Hypocholesterolemic Effect of Oral Administration of β-Carotene and Carrot Juice in Exogenous Hypercholesterolemic Mice

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I Introduction

Moderately and regularly exercising, adequate eating and sufficient sleeping are generally thought to be necessary for the prevention of degenerative diseases. With the increase in the lifestyle-related diseases, the aspect of food has been changed from the supply source of nutritional elements to the bio-functional factors to prevent diseases with the use of the functional foods in Japan and the designer foods in the USA.

Carotenoids are widely distributed over the face of the earth mainly in plants, and more than 600 compounds have been recognized. These carotenoids can act as antioxidants by quenching singlet oxygen and scavenging free radicals. Much attention has been paid to the role of dietary carotenoids in the prevention of cancer. An inverse association between dietary intake and the serum level of carotenoids such as β-carotene and lycopene, and the risk of human cancer in various organs has been observed in epidemiological studies. However, there have been contradictory reports concerning the supplementation with β-carotene for cancer prevention.

On the other hand, much attention has also been given to the role of carotenoids in the prevention of cardiovascular disease. Arteriosclerosis, which is one of the critical risk factors in lifestyle-related diseases, such as myocardial infarction and cerebral stroke, is accelerated by hypercholesterolemia. Oxidative modification of plasma low-density lipoprotein (LDL) is suggested to lead to the formation of lipid-laden foam cells in atherosclerotic lesions.

We previously reported that supplementation of carotenoids inhibited singlet oxygen-mediated oxidation of human plasma LDL and that dietary ingestion of tomatoes attenuated the endothelial dysfunction elicited by an atherogenic diet in mice. Therefore, dietary carotenoids are thought to exert beneficial effects in the prevention of cardiovascular disease by protecting LDL from the attack of singlet oxygen. Furthermore, β-carotene was reported to reduce the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase; mevalonate: NADP oxidoreductase (CoA-acylating), EC 1.1.1.34) in macrophages dose-dependently and inhibit the synthesis of cholesterol. But, there has been no report showing that dietary ingestion of foods including a substantial amount of carotenoids inhibits HMG-CoA reductase activity and suppresses the cholesterol synthesis. We report here the effects of ingestion of carrot products on mRNA levels of HMG-CoA reductase in the liver and plasma lipid levels in mice.

II Materials and Methods

1. Animals and diets

Male ICR strain mice were obtained from Charles River Japan (Yokohama, Kanagawa, Japan) at 5 weeks old. The mice were housed in a temperature (23-25°C)- and humidity (40-60%)-controlled room with a 12-hour cycle of light and dark (lights on from 06:00 to 18:00). Animals were allowed free access to water and a powdered diet throughout the experiment. Two diets were utilized in this study: a diet with or without cholesterol. The diet without cholesterol consisted of the following (g/kg): casein, 200; β-cornstarch, 397.486; α-cornstarch, 132; sucrose, 100; soybean oil, 70; cellulose powder, 50; AIN-93G mineral mixture, 35; AIN-93 vitamin mixture, 10; L-cystine, 3; choline bitartrate, 2.5; t-butyl-hydroquinone, 0.014. A 1% cholesterol was added instead of the same weight of β-cornstarch. The experimental procedures used in this study met the standards set forth in the Guidelines for the Care and Use of Laboratory Ani-
mals of the Experimental Animal Facility, the Japanese Society of Nutrition and Food Science.

2. Reagents and chemicals

All reagents and chemicals used in this study were of analytical grade. Fructose was purchased from Kanto Chem., Tokyo, Japan. Oleic acid, β-carotene, cholesterol, glucose and sucrose were purchased from Wako Pure Chem. Ind., Osaka, Japan.

3. Experiment 1

At first, we confirmed the effect of dietary β-carotene on the plasma cholesterol levels in ICR mice, as there had been no report to examine the hypocholesterolemic effect of β-carotene using the ICR strain mice. We divided the mice into 4 groups of 7 mice each. One group was given the diet without cholesterol and ingested oleic acid (5 ml/kg/d) for 14 days per os. The other three groups fed the diet with 1% cholesterol orally administrated oleic acid (5 ml/kg/d), β-carotene (50 mg/kg/d) and β-carotene (5 mg/kg/d), respectively for 14 days. β-Carotene was dissolved in oleic acid and administered per os at the volume of 5 ml/kg. The mice were sacrificed by decapitation under light anesthesia with diethyl ether. Collected blood was placed in a plastic tube containing 10 U of heparin and the plasma was separated from the whole blood by centrifugation at 2,000 × g for 20 min at 4°C. The plasma was stored at −80°C until used.

4. Experiment 2

Then, we assessed the effect of foodstuffs rich in β-carotene on the plasma cholesterol levels in mice. Eighty ICR mice were assigned to 4 groups of 20 mice each, and fed ad libitum the diet with 1% cholesterol. One group orally received concentrated carrot juice of which composition of ingredients was shown in the Table 1. Another groups received the carbohydrate solution (glucose, 64.0 mg/ml; fructose, 54.5 mg/ml; sucrose, 180 mg/ml) as a control solution, carrot juice and β-carotene (1 mg/ml in oleic acid), respectively. The carbohydrate composition of the control solution was mimicked to that of the concentrated carrot juice. The carrot juice was confected by diluting the concentrated carrot juice with 4-fold weight of distilled water. All solutions were given twice a day (09:00-10:30 and 16:00-17:30) for 14 days. The ingestion volume of these solutions was 12.5 ml/kg at a time except for β-carotene (2.5 ml/kg). After the feeding experiment for 14 days, fourteen mice from each group were killed by decapitation (13:00-15:00). Plasma was obtained as described above, and the liver was removed and weighed. The plasma and the liver were stored at −80°C until measurement of the lipids. Another six mice from each group were anesthetized with pentobarbital and the liver removed (09:30-11:30) for measuring the liver carotenoid levels and the mRNA levels of HMG-CoA reductase.

5. Measurement of plasma- and liver lipid levels

The plasma concentrations of total cholesterol and triacylglycerol (Experiments 1 and 2), and HDL-cholesterol and free cholesterol (Experiment 2) were measured enzymatically with commercial diagnostic kits (Cholesterol E-Test, Triglyceride E-Test, HDL-cholesterol E-test and Free cholesterol E-Test respectively, Wako Pure Chem. Ind.). The difference between total cholesterol and HDL-cholesterol or free cholesterol was assumed to be VLDL + LDL cholesterol or esterified cholesterol, respectively. The lipids of the liver were extracted by the method of Folch et al. (1957), and the cholesterol in the extracts from the liver was measured according to the methods of Zak (1960).

6. Measurement of carotenoids of liver

The assays of α- and β-carotene in the liver were performed by HPLC as previously described (1993). The liver tissues were homogenized and saponified by addition of 60% KOH and 3% butylated hydroxytoluene in ethanol, followed by heating at 50°C for 30 min. After extraction with hexane and dichloromethane (4:1, v/v, 5.0 ml) twice, the samples were dried and then reconstituted in a sufficient quantity of the mobile phase. The samples were analyzed by HPLC, using a Shimadzu SPD-M10A VP diode array detector (Shimadzu, Kyoto, Japan) and a LiChospher RP18-5 column (E. Merk, Darmstadt, Germany) (4.6 × 250 mm). The mobile phase was acetonitrile and methanol (1:1, v/v), and the flow rate was 2.0 ml/min.

7. Measurement of mRNA levels of HMG-CoA reductase

The principle of the real-time quantitative PCR was described by Heid et al. (1996). A 1.2 µg portion of total mRNA of liver tissues was isolated with ISOGEN solution (Nippon Gene Co., Tokyo, Japan), and the cDNA was synthesized with TaqMan Reverse Transcription Reagents (PE Applied Biosystems, CA, USA). The synthesized cDNA was used for amplification by the real-time PCR system (ABI Prism 7700 Sequence Detection System; PE Applied Biosystems). The forward primer (HMG-37F, 5'
GAC ATG CAG ATT CTG GCA GTC A-3'), reverse primer (HMGS-13R, 5'-CGT CCT TCG ATC CAA TTT ATG G-3'), and Taqman probe (HMGS-60T, 5'-TGG GAA CTA TTT GAC CGA CAAGA GCC-3') for real-time PCR amplification were designed with the PrimerExpress software (PE Applied Biosystems) to specifically amplify the HMG-CoA reductase gene. Briefly, cDNA (10 ng) was added to a reaction mixture containing 12.5 µl of 2× Taqman Universal PCR Master Mix, 0.75 µl of the forward primer HMGS-57F (10 µM), 0.75 µl of the reverse primer HMGS-113R (10 µM), and Taqman probe HMGS60T (20 µM) in a final volume of 25 µl. The PCRs were performed with the ABI PRISM 7700 sequence detection system (PE Applied Biosystems). After initial activation at 50°C for 2 min and at 95°C for 10 min, 50 PCR cycles of 95°C for 15 s and 60°C for 1 min were performed. All samples were measured in triplicate. The levels of 18S rRNA, a housekeeping gene as an internal standard, were also measured. The number of PCR cycles to reach the fluorescence threshold was determined as the cycle threshold (Ct), indicative of the quantity of the target gene. The Ct of HMG-CoA reductase gene in each sample was determined with the Sequence Detection System 1.6.3 (PE Applied Biosystems). The level of the HMG-CoA reductase gene was corrected with the Ct of 18S rRNA and expressed as the ratio to the initial sample.

8. Statistics
Data are expressed as means and SE. Values were subjected to an analysis of variance, and differences between the means were considered significant at p < 0.05 by Tukey's test using commercially available statistical analysis program, Visual Stat for Windows 4.51 (Stat Soft Inc., OK, USA).

III Results
1. Effect of β-carotene on growth and plasma lipids
Oral administration of β-carotene exerted no effect on the body weight of the mice throughout the experiment 1 (Table 2). The addition of cholesterol (1%) in the diet significantly increased the plasma total cholesterol of mice (Fig. 1). For three groups fed on the cholesterol additive diet, not a high dose (50 mg/kg/d) but a low dose (5 mg/kg/d) supplementation with β-carotene decreased the plasma total cholesterol level compared to the mice who ingested oleic acid alone. The plasma triacylglycerol level was slightly lowered by supplementation with β-carotene but not significant statistically.

![Fig. 1 Effect of β-carotene on plasma lipid levels](image)

2. Effect of carrot juice and β-carotene on growth,
   liver weight and the levels of total cholesterol and carotenoids in the liver
There was no difference in the body weight and growth of mice throughout the experiment 2 (Table 3). Though oral ingestion of concentrated carrot juice, carrot juice or β-carotene slightly reduced the liver weight and the liver total cholesterol level compared to the control, there was no statistical difference except the lowering effect of the carrot juice on the liver weight and the decrease of liver total cholesterol level by supplementation with β-carotene. The ingestion of concentrated carrot juice or carrot juice dose-dependently increased the liver concentrations of α- and β-carotene. The supplementation with β-carotene increased its level in the liver stronger than that with carrot juice containing same dose of β-carotene.

3. Effect of carrot juice and β-carotene on plasma lipid levels
The plasma levels of total cholesterol and esterified cholesterol were decreased by ingestion of concentrated carrot juice, carrot juice or β-carotene compared to the control given the carbohydrate solution (Table 4). Administration of the carrot juice or β-carotene also reduced plasma levels of HDL-cholesterol and free cholesterol. Only the carrot juice attenuated plasma triacylglycerol levels, and none of the three agents statistically affected plasma LDL+VLDL cholesterol levels.
Table 3  Effect of oral administration of carrot juice or β-carotene on body weight gain, liver weight and total cholesterol- and carotenoids- level in mice in the experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight gain (g/14 days)</th>
<th>Liver wet weight (g)</th>
<th>Liver total Chol. (μmol/g)</th>
<th>Liver carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.26 ± 0.25</td>
<td>1.77 ± 0.06</td>
<td>6.53 ± 0.13</td>
<td>α-Carotene (ng/g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.24 ± 0.55</td>
</tr>
<tr>
<td>CJ5</td>
<td>1.97 ± 0.17</td>
<td>1.63 ± 0.03</td>
<td>6.16 ± 0.18</td>
<td>19.18 ± 4.33</td>
</tr>
<tr>
<td>CJ1</td>
<td>2.02 ± 0.23</td>
<td>1.60 ± 0.03</td>
<td>6.16 ± 0.10</td>
<td>7.13 ± 0.83</td>
</tr>
<tr>
<td>BCS</td>
<td>2.04 ± 0.59</td>
<td>1.67 ± 0.04</td>
<td>6.06 ± 0.13</td>
<td>5.37 ± 1.61</td>
</tr>
</tbody>
</table>

Body weight gain, liver wet weight and liver concentrations of total cholesterol, α-carotene and β-carotene of mice injected the carbohydrate solution (C), concentrated carrot juice (β-carotene, 5 mg/kg/d; CJ5), carrot juice (β-carotene, 1 mg/kg/d; CJ1), and β-carotene (5 mg/kg/d; BCS) are shown as mean and SE (n=20, body weight gain; n=14, others). Within a column, values with different superscripts are significantly different, P < 0.05 (Tukey’s test). Abbreviation used: Chol, cholesterol.

Table 4  Effect of oral administration of carrot juice and β-carotene on plasma lipid levels in mice in the experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Chol. (mmol/L)</th>
<th>HDL Chol. (mmol/L)</th>
<th>LDL+VLDL (mmol/L)</th>
<th>Free Chol. (mmol/L)</th>
<th>Estertified Chol. (mmol/L)</th>
<th>TG (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>4.50 ± 0.18</td>
<td>2.70 ± 0.10</td>
<td>1.82 ± 0.13</td>
<td>1.04 ± 0.05</td>
<td>3.46 ± 0.16</td>
<td>0.81 ± 0.06</td>
</tr>
<tr>
<td>CJ5</td>
<td>3.90 ± 0.12</td>
<td>2.44 ± 0.09</td>
<td>1.46 ± 0.10</td>
<td>0.94 ± 0.05</td>
<td>2.96 ± 0.10</td>
<td>0.70 ± 0.03</td>
</tr>
<tr>
<td>CJ1</td>
<td>3.92 ± 0.14</td>
<td>2.26 ± 0.09</td>
<td>1.56 ± 0.12</td>
<td>0.86 ± 0.02</td>
<td>2.99 ± 0.13</td>
<td>0.61 ± 0.03</td>
</tr>
<tr>
<td>BCS</td>
<td>3.74 ± 0.13</td>
<td>2.21 ± 0.07</td>
<td>1.56 ± 0.05</td>
<td>0.88 ± 0.03</td>
<td>2.89 ± 0.08</td>
<td>0.76 ± 0.06</td>
</tr>
</tbody>
</table>

Plasma lipid levels of mice in each group were shown as mean and SE (n=14). Within a column, values with different superscripts are significantly different, P < 0.05 (Tukey’s test). Abbreviation used: Chol, cholesterol; LDL + VLDL, LDL + VLDL cholesterol; F Chol, free cholesterol; TG, triacylglycerol.

4. Effect of carrot juice and β-carotene on mRNA expression of HMG-CoA reductase

The relative amount of mRNA of HMG-CoA reductase normalized to 18S rRNA in the liver was attenuated by ingestion of concentrated carrot juice, carrot juice or β-carotene compared to the control (Fig. 2). On the other hand, the level of 18S rRNA, a housekeeping gene as an internal standard, was unaffected by supplementation with these agents.

![Fig. 2](image)

**Fig. 2** Effect of dietary carrot and β-carotene on the mRNA levels of HMG-CoA reductase in mice liver

Mean values of each group are shown with SE (n=6). Columns with different superscripts are significantly different, P < 0.05 (Tukey’s test). Abbreviations are shown in footnote of table 3.

**IV Discussion**

We used a mouse as an experimental animal in this study to administer maximal dose of β-carotene and carrot juice, because carotenoids were thought to be less absorbable in rodents than in humans. In the present study, the oral administration of β-carotene or carrot juice rich in β-carotene increased the liver concentrations of β-carotene, attenuated the expression of HMG-CoA reductase gene in mice compared to the control fed on the diet containing 1% cholesterol and resulted in a suppression of the increase in plasma and liver total cholesterol levels. However, there was no relation between the β-carotene levels in the liver and the hypocholesterolemic effects, and in the experiment 1, a high dose (50 mg/kg/d) supplementation with β-carotene was ineffective to attenuate the plasma cholesterol levels. Ito et al.21 also showed that the overloaded β-carotene did not suppress the plasma cholesterol levels in ddY mice. We thought that there was the upper limit of the β-carotene dosage for the hypocholesterolemic effects. These results suggest that β-carotene is one of the factors to induce the amelioratory effects of carrot juice on the plasma cholesterol levels.

Dietary fiber containing in carrot juice is also a candidate to elicit the hypocholesterolemic effects. Robertson et al.22 has reported that 200 g of raw carrot eaten each day for 3 weeks significantly reduced serum cholesterol by 11% in humans and that dietary fiber was supposed to be a hypocholesterolemic agent. The mechanism of the hypocholesterolemic effect of dietary fiber is thought to be mainly induced by increasing fecal bile acid excretion. But, ingestion of dietary fiber has been reported to enhance the activity of HMG-CoA reductase23. The mice used in this study received about 6 g/kg dietary fiber per day if they fed the diet about 4 g/d. Then we thought that the dietary fiber
derived from carrot juice (50 mg/kg/d) in this study was negligible and that the beneficial effects of oral ingestion of carrot juice on plasma cholesterol levels mainly depended on non-dietary fiber but other compounds such as β-carotene.

We added the cholesterol in the diet to make a model mouse of a mild hyperlipidemia. The maintenance of cholesterol homeostasis is precisely controlled by multiple feedback mechanisms and the translational efficiency of HMG-CoA reductase mRNA is diminished through the action of dietary cholesterol, and high dose of carbohydrate also suppresses the cholesterol synthesis. In the present study, oral administration of β-carotene, carrot juice or concentrated carrot juice attenuated the expression of HMG-CoA reductase gene in mice compared to the control fed on the diet containing 1% cholesterol and received the carbohydrate solutions. The down-regulation of the expression of HMG-CoA reductase may not affect so much on the plasma cholesterol levels in the exogenous hypercholesterolemic mice, it may be one of the mechanisms by which β-carotene reduces the concentration of the plasma cholesterol. We did not examine the mRNA levels of LDL receptors, but Fuhrman et al. showed carotenoids such as β-carotene and lycopene inhibited the activity of HMG-CoA reductase and augmented the activity of LDL receptors in macrophages. Moreno et al. suggested that supplementation with β-carotene suppressed the gene expression of HMG-CoA reductase in rat liver and that β-carotene could induce the synthesis of a less stable mRNA due to differential splicing. In addition, dietary supplementation with β-carotene derived from Dunaliella bardawil was reported to reduce the plasma cholesterol concentrations in exogenous hyperlipidemic mice. Thus, β-carotene may affect the concentration of the plasma cholesterol, and our study is the first report on the attenuation of HMG-CoA reductase gene expression in the liver induced by oral administration of foodstuffs rich in carotenoids.

Carotenoids such as β-carotene, α-carotene and lycopene exert antioxidative activity and supplementation with them is thought to be beneficial for the prevention of lifestyle-related diseases such as cancer and cardiovascular diseases. Consequently, dietary ingestion of foodstuffs rich in carotenoids as typified by carrots may lower the risk of atherosclerosis, a critical risk factor in myocardial infarction and cerebral stroke by reducing the plasma cholesterol levels and by inhibiting the generation of oxidized low-density lipoprotein, which accelerates atherosclerosis. We have now designed a feeding experiment with carrot juice using volunteers with borderline hyperlipidemia.

V Synopsis

The effect of oral administration of β-carotene (5 mg/kg/d), concentrated carrot juice (β-carotene, 5 mg/kg/d) and carrot juice (β-carotene, 1 mg/kg/d) on plasma cholesterol levels in mice fed on the diet containing 1% cholesterol (w/w) were investigated. Supplementation with these samples for 14 days attenuated the increase in plasma concentrations of total cholesterol and esterified cholesterol. Ingestion of β-carotene and the carrot juice suppressed the increase of plasma levels of high-density lipoprotein (HDL) cholesterol and free cholesterol, and only the carrot juice inhibited the increase in plasma triacylglycerol. The mRNA expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) in mice liver was attenuated by ingestion of β-carotene, concentrated carrot juice and carrot juice. These observations suggest that dietary ingestion of β-carotene or foodstuffs rich in β-carotene decreases the plasma cholesterol levels, and the attenuation of the expression of HMG-CoA reductase in the liver might be involved in it.

VI References


β-カロテン及びニンジンジュース摂取による食餌性高脂血症マウスのコレステロール上昇抑制作用

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キーワード：ニンジン、β-カロテン、コレステロール、HMG-CoA還元酵素

概要
β-カロテン（BC）には、コレステロール合成系の律速酵素であるHMG-CoA還元酵素の働きを抑える、コレステロール合成を抑制する作用があることが報告されていることから、血中脂質濃度を抑制する作用が期待された。そこで、本実験においては、食餌中に1％コレステロールを負荷して飼育したICR系マウスに対して、まず、BCをオレイン酸に溶解し、強制経口投与（5及び50 mg/kg/dで14日間）し、血中脂質に及ぼす影響を評価したところ、5 mg/kg/dの群において、オレイン酸を摂取させた対照と比較して、有意に総コレステロール濃度が低下した。次いで、BC（5 mg/kg/d）または、濃縮ニンジンジュース（BC；1 mg/kg/d）を経口投与し、その血中脂質濃度と、肝臓のHMG-CoA還元酵素のmRNA量を測定し、脂質代謝に及ぼす影響を評価した。

14日間の投与試験後、血漿中の脂質濃度を比較したところ、BCを含まない糖液（濃縮ニンジンジュースの糖含用量に調製）を投与した対照と比較して、BCを含有する3種類の試料を投与した群では、いずれも、総コレステロール及びコレステロールエステルの濃度が有意に低値を示した（Table 4）。
また、BCまたはニンジンジュースを摂取した群では、HDL-コレステロール及び遊離コレステロール濃度も、対照と比較して低値であった。このことから、BCまたはBCを多く含むニンジンの摂取により、外因性コレステロールによる血漿脂質濃度の上昇が抑制されることが示唆された。

また、RT-PCRにより、肝臓におけるHMG-CoA還元酵素のmRNA量を比較したところ、BCを含むいずれの試料を投与した群も、対照と比較して有意に発現量が低く（Fig. 2）、本酵素の発現量の抑制が、血漿コレステロール濃度の上昇を抑制した機序の一つであることが推測された。

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