥ 6.5% or those under treatment. Patients with dyslipidemia were defined as those with fasting cholesterol ≥ 220 mg/dL, fasting triglyceride ≥ 150 mg/dL, or fasting HDL-cholesterol ≤ 40 mg/dL. Patients with hypertension were defined as those with systolic blood pressure ≥ 130 mmHg and/or diastolic pressure ≥ 85 mmHg. The following blood laboratory values were obtained in fasting conditions within 1 month of diagnosis: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), total protein, albumin, total cholesterol, HDL-cholesterol, triglyceride, plasma glucose (FPG), serum insulin (IRI), and HbA1c. Insulin resistance was assessed by HOMA-R calculated as FPG (mg/dL) × IRI (mU/L) / 405. Concentrations of six serum carotenoids, lutein, lycopene, a-carotene, b-carotene, b-cryptoxanthin and zeaxanthin, were analyzed by reverse-phase HPLC using b-apo-8'-carotenal as an internal standard at Kyoto Biken Laboratories Inc. (Kyoto, Japan), as described previously [25].

Clinical trial 1: Eighteen patients with NASH and 22 patients with NAFL, diagnosed histologically in the Department of Gastroenterology and Metabology of Ehime University Hospital from 2011 to 2012, were enrolled in this study. A priori approval for the study was obtained from the Ehime University Hospital Research Ethics Board (#1104003). We randomly assigned patients to receive the trial beverage containing 3 mg of b-cryptoxanthin (b-crypt group, n=20) or placebo (placebo group, n=20) for 12 weeks. Both trial beverages had 55 kcal in a 125 ml pack and were consumed daily. Three mg of b-cryptoxanthin is approximately the same amount found in three pieces of Japanese mandarin orange. The trial beverages were provided by Ehime Beverage Inc. (Ehime, Japan).

The following causes of liver diseases were ruled out by appropriate examination: daily alcohol consumption >30 g in men and >20 g in women, drug-induced hepatotoxicity, infection with hepatitis B virus or hepatitis C virus, genetic metabolic diseases, autoimmune liver diseases, biliary diseases, and thyroid diseases. Patients who had taken medications for hepatic steatosis, such as adrenocortical steroid hormones, anti-estrogen hormones, or tetracycline antibiotics, medications for appetite suppressing or weight loss, such as noradrenergic neurotransmitter agents or serotonin neurotransmitter agents, or who had undergone surgery of the gastrointestinal tract were excluded from this study. Cirrhotic patients were also excluded from this study.

The primary outcome was a decrease in serum liver enzymes (AST, ALT and GGT). Secondary outcomes were an improvement in glucose and lipid metabolism, as well as in oxidative and inflammatory markers.

Clinical trial 2: Forty patients with NAFLD diagnosed histologically in the Department of Gastroenterology and Metabology of Ehime University Hospital from January 2014 to April 2015 were enrolled in this study. A priori approval for the study was obtained from the Ehime University Hospital Research Ethics Board (# 1407005) and UMIN Clinical Trials Registry (# 000014736). We randomly assigned NAFLD patients to receive either an enriched trial beverage containing 3 mg of b-cryptoxanthin, 12 mg of Zn and 30 mg of a-tocopherol (b-cryp plus group, n=20) or an enriched trial beverage containing only 3 mg of b-cryptoxanthin (b-crypt group, n=20). Both trial beverages had 55 kcal in 125 ml pack and were consumed daily. The amount of 12 mg of Zn and 30 mg of a-tocopherol is the same amount found in a standard healthy supplement beverage in Japan. The trial beverages were provided by Ehime Beverage Inc. (Ehime, Japan). Inclusion criteria and exclusion criteria were same as the above clinical trial 1.

The primary outcome was a decrease in serum liver enzyme (AST, ALT and GGT). Secondary outcomes were an improvement in glucose and lipid metabolism, as well as in oxidative and inflammatory markers.

Statistical analysis

All observations were expressed as the means ± standard deviation (SD). Statistical analysis was performed using SPSS software version 14 (SPSS Japan Inc., Tokyo, Japan). Data were analyzed using Kruskal-Wallis test or Wilcoxon test. $P<0.05$ was considered significant.

Results

Cross-sectional study

Clinical data for all patients and healthy volunteers (HC) are shown in Table 1. There were 30 patients (12 male and 18 female) with NASH, 30 patients (14 male and 16 female) with NAFL, and 15 HC (4 male and 11 female). The mean age was 51 years (range, 20-74 years) in the NASH group, 50 years (range, 20-78 years) in the NAFL group, and 49 years (range, 28-62 years) in HC group. BMI and waist circumference were significantly higher in the NASH and NAFL groups than in the HC group. There were no differences in BMI, waist circumference, VFA or SFA between the NASH and NAFL groups. There were no differences in the prevalence of diabetes, dyslipidemia, or hypertension between the NASH and NAFL groups. Concerning blood chemistry, only AST, ALT, TG, FPG, HbA1c, IRI

Table 1: Baseline characteristics of NASH and NAFL patients and healthy volunteers.

	NASH patients (n=30)	NAFL patients (n=30)	Healthy volunteers (n=15)
Age (y)	51 ± 17	50 ± 14	49 ± 6
Sex (M/F)	16/12	14/16	4/11
BMI (kg/m^2)	28 ± 6[a]	27 ± 5[a]	23 ± 2
Waist (cm)	86 ± 12	86 ± 11	80 ± 12
VFA (cm^2)	147 ± 75	120 ± 89	ND
SFA (cm^2)	189 ± 123	157 ± 66	ND
Steatosis (%)	52 ± 21	44 ± 20	-
Grading (1/2/3)	16/12/2	-	-
Staging (1/2/3/4)	22/3/2/3	-	-
Complication of Diabetes (%)	42	37	0
Complication of Dyslipidemia (%)	68	60	0
Complication of hypertension (%)	63	53	0
(AST (IU/L)	60 ± 37[ab]	28 ± 11	20 ± 12
ALT (IU/L)	82 ± 76[ab]	41 ± 29	21 ± 14
GGT (IU/L)	79 ± 69[a]	79 ± 58	39 ± 16
Total protein (g/dL)	7.4 ± 0.6	7.3 ± 0.5	7.4 ± 0.5
Albumin (g/dL)	4.4 ± 0.5	4.6 ± 0.4	4.6 ± 0.3
Total cholesterol (mg/dL)	202 ± 38	198 ± 47	181 ± 40
HDL-cholesterol (mg/dL)	48 ± 14	56 ± 25	59 ± 21
Triglyceride (mg/dL)	162 ± 100[ab]	117 ± 51	108 ± 47
Uric acid (mg/dL)	6.1 ± 1.6[a]	6.0 ± 1.9[a]	5.0 ± 0.7
FPG (mg/dL)	128 ± 64[ab]	112 ± 35[a]	96 ± 11
HbA1c (%)	6.8 ± 1.5[ab]	6.4 ± 1.8[a]	5.1 ± 0.5
IRI (mU/L)	18.6 ± 16.1[ab]	8.8 ± 5.2[a]	5.0 ± 1.2
HOMA-R	5.8 ± 2.6[ab]	2.4 ± 1.6[a]	1.2 ± 0.7

Data are presented as mean ± SD

ND: not determined

a: Significant difference from the healthy volunteers group ($p<0.05$)

b: Significant different from the NAFL group ($p<0.05$)

and HOMA-R levels were significantly higher in the NASH group than in the NAFL group.

Concerning dietary habits, there were no differences in the frequency or duration of eating among the groups. The dietary intake of energy, major nutrients and b-cryptoxanthin in each group are shown in Table 2. There were no marked differences in the intake of energy, carbohydrates, protein or fat between the NASH and NAFL groups. Intake of vitamins C, retinol, b-cryptoxanthin and zinc in the female NASH group was significantly lower than in the female NAFL group.

Serum carotenoid concentration levels are shown in Table 3. Only b-cryptoxanthin levels were significantly lower in the NASH and NAFL groups than in HC group. Retinol and b-cryptoxanthin levels were significantly lower in the female NASH group than in the female NAFL group.

Clinical trail 1: Clinical data of patients who completed 12 weeks with the trial beverage are presented in Table 4. Among the 40 patients included in the study, one NASH patient receiving the placebo beverage dropped out. Of the 17 NASH patients, 9 patients received b-crypt treatment and 8 received placebo. Of the 22 NAFL patients, 11 patients received b-crypt treatment and 11 received placebo. There were no differences in BMI, waist circumference, VFA or SFA between groups. There were no differences in staging or grading of NASH between the b-crypt and placebo groups. Concerning baseline blood chemistry, there were no differences between NASH patients receiving b-crypt and those receiving placebo, or between NAFL patients receiving b-crypt and those receiving placebo (Table 5). None of 39 patients who completed this study changed their medications during this study period.

Serum b-cryptoxanthin levels after b-crypt treatment were significantly increased from 0.19 to 3.68 μg/mL in the NASH group and were significantly increased from 0.30 to 3.92 μg/mL in the NAFL group, while there was no change in the placebo group.

In NASH patients, serum AST, ALT and GGT levels were significantly decreased with b-crypt treatment as compared with placebo. In NAFL patients, only serum GGT level was decreased with b-crypt treatment as compared with placebo. Serum oxidative LDL and interleukin (IL)-6 levels were significantly decreased and serum superoxide dismutase (SOD) and IL-10 levels were significantly increased in both NASH and NAFL patients who received b-crypt treatment (Table 5).

Clinical trial 2: Clinical data for all patients are presented in Table 6. All patients completed 12 weeks with the trial beverage. In the b-crypt group, 4 patients were NASH and 16 were NAFL. In the b-crypt plus group, 3 were NASH and 17 were NAFL. There were no differences in BMI, waist circumference, VFA or SFA between groups. There were no differences in staging or grading of NASH between groups. Concerning baseline blood chemistry, there was no marked difference between the b-crypt and b-crypt plus groups (Table 7). Patients who completed this study did not change their medications during this study period.

Serum b-cryptoxanthin and a-tocopherol levels after b-crypt plus treatment were significantly increased from 0.39 to 3.24 μg/mL and from 4.9 to 5.9 μg/mL, respectively, while there was no change in serum Zn levels. Serum b-cryptoxanthin levels after b-crypt treatment were significantly increased from 0.23 to 2.61 μg/mL, while there were no changes in serum a-tocopherol or Zn levels.

Serum GGT levels were decreased in b-crypt and b-crypt plus groups. Serum oxidative LDL and IL-6 levels were decreased and serum SOD and IL-10 levels were increased in both groups (Table 7).

Discussion

Whereas most cases of NAFLD remain free of inflammation, 10-20% of patients develop inflammation and fibrosis, as NASH. Inflammation may precede steatosis in certain instances. Therefore, NASH could reflect a disease where inflammation is followed by steatosis. In contrast, NASH subsequent to simple steatosis may be the consequence of a failure of antilipotoxic protection. In both situations, many parallel hits derived from the gut and/or the adipose tissue may promote liver inflammation. Endoplasmic reticulum stress and related signaling networks, adipokine, cytokine, and innate immunity are emerging as central pathways that regulate key features of NASH [13].

The main pathogenesis and progression of NASH are thought to be based on insulin resistance and increased oxidative stress [13]. Increased body weight, especially visceral fat, causes insulin resistance and increment of free fatty acids in the portal vein or systemic circulation and hepatic fat deposits. Insulin resistance and hepatic steatosis also lead to increase oxidative stress in the hepatocytes. Diet is strongly related to fat deposit and insulin resistance. We previously

Table 2: Daily intake of main dietary constituents in NASH and NAFL patients and healthy volunteers.

	Male (n=30)			Female (n=45)		
	NASH (n=12)	NAFL (n=14)	Healthy (n=4)	NASH (n=18)	NAFL (n=16)	Healthy (n=11)
Total energy intake (kcal)	3240 ± 653[a]	3594 ± 597[a]	2213 ± 560	2944 ± 1947[a]	2961 ± 922[a]	1793 ± 532
Dietary carbohydrate (g)	443 ± 93[a]	394 ± 86[a]	297 ± 88	390 ± 260[a]	371 ± 153[a]	246 ± 116
Dietary fat (g)	116 ± 28[a]	154 ± 25[a]	59 ± 21	107 ± 85[a]	115 ± 42[a]	54 ± 22
Dietary protein (g)	93 ± 25[a]	122 ± 24[a]	80 ± 25	88 ± 49[a]	97 ± 26[a]	68 ± 19
Zn (mg)	8.4 ± 3.5	8.6 ± 3.1	8.3 ± 3.0	9.4 ± 2.3[b]	11.8 ± 9.1	8.1 ± 1.7
Vitamin C (mg)	80 ± 138	77 ± 99	102 ± 112	113 ± 124	197 ± 173	121 ± 63
Vitamin E (mg)	11 ± 18	12 ± 27	9 ± 30	7 ± 5	10 ± 7	12 ± 2
Retinol (μgRE)	834 ± 925	820 ± 817	842 ± 827	858 ± 742[b]	1037 ± 894	869 ± 912
b-Cryptoxanthin (μg)	237 ± 208	263 ± 157	308 ± 121	299 ± 173[b]	537 ± 235	352 ± 123

Data are presented as mean ± SD
a: Significant difference from the healthy volunteers group ($p<0.05$)
b: Significant difference from the NAFL group ($p<0.05$)

Table 3: Serum carotenoid concentration levels in NASH and NAFL patients and healthy volunteers.

	Male (n=30)			Female (n=45)		
	NASH (n=12)	NAFL (n=14)	Healthy (n=4)	NASH (n=18)	NAFL (n=16)	Healthy (n=11)
Retinol (μg/dL)	90 ± 26[b]	110 ± 17	109 ± 32	79 ± 25[b]	114 ± 32	90 ± 21
Lutein (μg/mL)	0.20 ± 0.10	0.19 ± 0.03	0.20 ± 0.05	0.28 ± 0.07	0.20 ± 0.06	0.22 ± 0.06
Lycopene (μg/mL)	0.19 ± 0.09	0.24 ± 0.10	0.29 ± 0.08	0.33 ± 0.18	0.34 ± 0.12	0.29 ± 0.11
a-Carotene (μg/mL)	0.05 ± 0.04	0.06 ± 0.01	0.07 ± 0.01	0.09 ± 0.08	0.09 ± 0.03	0.09 ± 0.07
b-Carotene (μg/mL)	0.16 ± 0.10	0.21 ± 0.10	0.17 ± 0.06	0.47 ± 0.29	0.32 ± 0.15	0.34 ± 0.20
Zeaxanthin (μg/mL)	0.13 ± 0.06	0.11 ± 0.01	0.11 ± 0.03	0.15 ± 0.04	0.13 ± 0.04	0.12 ± 0.04
b-Cryptoxanthin (μg/mL)	0.13 ± 0.12[a]	0.14 ± 0.07[a]	0.21 ± 0.02	0.21 ± 0.10[ab]	0.31 ± 0.14[a]	0.45 ± 0.11

Data are presented as mean ± SD
a: Significant difference from the healthy volunteers group ($p<0.05$)
b: Significant difference from the NAFL group ($p<0.05$)

Table 4: Baseline characteristics of NASH and NAFL patients in clinical trial 1.

	NASH (n=17)		NAFL (n=22)	
	b-crypt (n=9)	placebo (n=8)	b-crypt (n=11)	placebo (n=11)
Age (y)	63 ± 11	63 ± 8	63 ± 7	62 ± 9
Sex (M/F)	3/6	5/3	3/8	4/7
BMI (kg/m^2)	26 ± 4	26 ± 4	27 ± 6	28 ± 5
Waist (cm)	92 ± 8	92 ± 11	94 ± 14	97 ± 12
VFA (cm^2)	105 ± 35	137 ± 28	104 ± 42	119 ± 62
SFA (cm^2)	214 ± 79	159 ± 35	138 ± 36	178 ± 64
Complication of diabetes (%)	44	50	36	36
Complication of dyslipidemia (%)	55	62	54	54
Complication of hypertension (%)	66	75	54	54

Data are presented as mean ± SD

reported that in patients with NASH, there was an excess intake of carbohydrates and low intakes of protein, polyunsaturated fatty acids and zinc [16]. Excess carbohydrates lead to insulin resistance, whereas sufficient polyunsaturated fatty acids increase insulin sensitivity in the liver, and zinc has an antioxidant effect in the liver.

There are many reports on intake or serum levels of another antioxidant, vitamin E and carotenoids, in NAFL and NASH [25-27,29,30]. These micronutrient antioxidant deficiencies may contribute to the development of greater adiposity and comorbidities, such as insulin resistance and chronic liver diseases. Carotenoids are as potent in inhibiting lipid peroxidation as vitamin E. A-carotene, b-carotene and b-cryptoxanthin are provitamin A carotenoids which are converted to retinol in the body. Retinoic acid is synthesized intracellular from retinol and plays a regulatory role in lipid/glucose homoeostasis and type 2 diabetes [31]. Among the six main carotenoids, provitamin A carotenoids might be more effective against liver dysfunction and its related metabolic disorders than other non-provitamin A carotenoids, such as lycopene, lutein or zeaxanthin.

B-cryptoxanthin is a xanthophyll carotenoid that is particularly abundant in the Japanese mandarin orange [32,33] and is relatively abundant in human plasma [25,26]. Serum b-cryptoxanthin concentrations have been found to be inversely associated with indices of oxidative DNA damage and lipid peroxidation [34]. Animal experiments and in vitro studies have shown that b-cryptoxanthin has anti-inflammatory effects, primarily by modulating the innate

Table 5: Changes in serum biomarkers at baseline and after trial beverage intervention in clinical trial 1.

	NASH (n=17)		NAFL (n=22)	
	b-crypt (n=9)	**placebo (n=8)**	**b-crypt (n=11)**	**placebo (n=11)**
AST (IU/L)				
Baseline	52 ± 30	38 ± 30	27 ± 9	27 ± 10
After	43 ± 20[a]	41 ± 36	30 ± 9	27 ± 12
ALT (IU/L)				
Baseline	56 ± 33	47 ± 26	29 ± 11	27 ± 16
After	46 ± 21[a]	47 ± 35	31 ± 11	26 ± 18
GGT (IU/L)				
Baseline	48 ± 38	37 ± 30	73 ± 127	36 ± 24
After	45 ± 27[a]	54 ± 63	62 ± 86[a]	39 ± 25
FPG (mg/dL)				
Baseline	122 ± 31	131 ± 37	118 ± 15	112 ± 17
After	123 ± 25	128 ± 34	117 ± 20	123 ± 21
HbA1c (%)				
Baseline	6.8 ± 1.4	6.8 ± 0.7	6.6 ± 0.7	6.4 ± 0.4
After	6.7 ± 1.1	7.1 ± 0.6	6.8 ± 0.8	6.2 ± 0.5
HOMA-R				
Baseline	3.9 ± 2.5	3.0 ± 2.4	2.6 ± 1.5	3.1 ± 2.9
After	3.7 ± 1.9[a]	2.9 ± 1.5	2.7 ± 2.2	3.8 ± 2.4
Total cholesterol (mg/dL)				
Baseline	175 ± 25	202 ± 44	176 ± 30	187 ± 21
After	171 ± 37	202 ± 49	176 ± 33	191 ± 35
HDL-cholesterol (mg/dL)				
Baseline	48 ± 7	45 ± 9	51 ± 12	49 ± 9
After	46 ± 8	44 ± 8	50 ± 13	52 ± 11
Triglyceride (mg/dL)				
Baseline	119 ± 41	119 ± 21	115 ± 68	129 ± 39
After	111 ± 64	135 ± 31	113 ± 64	131 ± 60
Uric acid (mg/dL)				
Baseline	5.5 ± 1.4	6.5 ± 1.7	5.1 ± 1.2	4.9 ± 0.7
After	5.6 ± 1.6	6.2 ± 1.7	4.9 ± 1.1	5.0 ± 0.9
oxLDL (ng/mL)				
Baseline	236 ± 97	53 ± 28	78 ± 77	117 ± 124
After	205 ± 121[a]	52 ± 22	72 ± 72[a]	125 ± 135
SOD (ng/mL)				
Baseline	8.2 ± 2.2	12.6 ± 5.0	8.4 ± 2.4	11.0 ± 1.6
After	13.1 ± 3.7[a]	9.6 ± 3.6	11.0 ± 1.7[a]	11.4 ± 2.9
IL6 (pg/mL)				
Baseline	3.1 ± 2.9	2.0 ± 0.6	4.0 ± 2.5	2.7 ± 1.6
After	2.1 ± 0.5[a]	2.5 ± 0.3	2.8 ± 2.6[a]	2.6 ± 1.6
IL10 (pg/mL)				
Baseline	8.1 ± 5.2	10.4 ± 7.2	7.7 ± 6.2	8.4 ± 4.8
After	11.0 ± 6.5[a]	6.8 ± 5.3	11.0 ± 7.0[a]	6.4 ± 7.4

Data are presented as mean ± SD

a: significant different from baseline ($p<0.05$, Wilcoxon test)

immune response induced by macrophages [35]. B-cryptoxanthin ameliorates diet-induced NASH in mice (mice fed a high-cholesterol and high-fat diet) by suppressing inflammatory gene expression [36]. Ni et al. [37] reported b-cryptoxanthin prevented and even reversed insulin resistance and steatohepatitis by regulating both macrophage accumulation and M1/M2 status in a diet-induced lipotoxic model of NASH.

Concerning humans, Ruhl et al. [29] revealed in a large cross-sectional study that the risk for apparent liver injury was associated with decreased antioxidants, particularly carotenoids, in NAFLD patients in the Third National Health and Nutrition Examination Survey in the USA. High serum b-cryptoxanthin levels lower the risk of insulin resistance and alcohol-induced increases in serum GGT levels in non-diabetic subjects [25,26]. The longitudinal cohort study among middle-aged and older Japanese subjects showed that the risk of developing elevated serum ALT levels was inversely associated with baseline serum a- and b-carotene and b-cryptoxanthin levels [27]. However, these reports are analyses of NAFL, not NASH. In Germany serum levels of a-tocopherol, lutein, zeaxanthin, lycopene,

Table 6: Baseline characteristics of NAFLD patients in clinical trial 2.

	b-crypt plus (n=20)	b-crypt (n=20)
Age (y)	67 ± 9	65 ± 9
Sex (M/F)	9/11	8/12
BMI (kg/m^2)	25 ± 4	29 ± 4
Waist (cm)	94 ± 12	97 ± 14
VFA (cm^2)	109 ± 42	120 ± 65
SFA (cm^2)	146 ± 51	181 ± 67
Complication of diabetes (%)	25	30
Complication of dyslipidemia (%)	30	30
Complication of hypertension (%)	35	35
NAFL/NASH	17/3	16/4

b-crypt plus: b-crypt, a-tocopherol and Zn enriched beverage
b-crypt: b-crypt enriched beverage
Data are presented as mean ± SD

a-carotene and b-carotene were significantly lower in NASH patients than in controls [30]. Our current cross-sectional study was conducted with NASH, NAFL and healthy volunteers. Only serum b-cryptoxanthin levels were significantly lower in the NASH and NAFL groups than in the healthy volunteer group, and serum retinol and b-cryptoxanthin levels were significantly lower in the female NASH group than in the female NAFL group. It is generally accepted that serum b-cryptoxanthin levels are correlated with b-cryptoxanthin intake [38]. Given the decreased levels of lipophilic antioxidant supplementation, b-cryptoxanthin might be an especially rational treatment option for patients with NASH.

Given the high prevalence of obesity in NAFLD patients, ameliorating insulin resistance through weight loss remains the cornerstone of therapy for NAFLD. We previously reported that in 18 patients who completed 24 months of dietary intervention, the biological parameters, such as mean AST, ALT, GGT and HbA1c, were significantly decreased with improvement of BMI and VFA and insulin resistance [17]. In our previous study, in the patients who completed the diet intervention, intakes of total energy, intermittent snack energy and fat were decreased, and intake of protein was increased. Also energy consumption from exercise was increased. As a result, in patients who completed the diet intervention, BMI was decreased as about 5 % and VFA was decreased as 11 %, and serum transaminase and GGT were improved.

However, sustained lifestyle modification may be difficult in modern society. Many agents, including insulin sensitizers, have been tested with disappointing results in the management of NASH. Only a-tocopherol has yielded some promise in the treatment of patients with NASH. Eight hundred IU daily of lipophilic antioxidant a-tocopherol was associated with reduced hepatic steatosis, lobular inflammation and fibrosis [23]. However, the need for additional and more effective therapies for patients with NASH still remains. We hypothesized that the administration of b-cryptoxanthin would exert a beneficial effect on the prevention and therapy of NASH by suppressing oxidative stress. In this study, in both NASH and NAFL patients, serum GGT levels were decreased with additional b-crypt 3 mg treatment, as compared with placebo. Twelve mg of Zn and 30 mg of a-tocopherol in addition to the 3 mg of b-cryptoxanthin showed no additional effects in NAFL or NASH patients. Amount of 12 mg of Zn and 30 mg of a-tocopherol is the same amount found in a standard healthy supplement beverage in Japan. Referring The Dietary Reference Intakes for Japanese [39], amount of recommended dietary allowance of Zn is 10 mg daily in male and 8 mg daily in female, and amount of adequate intake of a-tocopherol is 6.5 mg daily in male and 6 mg daily in female. More amount of Zn and a-tocopherol than those in the current study may be effective for NAFL and NASH improvement, however, excessive intake of a-tocopherol causes adverse health effects because of its character storing in adipose tissues.

Gene expression analysis showed that although b-cryptoxanthin histochemically reduced steatosis, it was more effective in inhibiting the inflammatory gene expression change in NASH. In fact,

Table 7: Changes in serum biomarkers at baseline and after trial beverage intervention in clinical trial 2.

	b-crypt plus (n=20)	b-crypt (n=20)
AST (IU/L)		
Baseline	29 ± 13	33 ± 27
After	26 ± 6[a]	32 ± 20
ALT (IU/L)		
Baseline	31 ± 18	35 ± 27
After	27 ± 8[a]	36 ± 27
GGT (IU/L)		
Baseline	46 ± 36	48 ± 37
After	39 ± 16[a]	44 ± 34[a]
FPG (mg/dL)		
Baseline	136 ± 41	122 ± 26
After	133 ± 51	123 ± 33
HbA1c (%)		
Baseline	6.9 ± 0.9	6.7 ± 1.1
After	6.8 ± 0.8	6.6 ± 1.0
HOMA-R		
Baseline	3.6 ± 2.3	4.0 ± 2.2
After	3.7 ± 2.9	4.2 ± 2.3
Total cholesterol (mg/dL)		
Baseline	189 ± 36	190 ± 25
After	188 ± 36	189 ± 19
HDL-cholesterol (mg/dL)		
Baseline	54 ± 11	54 ± 11
After	55 ± 11	54 ± 11
Triglyceride (mg/dL)		
Baseline	133 ± 76	118 ± 47
After	135 ± 66	120 ± 50
Uric acid (mg/dL)		
Baseline	5.5 ± 1.3	5.6 ± 1.1
After	5.6 ± 1.1	5.9 ± 1.3
oxLDL (ng/mL)		
Baseline	381 ± 1006	351 ± 603
After	315 ± 839[a]	285 ± 516[a]
SOD (ng/mL)		
Baseline	5.4 ± 3.2	4.6 ± 2.2
After	6.6 ± 3.6[a]	5.8 ± 2.2[a]
IL6 (pg/mL)		
Baseline	2.9 ± 2.1	3.3 ± 1.5
After	1.8 ± 1.1[a]	2.4 ± 1.3[a]
IL10 (pg/mL)		
Baseline	4.7 ± 2.0	5.4 ± 1.6
After	5.3 ± 2.2[a]	6.9 ± 2.5[a]

Data are presented as mean ± SD
a: significant different from baseline ($p<0.05$, Wilcoxon test)

b-cryptoxanthin reduced the induction of the markers of macrophages, T helper cells, and cytotoxic T cells. The expression of LPS-inducible and/or TNFa-inducible genes was suppressed by b-cryptoxanthin, probably via the inhibition of macrophage activation. Thus, b-cryptoxanthin suppresses inflammation and subsequent fibrosis primarily by suppressing the increase and activation of macrophages and other immune cells. Reduction of oxidative stress is likely to be a major mechanism of suppression of inflammation and injury in the livers of mice with NASH [36]. Concerning oxidative stress and antioxidant markers in NAFLD, there are a few reports that serum oxidative LDL levels are higher in NASH than in NAFL, and serum antioxidant SOD levels are lower in NASH than in NAFL [12]. Concerning inflammatory cytokines in NAFLD patients, there are a few reports that serum pro-inflammatory IL-6 levels were trend to increased, and anti-inflammatory IL-10 levels were trend to decrease from NAFL to NASH [40]. In our b-crypt interventional study in NAFLD patients, additional b-crypt 3 mg/day improved serum GGT levels while also improving serum oxidative LDL, SOD, IL-6 and IL-10 levels.

Treatment regimens for NASH patients targeting insulin resistance, oxidative stress, obesity, diabetes, dyslipidemia, and hepatic fibrosis all warrant critical appraisal. Multiple modalities including diet, exercise, and pharmacotherapy, require evaluation to determine the most effective treatment algorithms.

Conclusion

In conclusion, our results suggest that beta-cryptoxanthin is effective in improving biological parameters in patients with NAFLD.

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