Dietary Antioxidants Do Not Reduce Fatty Streak Formation in the C57BL/6 Mouse Atherosclerosis Model

John S. Munday, Keith G. Thompson, Kerry A.C. James, B. William Manktelow

Abstract—Epidemiological studies and animal trials have suggested that dietary antioxidants protect against atherosclerosis. To test this hypothesis, C57BL/6 mice were fed atherogenic diets supplemented with either vitamin E or butylated hydroxytoluene (BHT). Three groups of 20 mice were fed for 15 weeks on diets containing 1% cholesterol and 0.5% cholic acid. The diet of two groups was supplemented with either 2% vitamin E or 1% BHT. The control group received no antioxidant supplements. The lowest mean serum cholesterol concentration was measured in mice supplemented with vitamin E. Mean serum HDL cholesterol concentrations were highest in the control group, which also had the highest ratio of HDL cholesterol to total cholesterol. Mice fed BHT developed a significantly greater area of aortic fatty streak lesions than the other two groups. However, despite having a more atherogenic lipoprotein profile, mice fed vitamin E developed a level of fatty streak formation similar to the control group. At the end of the trial, mice consuming the vitamin E- and BHT-supplemented diets had higher serum total antioxidant levels than the control mice. Because of changes to lipid metabolism caused by both vitamin E and BHT, the results of this study cannot be used to support the hypothesis that antioxidants confer protection against atherosclerosis. The results do, however, raise the possibility that other studies demonstrating an antiatherogenic action of vitamin E and BHT may have been influenced by their effects on lipid metabolism. (Arterioscler Thromb Vasc Biol. 1998;18:114-119.)

Key Words: atherosclerosis ■ antioxidants ■ BHT ■ vitamin E ■ mice

The earliest lesion of atherosclerosis is believed to be the fatty streak,¹ which consists of a subendothelial collection of foam cells (macrophages containing lipid droplets), small amounts of extracellular lipid, and increased numbers of smooth muscle cells.² Oxidation of LDL is considered to be an important step in the development of fatty streaks.³ Macrophages do not engulf native LDL quickly enough to form foam cells, but oxidized LDL is recognized by macrophage scavenger receptors,⁴ leading to fast, unregulated LDL uptake and foam cell formation.³,⁶ Oxidation of LDL occurs after it has become trapped in the subendothelial matrix because at that site it loses the protection of plasma antioxidants.⁶,⁷ Dietary antioxidants may, however, delay the oxidation of LDL,⁶,⁸ enabling it to leave the subendothelial space before oxidation occurs.⁹ This may reduce incorporation of LDL into foam cells and therefore reduce the atheroma development and progression.⁶ This concept has led to the hypothesis that low plasma antioxidant status is a risk factor for atherosclerosis.¹²

Studies on the possible beneficial effects of vitamin E on heart disease began in 1949,¹³ but the results of such studies have been inconclusive. Vitamin E was reported to decrease the severity of atherosclerosis in a Dutch-belted rabbit model,¹⁴,¹⁵ and in primates,¹⁶ but had no effect in a WHHL rabbit model.⁵,¹⁷,¹⁸ In a rabbit model in which atheroma formation was induced by damaging the endothelium, vitamin E increased the severity of atherosclerosis.¹⁹ The results of epidemiological studies investigating the relationship between vitamin E and atherosclerosis have also been inconclusive.²⁰-²⁸

The antioxidant BHT has been shown to reduce atherosclerosis in a model using WHHL rabbits.²⁹ This chemical is widely used at low levels in foods and cosmetics,³⁰,³¹ although its chronic toxicity makes it unlikely to be used as an antioxidant supplement in human diets.³² Another synthetic antioxidant, diphenyl-1,4-phenylenediamine, has also been shown to decrease atherosclerosis in rabbit³² and mouse³³ atherosclerosis models.

C57BL/6 mice fed an atherogenic diet for 15 weeks develop fatty streaks in their aortic sinus.³⁴,³⁵ This well-established mouse model was used in the present study to examine the effect on subendothelial fatty streak formation of dietary supplementation with the antioxidants vitamin E and BHT.

Materials and Methods

Experimental Animals

Sixty 6-week-old female C57BL/6 mice were obtained from the Animal Breeding Station, Department of Laboratory Animal Sciences, University of Otago, New Zealand. Five mice were housed in each solid-floored cage and kept in a room maintained at 22°±1°C with a
humidity level of 60%±5% and air exchange 12 times per hour on a 12-hour light-dark cycle. The mice were acclimatized for 20 weeks, during which time they were fed a normal commercial mouse diet. At the start of the trial the mean mouse weight was 26.3±2.6 g. Throughout the experiment, the mice were given free access to food and water.

Experimental Design
Twenty mice were randomly allocated into one of the three dietary groups. Two treatment groups received diets supplemented with either 2% vitamin E acetate (10 IU/g diet) or 1% BHT by weight. The control group received the same diet but without supplementary antioxidant.

The mice were fed ad libitum for 15 weeks and were weighed weekly. At the end of the trial period, the mice were fasted for between 8 and 12 hours, then killed by carbon dioxide inhalation.

All procedures involving animals in this study were conducted under guidelines established by the Massey University Animal Ethics Committee and with their prior approval.

Experimental Diets
The composition of the experimental diets was based on that of Nishina et al.34 All mice were fed diets containing 5% corn oil, 5% olive oil, 5% anhydrous milk fat, 1% cholesterol, and 0.5% cholic acid (Table 1). The vitamin and mineral mixes were formulated by staff at the Food Evaluation Unit, Crop and Food Research Ltd, Palmerston North according to guidelines established by the United States National Research Council.36 Dry vitamin E 50%, type CWS/F containing 500 IU of vitamin E per gram, in the form of DL-α-tocopherol acetate was obtained from Roche Products (NZ) Ltd. All other chemicals were purchased from either Sigma Chemical Co or BDH Chemicals New Zealand Limited. To prepare the diets, the dry ingredients were mixed together and then water was added to form a dough. The dough was cut into strips, dried in an oven at 29°C for 3 days and then stored at −20°C until used. One week's supply of diet was removed from the freezer at a time and kept refrigerated at 4°C until used. Mice were provided daily with fresh diet from the refrigerator.

TABLE 1. Composition of Diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>BHT</th>
<th>Vitamin E</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>477</td>
<td>472</td>
<td>482</td>
</tr>
<tr>
<td>Casein</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>Corn oil</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Olive oil</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Anhydrous milk fat</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Salt mix</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>BHT</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin E acetate</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Composition of the antioxidant-containing and the control diet fed to CS75BL/6 mice for 15 weeks.

Statistical Analysis
Differences among dietary groups were analyzed using analysis of variance techniques. Because the groups contained an uneven number of animals by the completion of the trial, differences between means were analyzed using an unbalanced design. Lesion size, total serum cholesterol, total antioxidant status, triglyceride, and lipoprotein cholesterol concentrations were modeled using linear regression. All statistics were calculated using the SAS statistics package (SAS Institute Inc).

Results
During the trial, three mice were removed from the control and vitamin E groups, and two from the BHT group after failing to adapt to the trial diet and losing weight. The mean weights of the remaining mice in each trial group are shown in Table 2. At the completion of the trial, the mice in the BHT group were significantly lighter than those in the control group.

Antioxidant Status
Total antioxidant status was significantly higher in serum from mice fed diets containing either BHT or vitamin E than from control mice (Table 2).

Selected Abbreviations and Acronyms
ACAT = acyl-CoA:cholesterol acyltransferase
BHT = butylated hydroxytoluene
WHHL = Watanabe heritable hyperlipidemic rabbit model

Sample Collection and Processing
Immediately after euthanasia, between 0.5 and 1.0 mL of blood was collected from each mouse by cardiac puncture. The blood was transferred to a test tube and the serum separated by centrifugation at 1500×g for 15 minutes. Serum cholesterol and triglyceride concentrations were determined using a Hitachi 704 autoanalyzer (Boehringer Mannheim GmbH, Mannheim, Germany). Because the densities of both mouse and human lipoproteins are the same,37 it was possible to determine serum HDL cholesterol concentrations after selective precipitation of VLDL and LDL using polyethylene glycol 6000.36–42 The LDL cholesterol concentration could not be calculated from triglyceride and HDL cholesterol concentrations because the Friedewald formula31 has been found to be inaccurate in mice.44

Serum total antioxidant status was measured using a commercial kit (Randox Laboratories Ltd) run on a Hitachi 704 autoanalyzer. The kit contains a reagent that is oxidized at a known rate by a peroxidase. The ability of the test sample to inhibit this reaction is determined by measuring the formation of oxidation products of the reagent.

The liver from one mouse in each cage was fixed in neutral buffered formalin, embedded in paraffin, then sectioned and stained with hematoxylin and eosin for routine histological examination.

Morphological Evaluation of Fatty Streaks
The size of fatty streaks in the aortic sinus was quantified using a method similar to that previously described.35 After euthanasia, the heart was placed in 0.9% saline for 1 hour, then in 0.9% saline containing 4% formalin for 1 to 7 days. The ventricles were removed by cutting across the heart in a plane that included the base of both auricles. The hearts were then passed through ascending concentrations of gelatin at 37°C for 18 hours, frozen, and sectioned at 40 μm with a cryostat. Sections were discarded until the aortic sinus was recognized by the appearance of aortic valves and the rounded appearance of the aortic wall. Twenty-four consecutive 10-μm sections were then cut, mounted onto gelatin-coated slides, and stained using oil red O and Meyer’s hematoxylin method with light green counterstaining. The areas of intimal lipid deposition stained red with oil red O were quantified using the SigmaScan Scientific Measurement System (Jandel Scientific, San Rafael, Calif.). The cross-sectional area of lesions in every second section was measured so that a total of 12 sections from a 240-μm segment of aorta were examined per heart. All lesion assessment was performed blind by the same researcher.

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Serum Lipids
The mean serum lipoprotein and triglyceride concentrations of each group of mice are shown in Table 2. The mean total cholesterol concentration of mice supplemented with vitamin E was significantly lower than that of the other two groups. The mean serum HDL cholesterol concentrations of mice in the control group was significantly higher than those in the BHT and vitamin E groups. The highest mean ratio of serum HDL cholesterol to total cholesterol was observed in the control group, whereas the BHT group had a significantly lower ratio than the other groups. The ratio of serum HDL cholesterol to total cholesterol was negatively correlated with the serum total antioxidant status ($r^2 = .16$, $P = .005$; $n = 52$) (Fig 1).

Mean serum triglyceride concentrations in mice from the BHT group were higher than in mice from the control group.

Pathology
After 15 weeks, all mice developed lipid-containing lesions in the intima of the aortic sinus, similar to those previously described. The lesions were most common close to the origins of the coronary arteries and at the base of the aortic valves, and consisted of subendothelial collections of macrophages containing numerous oil red O–positive lipid globules. These globules were found extracellularly around areas of macrophage accumulation and were also present in the underlying media.

The mean area of the aortic lesions in each group of mice is shown in Table 2. The mean lesion area in mice receiving the diet containing BHT was significantly greater than in mice in the control and vitamin E groups. The ratio of HDL to total cholesterol was not significantly correlated with mean lesion area for individual mice ($r^2 = .07$, $P = .07$; $n = 52$) or when mean values of the dietary groups were considered ($r^2 = .75$, $P = .33$; $n = 3$) (Fig 2).

The livers of mice from all groups were enlarged and pale tan in color. Histological examination revealed diffuse hepatic lipidosis that did not differ in severity among groups on the basis of subjective assessment.

Discussion
The addition of either vitamin E or BHT to an atherogenic ration fed to C57BL/6 mice did not reduce the severity of fatty streak formation in the aortic sinus. Both antioxidants did, however, significantly change the serum lipoprotein profile.

The failure to observe an effect of vitamin E on aortic fatty streak reduction in this trial is in agreement with previous studies that used cholesterol-fed and WHHL rabbits. In contrast, two studies in which a low cholesterol, atherogenic diet was fed to rabbits for 10 months reported an atherosclerosis-reducing effect of vitamin E. In these last two studies, the vitamin E-supplemented diet was associated with lower serum cholesterol concentrations, and the beneficial effect of vitamin E observed may have reflected exposure to this, rather than an antioxidant effect. In a further study, in which vitamin E was added to an atherogenic diet fed to nonhuman primates, vitamin E resulted in significant reductions in arterial stenosis at some sample sites, although no significant differences were observed in the aorta.

In our study, mice receiving the diet supplemented with vitamin E had a significantly lower mean total serum chole-

### Table 2. Summary of Measurements Made After 15 Weeks on an Atherogenic Diet

<table>
<thead>
<tr>
<th>Dietary Group</th>
<th>1% BHT (n=18)</th>
<th>2% Vitamin E Acetate (n=17)</th>
<th>Control (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total aortic fatty streak area (mm²)</td>
<td>0.483 (0.265)a</td>
<td>0.312 (0.105)b</td>
<td>0.300 (0.156)c</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/L)</td>
<td>6.13 (0.81)a</td>
<td>4.68 (0.85)b</td>
<td>5.53 (1.54)c</td>
</tr>
<tr>
<td>Serum HDL cholesterol (mmol/L)</td>
<td>2.00 (0.42)a</td>
<td>1.96 (0.51)b</td>
<td>2.70 (0.59)c</td>
</tr>
<tr>
<td>HDL cholesterol: total cholesterol</td>
<td>0.33 (0.08)a</td>
<td>0.42 (0.10)b</td>
<td>0.50 (0.08)c</td>
</tr>
<tr>
<td>Serum triglyceride (mmol/L)</td>
<td>0.48 (0.24)a</td>
<td>0.58 (0.17)b</td>
<td>0.62 (0.69)c</td>
</tr>
<tr>
<td>Serum total antioxidant status (mmol/L)</td>
<td>1.40 (0.22)a</td>
<td>1.43 (0.29)b</td>
<td>1.22 (0.26)c</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>24.21 (2.21)a</td>
<td>25.62 (1.93)b</td>
<td>26.35 (2.52)c</td>
</tr>
</tbody>
</table>

The vitamin E group was fed a diet containing 2% vitamin E acetate, the BHT diet contained 1% butylated hydroxytoluene. The control group was fed a diet containing no antioxidants. All figures are the group means with the standard deviation contained in brackets. Values which do not have the same letter are significantly different ($P < .05$).

*Indicates that the differences between the means are significant at $P < .01$. 
terol concentration than controls. A hypocholesterolemic effect of vitamin E has been previously reported in rats and rabbits. Significant changes in serum cholesterol concentrations have not been reported in human subjects receiving vitamin E supplements; however, the doses of vitamin E used in human studies (600 to 800 mg/d per person) were far lower than those used in animal studies. Vitamin E probably lowers serum cholesterol by increasing the activity of cholesterol 7α-hydroxylase, the enzyme responsible for controlling the rate at which hepatic cholesterol is converted to bile acids.

Mean serum HDL cholesterol concentrations in the mice supplemented with either vitamin E or BHT were significantly lower than in controls. This is consistent with previous observations in which vitamin E has been shown to reduce serum HDL cholesterol concentrations in rabbits. In another rabbit study, BHT also showed a definite, if not significant, trend toward lowered HDL. In our study, the ratio of HDL to total cholesterol was negatively correlated to serum total cholesterol, suggesting that the proportion of cholesterol in the HDL fraction may be reduced by dietary antioxidants.

The addition of 1% BHT to the atherogenic diets of C57BL/6 mice resulted in significantly more aortic fatty streak development than in controls. This is in contrast to previous trials using rabbits, which reported either a reduction in atherosclerosis or no change in animals given BHT. Furthermore, total serum cholesterol concentrations in the C57BL/6 mice fed BHT were not significantly different from controls. Again, this is in contrast to previous rabbit, rat, and mouse BHT studies that reported an increase in serum cholesterol concentrations because of an inhibition of acyl-CoA:cholesterol acyltransferase (ACAT) activity. Because of the presence of cholic acid in the atherogenic diets, ACAT has little influence in determining serum cholesterol concentrations in the C57BL/6 mouse model. This may explain why BHT failed to increase serum cholesterol concentrations in this model.

The inhibition of ACAT activity by BHT may also reduce foam cell production. Foam cells develop after cholesterol contained in modified LDL is phagocytosed by a macrophage. In the macrophage, ACAT is responsible for forming cytoplasmic cholesterol droplets by esterifying cholesterol, and evidence suggests that the excretion of cholesterol from macrophages is most rapid when ACAT activity is low. It is possible, therefore, that BHT reduces foam cell formation by decreasing ACAT activity and so promoting the clearance of cholesterol from macrophages rather than as a result of any antioxidant action. Macrophages from C57BL/6 mice, when compared with macrophages from atherosclerosis-resistant mice, posses high ACAT activities when challenged with a high cholesterol diet. BHT may not depress ACAT activity sufficiently in this model to cause the reduction in foam cell formation that has been observed in rabbits.

Serum triglyceride concentrations were significantly lower in mice consuming a diet containing BHT than in controls. This does not support findings of previous rabbit trials in which dietary BHT greatly increased serum triglyceride concentrations. However, in our trial the mice that were fed diets supplemented with BHT for 15 weeks weighed less than those fed the control diet. Obesity is thought to be a cause of hypertriglyceridemia, and this may explain the lower levels reported here. Decreased weight gain because of 1% BHT has also been observed in trials with rats. In the rabbit studies in which increased triglyceride concentrations were reported, no differences in weight gain between the BHT and control groups were reported.

In a previous trial using the C57BL/6 mouse atherosclerosis model to examine the effect on fatty streak formation of diets containing different proportions of saturated fat, the lesion area was correlated to the HDL to total cholesterol ratio ($r^2=0.1$, $P=.01$; $n=73$) (unpublished data, 1996). This relationship was not observed in the present trial, ($r^2=.07$, $P=.07$; $n=52$), despite the fact that all the mice were fed essentially the same diet. These results could perhaps be interpreted as evidence of an antithrombotic effect of antioxidants. As illustrated in Fig 2, despite having a significantly lower HDL to total cholesterol ratio, which would be expected to result in greater fatty streak formation, mice fed vitamin E had a similar mean lesion area to the controls. However, because both antioxidants studied in this trial alter lipid metabolism their effect on foam cell formation may be independent of their antioxidant action.

No published study examining the effect of dietary vitamin E supplementation on atherosclerosis has demonstrated a decrease in atherosclerosis without a concurrent decrease in serum cholesterol. This is despite LDL particles from animals fed vitamin E becoming enriched with antioxidant and being more resistant to in vitro oxidation.

Because of the apparent effects on lipid metabolism of the antioxidants investigated in this trial, our results cannot be used to support the hypothesis that antioxidants confer protection from atherosclerosis. As most antioxidants appear to have an
Effect on blood lipoprotein profiles or body weight, it is difficult to evaluate the benefits of antioxidants from animal feeding trials. In vitro investigations into the oxidizability of LDL have been performed, but convincing data on a causal relationship between LDL oxidizability and atherosclerosis development is lacking. Results of epidemiological studies examining the relationship between antioxidants and atherosclerosis are also difficult to interpret because of inconsistency of results and the presence of confounding factors. Therefore, until more conclusive proof of a protective action of antioxidants is produced, discretion is needed before dietary antioxidant supplementation can be recommended to reduce the risk of atheroma-associated coronary heart disease.

Acknowledgments
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References


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