Fish Oil Inhibits Development of Atherosclerosis in Rhesus Monkeys

Harry R. Davis, Robert T. Bridenstine, Dragoslava Vesselinovitch, and Robert W. Wissler

The effect of feeding fish oil (Menhaden) on the progression of rhesus monkey atherosclerosis was determined by feeding diets containing 2% cholesterol and either 25% coconut oil (Group I), 25% fish oil/coconut oil (1:1) (Group II), or 25% fish oil/coconut oil (3:1) (Group III) for 12 months (n=8/group). The average serum cholesterol levels were 875 mg/dl for Group I, 463 mg/dl for Group II, and 405 mg/dl for Group III. HDL cholesterol levels were 49 mg/dl for Group I, 29 mg/dl for Group II, and 20 mg/dl for Group III. An average of 79% of the aortic intima was involved with atherosclerosis in Group I, 48% in Group II, and 36% in Group III. The aortas of both fish-oil groups (II or III) contained significantly less cholesterol (total, free, and esterified), as well as less acid lipase, cholesteryl esterase, and ACAT activities when compared to the coconut-oil group (I) (p < 0.05). Microscopically, the aortic and carotid artery lesions were smaller in cross-sectional area and in thickness, and contained less macrophages in the fish-oil groups (II and III) when compared to the coconut-oil group (I) (p < 0.05). This protective effect was not consistently enhanced by increasing the proportion of fish oil to 3:1 (Group III) over 1:1 (Group II). The results indicate that fish oil-containing diets reduce serum cholesterol levels and inhibit atherosclerosis even in the face of lowered HDL cholesterol levels when compared to a pure coconut oil/cholesterol diet in rhesus monkeys. Therefore, fish-oil diets exert effective protective control of progression of atherosclerosis during severe atherogenic stimuli. (Atherosclerosis 7:441–449, September/October 1987)

Epidemiologic studies which compared Greenland Eskimos and mainland Danes as well as a recent study of men from Zutphen, The Netherlands have indicated that a reduction in coronary heart disease may be associated with the consumption of fish. Controlled human dietary studies have shown that consumption of fish oil, which contains high levels of long-chain, highly unsaturated omega-3 fatty acids causes a reduction in serum cholesterol and triglyceride levels. These dietary studies also demonstrated a reduction in both low density (LDL) and very low density lipoprotein (VLDL) levels and synthesis, with varying responses in high density lipoprotein (HDL) levels.

There is a general lack of information concerned with the effect of fish oil alone or in combination with other oils on atherosclerotic lesions. In addition to epidemiological studies, most investigations in which fish oil has been given in definite amounts with unrestricted diets have focused mainly on serum lipid levels, the effects on platelet function, variations in the cyclooxygenase and lipoxygenase pathways, and changes in human monocytes and polymorphonuclear. This lack of data on the effect of fish oil at the vessel wall level prompted us to undertake this investigation in the rhesus monkey, which is an excellent model for this type of study of atherogenesis. The experiment was designed to compare the effects on these monkeys of 12 months of a high-cholesterol diet supplemented with coconut oil alone to similar diets supplemented with Menhaden fish oil and coconut oil in a 1:1 or a 3:1 ratio.

Methods

**Animals and Diets**

Twenty-four adult (2.5 to 3 years old), male rhesus monkeys (Macaca mulatta) were purchased from the National Institute of Health (Alice, Texas). The monkeys were divided into three groups of eight each and fed the diets presented in Table 1 for 12 months. Custom-made constant formula, low-fat Purina monkey chow was used as the basic ration to which was added 25% fat and 2% cholesterol. The 25% fat consisted of coconut oil for Group I, coconut oil/Menhaden fish oil (1:1) for Group II, and coconut oil/Menhaden fish oil (1:3) for Group III. The fish oil contained cholesterol (432 mg/dl); therefore, the monkeys in Groups II and III consumed slightly more cholesterol than Group I (Table 1). The fatty acid content of the fat supplemented in each diet is detailed in Table 2. Each chow-based diet supplied sufficient essential fatty acids and other nutrients. All animals were treated and cared for according to NIH guidelines for humane treatment of laboratory animals. Fasting serum lipid values were determined at the onset and at monthly intervals throughout the
Table 1. Diet Composition

<table>
<thead>
<tr>
<th>Diet ingredients (g/100)</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III*</th>
<th>Group III†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monkey meal‡</td>
<td>71.25</td>
<td>71.25</td>
<td>71.25</td>
<td>71.25</td>
</tr>
<tr>
<td>Coconut oil§</td>
<td>25.00</td>
<td>12.50</td>
<td>0.00</td>
<td>6.25</td>
</tr>
<tr>
<td>Fish oil∥</td>
<td>0.00</td>
<td>12.50</td>
<td>25.00</td>
<td>18.75</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>2.000</td>
<td>2.054</td>
<td>2.108</td>
<td>2.081</td>
</tr>
<tr>
<td>Ausman-Hayes vitamin mix</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Gelatin</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Orange juice¶</td>
<td>15.00</td>
<td>15.00</td>
<td>15.00</td>
<td>15.00</td>
</tr>
</tbody>
</table>

*Diet fed for 1.5 months initially. † Dated fed for the remainder of the study. ‡ Purina Monkey Chow, specially formulated without added fat. § Durkee Foods, Chicago, Illinois. ||Menhaden, Zapata Haynie, Reedville, Virginia. ¶ Excluded from total dry weight.

Table 2. Fatty Acid Content of Dietary Fat Supplements

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:0</td>
<td>0.12</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>8:0</td>
<td>1.75</td>
<td>0.88</td>
<td>0.44</td>
</tr>
<tr>
<td>10:0</td>
<td>1.50</td>
<td>0.75</td>
<td>0.38</td>
</tr>
<tr>
<td>12:0</td>
<td>12.00</td>
<td>6.00</td>
<td>3.00</td>
</tr>
<tr>
<td>14:0</td>
<td>4.75</td>
<td>3.42</td>
<td>2.75</td>
</tr>
<tr>
<td>16:0</td>
<td>2.25</td>
<td>3.02</td>
<td>3.41</td>
</tr>
<tr>
<td>16:1</td>
<td>—</td>
<td>1.45</td>
<td>2.18</td>
</tr>
<tr>
<td>16:2—4</td>
<td>—</td>
<td>0.76</td>
<td>1.14</td>
</tr>
<tr>
<td>18:0</td>
<td>0.75</td>
<td>0.71</td>
<td>0.69</td>
</tr>
<tr>
<td>18:1</td>
<td>1.50</td>
<td>1.94</td>
<td>2.16</td>
</tr>
<tr>
<td>18:2</td>
<td>0.38</td>
<td>0.41</td>
<td>0.43</td>
</tr>
<tr>
<td>18:3</td>
<td>—</td>
<td>0.23</td>
<td>0.34</td>
</tr>
<tr>
<td>18:4</td>
<td>—</td>
<td>0.43</td>
<td>0.65</td>
</tr>
<tr>
<td>20:1—3</td>
<td>—</td>
<td>0.25</td>
<td>0.38</td>
</tr>
<tr>
<td>20:4</td>
<td>—</td>
<td>0.29</td>
<td>0.43</td>
</tr>
<tr>
<td>20:5</td>
<td>—</td>
<td>2.00</td>
<td>3.01</td>
</tr>
<tr>
<td>22:1—4</td>
<td>—</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>22:5</td>
<td>—</td>
<td>0.49</td>
<td>0.74</td>
</tr>
<tr>
<td>22:6</td>
<td>—</td>
<td>1.35</td>
<td>2.03</td>
</tr>
<tr>
<td>Others</td>
<td>—</td>
<td>0.48</td>
<td>0.69</td>
</tr>
<tr>
<td>Total</td>
<td>25.00</td>
<td>25.00</td>
<td>25.00</td>
</tr>
<tr>
<td>Total n-6</td>
<td>0.38</td>
<td>0.70</td>
<td>0.86</td>
</tr>
<tr>
<td>Total n-3</td>
<td>0</td>
<td>4.50</td>
<td>6.77</td>
</tr>
</tbody>
</table>

Values are given in grams per 100 g dry weight. The Group I diet contained 25% coconut oil; the Group II diet contained 12.5% coconut oil and 12.5% fish oil; the Group III diet contained 6.25% coconut oil and 18.75% fish oil.

12-month experimental period by previously described methods.

**Laboratory Techniques**

The animals were autopsied after 12 months following exsanguination with thiamyal sodium anesthesia. Aorta, carotid, and femoral arteries were cleaned of adventitia and were opened, and the extent and severity of atherosclerosis was evaluated independently by two observers using a point counting technique. Standard samples from aorta, carotid, and femoral arteries less than 3 mm in thickness were fixed at 4° C for 24 hours in 4% paraformaldehyde (wt/vol) containing 7.5% sucrose (wt/vol), followed by a 24-hour washing at 4° C in 1% gum acacia (wt/vol) containing 30% sucrose (wt/vol). Serial frozen sections (10 μm) were cut and stained for lipid with oil red O and for macrophages by histochemical localization of acid lipase activity. The atherosclerotic lesions and their components were quantitated by computer-assisted morphometric analysis. The system used has been previously described by Glagov et al. and other investigators. Standard samples of the thoracic and abdominal aortas were analyzed biochemically for composition and enzyme activities. The aorta was split longitudinally, and adjacent samples from the right side of the thoracic aorta beginning at the second intercostal were sampled for enzyme assays (2 cm) and chemical composition (1 cm). The abdominal aorta was sampled 3 cm distal to the renals for chemical composition (1 cm) and enzyme assay (2 cm). Aortic lipids were extracted by the method of Bligh and Dyer with coprostanol as an internal standard. The quantitation of total, free, and esterified cholesterol was performed according to the method of Ishikawa et al. as modified by Bates and Wissler. Briefly, the relative volume of the cholesterol peak to the nearby coprostanol peak was used to demonstrate the free cholesterol content of the sample using gas liquid chromatography. After the free cholesterol content of a sample aliquot was determined, the entire sample was saponified. Chromatographic determination on this saponified sample provided quantitation of the total cholesterol in the original sample. The cholesterol ester content was determined by subtraction of the free cholesterol content from the post-saponified determination.

Samples for enzyme assay were homogenized in distilled, deionized water at 0° C in an all-glass homogenizer (1/10, wt/vol). The homogenate was then centrifuged at 5000 g for 10 minutes, and the supernatant was collected for enzyme activity studies.

Cholesteryl ester hydrolytic activity was determined using a radioactively labelled vesicle substrate system modified from the method of Brecher et al. Vesicles were prepared with egg-yolk lecithin containing unlabelled cholesteryl oleate and cholesteryl-1-14C oleate in a 66:1 phospholipid/substrate molar ratio. These mixtures were dried and lyophilized, mixed with 0.01 M Tris HCl buffer (pH 7.4), containing 0.1 M NaCl and 0.02% sodium azide, and sonicated. The vesicle mixture (100 μl) was then added to an equal volume of 0.15 M acetate buffer (pH 4.5) (100 μl), and 50 μl of tissue homogenate was assayed. The final substrate concentration in the incubation mixture was 1 mg of egg-yolk lecithin and 14 μg of cholesteryl oleate. The reaction was terminated after a 4-hour incubation by add-
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Figure 1. Serum total cholesterol and low density lipoprotein (LDL) cholesterol levels of the three groups of monkeys fed high fat, high cholesterol diets for 12 months. The mean total cholesterol levels ± SEM are represented by the total bars and the mean LDL cholesterol levels by the hatched portion.

Acid lipase substrate micelles were prepared using 1.0 mM alpha-naphthyl palmitate and 10 mM Triton X-100 in 0.1 M sodium acetate buffer (pH 4.2) containing 0.1% fatty acid-poor bovine serum albumin. \(^{29}\) Fifty µl of homogenized tissue supernatant was assayed in 1 ml of substrate at 25°C with continuous agitation for 1 to 4 hours. The reaction was stopped by placing the samples in boiling water for 2 minutes. One ml of 1.0 M sodium acetate buffer (pH 4.2) containing 10% Tween 20 (wt/vol) and 0.5 mg fast garnet GBC salt was added to each sample. Diazocoupling of liberated alpha-naphthol was performed at 25°C for 16 hours. Absorbance of samples, heat-inactivated blanks, and alpha-naphthol standards were then measured at 535 nm.

Acyl-CoA cholesterol acyl transferase (ACAT) activity was determined by a modified method of Brecher and Chobanian. \(^{39}\) The incubation medium consisted of 40 µM (1-14C) palmitoyl-CoA, 2 mg/ml fatty acid-free bovine serum albumin (BSA) and 2 mM dithiothreitol in 0.1 M potassium phosphate buffer (pH 7.4). The aortic supernatants (20 µl) were assayed for 60 minutes at 37°C and the reaction was stopped by the addition of methanol. The lipids were extracted by the method of Bligh and Dyer. \(^{34}\) The solvents were evaporated under N\(_2\); the lipids were resuspended in chloroform and spotted on silica gel IIB2 (Baker-flex) thin-layer chromatography plates. The plates were developed in hexane/ethyl ether/acetic acid, 83:16:1; the spots were visualized in I\(_2\) vapors; the lipid classes were cut out and counted by liquid scintillation spectrometry.

Figure 2. Serum high density lipoprotein (HDL) cholesterol levels of the three groups of monkeys over the 12-month dietary period. Values are means ± SEM at selected time points.

Statistical Analysis

Statistical differences between groups were analyzed using the Wilcoxon test and significance was assumed for \(p\) values less than 0.05.

Results

Serum Lipids

The average total cholesterol for all three groups at the start of the experiment was 172 mg/dl. The animals in Group I which were fed 25% coconut oil and 2% cholesterol rapidly increased their average serum cholesterol levels to 683 mg/dl after 1 month and maintained a running average of 875 mg/dl over the twelve months (Figure 1). The animals in Group II which were fed coconut oil/fish oil (1:1) increased their average serum cholesterol levels to 571 mg/dl at 1 month which fell to a 12-month running average of 463 mg/dl which was significantly lower than Group I (\(p<0.05\)). Group III with a 1:3 coconut oil/fish oil diet had similar changes in serum cholesterol as seen in Group II (Figure 1) with a 12-month average of 405 mg/dl.

The HDL cholesterol levels in all three groups were reduced by the high-fat diets (Figure 2). Group I demonstrated the least reduction from a baseline level of 83 mg/dl to an average of 49 mg/dl over the 12 months. Fish oil in the diets of Groups II and III caused a more significant reduction in HDL cholesterol to average levels of 29 mg/dl and 20 mg/dl, respectively, over the 12 months.

Serum triglyceride levels were never increased over normal and were below 40 mg/dl, and there were no differences among the three groups.

Atherosclerotic Lesion Distribution

The degree of aortic, carotid, and femoral artery atherosclerosis involvement was evaluated and was expressed as the percentage of total surface area covered by lesions (Table 3). The aortas of Group I were more extensively involved with atherosclerosis with an average of 79% of
Table 3. Percent Surface Involvement with Intimal Lesions

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Supplements</th>
<th>Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thoracic</td>
</tr>
<tr>
<td>I</td>
<td>Coconut oil and cholesterol</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>II</td>
<td>Coconut oil/fish oil (1:1) and cholesterol</td>
<td>38 ± 7*</td>
</tr>
<tr>
<td>III</td>
<td>Coconut oil/fish oil (1:3) and cholesterol</td>
<td>29 ± 11*</td>
</tr>
</tbody>
</table>

Values are the means ± SEM. A two-observer point-counting method was used.

*p < 0.01; †p < 0.001 less than Group I.

Aortic Cholesterol

The cholesterol concentration of the aortas of all three groups was increased over that normally found in rhesus monkey aortas. Group I which was fed coconut oil alone had more cholesterol (total, free, and esterified) in their aortas when compared to either Group II or III (p < 0.01). The total cholesterol content (µg/mg wet weight) values for the aortas were 19.3 ± 0.8 for Group I, 7.6 ± 1.6 for Group II, and 5.3 ± 0.8 for Group III. Of the total cholesterol, 34% was in the esterified form in Group I, 29%, in Group II, and 15%, in Group III.

Microscopic Morphometry

The atherosclerotic lesions that resulted from the 12 months of dietary induction were distributed throughout the aorta and most severely in the carotid bifurcation and intermittently in the femoral arteries. The lesions in the coconut-oil alone Group I were similar to those that we have previously reported. Figure 3A shows a representative aortic lesion with numerous macrophage-derived foam cells and a necrotic core containing an area of calcification. The lesions that developed in the fish-oil Groups II and III were similar in appearance and were less severe fatty plaques containing a mixture of proliferated cells, some with lipid droplets, foam cells, and connective tissue. A representative aortic lesion from the fish-oil groups is shown in Figure 3B.

Morphometric analysis of the lesions in the thoracic aortas revealed that the fish-oil groups (II and III) developed lesions that were significantly smaller in cross-sectional area and thickness than the lesions in the coconut-oil Group I (Table 4) (p < 0.001). The lesions in the abdominal aorta of all the groups were larger and more severe than the thoracic lesions. This topographic differential is similar to the distribution of human atherosclerotic lesions. The fish-oil groups (II and III) also developed abdominal lesions that were significantly smaller in area than the coconut-oil group (Table 4) (p < 0.05). No differences were found between the aortic lesions in Group II and Group III.

The atherosclerotic lesions just proximal to the carotid bifurcation were the most severe and complex that developed. The fish oil added to the diets also reduced the carotid lesion induction when compared to the coconut-oil alone group. Both Groups II and III had carotid lesions that were significantly less stenotic (p < 0.001), smaller in area (p < 0.02), and less thick (p < 0.001) than Group I.

Figure 3. Aortic sections stained for acid lipase activity from the coconut-oil Group I (A) and a representative section from the fish-oil, combined Groups II and III (B). A. An advanced intimal plaque from Group I with many acid lipase-positive macrophages (darkly stained cells) and a necrotic core, containing an area of calcification. Bar = 200 µm. B. A less severe intimal lesion from the fish-oil groups with a few acid lipase-positive macrophages and a mixture of smooth muscle cells and connective tissue. Methyl green counterstain, bar = 100 µm.
Table 4. Aortic Microscopic Data

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Supplements</th>
<th>Lesion area (mm²)</th>
<th>Lesion thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thoracic</td>
<td>Abdominal</td>
</tr>
<tr>
<td>I</td>
<td>Coconut oil and cholesterol</td>
<td>0.67 ± 0.07</td>
<td>1.24 ± 0.18</td>
</tr>
<tr>
<td>II</td>
<td>Coconut oil/fish oil (1:1) and cholesterol</td>
<td>0.20 ± 0.05†</td>
<td>0.52 ± 0.10*</td>
</tr>
<tr>
<td>III</td>
<td>Coconut oil/fish oil (1:3) and cholesterol</td>
<td>0.20 ± 0.04†</td>
<td>0.46 ± 0.16*</td>
</tr>
</tbody>
</table>

Thoracic: 0.20 ± 0.04 0.26 ± 0.02
Abdominal: 0.08 ± 0.01† 0.13 ± 0.02

Values are the means ± SEM.
*p < 0.01; †p < 0.001 less than Group I.

Table 5. Carotid Microscopic Data

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Supplements</th>
<th>Percent luminal stenosis</th>
<th>Lesion area (mm²)</th>
<th>Lesion thickness (mm)</th>
<th>Macrophage area (mm²)</th>
<th>Percent macrophages</th>
<th>Intracellular lipid area (mm²)</th>
<th>Percent intracellular lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Coconut oil and cholesterol</td>
<td>32 ± 5</td>
<td>0.64 ± 0.22</td>
<td>0.28 ± 0.05</td>
<td>0.23 ± 0.06</td>
<td>35 ± 3</td>
<td>0.21 ± 0.05</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>II</td>
<td>Coconut oil/fish oil (1:1) and cholesterol</td>
<td>10 ± 1†</td>
<td>0.13 ± 0.02</td>
<td>0.08 ± 0.01†</td>
<td>0.03 ± 0.01*</td>
<td>21 ± 5</td>
<td>0.03 ± 0.01†</td>
<td>14 ± 3*</td>
</tr>
<tr>
<td>III</td>
<td>Coconut oil/fish oil (1:3) and cholesterol</td>
<td>4 ± 2†</td>
<td>0.06 ± 0.03*</td>
<td>0.04 ± 0.01*</td>
<td>0.02 ± 0.01*</td>
<td>11 ± 4*</td>
<td>0.02 ± 0.01†</td>
<td>9 ± 4†</td>
</tr>
</tbody>
</table>

Values are means ± SEM for each group.
*p < 0.05; †p < 0.001 less than Group I.

Table 6. Lipids and Macrophages in Aortic Intimal Lesions

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Supplements</th>
<th>Percent intracellular lipid</th>
<th>Percent macrophages</th>
<th>Area intracellular lipids (mm²)</th>
<th>Area macrophages (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thoracic</td>
<td>Abdominal</td>
<td>Thoracic</td>
<td>Abdominal</td>
</tr>
<tr>
<td>I</td>
<td>Coconut oil and cholesterol</td>
<td>43 ± 7</td>
<td>27 ± 4</td>
<td>37 ± 5</td>
<td>17 ± 4</td>
</tr>
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<td>II</td>
<td>Coconut oil/fish oil (1:1) and cholesterol</td>
<td>36 ± 8</td>
<td>15 ± 5</td>
<td>22 ± 5</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>III</td>
<td>Coconut oil/fish oil (1:3) and cholesterol</td>
<td>30 ± 10</td>
<td>18 ± 9</td>
<td>17 ± 7</td>
<td>6 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
*p < 0.001; †p < 0.01 less than Group I.

Table 7. Aortic Enzyme Activities

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Supplements</th>
<th>Acid lipase* (μM/min/g)</th>
<th>Cholesteryl esterase (CPM/min/g)</th>
<th>ACAT (CPM/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Thoracic</td>
<td>Abdominal</td>
<td>Thoracic</td>
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<tr>
<td>I</td>
<td>Coconut oil and cholesterol</td>
<td>103 ± 23</td>
<td>125 ± 54</td>
<td>11,719</td>
</tr>
<tr>
<td>II</td>
<td>Coconut oil/fish oil (1:1) and cholesterol</td>
<td>26 ± 7*</td>
<td>45 ± 10</td>
<td>2878</td>
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<tr>
<td>III</td>
<td>Coconut oil/fish oil (1:3) and cholesterol</td>
<td>38 ± 14*</td>
<td>39 ± 5</td>
<td>5267</td>
</tr>
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</table>

Values are the means ± SEM.
*p < 0.05; †p < 0.01 less than Group I.
(Table 5). Although the carotid lesions in Group III were smaller than in Group II, this difference was not statistically significant.

The amount of lipid in the lesions was morphometrically quantitated on a percentage of lesion area and absolute cross-sectional area basis. The percentage of the aortic lesions occupied by intracellular lipid was less in the fish-oil groups (II and III) when compared to Group I but this difference was not statistically significant (Table 6). The percentage of the carotid lesions occupied by intracellular lipid was significantly less in the fish-oil groups (II and III) when compared to Group I (p < 0.01) (Table 5). The area occupied by intracellular lipid was significantly less in both the thoracic and abdominal aortic lesions, as well as the carotid lesions, in Groups II and III when compared to Group I (Tables 5 and 6). No differences were found between Groups II and III.

Macrophages were identified by staining for acid lipase activity, and the acid lipase-positive macrophage lesions were quantitated in cross-sectional area and by the percentage of the intimal lesion. The percentage of the aortic and carotid lesion areas occupied by macrophages was less in the fish-oil groups (II and III) when compared to Group I, but this difference was only statistically significant in the Group III carotid lesions (p < 0.001) (Tables 5 and 6). The actual cross-sectional area of the lesions occupied by acid lipase-positive macrophages was significantly smaller in most lesions examined in Groups II and III when compared to Group I. The macrophage involvement was not found to be different when Groups II and III were compared (Tables 5 and 6).

Aortic Enzymes

Atherosclerosis-associated increases in lipid-related enzyme activities were measured in the thoracic and abdominal aortas of the monkeys, and the results are seen in Table 7. Acid lipase and cholesteryl esterase hydrolytic activities were reduced in the fish-oil groups (II and III) when compared to Group I. The difference in lipid hydrolytic activity was significantly less in the thoracic aorta (p < 0.05) and because of the large variance in the abdominal activities, only the cholesteryl esterase of Group III was significantly less (p < 0.01).

Acyl-CoA cholesterol acyl transferase (ACAT) activity increased to a lesser extent in the fish-oil groups (II and III) when compared to Group I (Table 7). These differences were also only statistically significant in the thoracic aorta (p < 0.05) due to the large variance in the abdominal aorta ACAT activities. No differences were found between the aortic lipid-related enzyme activities of Group II when compared to Group III. In general, the samples with the smaller lesions had the least activity.

Discussion

This study demonstrates that the mixing of substantial quantities of fish oil with coconut oil and cholesterol reduces both the hyperlipidemia and atherosclerotic response measured when a ration containing only coconut oil and cholesterol is fed to rhesus monkeys for 12 months. The combination of coconut oil and cholesterol has been shown to be one of the most atherogenic diets in several experimental models of atherosclerosis, including nonhuman primates, rabbits, and dogs. In fact, coconut oil is the only dietary fat that has been consistently shown to support the development of canine atherosclerosis. The rhesus monkey is one of the best animal models in which to study the effects of fish oil on serum lipids and atherogenesis since their digestive system, serum lipoprotein pattern, and the induced atherosclerosis appear to be the most similar to humans of all the presently tested animal models.

The supplementation of Menhaden fish oil, which contains a large amount of long-chain polyunsaturated omega-3 fatty acids, to the coconut oil/cholesterol atherogenic diet led to a marked decrease in the development of hypercholesterolemia even though the fat content and the caloric intake of the animals remained the same and the cholesterol consumption was substantially increased due to the cholesterol in the fish oil (432 mg/dl). This inhibition of the increase in serum lipids was primarily due to the reduction of the LDL cholesterol fraction in these monkeys. Many of the human dietary studies with fish oil have attributed its hypocholesterolemic effect to the reduction of LDL and VLDL levels. Harris et al. and others have shown that fish oil lowers serum cholesterol, LDL, VLDL, and triglycerides in normal humans. Phillipson et al. extended these studies to hypertriglyceridemic patients (Type IIb and V) and found that salmon and/or Max elcosapentaenoic acid (EPA) reduced serum cholesterol, triglyceride, VLDL, and apoprotein E levels. Similar studies of hyperlipidemias by Simon et al. and Sanders et al. demonstrated only a reduction in serum triglycerides, while Saynor et al. reported a rapid reduction in triglycerides, a slow fall in cholesterol, and a rise in HDL cholesterol. Many of these investigators have attributed the hypolipidemic effects of fish oil to the reduction of VLDL synthesis in the liver, leading to a reduction in LDL synthesis. In the present rhesus monkey study, the increase in the ratio of fish oil to coconut oil from 1:1 to 3:1 did not significantly increase the hypolipidemic effect of the fish oil. This amount of fish oil consumption per day was from half of to equivalent to the amount fed in the above human studies (10 to 20 g Max EPA/day), but the monkeys only weighed between 4.5 and 5 kg. Potentially, a smaller dose of fish oil that more closely mimics the human studies may be as effective in lowering serum cholesterol levels. The most consistent effect of dietary fish oil in humans is the reduction of serum triglyceride levels. Rhesus monkeys rarely increase their serum triglyceride levels in response to an atherogenic diet challenge and the serum triglycerides never rose above 40 mg/dl in this experiment, and no differences were found between the groups. The mechanism by which the fish oil reduced serum LDL levels in rhesus monkeys may be due in part to a decrease in VLDL synthesis, even though this is not reflected in the fasting serum triglyceride levels. Several other possible mechanisms may also be operative, although not directly tested in this study. Since a majority of the serum cholesterol was in the LDL fraction, any changes in serum lipids and atherogenesis since their digestive system, serum lipoprotein pattern, and the induced atherosclerosis appear to be the most similar to humans of all the presently tested animal models.
likely mechanism is an increase in lipolytic enzyme activities and LDL turnover due to increases in fluidity of both lipoproteins and cell membranes. Additional studies addressing the mechanism(s) of the hypocholesterolemic effects of fish oil are necessary to clarify this issue.

High density lipoprotein cholesterol (HDL-C) levels were depressed slightly to 49 mg/dl by the coconut-oil diet, and the addition of fish oil caused a significant reduction of the HDL-C levels to 29 mg/dl and 20 mg/dl with 1:1 and 3:1 ratios of fish-oil to coconut-oil mixtures. HDL-C levels reported in human studies have been inconsistent, with reports of increases, \(^5\), \(^6\), \(^9\)–\(^10\) no effects, \(^7\) and reductions. \(^7\), \(^11\)

The reduction of HDL-C levels by the fish-oil diets could be a matter of concern, since HDL is thought to be protective against atherosclerosis. \(^51\) but it is becoming increasingly evident that there are many exceptions to this formulation.

The effect of fish-oil-containing diets on atherosclerosis in humans is primarily based on epidemiological studies that have looked at the average diets of populations and compared cardiovascular events. These populations have included Greenland Eskimos and mainland Danes, \(^1\), \(^2\) Japanese, \(^52\) and the Netherlands Zutphen study in males. \(^3\) Overall, the results of these studies indicate a beneficial effect of fish-oil-containing diets. There are very few experimental atherosclerosis studies that examine the effect of fish oil on the arterial wall. Two recent studies by Thierry and Seidel in rabbits \(^23\) and Rogers and Karnovsky in rats \(^24\) demonstrated that fish-oil diets actually enhanced the atherosclerotic response. The only previous reports of a preventive effect of fish oil on atherosclerosis were a 1957 study by Hegsted et al. \(^25\) which demonstrated a reduction of aortic sudanophilia in rats fed fish oil and a very recent study of the effects of cod liver oil in swine. \(^26\) Weiner et al. \(^26\) demonstrated a reduction in coronary atherosclerosis in hypercholesterolemic swine supplemented with cod liver oil without reducing serum lipid levels. In this swine study they attributed the fish-oil effect on lesions to the reduction of serum thromboxane B\(_2\) levels and the enrichment of platelet fatty acids with eicosapentaenoic acid. It is not clear why fish oil reduced serum cholesterol in the rhesus monkeys and not in swine except that it may be due to species differences and that the rhesus monkey is probably a better animal model to study serum lipid effects. The present study in the rhesus monkey, an excellent model for this type of diet study, demonstrated an inhibition of atherogenesis by fish oil with numerous methods of evaluation of atherogenesis. The fish-oil groups had less femoral and carotid artery and aortic surface area involved with lesions. Their aortas contained less cholesterol (total, free, and esterified). Microscopically, the lesions were smaller in cross-sectional area, were less thick and had less intracellular lipid in the aorta and carotid arteries of the fish-oil groups. The reduced atherosclerosis in the fish-oil groups occurred even though the HDL cholesterol levels were reduced when compared to normal values and to the values in the coconut-oil group.

The finding that the fish-oil group had fewer macrophages in their lesions may be associated with the anti-inflammatory effects attributed to fish oil. Lee et al. \(^22\), \(^23\) have shown that neutrophils and monocytes from humans fed fish oil have their S-lipoxygenase pathway inhibited, are less likely to adhere to leukotriene B\(_4\)-treated endothelial cells, and have their chemotactic response inhibited. Other investigators have been studying the anti-inflammatory effect of fish oil in the treatment of rheumatoid arthritis. \(^57\) The reduction of the number of macrophages in the fish-oil group lesions were probably the reason that the lesions had less acid lipase and cholesterol esterase activity, since we have previously found that the macrophages in both human and experimental lesions were responsible for most of these lesion lipolytic activities. \(^58\) In addition to acid lipase and cholesterol esterase, acyl-CoA cholesterol acyl transferase (ACAT) also increases in atherosclerotic arteries when compared to normal vessels. \(^59\) The increase in acyclic ACAT activity was significantly less pronounced in both of the fish-oil groups when compared to the coconut-oil group. Therefore, the results indicate that the arterial cells were reesterifying less cholesterol for storage in arterial cells in the fish-oil groups. \(^60\)

The overall results of this study demonstrate that replacing only 50% of coconut oil with fish oil reduced serum cholesterol and inhibited atherosclerosis even though HDL cholesterol levels were depressed. The beneficial effect was not directly related to the amount of fish oil fed, since replacing 75% of the coconut oil with fish oil did not consistently enhance its beneficial effect further. In fact, the only statistically significant reduction in lesions of Group III over Group II was the surface area of the carotid arteries. The effective dose of fish oil required to inhibit atherogenesis may be far less than that used in this study, since the consumption of only a modest amount of fish by humans has been shown to reduce coronary heart disease. \(^5\) Many investigators have attributed the beneficial effect of fish oil to its EPA content; whether concentrated EPA supplements in human diets will inhibit atherosclerosis has yet to be determined. This report presents tangible experimental evidence supporting previous results from epidemiologic studies, as well as clinical and experimental studies, which indicate that fish oil containing omega-3 fatty acids can reduce serum cholesterol by decreasing both low density and very low density lipoproteins and can thus inhibit atherogenesis. Fish-oil-containing diets may be a useful part of a therapeutic regimen for prevention or control of atherosclerosis progression in humans.

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