The official journal of the Japan Atherosclerosis Society and
the Asian Pacific Society of Atherosclerosis and Vascular Diseases

Original Article

J Atheroscler Thromb, 2022; 29: 881-893. http://doi.org/10.5551/jat.62832

Effects of Dietary Education Program for the Japan Diet on Cholesterol Efflux Capacity: A Randomized Controlled Trial

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Aim: Improving cholesterol efflux capacity (CEC) of high-density lipoprotein (HDL) has been regarded as a novel target for preventing cardiovascular disease. HDL reportedly has antioxidant properties which may contribute to its functions. We investigated changes in CEC with intake of the Japan Diet (JD) recommended by the Japan Atherosclerosis Society and the relationship of these changes to serum antioxidant concentrations.

Methods: A randomized parallel controlled clinical trial on JD intake was performed in Japanese patients with dyslipidemia. Ninety-eight participants were randomly divided into the JD ($n=49$) or the partial JD (PJD) ($n=49$) group. Nutrition education, based on each diet at baseline and at 3 months, was provided and the participants were followed up for 6 months.

Results: Mean CEC was 1.05 in total and correlated positively with HDL-cholesterol ($p<0.001$) at baseline. CEC did not change while oxygen radical absorbance capacity (ORAC) was decreased in both groups ($p<0.001$). Although serum total carotenoid increased in both groups, serum α-tocopherol decreased in the JD group as compared to the PJD group ($p<0.05$). CEC correlated positively with HDL ORAC at baseline ($p=0.021$) and with serum total carotenoid at 3 and 6 months ($p=0.005$, 0.035). Changes in CEC correlated positively with changes in HDL ORAC at 3 months and serum total tocopherol at 3 and 6 months ($p<0.001$).

Conclusion: CEC was not changed by JD education in Japanese patients with dyslipidemia who already had normal CEC at baseline. CEC was suggested to be positively associated with serum α- and γ-tocopherol and HDL ORAC.

Clinical trial registration number: UMIN000022955

Key words: Cholesterol efflux capacity, Diet, Oxygen radical absorbance capacity, Tocopherol, Carotenoid

Introduction

A low level of plasma high-density lipoprotein cholesterol (HDL-C) has been regarded as a strong and independent negative risk factor for cardiovascular disease (CVD)[1]. However, the classic hypothesis that high HDL-C concentrations lead to CVD risk reduction has been challenged by intervention studies[2-4]. Cholesterol efflux capacity (CEC) is one of the major functions of HDL, which contributes to the first step of the reverse cholesterol transport pathway by promoting efflux of excess cellular cholesterol.

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Received: January 26, 2021 Accepted for publication: April 13, 2021

CEC has been demonstrated to be an important factor preventing CVD[5, 6]. Therefore, improving CEC of HDL particles, rather than increasing HDL-C levels, is considered to be a novel target for the prevention of CVD. Although HDL has been shown to have antioxidant properties[7, 8] and certain antioxidant foods reportedly increase CEC[9, 10], there have been few studies focusing on the relationships of CEC with oxygen radical absorbance capacity (ORAC) and lipophilic dietary antioxidant levels in serum[11, 12].

Healthy dietary patterns are considered to be beneficial for the prevention of cardiovascular events. The Mediterranean diet recommends olive oil, nuts and vegetables, and emphasizes consuming large amounts of foods containing antioxidants such as α -tocopherol, polyphenols and carotenoids to protect against oxidation, and this diet has been reported to be beneficial for both preventing CVD[13] and increasing CEC[14].

The Japan Diet (JD) recommended by the Japan Atherosclerosis Society (JAS) for the prevention of atherosclerotic cardiovascular diseases[15, 16] features consuming more fish, soy and soy-products, vegetables, seaweed, mushrooms, konjak, and unpolished cereals instead of refined cereals, while concurrently consuming less animal fat and fatty meat and poultry, sweets and alcoholic drinks. We previously reported that a nutritional education program focusing on JD intake improved metabolic parameters in patients with dyslipidemia[17]. However, there have been no reports on the effects of the JD on CEC.

Aim

We investigated changes in CEC and the relationships of CEC with ORAC, and serum concentrations of tocopherols and carotenoids as lipid-soluble antioxidants, during a JD education program in patients with hyperlipidemia.

Materials and Methods

The study design, methods, and subjects were described in detail in our previous report[17]. Briefly, the recruited subjects were Japanese patients with dyslipidemia, between 30 and 65 years of age, with a body mass index (BMI) over 18.5 kg/m^2, non-smokers, receiving consistent drug regimens and permitted to participate in the program by doctors certified by the JAS.

This study was conducted at Japan Women's University according to the guidelines of the Declaration of Helsinki, and all procedures were approved by The Ethics Committee for Experimental Research involving Human Subjects of Japan Women's University (No.246). Written informed consent was obtained from all subjects prior to participation. The clinical trial registration number is UMIN000022955.

This was a 6-month randomized parallel-group clinical trial. Participants were allocated to either the JD group or the Partial JD (PJD) group. Face-to-face nutritional education for each diet, at baseline and at 3 months, was provided by three registered dietitians from Japan Women's University who had been specially trained for this study and we followed up the participants at 3 and 6 months.

For both diet groups, reductions in the intakes of animal fat, meat and poultry with fat, sweets, desserts and snacks, and alcoholic drinks were recommended. In addition, consuming more fish (especially fatty fish), soybeans and soy products (especially natto, a fermented soy product), vegetables including green and yellow vegetables, seaweed, mushrooms, konjak and unrefined cereals including barley were recommended for the JD group.

Three-day (two weekdays and 1 weekend day) weighted dietary records were kept at baseline, and at the ends of the 3- and 6-month follow-up periods. Nutrient intakes were calculated employing Excel-Eiyokun Ver.8.0, (Kenpaku-sha Co., Ltd., Tokyo, Japan) software.

At baseline, 3 months and 6 months, height, weight and blood pressure were measured and BMI was calculated as weight(kg)/height(m^2). Fasting blood collections were conducted in the morning following a 12-hour fast and the samples were centrifuged to obtain serum or plasma at each facility. Then, the serum or plasma samples were stored at −80℃ until analyses. The lipid parameters measured were total cholesterol (TC), low density lipoprotein-cholesterol (LDL-C), HDL-C, triglyceride (TG) and malondialdehyde-modified low-density lipoprotein (MDA-LDL).

Analysis

Preparation of Apolipoprotein B (ApoB)-Depleted Plasma/Serum

Plasma/serum samples were incubated with 20% polyethylene glycol solution in 0.2 mol/L glycine buffer (pH 7.4) for 20 minutes and these samples were centrifuged at 10,000 rpm and 4℃ for 10 minutes. The supernatants were apoB-depleted plasma/serum.

Cholesterol Efflux Capacity

The protocol for CEC was previously described[5, 18]. J774.1 cells (RIKEN, Saitama, Japan) cultured in Roswell Park Memorial Institute media containing

10% fetal bovine serum were kept under constant conditions of 5% carbon dioxide and 37℃. After the J774.1 cells had been seeded into 24-well plates and grown to 80% confluence, they were radiolabeled with 2 μCi/mL ^{3}H-cholesterol in the presence of acyl-CoA: cholesterol acyltransferase (ACAT) inhibitor (2 μg/mL Sandoz 58-035, Sigma-Aldrich Corp., St. Louis, Missouri, USA) and 0.3 mM 8-Br-cAMP (Sigma-Aldrich) for up-regulating ATP-binding cassette transporter (ABCA1). Subsequently, an efflux medium containing 2.8% apoB-depleted plasma was added, followed by incubation for 4 hours. A liquid scintillation counter was used to quantify the efflux of radioactive cholesterol from the cells. The quantity of radioactive cholesterol incorporated into the cells was counted twice, through hexane: isopropanol (v:v, 1:1) extraction in control wells not exposed to the serum. Relative efflux was calculated using the following formula: (cpm of ^{3}H-cholesterol in media containing 2.8% apoB-depleted serum - cpm of ^{3}H-cholesterol in serum-free media)/(cpm of ^{3}H-cholesterol before the efflux step). All assays were performed in duplicate. The CEC of patient plasma samples were expressed as the relative values to those of the pooled serum from six healthy volunteers.

Oxygen Radical Absorbance Capacity

The ORAC assay estimates the ability of serum to resist oxidative damage, reflecting the combined effects of all antioxidants in the serum, rather than any individual antioxidant. We measured serum ORAC and HDL ORAC using apoB-depleted serum.

Serum or apoB-depleted serum samples were diluted with phosphate buffer. Fluorescein (3', 6'-dihydroxy-spiro-3-one; 0.15 μmol/L, Sigma-Aldrich Corp., St. Louis, Missouri, USA) was added to the serum or apoB-depleted sera as a target of free radical attack with the water soluble peroxyl radical generator 2, 2'-azobis dihydrochloride (AAPH; 60 μmol/L, Sigma-Aldrich). Starting immediately after the addition of AAPH, fluorescence was measured every 2 minutes using the fluorescence spectrophotometer (TriStar LB942 Multimode Reader, Berthold Technologies GmbH & Co., KG, Bad Wildbad, Germany) for 90 minutes. The excitation wavelength was 485 nm, the emission wavelength 535 nm and the experimental temperature 37℃. A water-soluble Vitamin E analog, Trolox (Sigma-Aldrich), was used to establish a standard curve and the data were expressed as Trolox equivalents per volume. All assays were performed six times each.

Tocopherols and Carotenoids

Serum tocopherols and carotenoids were extracted according to the method reported by Johnson *et al.*, and by Khachik *et al.*[19, 20]. Serum was treated with ethanol to precipitate the proteins. Echinenone and tocol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) dissolved in ethanol were added as internal standards for the carotenoid and tocopherol analyses, respectively. The mixture was extracted twice with hexane containing 20 mg/L butylated hydroxytoluene. The serum extracts were redissolved in 200 μL of ethanol, vortexed and passed through a 0.45 μm membrane filter. A 20 μL aliquot was used for HPLC analysis.

The HPLC system consisted of a Chromaster 5110 Pump (Hitachi Co., Tokyo Japan), Chromaster Oven (Hitachi), Chromaster 5440 FL Detector (Hitachi) (α-tocopherol and γ-tocopherol were monitored at Ex 297 and Em 327 nm, respectively), Chromaster 5430 Diode Array Detector (Hitachi) (lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene were monitored at UV 450 nm and lycopene was monitored at UV 480 nm), a C30 carotenoid column (3 μm 150×4.6 mm, YMC), and a guard column (YMC-PARTS XPGCH-W1). The HPLC mobile phase and gradient procedure according to Yeum *et al.*[21] was methanol: methyl-tert-butyl ether: water (83:15:2, by vol, with 1.5% ammonium acetate in the water; solvent A) and methanol: methyl-butyl-ether: water (8:90:2, by vol, with 1% ammonium acetate in the water; solvent B). The gradient procedure, at a flow rate of 1 mL/min was as follows: 1) 90% solvent A and 10% solvent B for 5 min, 2) a 12-min linear gradient to 55% solvent A, 3) a 12-min linear gradient to 95% solvent B, 4) a 5-min hold at 95% solvent B, and 5) a 1-min gradient back to 90% solvent A and 10% solvent B.

Total tocopherol and carotenoid concentrations were calculated as the sum of α-tocopherol and γ-tocopherol and the sum of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene, respectively.

Statistical Analysis

Statistical analyses were carried out using SPSS for Windows (version 26; IBM Japan, Inc.). Normality was assessed by applying the Shapiro-Wilk test. All values are presented as means with standard deviation for the normal distribution and as medians (25^{th} percentile, 75^{th} percentile) for non-normal distributions. The *t* test or the Mann-Whitney *U* test was used to compare mean differences between the PJD and JD groups. The paired *t* test or Wilcoxon's signed-rank test was used to analyze differences from the baseline concentrations. The statistical significances of correlations were assessed using the Pearson or non-parametric Spearman's ranked test. *P*

Table 1. Changes in cholesterol efflux capacity and oxygen radical absorbance capacity

	PJD group ($n=49$)			JD group ($n=49$)			Between-group comparisons at 3 and 6 months (JD - PJD)	
	Mean	(SD)	$p^{\dagger}$	Mean	(SD)	$p^{\dagger}$	Difference (95% CI)	$p^{\ddagger}$
CEC[§]								
Baseline	1.05	(0.14)		1.04	(0.16)			
3-month	1.06	(0.17)	0.38	1.04	(0.16)	0.93	-0.01 (-0.05, 0.03)[‖]	0.58
6-month	1.03	(0.18)	0.31	1.02	(0.15)	0.31	0.00 (-0.05, 0.05)	0.91
HDL ORAC (TE μmol/L)								
Baseline	176	(26)		176	(24)			
3-month	173	(30)	0.23	174	(28)	0.46	1 (-6, 8)	0.73
6-month	161	(33)	0.000	160	(29)	0.000	-1 (-10, 8)	0.84
Serum ORAC (TE μmol/L)								
Baseline	276	(30)		276	(35)			
3-month	271	(29)	0.097	265	(36)	0.000	-6 (-13, 2)	0.15
6-month	257	(39)	0.000	253	(40)	0.000	-3 (-14, 8)	0.54

CEC, Cholesterol efflux capacity; ORAC, oxygen radical absorbance capacity; TE, trolox equivalent.
[†] *P* values within-group comparisons from baseline are analyzed by the paired *t* test.
[‡] *P* values between-group comparisons are analyzed by the unpaired *t* test.
[§] CEC is expressed as the ratio of efflux in the sample, normalized to a reference sample.
[‖] Values are expressed as means (95% CI).

<0.05 was considered to indicate a statistically significant difference.

Results

The characteristics and clinical backgrounds of the 98 participants (PJD: $n=49$, JD: $n=49$, imputations were used to replace missing data, employing the last observation carried forward method) with dyslipidemia at baseline and the changes in clinical parameters after the dietary interventions were detailed in our previous report[17]. At the 6-month follow-up, 43 participants (87.8%) in each of the diet groups had completed the study. Of these 43 participants, 79% answered that they had adhered to the recommended diet. Briefly, mean HDL-C at baseline was 62 mg/dL (range: 24 to 114 mg/dL) and 7% of participants had hypo-alpha lipoproteinemia defined as HDL-C levels less than 40 mg/dL, representing a minority with abnormal HDL metabolism based on quantity. After the intervention, BMI decreased in both groups ($p<0.001$). TC, LDL-C and TG concentrations were decreased at 6 months in the JD group as compared to the PJD group ($p<0.01$). HDL-C did not change in either group **(Supplemental Table 1)**.

Mean CEC was 1.05 (min 0.76, max 1.62) and the number of participants whose CEC was equal to or greater than 1.0 was 58 (60%) in total at baseline. CEC at baseline was independent of the presence/absence of lipid lowering therapy. CEC did not change in response to the intervention, though HDL ORAC and serum ORAC were decreased in both groups ($p<0.001$) and there was no difference between the two groups **(Table 1)**.

Serum total tocopherol, especially α-tocopherol, was decreased at 6 months in the JD group as compared to the PJD group ($p<0.05$). Serum total carotenoid increased in both groups after the dietary interventions. Changes in serum β-carotene, which accounted for more than 40% of all carotenoids, tended to be larger in the JD group than in the PJD group at 3 months ($p=0.055$) **(Table 2)**.

Food and nutrient intakes during the intervention were described in detail in our previous report. Briefly, intakes of energy, protein and fat were decreased at 6 months in both groups. There were no changes in either group's tocopherol intake. Though α and β-carotene intake was lower at baseline in the JD group than in the PJD group, changes in β-carotene ($p<0.001$), α-carotene and β-cryptoxanthin ($p<0.05$) intakes in the JD group ware larger than those in the PJD group at 3 months **(Supplemental Table 2)**.

In total, CEC showed a positive correlation with HDL-C at baseline, 3 months, and 6 months (r=0.72, 0.75 and 0.63, respectively). Furthermore, CEC correlated negatively with BMI and TG at all cross-sectional points and MDA-LDL at 3 and 6 months. CEC correlated positively with serum β-cryptoxanthin, α-carotene, and β-carotene at baseline ($p<0.05$), and the correlations with serum lutein+zeaxanthin and total carotenoid became significant at 3 and 6 months.

Table 2. Changes in serum tocopherol and carotenoid concentrations

	PJD group (n=49)			JD group (n=49)			Between-group comparisons at 3 and 6 months (JD - PJD)	
	Median	(25th percentile, 75th percentile)	p†	Median	(25th percentile, 75th percentile)	p†	Difference (95% CI)	p‡
Total tocopherol‖ (μmol/L)								
Baseline	32.3	(25.7, 37.0)		30.2	(28.1, 34.4)			
3-month	32.6	(26.6, 36.9)	0.97	29.8	(26.8, 32.2)	0.008	-1.3 (-3.0, 0.5)§	0.055
6-month	30.6	(26.7, 36.3)	1.00	28.4	(26.4, 30.9)	0.000	-1.6 (-3.6, 0.4)	0.010
α-tocopherol (μmol/L)								
Baseline	28.5	(23.4, 33.0)		27.3	(24.8, 30.4)			
3-month	28.4	(24.0, 33.0)	0.65	26.6	(23.5, 29.1)	0.008	-1.1 (-2.7, 0.6)	0.091
6-month	28.1	(24.4, 32.2)	0.73	25.6	(23.5, 27.1)	0.000	-1.6 (-3.4, 0.3)	0.012
γ-tocopherol (μmol/L)								
Baseline	3.1	(2.6, 4.2)		3.3	(2.5, 4.0)			
3-month	3.3	(2.4, 4.1)	0.47	3.2	(2.6, 3.9)	0.31	-0.2 (-0.6, 0.2)	0.22
6-month	3.0	(2.4, 4.3)	0.95	3.3	(2.6, 4.0)	0.16	-0.1 (-0.5, 0.3)	0.28
Total carotenoid¶ (μmol/L)								
Baseline	2.97	(2.29, 4.64)		2.73	(2.06, 4.26)			
3-month	3.44	(2.29, 4.89)	0.11	3.25	(2.21, 4.63)	0.000	0.27 (-0.18, 0.72)	0.14
6-month	3.97	(2.41, 5.57)	0.001	3.04	(2.04, 4.74)	0.005	-0.01 (-0.57, 0.55)	0.63
Lutein + zeaxanthin (μmol/L)								
Baseline	0.59	(0.53, 0.77)		0.55	(0.48, 0.64)			
3-month	0.62	(0.48, 0.89)	0.021	0.58	(0.47, 0.74)	0.063	0.00 (-0.07, 0.06)	0.81
6-month	0.70	(0.56, 0.90)	0.000	0.62	(0.49, 0.72)	0.061	-0.05 (-0.12, 0.01)	0.13
β-cryptoxanthin (μmol/L)								
Baseline	0.23	(0.13, 0.36)		0.20	(0.13, 0.34)			
3-month	0.24	(0.15, 0.37)	0.99	0.21	(0.15, 0.46)	0.069	0.12 (-0.02, 0.26)	0.17
6-month	0.29	(0.18, 0.45)	0.14	0.20	(0.16, 0.35)	0.16	-0.05 (-0.21, 0.11)	0.64
α-carotene (μmol/L)								
Baseline	0.30	(0.20, 0.45)		0.30	(0.19, 0.43)			
3-month	0.32	(0.21, 0.63)	0.071	0.31	(0.20, 0.51)	0.63	-0.01 (-0.09, 0.07)	0.11
6-month	0.38	(0.23, 0.54)	0.11	0.26	(0.18, 0.45)	0.44	0.00 (-0.11, 0.12)	0.10
β-carotene (μmol/L)								
Baseline	1.28	(0.61, 2.00)		0.98	(0.68, 1.94)			
3-month	1.32	(0.64, 2.42)	0.17	1.25	(0.72, 2.27)	0.000	0.15 (-0.07, 0.38)	0.055
6-month	1.59	(0.67, 2.47)	0.001	1.27	(0.62, 2.51)	0.000	0.18 (-0.15, 0.50)	0.66
Lycopene (trans+cis) (μmol/L)								
Baseline	0.58	(0.36, 0.97)		0.55	(0.30, 0.84)			
3-month	0.63	(0.35, 0.94)	0.78	0.51	(0.33, 0.79)	0.81	0.01 (-0.15, 0.16)	0.88
6-month	0.68	(0.41, 0.90)	0.19	0.51	(0.32, 0.81)	0.36	-0.08 (-0.25, 0.08)	0.11

† P values within-group comparisons from baseline are analyzed by the Wilcoxon signed-rank test.
‡ P values between-group comparisons are analyzed by the Mann–Whitney U test.
§ Values are expressed as means (95% CI).
‖ Total tocopherols are the sum of α-tocopherol and γ-tocopherol.
¶ Total carotenoids are the sum of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene.

The CEC change correlated positively with those in HDL-C, TG, both serum α- and γ-tocopherol, and HDL ORAC **(Table 3, Fig. 1)**. The HDL ORAC correlated positively with HDL-C at baseline and at 3 months. Change in HDL ORAC correlated positively with changes in serum total tocopherol at 3 and 6 months and with α-tocopherol at 3 months **(Table 4)**. Serum α-tocopherol showed positive correlations with TC, LDL-C, TG, and MDA-LDL at all cross-sectional points ($p<0.01$) and α-tocopherol change correlated positively with all lipid parameters including HDL-C ($p<0.01$).

Table 3. Correlation with CEC at baseline, 3 and 6 months and their changes

	baseline	3-month	6-month	⊿3-0 month	⊿6-0 month
Body Mass Index	-0.319**	-0.359**	-0.291**	0.117	0.023
LDL-cholesterol	0.113	0.058	-0.031	-0.045	0.042
HDL-cholesterol	0.720**	0.749**	0.627**	0.418**	0.463**
Triglyceride	-0.281**	-0.286**	-0.265**	0.217*	0.191
MDA-LDL	-0.036	-0.214*	-0.337**	-0.051	0.051
Total tocopherol†	0.184	0.123	0.173	0.428**	0.346**
α-tocopherol	0.168	0.131	0.162	0.361**	0.334**
γ-tocopherol	0.105	0.051	0.122	0.446**	0.231*
Total carotenoid‡	0.185	0.281**	0.215*	0.134	0.026
Lutein + zeaxanthin	0.196	0.309**	0.250*	0.138	0.047
β-cryptoxanthin	0.241*	0.201*	0.146	-0.153	0.029
α-carotene	0.265**	0.304**	0.221*	0.159	0.123
β-carotene	0.219*	0.313**	0.212*	0.094	0.035
Lycopene (trans+cis)	-0.080	-0.017	0.065	0.103	-0.027
HDL ORAC	0.232*	0.186	0.040	0.289**	0.188
serum ORAC	-0.022	-0.010	-0.079	-0.004	0.109

$n=98$ (PJD group, $n=49$; JD group, $n=49$)
CEC, Cholesterol efflux capacity; ORAC, oxygen radical absorbance capacity; MDA-LDL, malondialdehyde modified-low density lipoprotein.
Values are expressed correlation coefficients; r or rs (p values).
Values are analyzed using Pearson's or Spearman's rank correlation. *$p<0.05$; **$p<0.01$
†Total tocopherols are the sum of α-tocopherol and γ-tocopherol.
‡Total carotenoids are the sum of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene.

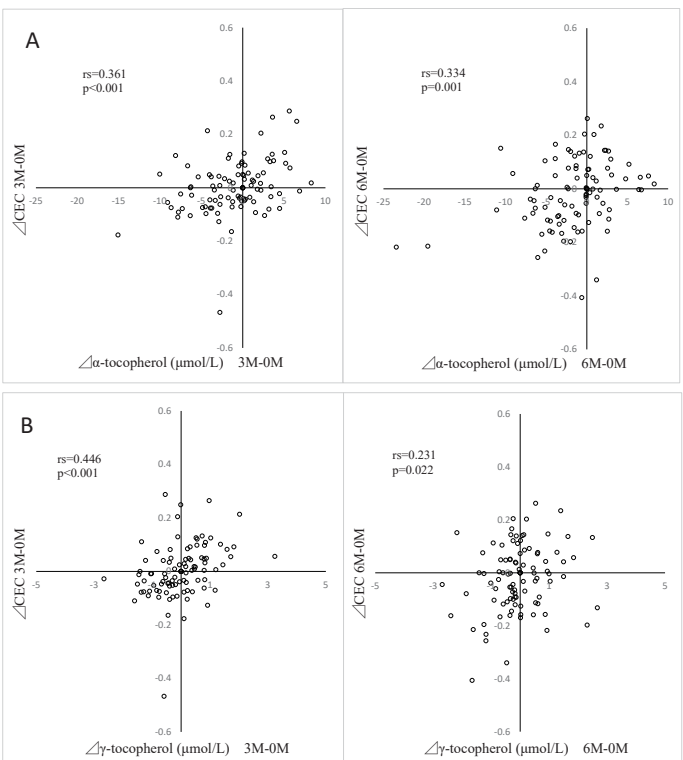

Fig. 1. Correlation between changes in CEC and serum α and γ- tocopherol concentrations at 3 and 6 months

$n=98$, Correlation between changes in CEC and (A) serum α-tocopherol and (B) γ-tocopherol.
The statistical significances of correlations were assessed using the non-parametric Spearman's ranked test. Changes in CEC correlated positively with changes in serum α and γ-tocopherol.

Table 4. Correlation with HDL ORAC at baseline, 3 and 6 months and their changes

	baseline	3-month	6-month	⊿3-0 month	⊿6-0 month
Body Mass Index	0.018	0.007	0.007	-0.046	0.134
LDL-cholesterol	0.182	-0.039	-0.033	0.079	0.064
HDL-cholesterol	0.217*	0.207*	0.150	0.222*	0.118
Triglyceride	0.088	0.058	0.058	0.051	0.069
MDA-LDL	0.082	0.065	0.030	0.088	0.071
Total tocopherol†	0.087	0.100	0.035	0.256*	0.202*
α-tocopherol	0.083	0.106	0.003	0.255*	0.196
γ-tocopherol	0.093	-0.030	0.098	0.119	0.185
Total carotenoid‡	-0.038	0.023	-0.116	0.216*	-0.056
Lutein + zeaxanthin	0.023	0.139	-0.032	0.161	-0.034
β-cryptoxanthin	-0.019	0.157	-0.040	0.022	0.020
α-carotene	-0.043	-0.128	-0.179	0.025	0.092
β-carotene	-0.047	-0.019	-0.113	0.200*	-0.074
Lycopene (trans+cis)	0.040	-0.052	-0.160	-0.021	-0.060
serum ORAC	0.380**	0.473**	0.382**	0.175	0.202*

$n=98$ (PJD group, $n=49$; JD group, $n=49$)
CEC, Cholesterol efflux capacity; ORAC, oxygen radical absorbance capacity; MDA-LDL, malondialdehyde modified-low density lipoprotein.
Values are expressed correlation coefficients; r or rs (p values).
Values are analyzed using Pearson's or Spearman's rank correlation. *$p<0.05$; **$p<0.01$
†Total tocopherols are the sum of α-tocopherol and γ-tocopherol.
‡Total carotenoids are the sum of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene.

Discussion

This is the first study to examine the effects of the JD on CEC in Japanese patients with dyslipidemia. We found that JD did not change CEC despite significant reductions in serum lipids in response to the JD[17].

CEC has been measured by several methods, which differ in the cells used, the transporters up-regulated such as ABCA1, ATP binding cassette subfamily G member 1 (ABCG1) and scavenger receptor class B member 1 (SRB1), and the types of reagents used for labeling. In this study, J774 cells were radiolabeled with ^{3}H-cholesterol in the presence of an ACAT inhibitor and 8-Br-cAMP to up-regulate ABCA1. Mean CEC at baseline was greater than 1.0, and CEC showed a high positive correlation with HDL-C, indicating CEC to not be impaired or to even be better than in healthy controls. We found that the JD did not change CEC despite serum lipids being significantly reduced by the JD[17], because the lack of change in CEC in response to the diet was partly due to the relatively good CEC at baseline. In previous reports employing the same method as this study, a PPAR-α agonist[22] and a PPAR-γ agonist increased CEC, while an HMG-CoA reductase inhibitor had no effect on CEC[5]. In this study, CEC did not differ among the participants regardless of whether or not a PPAR-α agonist was being taken, and none of the subjects had been prescribed a PPAR-γ agonist at baseline.

Though no difference in mean CEC was observed, MDA-LDL and TG were shown to correlate negatively with CEC at cross-sectional points in this study. Oxidized LDL measured by the ELISA method using an oxidized LDL antibody was revealed to have a negative association with CEC in obese Austrian adults[23], an observation consistent with the MDA-LDL results in this study. Reported correlations between CEC and circulating TG are not consistent. TG was reported to be positively associated with CEC in subjects with HDL-C <40 mg/dL but there was no association in subjects with HDL-C ≥ 40 mg/dL in the PHINOX study[24]. On the other hand, TG did not differ between groups of Japanese coronary artery disease patients with impaired versus enhanced CEC[25] and were not related in control participants in the EPIC-Norfolk study[26]. ABCA1-CEC, which is calculated as ABCA1 up-regulated CEC minus non up-regulated CEC, was higher in patients with TG $\geq$ 300 mg/dL than in healthy controls[27]. A positive association between TG and ABCA1-CEC was also reported in participants including those with coronary heart disease[28]. Although reasons for the mixed results due to lipid levels are unclear, different CEC methodologies, few subjects with TG $\geq$ 300 mg/dL in

the present study, and higher CEC values at baseline may account for the differences.

Regarding nutrients, serum α-tocopherol concentrations decreased in the JD group without changes in tocopherol intake. Around 60~90% of serum α-tocopherol is distributed to very low-density lipoprotein (VLDL) and LDL[29] and reportedly correlates positively with both LDL-C and TG[30]. Thus, the observed decrease in the serum α-tocopherol concentration might be due to the decreases in LDL-C and TG in response to JD intake. α-tocopherol is consumed as an antioxidant for prevention of lipid peroxidation[31]. It has also been reported that the higher the degree of unsaturation, the greater the need for α-tocopherol[32]. The JD group participants were recommended to increase intake of fish, which contains polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, possibly resulting in the need for more α-tocopherol for protection from oxidation than in the PJD group. Furthermore, the serum α-tocopherol concentration might have been decreased in the JD group.

As previously reported, supplementation of α-tocopherol increased CEC in patients with end-stage renal disease[11], a finding consistent with that in patients with type 1 diabetes[12]. CEC changes correlated positively with serum α-tocopherol and γ-tocopherol in this study. In cell and animal experiments, ABCA1[33, 34] and SR-B1[35, 36] were reported to be involved in the secretions of α-tocopherol and γ-tocopherol from tissues and in their intestinal absorption. In addition, α-tocopherol has been shown to activate the ABCA1 transporter in apoE-/- mice, which may affect the capacity for cholesterol efflux via ABCA1[37]. We consider our results to be supported by these previous studies.

It is noteworthy that, in this study, not only serum α-tocopherol but also γ-tocopherol appeared to have a positive correlation with CEC. The serum γ-tocopherol concentration did not change whereas α-tocopherol decreased with serum lipid reduction. The usefulness of γ-tocopherol has not been emphasized in terms of its physiological activity or lipid peroxidation reactivity[38, 39]. However, the γ-tocopherol level has been reported to be inversely correlated with coronary heart disease[40] and supplementation of γ-tocopherol alone or with α-tocopherol reduced biomarkers of oxidative stress in patients with metabolic syndrome[41]. Intake of γ-tocopherol, which is contained in vegetable oils, nuts and seeds, soybeans, and so on, was the highest among tocopherol analogues such as α, β, γ and δ. Therefore, serum γ-tocopherol might serve a compensatory function in response to decreased α-tocopherol in states of low LDL without α-tocopherol supplementation.

Higher circulating ORAC has been reported in subjects on the Mediterranean[42] and Dietary Approaches to Stop Hypertension (DASH) diets[43], and ORAC increases with phytochemical supplementation from oat porridge, white mushrooms and coffee[44-46]. We predicted that JD intake would improve circulating oxidative stress in patients with dyslipidemia. Although a weak but positive correlation between CEC and HDL ORAC was present at baseline, total and HDL ORAC decreased in both of our study groups after the interventions. Decreases in ABCA1-CEC and HDL ORAC have been reported after weight loss in overweight and obese women[47], findings consistent with those of our present study. Nutrients such as tocopherol, vitamin C and flavonoids are well known to have radical scavenging capacity, as reflected by ORAC[48-50]. The oxidized state of HDL has been suggested to be related to CEC[51], but only serum tocopherol concentrations were measured in this study. We found HDL ORAC to not be related to the serum tocopherol concentration at cross sectional points but we did detect relationships between changes in HDL ORAC which showed moderately positive correlations with tocopherol and β-carotene. Changes in serum α-tocopherol and ORAC were reported to be positively related in the DASH-sodium trial[43], while the diet high in fruits and vegetables trial showed no relationship between serum α-tocopherol and ORAC[52].

Multiple antioxidant systems might work synergistically to enhance total antioxidant capacity[53]. Further study is needed to elucidate the relationship between antioxidant capacity and CEC.

Carotenoids were measured as parameters of vegetable intake, and it was confirmed that green-yellow vegetables and serum carotenoids increased due to the JD intervention. Serum carotenoid levels correlated positively with CEC at cross-sectional measurements. In a previous study on the relationship between CEC and carotenoids, using RAW264.7 cells, β-carotene was shown to be associated with increased CEC, and 9-cis β-carotene in particular increased the RNA expression levels of ABCA1 and ABCG1[54]. It has been suggested that CEC might be increased by retinoic acid converted from β-carotene, which reportedly improves CEC[55, 56]. On the other hand, in an *in vivo* trial, β-cryptoxanthin or lycopene, which are non-provitamin A carotenoids, increased ABCA1[57, 58]. Although it can be said that carotenoids are related to CEC, the carotenoid concentration in our study was lower than those in the aforementioned *in vivo* studies and the correlation with CEC was weak.

This study has major limitations. First, our CEC measuring method employed a variety of cells, up-regulated transporters and reagent types used for labeling. Second, CEC values in our participants were normal at baseline. Third, since we did not measure antioxidants closely related to HDL such as PON1 or the antioxidants reflected in ORAC such as vitamin C and flavonoids, the relationship between CEC and antioxidant capacity could not be fully evaluated. Finally, our study's sample size was not sufficient for multiple regression analysis. However, we did obtain results suggesting that dietary components such as tocopherol and carotenoids affect CEC in patients with dyslipidemia.

Conclusion

HDL function, i.e., CEC, was not changed by the JD in Japanese patients with dyslipidemia who already had normal CEC at baseline. CEC has been suggested to be positively associated with serum α and γ-tocopherol concentrations and HDL ORAC. Further studies are needed on the JD education program to improve CEC in subjects with severe dyslipidemia.

Acknowledgements

The authors thank all patients who participated in the present study, and all staff members who supported the survey. The authors also appreciate all nutrition study team members of the laboratory of Nutrition Education and Clinical Nutrition of Japan Women's University, including Ms. Kanako Kamoshita, Ms. Seina Komine, Ms. Sayaka Hasegawa, Ms. Rina Ichiki, Ms. Kanako Chibai, Ms. Chieko Fukuda, Ms. Miyu Oshika, Ms. Sia SuHuai, Ms. Moe Matsumoto, Ms. Saaya Yamada, Ms. Mariko Nakazawa, Ms. Yui Nishikata, Ms. Sayuri Igawa, Ms. Hazuki Kitayama, Ms. Mayuka Kodama, Ms. Kirika Fujitani, Ms. Aoi Tokunaga, Ms. Akari Yasuda, Ms. Hinako Omata, Ms. Saori Toyota, Ms. Kana Kinugawa, Ms. Mai Murano, Ms. Chisato Ogawa, and Ms. Nana Mihara, all of whom made major contributions to calculating the nutrient intakes in this study.

Financial Support

This study was supported by a research grant from the SKYLARK Food Science Institute and Rice Stable Supply Support Organization. This study was also supported by gifts of canned mackerel from Maruha Nichiro corporation, Tokyo, retort-packed rice with barley, Mochimugi Gohan from Hakubaku Co., Ltd. Tokyo and Omugi Gohan from Otsuka Pharmaceutical Co., Ltd. The SKYLARK Food Science Institute, Rice Stable Supply Support Organization, Maruha Nichiro corporation and Hakubaku Co., Ltd. had no role in the design, analysis or writing of this article.

Conflict of Interest

The authors report the following disclosures: Masako Waki has received clinical research funding from AstraZeneca KK and Eli Lilly Japan KK. Tamio Teramoto has received clinical research funding from Dai-ichi Sankyo KK. Chizuko Maruyama has received scholarship grant from Rice Stable Supply Support Organization. Ariko Umezawa, Yasuhiro Endo, Yumiko Suenaga, Yuri Shijo, Noriko Kameyama, Aisa Sato, Ai Nishitani, Makoto Ayaori, Katsunori Ikewaki have no conflicts of interest.

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Supplemental Table 1. Changes in anthropometric variables and lipid parameters of the patients in the Partial Japan Diet and Japan Diet groups

	PJD group ($n=49$)			JD group ($n=49$)			Between-group comparisons at 3 and 6 months (JD - PJD)	
	Mean	(SD)	$p^{\dagger}$	Mean	(SD)	$p^{\dagger}$	Difference (95% CI)	$p^{\ddagger}$
Body Mass Index (kg/m^2)								
Baseline	23.9	(3.3)		24.4	(3.7)			
3-month	23.5	(3.2)	0.000	24.0	(3.8)	0.000	0.1 (-0.2, 0.4)[§]	0.50
6-month	23.3	(3.3)	0.000	24.0	(3.7)	0.001	0.1 (-0.2, 0.5)	0.43
Total cholesterol (mg/dL)								
Baseline	224	(38)		213	(29)			
3-month	219	(38)	0.22	204	(25)	0.004	-4 (-14, 5)	0.38
6-month	223	(36)	0.78	202	(27)	0.000	-10 (-19, -1)	0.033
LDL-cholesterol (mg/dL)								
Baseline	128	(33)		123	(24)			
3-month	124	(30)	0.24	116	(20)	0.006	-3 (-11, 5)	0.45
6-month	129	(32)	0.74	115	(22)	0.005	-9 (-17, 0)	0.043
MDA-LDL[‖] (U/L)								
Baseline	94	(71, 126)		88	(74, 107)			
3-month	86	(71, 107)	0.041	86	(69, 105)	0.082	4 (-7, 16)	0.76
6-month	91	(67, 122)	0.23	79	(69, 98)	0.003	-4 (-16, 8)	0.29
HDL-cholesterol (mg/dL)								
Baseline	63	(18)		61	(17)			
3-month	63	(21)	0.60	60	(15)	0.25	-2 (-4, 1)	0.26
6-month	64	(20)	0.43	60	(16)	0.40	-2 (-4, 1)	0.25
Triglyceride[‖] (mg/dL)								
Baseline	90	(63, 173)		103	(77, 155)			
3-month	91	(61, 152)	0.18	92	(68, 127)	0.004	-9 (-30, 13)	0.21
6-month	87	(64, 153)	0.49	86	(60, 131)	0.000	-10 (-33, 13)	0.023

[†] *P* values within-group comparisons from baseline are analyzed by the paired *t* test or Wilcoxon signed-rank test.
[‡] *P* values between-group comparisons are analyzed by the unpaired *t* test or Mann–Whitney *U* test.
MDA-LDL, malondialdehyde modified-low density lipoprotein.
[§] Values are expressed as means (95% CI).
[‖] Values are expressed as medians (25th percentile, 75th percentile).

Supplemental Table 2. Changes in energy and nutrients in the Partial Japan Diet and Japan Diet groups

	PJD group (n = 49)			JD group (n = 49)			p[‡]	Between-group comparisons at 3 and 6 months (JD - PJD)	
	Median	(25th percentile, 75th percentile)	p[†]	Median	(25th percentile, 75th percentile)	p[†]		Difference (95% CI)	p[‡]
Energy[§] (kcal)									
Baseline	1873	(493)		1919	(440)		0.63		
3-month	1786	(446)	0.089	1749	(303)	0.001		-83 (-220, 54)[‖]	0.23
6-month	1726	(503)	0.001	1650	(415)	0.000		-121 (-258, 16)	0.082
Protein (g)									
Baseline	70.2	(62, 84)		70.2	(60, 84)		0.85		
3-month	72.8	(59, 84)	0.94	72.7	(62, 81)	0.48		0 (-7, 7)	0.45
6-month	64.7	(56, 81)	0.001	66.6	(60, 82)	0.56		3 (-3, 9)	0.075
Fat[§] (g)									
Baseline	63	(20)		65	(20)		0.72		
3-month	60	(19)	0.23	58	(18)	0.042		-3 (-11, 5)	0.47
6-month	55	(19)	0.000	55	(19)	0.000		-2 (-8, 5)	0.65
Carbohydrate[§] (g)									
Baseline	237	(73)		237	(59)		0.99		
3-month	225	(65)	0.12	219	(46)	0.004		-6 (-24, 11)	0.48
6-month	225	(72)	0.065	204	(56)	0.000		-21 (-40, -1)	0.037
α-tocopherol (mg)									
Baseline	8.2	(6.3, 9.2)		7.3	(5.4, 9.7)		0.44		
3-month	7.5	(6.1, 9.3)	0.71	8.0	(6.4, 9.7)	0.33		0.5 (-1.0, 1.9)	0.39
6-month	7.4	(6.3, 9.3)	0.27	7.6	(5.5, 9.6)	0.59		0.7 (-0.4, 1.8)	0.28
β-tocopherol (mg)									
Baseline	0.4	(0.3, 0.5)		0.4	(0.3, 0.5)		0.11		
3-month	0.4	(0.3, 0.5)	0.19	0.4	(0.3, 0.5)	0.81		0.0 (0.0, 0.1)	0.29
6-month	0.4	(0.3, 0.5)	0.97	0.4	(0.3, 0.5)	0.60		0.0 (-0.1, 0.1)	0.61
γ-tocopherol (mg)									
Baseline	12.0	(9.5, 15.5)		12.0	(9.3, 14.9)		0.92		
3-month	11.7	(8.6, 15.2)	0.47	11.9	(8.7, 15.0)	0.83		0.3 (-1.8, 2.5)	0.90
6-month	11.2	(7.9, 14.7)	0.32	10.8	(8.8, 15.7)	0.78		0.4 (-1.7, 2.5)	0.39
δ-tocopherol (mg)									
Baseline	2.8	(2.1, 3.6)		2.7	(1.7, 3.4)		0.60		
3-month	2.5	(1.8, 3.4)	0.47	2.7	(1.9, 4.0)	0.11		0.4 (-0.2, 0.9)	0.11
6-month	2.5	(2.1, 3.6)	0.91	3.0	(1.9, 3.7)	0.19		0.2 (-0.4, 0.9)	0.34
β-carotene (μg)									
Baseline	3434	(2064, 4541)		2293	(1458, 3118)		0.006		
3-month	2830	(1915, 4508)	0.36	4090	(2076, 5711)	0.000		1775 (770, 2780)	0.000
6-month	3002	(2116, 5143)	0.65	4073	(2426, 6276)	0.000		1960 (828, 3091)	0.003
α-carotene (μg)									
Baseline	570	(357, 1036)		426	(139, 795)		0.043		
3-month	480	(199, 971)	0.47	712	(303, 1306)	0.012		301 (-20, 622)	0.015
6-month	640	(360, 1086)	0.65	682	(271, 1384)	0.032		278 (-95, 652)	0.17
β-cryptoxanthin (μg)									
Baseline	72	(32, 129)		44	(27, 88)		0.25		
3-month	47	(27, 77)	0.059	54	(34, 146)	0.10		139 (-20, 299)	0.019
6-month	47	(34, 105)	0.31	45	(31, 80)	0.34		-37 (-229, 156)	0.86

[†] P values within-group comparisons from baseline are analyzed by the paired t test or Wilcoxon signed-rank test.
[‡] P values between-group comparisons are analyzed by the unpaired t test or Mann–Whitney U test.
[§] Values are expressed as means (SD).
[‖] Values are expressed as means (95% CI).