Differential Effect of Walnut Oil and Safflower Oil on the Serum Cholesterol Level and Lesion Area in the Aortic Root of Apolipoprotein E-deficient Mice

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Walnut oil (WO) is a good source of α-linolenic acid. We compared the effects of WO and high-linoleic safflower oil (HLSO) on the serum lipid level and atherosclerosis development in male and female apolipoprotein (apo) E-deficient mice. The WO diet resulted in a higher level of serum cholesterol than with HLSO. Female mice fed on the WO diet had a greater lesion area in the aortic root than did those on the HLSO diet. There was no diet-dependent difference in the level of cholesterol and its oxidation products in the abdominal and thoracic aorta. These results suggest that the unpleasant effects of the WO diet on apo E-deficient mice may be attributable to α-linolenic acid.

Key words: apolipoprotein E-deficient mice; atherosclerotic lesion; cholesterol; cholesterol oxidation product; linoleic acid

Epidemiological studies have suggested that frequent nut consumption may be protective against coronary heart diseases because of the beneficial effect on blood cholesterol. In clinical studies, diets supplemented with walnuts or almonds have decreased the serum concentration of low-density lipoprotein- and total-cholesterol. The potentially protective constituents in these nuts include high amounts of protein, Mg, vitamin E, fiber, potassium, and α-linolenic acid (primarily in walnuts). Epidemiological studies have indicated that ingesting α-linolenic acid might be beneficial to prevent humans from cardiac diseases. We compared the effect of walnut oil (WO) and high-linoleic safflower oil (HLSO) in the diet on the development of arterial lesions in apolipoprotein (apo) E-deficient mice that develop advanced lesions which are similar to those in humans. In addition, as risk factors for atherosclerosis, we determined the level of serum cholesterol and arterial cholesterol and its oxidation products.

Materials and Methods

Materials. 5α-cholestane, cholest-5-en-3β-ol-7-one (7-ketocholesterol), cholestan-3β,5α,6β-triol (cholestanetriol), cholestan-5α,6α-epoxy-3β-ol (α-epoxycholesterol), and cholestan-5-en-3β,25-diol (25-hydroxycholesterol) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Cholestan-5-en-3β,7α-diol (7α-hydroxycholesterol), cholestan-5-en-3β,7β-diol (7β-hydroxycholesterol), cholestan-5β,6β-epoxy-3β-ol (β-epoxycholesterol), and 25R-cholestan-5-en-3β,26-diol (27-hydroxycholesterol) were obtained from Steraloids, Wilton, NH, U.S.A. Cholestan-5-en-3β-ol (cholesterol) was purchased from Daiichikagaku, Tokyo, Japan, and pyridine, trimethylchlorosilane (TMCS), and 1,1,1,3,3,3-hexamethyldisilazane (HMDS) were products from Nacalai Tesque, Kyoto, Japan. All organic solvents were of guaranteed reagent grade.

Diets, animals and study protocol. The diets were according to the AIN93G formula and contained the following ingredients in weight%: 20 casein, 5 WO or HLSO, 13.2 α-cornstarch, 10 sucrose, 5 cellulose, 3.5 mineral mixture, 1 vitamin mixture, 0.3 L-cystein, 0.25 choline bitartrate, 0.0014 tert-butylhydroquinone, and 41.75 cornstarch. Each diet was kept at −30°C until used. WO and HLSO were obtained from Angria (England) and Nisshin Oil Co. (Kanagawa, Japan), respectively. The fatty acid composition of the dietary fats is shown in Table 1.

Each of the male and female apoE-deficient mice initially engineered at University of North Carolina (Chapel Hill, NC, U.S.A.) was purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A.) in 1994. These mice were maintained in the Laboratory of Animal Experiments at Kyushu University Faculty of Medicine (Fukuoka, Japan). Nine-week-old apo E-deficient mice were housed individually in
a temperature-controlled room at 22–25°C with a 12-h light/dark cycle (08:00–20:00, lights on) and given free access to one of the diets and nonionized water throughout the experimental period. The mice were divided into two dietary groups and fed for 9 weeks.

At the termination on the experiment period, the mice were deprived of food for 5 h (05:00–10:00) and killed by withdrawing blood from the left ventricle. The heart and entire aorta with its main branches were immediately dissected from each animal as a unit.

This experiment was carried out under the control of the guidelines for animal experiments of the Faculty of Agriculture and Graduate Course at Kyushu University and Law No. 105 and Notification No. 6 of the Government of Japan.

Tissue preparation and morphometric determination of atherosclerosis. The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g of body weight) and then sacrificed by withdrawing blood from the left ventricle. The circulatory system was perfused with 50 ml of phosphate-bufered saline at pH 7.4. The heart was removed and fixed in the 10% neutral formalin bufer solution. To determine the cholesterol and its oxidation products, the aortic roots were processed for a quantitative atherosclerosis assay of the aortic root by using a modification of the methods described by Paigen et al.15) Briefly, the heart was cut along a plane between the tip of two artia, the top half was embedded in paraffin, and consecutive sections (10 µm thick) were prepared from the ascending aorta to the aortic sinus until the aortic tissue had disappeared. The sections were mounted on slide glasses and stained with elastic Van Gieson and hematoxylin.15) Five sections of each heart were selected for an intimal area determination; the first and most distal section to the heart was taken where the aortic valve cusp were barely discernible. From this section, moving to the base of the heart, the 15th, 30th, 45th and 60th sections were also photographed together with the first one with a video camera mounted on an Olympus LX70 light microscope and analyzed by Adobe Photoshop and NIH image/68 k 1.57 software (National Institute of Health, Bethesda, MD, U.S.A.) with a Power Macintosh computer. The mean intimal area was calculated for each animal and subsequently for each group.

The adventitia of the aorta was removed as much as possible in situ, and the aorta was kept at ~80°C until the analyses of cholesterol and its oxidation products.

Determination of oxysterols in the aorta. The cholesterol oxidation products in the aorta were determined according to the method of Mori et al.,13) 5α-cholestan being used as an internal standard. Briefly, aortic lipids were extracted with 20 volumes of chloroform/methanol (2:1, v/v) containing 0.01% BHT according to the method of Folch et al.14) A screw-cap tube was flushed with argon (99.9% purity, Hakata Kyoudousannso Co., Fukuoka, Japan), and 4 ml of freshly prepared 1 M ethanolic potassium was added. Each sample was allowed to saponify overnight at room temperature in the dark.13) Cholesterol was determined by conventional gas liquid chromatography, as described.15) The cholesterol oxidation products were converted to trimethylsilyl ethers in a mixture of TMCS, HMDS, and dried pyridine (1:3:9 v/v/v) for 30 min at room temperature. Each sample was dried under N2 and then dissolved in hexane (50 µl) for the GC-MS analysis. GC-MS was performed with a Shimadzu GC-17A ver. 3 gas chromatograph coupled with a SPB-1 fused silica capillary column (60 m × 0.25 mm, 0.25 mm phase thickness; Supelco, Bellefonte, PA, U.S.A.), and connected to a Shimadzu QP5050A series mass-selective detector. The mass spectrometer was operated in the electron impact mode (70 eV). A quantitative analysis was performed by the internal standard method for mass spectrometry in the selected ion monitoring mode. Peak identification was confirmed by the relative retention time and a mass spectral comparison with authentic standards, as well as with the NIST/EPA/NIH mass spectral database library (Shimadzu, Kyoto).

Analyses of serum and liver lipids. Serum lipid levels were determined by commercially available kits (Cholesterol C test, Triglyceride G Test and Phospholipid B Test, all from Wako Pure Chemicals), as described.15) Serum fatty acid compositions were

<table>
<thead>
<tr>
<th>Table 1. Fatty Acid Composition of the Dietary Fats</th>
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<tbody>
<tr>
<td>Fatty acid</td>
</tr>
<tr>
<td>---------------</td>
</tr>
<tr>
<td>16:0</td>
</tr>
<tr>
<td>16:1</td>
</tr>
<tr>
<td>18:0</td>
</tr>
<tr>
<td>18:1</td>
</tr>
<tr>
<td>18:2</td>
</tr>
<tr>
<td>18:3</td>
</tr>
<tr>
<td>SFA</td>
</tr>
<tr>
<td>MFA</td>
</tr>
<tr>
<td>PFA</td>
</tr>
</tbody>
</table>

SFA: saturated fatty acid.  
MFA: monounsaturated fatty acid.  
PUFA: polyunsaturated fatty acid.
analyzed by gas-liquid chromatography (GC-14A, silar 10C column, He carrier gas; Shimadzu, Kyoto, Japan).15) Liver cholesterol, triacylglycerols and phospholipids were determined as described.15)

Statistical analyses. Statistics were obtained with Statcel (Excel 2000). The variance among groups was first evaluated according to the Bartlett test. Data were then analyzed by two factorial ANOVA (diet and gender), before Fisher’s PLSD method was applied when interaction was found.

Results

Growth parameters

The initial body weight, final body weight, body weight gain, food efficiency and relative liver weight were lower in the females than in the males (Table 2). There were no significant differences in the food intake and liver weight, and the two diets had no significant effect on the growth parameters.

Fatty acid composition of the serum total lipids

The difference in the proportion of dietary linoleic and α-linolenic acids was reflected in the proportion of their desaturation products (Table 3). The proportion of n-6 fatty acids (linoleic and arachidonic acid) was higher in the mice fed on HLSO than in those fed on WO. The proportion of n-3 fatty acids (α-linolenic, eicosapentaenoic and docosapentaenoic acid), but not docosahexaenoic acid, was higher in the WO group. There was also a gender effect on the fatty acid composition: the male mice had a greater proportion of palmitoleic, linoleic, α-linolenic and docosahexaenoic acids, while palmitic, stearic, arachidonic and docosapentaenoic acids were higher in the females.
Table 4. Lipid Concentrations in the Serum and Liver of Apo E-Deficient Mice Fed on the WO or HLSO Diet for 9 Weeks

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Diet</th>
<th>Sex</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>505.0 ± 57.3</td>
<td>707.2 ± 97.5</td>
<td>0.06</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>72.2 ± 15.7</td>
<td>107.7 ± 33.7</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipids</td>
<td>238.3 ± 39.8</td>
<td>236.2 ± 25.0</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (mg/g liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>5.6 ± 1.4</td>
<td>4.7 ± 1.4</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>25.4 ± 4.2</td>
<td>32.6 ± 6.7</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>26.9 ± 0.6</td>
<td>27.1 ± 0.7</td>
<td>NS</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

Each value is the mean ± SE.
NS: not significant.

Table 5. Lesion Area of the Aortic Root in Apo E-Deficient Mice Fed on the WO or HLSO Diet for 9 Weeks

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mm²)</td>
<td>HLSO (n = 4)</td>
<td>WO (n = 5)</td>
<td>HLSO (n = 3)</td>
</tr>
<tr>
<td>0.278 ± 0.048a</td>
<td>0.220 ± 0.045a</td>
<td>0.343 ± 0.029a</td>
<td>0.519 ± 0.035b</td>
</tr>
</tbody>
</table>

Each value is the mean ± SE.
a Different superscript letters show significant difference at p < 0.05.
NS: not significant.

Table 6. Cholesterol Oxidation Products in the Aorta of Apo E-Deficient Mice Fed on the WO or HLSO Diet for 9 Weeks

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Male</th>
<th>Female</th>
<th>Diet</th>
<th>Sex</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>(ng/mg aorta)</td>
<td>HLSO (n = 4)</td>
<td>WO (n = 5)</td>
<td>HLSO (n = 3)</td>
<td>WO (n = 4)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>3430 ± 629</td>
<td>3170 ± 479</td>
<td>2126 ± 401</td>
<td>2604 ± 492</td>
<td>NS</td>
</tr>
<tr>
<td>7α-Hydroxycholesterol</td>
<td>0.75 ± 0.24</td>
<td>0.67 ± 0.21</td>
<td>0.69 ± 0.07</td>
<td>0.96 ± 0.34</td>
<td>NS</td>
</tr>
<tr>
<td>7β-Hydroxycholesterol</td>
<td>0.78 ± 0.20</td>
<td>0.74 ± 0.24</td>
<td>0.66 ± 0.07</td>
<td>0.92 ± 0.28</td>
<td>NS</td>
</tr>
<tr>
<td>β-Epoxycholesterol</td>
<td>1.52 ± 0.40</td>
<td>1.39 ± 0.47</td>
<td>1.44 ± 0.13</td>
<td>1.58 ± 0.58</td>
<td>NS</td>
</tr>
<tr>
<td>α-Epoxycholesterol</td>
<td>1.21 ± 0.31</td>
<td>0.96 ± 0.27</td>
<td>1.19 ± 0.23</td>
<td>1.06 ± 0.40</td>
<td>NS</td>
</tr>
<tr>
<td>Cholestanetriol</td>
<td>0.67 ± 0.25</td>
<td>0.48 ± 0.13</td>
<td>0.62 ± 0.15</td>
<td>0.74 ± 0.32</td>
<td>NS</td>
</tr>
<tr>
<td>7-Ketocholesterol</td>
<td>1.15 ± 0.23</td>
<td>0.99 ± 0.28</td>
<td>0.98 ± 0.15</td>
<td>1.36 ± 0.38</td>
<td>NS</td>
</tr>
<tr>
<td>25-Hydroxycholesterol</td>
<td>0.19 ± 0.05</td>
<td>0.19 ± 0.06</td>
<td>0.12 ± 0.06</td>
<td>0.13 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>27-Hydroxycholesterol</td>
<td>0.11 ± 0.02</td>
<td>0.12 ± 0.06</td>
<td>0.10 ± 0.02</td>
<td>0.07 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Total oxysterol</td>
<td>6.38 ± 1.53</td>
<td>5.54 ± 1.61</td>
<td>5.80 ± 0.62</td>
<td>6.81 ± 2.30</td>
<td>NS</td>
</tr>
</tbody>
</table>

Each value is the mean ± SE.
NS: not significant.

**Serum and liver lipids**
The WO diet resulted in a higher concentration of serum cholesterol than the HLSO diet did (P = 0.06; Table 4). The female mice had a lower concentration of serum cholesterol than did the male mice. There were no significant effects of dietary fat on the levels of serum phospholipids and liver lipids.

**Lesion area in the aortic valve**
The lesion area in the aortic root was greater in the females than in the males (Table 5). The WO diet resulted in a higher lesion area in the female mice than the HLSO diet did.

**Cholesterol and its oxidation products in the aorta**
As shown in Table 6, the major sterol in the aorta was cholesterol. Prevalent cholesterol oxidation...
products were β- and α-epoxycholesterol, and 7-ketocholesterol. There was no diet or gender effect on these sterol levels.

Discussion

The present study has shown that the WO diet resulted in a higher level of cholesterol and lesion area in the aortic root of apo E-deficient mice than did the HLSO diet. The diet-induced difference in lesion area was more prominent in the female mice than in the males. However, the WO diets, which was rich in α-linolenic acid, had no significant effect on the serum and liver triacylglycerol levels in this animal, despite the hypotriglyceridemic action of α-linolenic acid that has been reported in other strains of rat\(^\text{10}\) and mouse.\(^\text{17}\) These results were unexpected, because previous animal and human experiments, including epidemiological studies, have presented evidence showing that α-linolenic acid, in comparison with linoleic acid, exerted equivalent or rather beneficial effects on the serum cholesterol level, atherosclerotic lesion development and coronary heart diseases.\(^\text{1–3,7,8}\)

A lack of apo E leads to hypercholesterolemia in apo E-deficient mice, since apo E is the ligand for serum triacylglycerol-rich lipoproteins (chylomicrons and VLDL) when they are taken up by the liver.\(^\text{19}\) Furthermore, apo E in the liver appears to play an important role in assembling and/or secreting VLDL from the liver, because apo E-deficient mice, in comparison with their wild strain, have a lower secretion rate of VLDL from the liver.\(^\text{19}\) These characteristics in apo E-deficient mice might have been involved in the diet-induced changes in serum cholesterol and serum and liver triacylglycerols that were different from those in the wild strain. However, it remains to be clearly determined why the WO diet resulted in an increased level of serum cholesterol and no reduction of triacylglycerol levels in the serum and liver.

In the present study, the serum cholesterol level was higher in the male mice than in the females, as has been reported previously.\(^\text{20}\) In contrast, the lesion area in the aortic root was greater in the females than in the males. This was confirmed by a separate experiment (unpublished observation). The present study has also shown that there was an interactive effect between the diet and gender in the lesion area in the aortic root. The WO diet, compared with the HLSO diet, led to an increased lesion area in the female mice, but not in the males. Accordingly, it seems that the WO diet directly affected the arterial metabolism of the female mice. Conversely, it is unlikely that the ability of the WO diet to raise the serum cholesterol level contributed to the development of the lesion area in the female mice.

We measured the level of cholesterol and its oxidation products in the abdominal and thoracic aorta as an index for atherosclerosis development in apo E-deficient mice, since the contents of cholesterol and its oxidation products had been found to increase with increasing lesion area in apo E-deficient mice (unpublished observation). The result of the present study showed no diet- and gender-dependent effects on the levels of those sterols, suggesting that there were no diet-dependent effects on the lesion area in the aorta. Therefore, the WO diet seemed to exert a site-specific effect, particularly in arteries smaller than the aorta.\(^\text{21}\)

α-Linolenic acid in comparison with linoleic acid is susceptible to lipid peroxide formation in vitro.\(^\text{22}\) This may raise the possibility that an α-linolenic acid-rich diet would accelerate the formation of lipid peroxides in the arterial wall, thereby facilitating the development of the arterial lesion area. However, this is not likely because there were no differences in the lesion area of the aorta or aortic root among apo E-deficient mice that had been fed on a diet rich in linoleic, eicosapentaenoic or docosahexaenoic acid (unpublished observation). Furthermore, our unpublished results showed that, when apo E-deficient mice were fed on the WO and HLSO diets, there were no differences in the serum titers of autoantibodies against aldehyde-modified albumin which are proportional to the development of the lesion area in the aortic root.\(^\text{23}\)

Epidemiological studies have suggested that ingesting α-linolenic acid might be beneficial to protect humans from cardiac diseases.\(^\text{7,8}\) This is partly due to the beneficial effect on the serum lipid profile, because, in clinical studies, a diet supplemented with walnuts that is rich in α-linolenic acid decreased the serum concentration of low-density lipoprotein and total cholesterol.\(^\text{1–5}\) Our present results with apo E-deficient mice are in contrast to those observations. In this context, apo E-deficient mice might not be an appropriate animal model for studying the role of α-linolenic acid in atherosclerotic lesion development.

In summary, the present results show that the WO diet resulted in an elevation of serum cholesterol and in the development of the atherosclerotic lesion area in apo E-deficient mice. These effects appear to have been due to α-linolenic acid.

References

3) Sabaté, J., Fraser, G. E., Burke, K., Knutsen, S., Bennett, H., and Lindsted, K. D., Effects of walnuts...


