A Lutein-Enriched Diet Prevents Cholesterol Accumulation and Decreases Oxidized LDL and Inflammatory Cytokines in the Aorta of Guinea Pigs

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Abstract

Lutein has been shown to be protective against age-related macular degeneration; however, the antiinflammatory and antioxidant effects of this carotenoid in aortas are less known. Guinea pigs were fed a hypercholesterolemic diet (0.25 g cholesterol/100 g) and randomly allocated to a control group (n = 9) or a lutein group (n = 10) (0.1 g/100 g lutein) and fed the experimental diets for 12 wk. Plasma LDL cholesterol and TG did not differ between groups; however, the lutein group had lower concentrations of medium size LDL (P < 0.05). As expected, guinea pigs from the lutein group had higher concentrations of plasma and liver lutein than those from the control group (P < 0.0001). Aortic cholesterol and malondialdehyde concentrations were lower in the lutein group (9.6 ± 2.8 mmol/g and 1.69 ± 1.35 nmol/mg protein) compared to the control group (15.5 ± 2.3 mmol/g and 2.98 ± 1.45 nmol/mg protein) (P < 0.05). Hematoxilin and eosin staining indicated that aortas from the control group presented focal intimal thickening, whereas either less thickness or no visible thickness was present in aortas from the lutein group. Oxidized LDL (oxLDL) was lower both in plasma and aorta in the lutein group compared to the control group (P < 0.001). Aortic cytokines were also lower in the lutein group (P < 0.05). Plasma lutein and oxLDL (r = −0.79; P < 0.0001) and plasma lutein and aortic oxLDL (r = −0.64; P < 0.0001) were negatively correlated. These data suggest that lutein exerts potent antioxidant and antiinflammatory effects in aortic tissue that may protect against development of atherosclerosis in guinea pigs.

Introduction

Lutein, a carotenoid found primarily in green leafy vegetables and egg yolk, is preferentially located in the retina (1). Lutein is thought to have a role in protecting the retina against light damage (2); hence, there has been a major focus of research on age-related macular degeneration (AMD) (2,3). AMD is a leading cause of vision loss in the elderly (4) and the prevalence of AMD continues to grow in the United States (5). In addition to its protective effect against AMD, lutein is emphasized as a beneficial antioxidant due to its structural characteristics. Lutein contains conjugated C = C double bonds, which can readily quench singlet oxygen species (6). Previous studies have shown that lutein is useful in the prevention of systemic inflammation in response to LPS treatment in animal models (7,8) and in RAW264.7 cells (7,9) by inhibiting NF-κB–dependent signaling pathways and the subsequent production of proinflammatory mediators. In addition, lutein treatment also decreased intracellular reactive oxygen species accumulation in RAW264.7 cells (9), which suggests an antioxidant effect of lutein.

Based on these properties, lutein may play a role in the prevention of atherosclerosis (10,11). One of the main features of atherosclerosis, the major cause of coronary heart disease, is the accumulation of lipids and fibrous elements in large arteries (12). The first step that initiates atherosclerosis is the presence of LDL in the subendothelial matrix and its subsequent oxidation. Oxidized LDL (oxLDL) in the vessel wall is a major contributor to foam cell formation (12,13). OxLDL stimulate the overlying endothelial cells to produce adhesion molecules, resulting in the recruitment of monocytes to the vessel wall (12,14). The recruited monocytes are differentiated into macrophages and macrophages release cytokines such as TNFα and IL-6 (12).

We hypothesized that lutein would reduce inflammation and oxidation in plasma and aorta and would prevent early atherosclerosis development. Therefore, we evaluated the effects of this carotenoid on circulating atherogenic lipoproteins and oxLDL as well as on cytokine production. The present study...
utilized guinea pigs as the animal model, because they present similar responses to dietary interventions and lipoprotein metabolism as humans (15). Guinea pigs were fed a hypercholesterolemic diet to develop early atherosclerosis and inflammatory responses in a short period of time (16).

Materials and Methods

Animals. Nineteen male Hartley guinea pigs, aged 18 mo old (Charles River Breeding Laboratories), were randomly assigned to 2 groups, the lutein group and the control group. All guinea pigs were fed high cholesterol (0.25 g/100 g of diet) and the lutein group was treated with lutein (0.1 g/100 g of diet) (FloraGLO trans Lutein, Kemin Industries) for 12 wk. The composition of the experimental diets is presented in Table 1. The composition of the vitamin and mineral mix was previously reported (17). Animal protocols were approved by the Institutional Animal Care and Use Committee from the University of Connecticut. After 12 h of being feed deprived, guinea pigs were killed and blood, liver, and aorta were collected.

Plasma and liver lutein concentration. Approximately 0.5 g of liver or 300 µL of plasma was prepared for carotenoid analysis by saponification and extraction with hexane and ethyl ether. Samples were prepared as previously reported (18).

Plasma lipids. Photometric measurements were used to measure plasma lipids including total cholesterol, LDL cholesterol (LDL-C), HDL cholesterol, and TG (19) by using Cobas c 111 analyzer (Roche-Diagnostics). Plasma LDL particle size and concentration.

Lipoprotein subfractions and size. NMR analysis was performed on a 400-MHz NMR analyzer (Bruker BioSpin) as previously described (20).

Cholesterol accumulation in aorta and arterial morphology. Total cholesterol was measured in the abdominal aorta by extraction with chloroform:methanol (2:1) and then analyzed as described by Leite et al. (21). Aortas from each guinea pig were fixed in 10% neutral buffered formalin for histopathologic examination. Hematoxylin and eosin-stained, formalin-fixed, paraffin-embedded sections of the aorta were examined for atherosclerosis by a certified pathologist who was unaware of the treatments. The method of Stary et al. (22) was used for the evaluation of atherosclerosis.

Aortic and plasma oxLDL. Aortic oxLDL concentrations were measured in homogenized descendent thoracic aorta. The vessel was dissected (0.05 g) and the surrounding tissues were removed as previously reported (23). Both aortic and plasma oxLDL were analyzed using a mouse competitive ELISA from Mercodia, which is based in the mouse monoclonal antibody 4E6, which is directed against a conformational epitope in oxidized ApoB-100. We have successfully used this method to measure oxLDL in guinea pigs (16).

Aortic malondialdehyde concentrations. Aortic malondialdehyde (MDA) was measured by TBARS assay using a commercially available assay kit (Cayman Chemical). Samples (150 µL) and standards were read by fluorescence at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. Detected MDA were normalized to aortic protein concentrations, which were measured using the Bradford assay (Bio-Rad Laboratories).

Inflammatory cytokine concentration in the aorta. Cytokines were evaluated from homogenates of aorta, such as described elsewhere (21) using the MILLIPLEX MAP Mouse Cytokine PREMIXED Immunoassay kit (Millipore) and Luminex system (Luminex 200 System). The following aortic cytokines were measured: TNFα, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, and IL-13.

Statistical analyses. Statistical analyses were performed by using SPSS 17.0. Values are reported as mean ± SD. Independent t test and bivariate correlations were used when appropriate. Results with P < 0.05 were considered to be significant.

Results

Plasma lipids. Plasma total cholesterol concentrations did not differ between the lutein group (4.45 ± 1.50 mmol/L) and control group (3.78 ± 1.06 mmol/L), nor did LDL-C or TG, which were 3.38 ± 1.0 mol/L and 3.06 ± 1.10 mol/L, and 0.90 ± 0.17 mol/L and 0.76 ± 0.27 mol/L, respectively.

Plasma and hepatic lutein concentration. Plasma lutein concentrations of guinea pigs from the lutein group were higher compared to the control group (P < 0.0001) (Table 2). Hepatic lutein concentrations in the lutein group (23.8 ± 5.6 nmol/g) were also higher than in the control group (< 1 nmol/g) (P < 0.0001).

Plasma LDL particle size and concentration. LDL particle size and LDL subfraction concentrations did not differ between groups (data not shown). The percent distribution of large and small LDL particles also did not differ between the groups, whereas the percent distribution of medium LDL particles was lower in the lutein group (19.2 ± 3.0%) compared to the control group (26.1 ± 8.5%) (P < 0.05).

TABLE 1 Composition of experimental diets of guinea pigs from the control or lutein groups

<table>
<thead>
<tr>
<th>Components</th>
<th>Lutein</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean protein</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>Fat</td>
<td>15.1</td>
<td>15.1</td>
</tr>
<tr>
<td>Vitamins</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Minerals</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>Cellulose</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Guar gum</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Lutein</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

1 Corn starch:sucrose ratio, 1:1.43.
2 Fat mix was olive oil–palm kernel oil–safflower oil (12:1:8), high in lauric and myristic acids.
3 Vitamin and mineral mixes were formulated to meet the NRC requirements for guinea pigs. A detailed composition of the vitamin and mineral mix has been reported elsewhere (17).
4 Lutein was provided by FloraGLO (Kemin Industries).

TABLE 2 Plasma and liver lutein and aorta total cholesterol and malondialdehyde (MDA) concentrations of guinea pigs fed lutein or control diets for 12 wk

<table>
<thead>
<tr>
<th>Variables</th>
<th>Lutein (n = 10)</th>
<th>Control (n = 9)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma lutein, nmol/L</td>
<td>75.1 ± 17.1</td>
<td>&lt;5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Liver lutein, nmol/g</td>
<td>23.8 ± 5.6</td>
<td>&lt;1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Aorta Cholesterol, nmol/g</td>
<td>9.6 ± 2.8</td>
<td>15.5 ± 2.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>MDA, nmol/mg protein</td>
<td>1.69 ± 1.35</td>
<td>2.98 ± 1.45</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

1 Values are mean ± SD.
Aortic total cholesterol and MDA concentration. Aorta total cholesterol concentrations were lower for the lutein group compared to the control group \((P < 0.001)\) (Table 2). In addition, the lutein group also had lower aortic MDA concentrations than the control group \((P < 0.05)\), indicating attenuated lipid peroxidation in the lutein group (Table 2).

OxLDL concentrations in plasma and aorta. Plasma oxLDL concentrations in the lutein group were lower than in the control group \((P < 0.0001)\) (Fig. 1A). Similarly, aortic oxLDL concentration in the lutein group was also lower compared to the control group \((P < 0.001)\) (Fig. 1B).

Correlations between lutein concentrations and aortic cholesterol and plasma and aortic oxLDL. Plasma \((r = -0.79; P < 0.001)\) and hepatic \((r = -0.73; P = 0.001)\) lutein concentrations were negatively correlated with aortic total cholesterol. In addition, plasma and aortic oxLDL and aortic total cholesterol were negatively correlated with plasma oxLDL \((r = -0.64, P < 0.01; r = -0.69, P < 0.001)\), indicating associations between lutein concentrations and oxLDL production.

Cytokine concentration in aorta. Guinea pigs from the lutein group had significantly lower concentrations of most of the inflammatory cytokines, including IFN\(\gamma\), TNF\(\alpha\), IL-1\(\beta\), IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, and IL-12 than guinea pigs from the control group (Fig. 2). The aorta IL-13 concentration did not differ between the groups.

Aortic morphology. For aortic morphology, 6 guinea pigs/group were examined. Two guinea pigs in the control group (33%) had focal intimal thickening due to the presence of layers of myointimal cells (Fig. 3A). Lipid vacuoles were present in some of these cells and in the extracellular matrix. These changes are features of atherosclerosis. Among the remaining guinea pigs, there was variation in the thickness of the proteoglycan matrix in the intima and rare macrophages, some of which contained lipid vacuoles (foam cells). In addition and with only one exception, the thickness of the proteoglycan layer was less in the lutein group and was not visible in 2 of the lutein-treated animals (Fig. 3B). Thus, 84% of the examined guinea pigs from the lutein group presented no thickness of the proteoglycan layer, whereas 100% of the control group presented either features of early atherosclerosis or increased thickness in the proteoglycan matrix in addition to macrophages and foam cells.

Discussion
In this study, we demonstrated that in guinea pigs, dietary lutein was beneficial in preventing early atherosclerosis development by lowering circulating atherogenic lipoproteins and oxLDL as well as decreasing MDA and cytokine production in aortas without changing plasma lipid profiles. Further hematoxylin and eosin staining indicated that aortas from the control guinea pigs presented thickened intima plaque with the presence of foam cells, which was not present in the majority of guinea pigs from the lutein group.

Because the absorption of carotenoids varies widely depending on the food matrix (6) and we were uncertain of the rate of lutein absorption in guinea pigs, we provided the equivalent of 25–30 mg lutein/d, which is higher than supplement intake in humans (6–10 mg/d) (24,25). Based on the results from this study, guinea pigs have slightly lower concentrations of plasma lutein compared to humans (6,18), suggesting that the provided amount of dietary lutein was adequate to ensure sufficient circulating levels of this carotenoid to determine its antioxidant and antiinflammatory effects in the aorta.

Lutein, lipoproteins, oxLDL, and aortic cholesterol. Lutein had no effect on plasma lipid profiles as expected. Similar to our findings, no significant change in plasma LDL-C concentration was observed in ApoE null mice with lutein treatment (0.2 g/100 g)
Although a subepithelial layer of proteoglycan matrix on its own does not appear to be sufficient for the formation of a thick proteoglycan layer and presence of some vacuoles and macrophages that indicate potential development of atherosclerosis, guinea pigs from the lutein group (84%) presented no proteoglycan layer (B).

FIGURE 3 Features of aortas of guinea pigs fed a control or lutein diet for 12 wk. Aortas from 6 pigs/group were stained with H and E. Two of the guinea pigs (33%) from the control group presented features characteristic of early atherosclerosis development (A). Arrowheads indicate lipid vacuolation and arrows indicate internal elastic lamina. The rest of the control group (66%) plus one guinea pig from the lutein-treated group (17%) presented a thick proteoglycan layer and presence of some vacuoles and macrophages that indicate potential development of atherosclerosis. Guinea pigs from the lutein group (84%) presented no proteoglycan layer (B).

In regards to atherosclerosis development, only 2 guinea pigs in the control group developed classical atherosclerotic lesions. Although a subepithelial layer of proteoglycan matrix on its own is insufficient to qualify as atherosclerosis, it is nonetheless interesting that this layer was generally thinner or nonexistent in the lutein group. It is possible that if the guinea pigs had been maintained on the high-cholesterol diet for a longer period of time, other histological features of atherosclerosis may have developed in the control group. Consistent with our findings, both ApoE null mice and LDL receptor null mice had smaller atherosclerotic lesions of the aortic arch in the lutein-supplemented condition relative to the control (10).

Oxidative modification of LDL in the vascular endothelium is considered a pivotal factor in the development of early atherosclerosis (33). OxLDL promotes atherosclerosis through different mechanisms: by increases in the expression of adhesion molecules, enhancing migration of macrophages and smooth muscle cells, and augmenting the release of cytokines (34). OxLDL is also removed by macrophages, leading to the formation of foam cells, the first step in the initiation of atherosclerosis (12,35). Furthermore, oxLDL also causes oxidative stress in endothelial cells, smooth muscle cells, and macrophages and substantially contributes to the formation of the atherosclerotic plaque (35). It has also been suggested that circulating oxLDL is a marker of the early stage of atherosclerosis (36).

In our findings, lutein treatment showed antioxidant effects by significantly lowering oxLDL both in plasma and aorta. Indeed, plasma and hepatic lutein concentrations were highly correlated with plasma and aortic oxLDL. Along with lower oxLDL concentrations, the lutein group also presented significantly lower aortic MDA, further supporting the role of lutein as an antioxidant. LDL-conjugated dienes have been used as a measurement of LDL oxidation (37,38). One study reported that plasma lutein concentration is a powerful antioxidant that determines serum LDL-conjugated dienes and reduces concentrations of oxLDL in circulation (38). Other studies support an antioxidant effect of lutein on LDL oxidation. When 100 nmol/L of lutein was added to endothelial and smooth muscle cells in human aortas, monocyte chemotactic activity induced by LDL oxidation was markedly reduced (10). Indeed, in vitro experiments of human LDL, lutein has been shown to work as a scavenger of peroxynitrite radicals, which is the product of the reaction between NO and superoxide (39). However, the inhibition of LDL oxidation with lutein has not been observed in all studies (40). The discrepancies in results can be due to the methods that have been used to quantify oxLDL (10,39–41). The utilization of an antibody to oxLDL such as we used in the present study appears to give more accurate results (16).

Lutein and inflammation. Inflammation is recognized as a major contributor to atherosclerosis through adverse effects on lipoprotein metabolism and arterial wall biology (42). OxLDL triggers the recruitment of monocytes and T-cells from circulation to the vessel wall by stimulating the production of adhesion molecules, chemotactic proteins, and growth factors, followed by monocyte proliferation and differentiation into macrophages (12,14), and finally production of inflammatory cytokines. In general, reduction in inflammatory aortic cytokines indicates a lower degree of arterial wall inflammation. Along with the reduced production of aortic oxLDL, guinea pigs fed with lutein produced lower levels of most inflammatory cytokines in aorta compared to the control group. An antiinflammatory effect of lutein as well as suppression of LPS-induced secretion of TNFα and IL-1β proteins and mRNA levels in primary cultured mouse peritoneal macrophages has been reported (9). It has also been shown that lutein directly decreases NF-κB activation and NF-κB promoter activity, which is involved in cytokine production (12). In addition, it has been demonstrated that lutein blocked the degradation of inhibitory κB-α from the cytosolic fraction and prevented NF-κB translocation (7). Therefore, another possible antiinflammatory mechanism of lutein is that this carotenoid suppresses NF-κB signaling pathways along with...
subsequent proinflammatory cytokine gene expression. The lutein group also had lower concentrations of IL-10 in the present study. IL-10, a pleiotropic cytokine produced by a variety of immune cells, can inhibit inflammatory responses and it is thought to be an atheroprotective cytokine (43). However, IL-10 is a modulator of IL-12 action (44) and the lutein group also had reduced IL-12 levels, suggesting that lower levels of IL-10 were required to modulate the inflammatory process. Similar results have been reported in guinea pigs in a study using A-002 (Varespladib), a phospholipase A2 inhibitor (21).

In summary, our findings suggest that lutein might contribute to the prevention of early atherosclerosis development by reducing cholesterol accumulation and features of atherosclerosis in aorta. Further, lutein might attenuate the inflammatory state in aorta by decreasing MDA and oxLDL levels and reducing inflammatory cytokines.

Acknowledgments
J.E.K. conducted the study, wrote the manuscript, and analyzed the data; J.O.L. and R.D. helped with laboratory assays and provided input in the writing of the manuscript; J.A.S. provided the evaluation of aortic morphology and took the pictures; R.M.C. analyzed hepatic and plasma lutein and provided input in data analysis and interpretation; and M.L.F. designed the experiment and analyzed and interpreted the data. All authors read and approved the final manuscript.

Literature Cited