Comparison of Atherogenicity of Soybean Oil and Peanut Oil, and Effect of Clentiazem on Diet-Induced Atherosclerosis in Rabbits

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ABSTRACT. Rabbits were fed with two kinds of atherogenic diet, one containing 0.5% cholesterol and 3% soybean oil and the other 0.5% cholesterol and 6% peanut oil, for three months to compare the atherogenic properties of the diets. The soybean oil diet seemed to be superior to the peanut oil diet for evaluation of the anti-atherogenic effect of drugs, because the former caused milder vascular lesions than the latter. Using this rabbit model for atherosclerosis, the anti-atherogenic effect of clentiazem, a new calcium antagonist, was examined. Clentiazem at an oral dose of 30 mg/kg/day significantly reduced the size of atheromatous lesion in the aortic arch and thoracic aorta, and lowered the collagen content of the aortic intima and media, although it did not decrease serum lipid levels. On the other hand, clentiazem showed no clear effect on reducing the coronary atherosclerotic lesions. These results suggest that clentiazem may inhibit the progression of diet-induced aortic atherosclerosis without normalizing the serum lipid levels. — KEY WORDS: atherosclerosis, calcium antagonist, hypercholesterolemia, peanut oil, soybean oil.


Long-term feeding of rabbits with a cholesterol-rich diet has been widely used as an animal model of atherosclerosis [2–4, 7, 22]. In most previous reports [8, 10, 11], researchers tried to reinforce the atherogenicity of the diet by annexing 4–8% vegetable oil to the 1–3% cholesterol loading. Rabbits fed with such a lipid-rich diet showed enormously high serum cholesterol levels and developed severe systemic lipidosis within several weeks [18]. However, in the case of human atherosclerosis [24], hyperlipidemia and systemic lipidosis are milder than those in rabbits. Therefore, an experimental model of atherosclerosis with milder hypercholesterolemia has been needed for the evaluation of anti-atherogenic drugs in experimental animals, especially in rabbits. For this purpose, the present study determined the concentrations of cholesterol and vegetable oil in the atherogenic diet, which was lower than those in the previous reports [8, 10, 11]. The cholesterol content was fixed at 0.5% in the diet and as for additional vegetable oil either 3% soybean oil or 6% peanut oil was applied according to the method of Rouleau [20] and Kritchovsky [12]. The atherogenicity of these two diets after three-month feeding was compared in rabbits. Then, this atherosclerosis model was used for evaluating the anti-atherogenic effect of clentiazem, a new calcium antagonist [9, 14, 16] which has shown a hypotensive effect in rabbits [21].

MATERIALS AND METHODS

Animals: Experiment 1: Seventeen male Japanese white rabbits (17 weeks old, Kitayama Labes, Kyoto), weighing 2.4 kg on the average, were divided into 3 groups. Five rabbits were fed with a restricted amount (100 g/head/day) of the standard diet. The remaining eight rabbits were fed with the same amount of the atherogenic diet containing 0.5% cholesterol and 3% soybean oil. Clentiazem, a calcium antagonist which had been newly synthesized in Tanabe Seiyaku Co., Ltd., Saitama, was dissolved in deionized water and given orally to ten rabbits of the atherogenic diet group every day for 12 weeks from the day when the atherogenic diet was started. According to the dose finding study in rabbits, clentiazem caused a dose-related decrease in mean blood pressure. The hypotensive effect of single oral administration was statistically significant at a dose of 30 mg/kg, but at a dose of 10 mg/kg its hypotensive effect was slight and insignificant [21]. From these preliminary data, we used an oral dose of 30 mg/kg clentiazem. To the remaining eight rabbits of the atherogenic diet group, water, instead of clentiazem solution, was administered in the same way.

Sampling for biochemical and histological analysis: Approximately 3 ml of blood was taken with a plastic syringe from the ear artery every month. Serum samples were then prepared and kept at −20°C until measurement of serum lipids. After exanguination under deep anesthesia with sodium pentobarbital, the entire length of the aorta was removed and a photo-copy of the aortic inner surface was taken. The aorta was then longitudinally divided into 2 parts. The intimal and medial tissues were
stripped off one part according to the method of Wolinsky and Daly [23]. Aortic lipids were extracted by a modification of Folch’s method [5]. The residual defatted aortic tissues were used for measurement of protein and collagen. The other part of the aorta was observed histopathologically. For electron microscopic examination, small tissue segments (3 mm in length) taken from the aortic arch and thoracic aorta of 2 rabbits in each group were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer and embedded in epoxy resin. Ultra-thin sections were cut and double-stained with uranyl acetate and lead citrate and observed under an electron microscope (JEM-100C, JEOL, Tokyo). The remaining tissue was fixed in 10% neutral buffered formalin solution and frozen. Frozen sections 6 μm in thickness were cut and stained with oil red O and hematoxylin, and hematoxylin and eosin (HE).

Morphometry: The ratio of the atheromatous area to the whole inner surface of the aortic arch and thoracic aorta on the photo-copy was measured by a computer-assisted image analyzing system (Yokogawa Hewlett-Packard 9845B and 9111A, Tokyo).

Determination of lipids and collagen: Serum lipids and aortic total cholesterol and triglycerides were enzymatically measured in an automatic analyzer (Hitachi 705, Tokyo) with enzymatic kits (Cholesteryne-V and Triglyzene-V, Eiken Chemical, Tokyo). The amount of phospholipids in the aortic tissues was calculated from the inorganic phosphorus value which had been measured according to Allen’s method [1] modified by Nakamura and Mori [15]. After being hydrolyzed with 6N HCl and then neutralized with NaOH, hydroxyproline in the defatted aortic tissues was measured by the method of Neuman and Logan [17]. Aortic protein was measured by the method of Lowry et al. [13].

Statistical analysis: The results were expressed as the means ± SEM. Statistical analysis was performed by analysis of variance and Scheffe’s test or Student’s t-test.

RESULTS

Clinical and biochemical profiles: Experiment 1: In the peanut oil feeding group, two rabbits lost their appetite in the last week of the experimental period and died 1 or 2 days before the end of the experiments. They both showed accumulation of pleural and ascitic fluids. Only histopathological examination was carried out on the dead animals.

In the remaining animals, no clinical sign or body weight loss was observed throughout the experimental period. Serum total cholesterol, triglycerides and phospholipids were markedly increased in the rabbits in the two atherogenic diet groups in comparison with the standard diet group (Table 1).

Experiment 2: No clinical sign or body weight loss was observed in any of the groups over the entire course of the experiments (Table 1). Serum total cholesterol, triglycerides and phospholipids were markedly increased with time in the rabbits fed with the soybean oil-supplemented diet. Serum concentrations of the three lipids were not lowered by clentiazem administration (Table 1).

Aortic lipids and collagen: Experiment 1: In the atherogenic diet feeding groups, the lipid content of the aortic arch was greater than that of the thoracic or abdominal aorta. The lipid content of the aortic walls were not markedly different in the two atherogenic diet groups (Table 2). The collagen content and the percentage of collagen to the total protein content of the aortic arch showed a tendency to decrease following the feeding of either atherogenic diet (Table 3).

Experiment 2: Total cholesterol, triglycerides and phospholipids were markedly increased in the aorta of the rabbits fed with the soybean oil-supplemented diet. The administration of clentiazem slightly reduced the amount of each lipid (Table 2).

The aortic content of collagen (μg/mg tissue dry weight) was decreased by feeding of the soybean oil-supplemented diet. On the other hand, the percentage of collagen to the total protein was slightly increased by feeding the atherogenic diet. The administration of clentiazem reduced both the collagen content and the percentage of collagen to the total protein content of the aorta (Table 3).

Morphometry: Experiment 1: Atheromatous plaques were observed on the aortic inner surface of all the rabbits fed with the soybean or peanut oil-supplemented diet but not in the control rabbits. The atheromatous area percentage was greater in the peanut oil feeding group (64±9%) than that in the soybean oil feeding group (36±10%) (Fig. 1).

The size of the atheromatous area in the aortic arch was the largest, followed by the thoracic and abdominal aorta in that order.

Experiment 2: The atheromatous area percentage of the

Table 1. Body weight and serum cholesterol, triglyceride and phospholipid levels at sacrifice

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>BW (g)</th>
<th>TC (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>PL (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard diet</td>
<td>6</td>
<td>3695±38</td>
<td>10±2</td>
<td>53±10</td>
<td>36±5</td>
</tr>
<tr>
<td>SBO diet</td>
<td>6</td>
<td>3392±130</td>
<td>2055±332**</td>
<td>295±36**</td>
<td>687±83**</td>
</tr>
<tr>
<td>PNO diet</td>
<td>3</td>
<td>3610±153</td>
<td>1691±497**</td>
<td>231±95*</td>
<td>587±139**</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard diet</td>
<td>5</td>
<td>3378±46</td>
<td>18±4</td>
<td>33±6</td>
<td>53±9</td>
</tr>
<tr>
<td>SBO diet</td>
<td>8</td>
<td>3368±78</td>
<td>201±134**</td>
<td>412±71**</td>
<td>793±60**</td>
</tr>
<tr>
<td>SBO diet+ Clentiazem</td>
<td>10</td>
<td>3322±65</td>
<td>2185±225**</td>
<td>383±51**</td>
<td>842±90**</td>
</tr>
</tbody>
</table>


a) Values are means±SEM.

b) 3% soybean oil+0.5% cholesterol in standard diet.
c) 6% peanut oil+0.5% cholesterol in standard diet.
d) 30 mg/kg/day, p.o.

*: p<0.05, **: p<0.01 vs. standard diet group (Scheffe’s method).
ANTI-ATHEROGENIC EFFECT OF CLENTIAZEM

Table 2. Aortic total cholesterol, triglyceride and phospholipid contents

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Total cholesterol</th>
<th>Triglycerides</th>
<th>Phospholipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(μg/mg of dry weight tissues)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AR</td>
<td>TH</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard diet</td>
<td>6</td>
<td>5.5±0.6 ⁰</td>
<td>4.5±0.3</td>
<td>4.6±0.3</td>
</tr>
<tr>
<td>SBO diet³</td>
<td>6</td>
<td>90.8±24.3 ⁴</td>
<td>30.0±16.9</td>
<td>51.9±17.0 ⁴</td>
</tr>
<tr>
<td>PNO diet³</td>
<td>3</td>
<td>94.4±43.2 ⁴</td>
<td>23.0±13.7</td>
<td>30.5±9.7 ⁴</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard diet</td>
<td>5</td>
<td>5.9±0.2 ²</td>
<td>5.5±0.3</td>
<td>5.6±0.3</td>
</tr>
<tr>
<td>SBO diet</td>
<td>8</td>
<td>195.9±12.7 ⁴**</td>
<td>43.8±8.3</td>
<td>90.8±9.9 ⁴**</td>
</tr>
<tr>
<td>SBO diet+</td>
<td>10</td>
<td>169.1±20.2 ⁴**</td>
<td>33.5±12.6</td>
<td>67.8±11.9 ⁴**</td>
</tr>
<tr>
<td>Clentiazem⁰</td>
<td></td>
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</table>

Abbreviations: AR: Aortic arch, TH: Thoracic aorta, AB: Abdominal aorta.
a) Values are means±SEM.
b) 3% soybean oil+0.5% cholesterol in standard diet.
c) 6% peanut oil+0.5% cholesterol in standard diet.
d) 30 mg/kg/day, p.o.
*: p<0.05, **: p<0.01 vs. standard diet group (Sheffe’s method).

Table 3. Aortic collagen content

<table>
<thead>
<tr>
<th>Collagen</th>
<th>(μg/mg of dry weight tissues)</th>
<th>(% of total protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AR</td>
<td>TH</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard diet</td>
<td>6</td>
<td>166.7±12.3 ³*</td>
</tr>
<tr>
<td>SBO diet³</td>
<td>6</td>
<td>118.0±17.9</td>
</tr>
<tr>
<td>PNO diet³</td>
<td>3</td>
<td>113.3±13.4</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard diet</td>
<td>5</td>
<td>154.9±10.2</td>
</tr>
<tr>
<td>SBO diet</td>
<td>8</td>
<td>91.5±6.2**</td>
</tr>
<tr>
<td>SBO diet+</td>
<td>10</td>
<td>87.8±7.1**</td>
</tr>
</tbody>
</table>

Abbreviations: AR: Aortic arch, TH: Thoracic aorta, AB: Abdominal aorta.
a) Values are means±SEM.
b) 3% soybean oil+0.5% cholesterol in standard diet.
c) 6% peanut oil+0.5% cholesterol in standard diet.
d) 30 mg/kg/day, p.o.
*: p<0.05, **: p<0.01 vs. standard diet group,
#: p<0.05 vs. SBO diet group (Sheffe’s method).

clentiazem-treated rabbits was significantly decreased (Fig. 1).

Microscopic findings in the aorta: Experiment 1: In the aortic arch and thoracic aorta of the atherogenic diet-fed rabbits, the intima showed a marked thickening with the accumulation of a large number of round or spindle-shaped foam cells and extracellular collagen fibers (Fig. 2). Lipid droplets were observed in the cytoplasm of the foam cells. Endothelial cells also had some lipid vacuoles (Fig. 3). These lipid deposits were observed not only in the intima but also in the upper layer of the media. Furthermore, smooth muscle cells (SMCs) migrating from the media to the intima were sporadically observed (Fig. 4). There were no distinct differences between the two atherogenic diet-fed groups in the microscopic findings in the aorta.

Experiment 2: Microscopic findings in the atheromatous plaques of all the rabbits fed with the atherogenic diet were nearly the same as those in Experiment 1. In some cases, spindle-shaped SMC-like cells proliferated in the subendothelial spaces of the plaques, forming so-called fibrous caps. Both intracellular and extracellular lipid droplets were observed in the aortic wall (Fig. 5). In the media just beneath the markedly thickened intima, lipid droplets were deposited both intrasonic and extracellularly.

There were no essential differences between the microscopic pictures of the atherogenic diet-fed control and the clentiazem-treated groups.

Microscopic findings in the heart: Experiment 1: Marked thickening of the intima was observed in the intramyocardial artery (IMA) walls of the left ventricle in all the rabbits fed with either of the two atherogenic diets.
Slightly eosinophilic lipid-containing amorphous materials were deposited in the subendothelial space (Fig. 6). In some animals, lipid droplets were observed within macrophage-like cells in the subendothelial space. There were few collagen fibers in these intimal lesions. Foci of slight myocardial degeneration and small areas of granulation were usually observed in the atherosclerotic diet-fed rabbits. Two rabbits fed with the peanut oil-supplemented diet which died during the experimental period had apparent myocardial infarction in the left ventricular wall and papillary muscles (Fig. 7).

**Experiment 2**: Microscopic changes in the heart of the atherosclerotic rabbit fed rabbits were the same as those in Experiment 1. There were no distinct differences between lesions in the atherosclerotic diet-fed control and clentiazem-treated rabbits.

**DISCUSSION**

Rabbits fed with a lipid-rich diet usually show an enormously high serum cholesterol level and develop severe systemic lipodisosis, which are rare findings in human atherosclerosis and are the cause of emaciation in this animal model [18]. We therefore tried to produce atherosclerosis with relatively mild hypercholesterolemia in rabbits. Kritchevsky et al. [12] reported that the atherogenicity of a cholesterol-rich diet was reinforced by the addition of certain vegetable oils, especially peanut oil. Recently, Rouleau et al. [20] reported that a 3% soybean oil-supplemented diet was suitable for producing atherosclerosis in rabbits. In Experiment 1, accordingly, the concentration of cholesterol in the diet was reduced to below that described in previous reports [8, 10, 11] and 6% peanut oil or 3% soybean oil was added to the diet.

The serum cholesterol level of the atherogenic diet-fed rabbits was approximately 2,000 mg/dl at the end of the experimental period, which was relatively lower than or similar to those in previous studies [8, 10, 11, 20] in which 1–5% cholesterol was used. The serum lipid levels in the peanut oil-loaded rabbits were somewhat lower than those in the soybean oil-loaded rabbits.

In the microscopic examination, atheromatous plaques of the aorta were characterized by a large number of lipid-laden SMC-like cells, macrophages and foam cells, and excessive extracellular collagen and lipid in the intima. On the other hand, the atherosclerotic lesion of the IMA was quite different from that of the aorta. The lesion consisted mainly of extracellular lipid deposits and its cellularity was very low. The difference between the atheromatous lesions of the aorta and the IMA might be due to the architecture of their vessel walls, that is, the aorta is an elastic type vessel and the IMA is of a muscle type [19]. The influence of direct physical force, that is, the effect of myocardial contraction may play an important role in forming the IMA lesion.

The area percentage and the degree of aortic atheromatous plaques in the peanut oil-loaded group were greater than those in the soybean oil-loaded group. These results suggest that the atherogenicity of peanut oil might be stronger than that of soybean oil as Kritchevsky et al. reported [12]. The soybean oil-supplemented diet seemed more suitable than the peanut oil-supplemented diet for an efficacy evaluation study of anti-atherogenic drugs over a period as long as 10 weeks. We therefore selected the soybean oil-supplemented diet for the study of the anti-atherogenic effect of clentiazem.

Clentiazem is a new benzoiazepine derivative which resembles diltiazem in chemical structure. Clentiazem has been found to be more potent than diltiazem in its calcium-antagonizing action [9, 14, 16]. Clentiazem significantly reduced the area percentage of atheromatous plaques in the aorta. Clentiazem also reduced aortic lipid and collagen and the collagen/protein ratio, although the decreases were both small. The serum lipid levels were almost the same in the lipid-loaded control rabbits and clentiazem-treated rabbits. These results suggest that clentiazem does not exhibit its anti-atherogenic effect through a serum lipid-lowering action. We have reported that clentiazem suppressed the balloon-induced intimal thickening in the aorta of rabbits fed with an atherogenic diet and this anti-atherogenic effect might be due to the prevention of SMC proliferation and the production of matrix proteins, particularly collagen, by SMCs in the aortic intima [21]. The preliminary study revealed that an oral dose of 30 mg/kg clentiazem also had a hypotensive effect on rabbits. The anti-atherogenic effect of clentiazem, therefore, may be attributable not only to the direct inhibitory action on SMC proliferation in the
vascular wall but also to its hypotensive action.

On the other hand, development of the atherosclerotic lesions in the IMA was not influenced by clentiazem administration. Moreover, there were no marked differences between myocardial lesions in the lipid-loaded control rabbits and clentiazem-treated rabbits. The lack of a suppressing effect on the IMA lesions has also been reported in the case of another calcium antagonist, diltiazem, by Ginsburg et al. [6]. It seems that the anti-atherogenic action of clentiazem is quite similar to that of diltiazem in cholesterol-loaded rabbits. Although the reason why these calcium antagonists had no inhibitory effect on the atheromatous lesions in the IMA was not fully understood, a very small number of target cells (SMCs) in the IMA wall may have to do with their ineffectiveness.
Consequently, it was concluded that the 3% soybean oil-supplemented diet was more suitable for an efficacy evaluation study of anti-atherogenic drugs because of relatively mild atherosclerosis induced. With this system, it was revealed that clentiazem suppressed aortic but not IMA atherosclerosis in rabbits.

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