THE EFFECTS OF DIETARY FLAXSEED ON VASCULAR CONTRACTILE FUNCTION AND ATHEROSCLEROSIS DURING PROLONGED HYPERCHOLESTEROLEMIA IN RABBITS

CMC Dupasquier1-3, A-M Weber1-3, BP Ander1-3, P Rampersad2,3, S Steigerwald1, JT Wigle2,6, RW Mitchell7, EA Kroeger3, JSC Gilchrist1-4, MM Moghadasian1,5, A Lukas2,3 and GN Pierce1-3

1Canadian Centre for Agri-Food Research in Health and Medicine, 2Institute for Cardiovascular Sciences, St. Boniface Hospital Research Centre, 3Departments of Physiology, 4Oral Biology, 5Human Nutritional Sciences, and 6Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Canada, and 7Section of Pulmonary and Critical Care Medicine, University of Chicago, Chicago, IL, USA

Running Title: Flaxseed and vascular function in hypercholesterolemia

Address for correspondence:
Grant N. Pierce, PhD
Executive Director of Research,
St. Boniface Hospital Research Centre,
351 Tache Ave.,
Winnipeg, Manitoba, Canada
R2H 2A6
Ph. #: (204) 235-3206; Fax #: (204) 231-1151; E-mail: gpierce@sbrc.ca
ABSTRACT

Dietary flaxseed has significant anti-atherogenic effects. However, the limits of this action and its effects on vascular contractile function are not known. We evaluated the effects of flaxseed supplementation on atherosclerosis and vascular function under prolonged hypercholesterolemic conditions in New Zealand White rabbits assigned to one of 4 groups for 6, 8 or 16 weeks of feeding: regular diet (RG), 10% flaxseed supplemented diet (FX), 0.5% cholesterol supplemented diet (CH), and 0.5% cholesterol and 10% flaxseed supplemented diet (CF). Cholesterol feeding resulted in elevated plasma cholesterol levels and the development of atherosclerosis. The CF group had significantly less atherosclerotic lesions in the aorta and carotid arteries following 6 and 8 weeks than the CH animals. However, the anti-atherogenic effect of flaxseed supplementation was completely attenuated by 16 weeks. Maximal tension induced in aortic rings by either KCl or norepinephrine (NE) was not impaired by dietary cholesterol until 16 weeks. This functional impairment was not prevented by including flaxseed in the high cholesterol diet. Aortic rings from the cholesterol-fed rabbits exhibited an impaired relaxation response to acetylcholine (ACh) at all time points examined. Including flaxseed in the high cholesterol diet completely normalized the relaxation responses at 6 and 8 weeks and partially restored it at 16 weeks. No significant changes in the relaxation response induced by sodium nitroprusside were observed in any of the groups. In summary, dietary flaxseed is a valuable strategy to limit cholesterol-induced atherogenesis as well as abnormalities in endothelial-dependent vasorelaxation. However, these beneficial effects were attenuated during prolonged hypercholesterolemic conditions.
Keywords: linseed, acetylcholine, nutrition, polyunsaturated fatty acids, vascular relaxation
INTRODUCTION

Atherosclerosis is the leading cause of cardiovascular morbidity and mortality in North America (77). Atherosclerosis induces two significant pathological processes: an ischemic event due to blood flow obstruction and vascular contractile dysfunction. It is well known that atherosclerosis is associated with elevated circulating cholesterol levels. Elevated plasma cholesterol concentrations induced by cholesterol feeding results in the development of atherosclerosis and an impairment in endothelium-dependent vasodilation in rabbits (9, 26, 29, 30, 36). The development of interventions to inhibit cholesterol-induced atherosclerosis and the associated vascular dysfunction has received much attention due to this strong association. For example, there is an increasing interest in nutritional interventions that may prevent the development of atherosclerosis and protect against the vascular function abnormalities induced by cholesterol consumption. Flaxseed is one such novel dietary intervention. Flaxseed is a good source of soluble and insoluble dietary fibre and is the richest plant source of α-linolenic acid (ALA; C18:3n-3, omega-3 [n-3] fatty acid), as well as the lignan secoisolariciresinol diglucoside (SDG) (39, 53, 68). Whole ground flaxseed or the derivatized components of flaxseed have exhibited cardioprotective and anti-atherogenic properties both clinically (7, 8, 12, 32, 42, 47) and in several animal models (41, 52, 54, 56, 59, 62, 64, 71, 76). However, these results were observed using rather short periods of cholesterol feeding. The effects of a dietary intervention with flaxseed during prolonged periods of cholesterol supplementation are uncertain. More importantly, it is not known if dietary flaxseed can prevent the negative effects on vascular function that are induced by cholesterol.
The objective of the present study was to determine the effects of dietary supplementation with flaxseed on vascular function and atherosclerotic lesion development during prolonged hypercholesterolemic conditions. We selected a rabbit model to test four different dietary conditions: a regular control diet, a flaxseed supplemented diet, a diet containing elevated cholesterol levels, and a diet containing both cholesterol and flaxseed. We hypothesized that dietary flaxseed would limit atherosclerotic development and demonstrate a protective effect against cholesterol-induced vascular contractile abnormalities.
MATERIALS AND METHODS

Animals and Dietary Interventions

Ninety-six male albino New Zealand White (NZW) rabbits (Southern Rose Rabbitry Farm, St. Claude, Canada), weighing 2.5-3 kg upon arrival, were individually housed in metal cages in a room with controlled temperature, humidity, and a 12-hour light cycle. Guidelines for the ethical care and treatment of animals from the Canadian Council on Animal Care were strictly followed (48). Animals were randomly assigned to 4 groups of 8 animals per feeding duration based on dietary treatment. Animals were fed for 6, 8 or 16 weeks. The four diets included a control diet (RG) of regular rabbit chow (CO-OP Complete Rabbit Ration, Federal Co-operatives Limited, Saskatoon, Canada), a 10% ground flaxseed supplemented chow (FX), a 0.5% cholesterol supplemented chow (CH), or a diet supplemented with 0.5% cholesterol and 10% ground flaxseed (CF). The Promega flaxseed, provided from Polar Foods Inc. in Fisher Branch, Canada, contained 71% alpha-linolenic acid (ALA). All diets were dry mixed and pelleted to incorporate the added components. Rabbits were fed 125 grams of the appropriate dietary treatment per day. Water was given ad libitum.

Blood Sampling and Analysis

Blood samples were taken at baseline (0 weeks) and following 6, 8, or 16 weeks of dietary intervention from the marginal ear vein prior to daily feeding. Plasma cholesterol and triglyceride levels were measured enzymatically using commercial kits (Thermo Electron Corporation). Total fatty acids were extracted from the plasma samples and derivatized as previously described (4, 38). Fatty acids were esterified into their corresponding methyl esters using an acetylchloride:methanol:benzene solution.
Subsequent analysis by gas chromatography with flame ionization detection (FID) yielded the amounts of fatty acid methyl esters (FAMEs) quantitatively. The fatty acid content of the samples was identified by comparison with authentic standards (NuChek Prep, Elysian, MN, USA).

**Preparation of tissues**

Following 6, 8 or 16 weeks of dietary treatment, rabbits were anesthetized with isofluorane (5%, in oxygen, 2L/min) and heparinized. The aorta and carotid arteries were excised and immediately placed in cold Krebs Henseleit 1.9 mM calcium solution (115 mM NaCl, 25mM NaHCO3, 1.38 mM KH2PO4, 2.5 mM KCl, 2.46 mM MgSO4, 1.9 mM CaCl2, 11.2 mM dextrose, pH 7.4). The aorta was carefully dissected from the distal end of the aortic arch to the base of the diaphragm. The aorta and carotids were cleaned of adventitial tissue and prepared for vascular function testing, gas chromatography (GC), sectioning or **en face** analysis.

**Assessment of atherosclerotic lesion formation**

Atherosclerotic lesions along the distal aorta and carotid artery were evaluated **en face** and by cross-sectional analysis. For **en face** analysis, the aorta and carotid arteries were cleaned of peripheral tissue, opened longitudinally, digitally photographed, and the luminal images were analyzed using the Silicon Graphics Imaging (SGI) software. The lesion area was calculated as a percent of the total luminal area covered by atherosclerotic lesions.

Aortic tissue, fixed in 4% buffered paraformaldehyde and rinsed with 30% sucrose solution buffered in 1x PBS, was embedded in tissue freezing medium (O.C.T. compound), frozen at -20°C, cut into 10 µm thick sections using a cryostat, and thaw-
mounted onto positive glass slides. Sections were stained with Oil Red O and
counterstained with hematoxylin as previously described (44).

Following *en face* atherosclerosis analysis, the aortae were stored at −80°C,
thawed, and homogenized in preparation for chloroform:methanol lipid extraction as
described in detail (4, 21). The extracted lipids were quantified as described above.

*Experimental protocol for assessing vascular response*

Aortic tissue, dissected into 3 mm width rings from the distal end of the aortic
arch, were fastened in an organ bath with surgical wire, perfused with the Krebs
Henseleit solution, aerated with 95% O₂ and 5% CO₂, and equilibrated at 37°C and pH
7.4. Vascular function was measured with a force transducer as mechanograms of tension
(tension (g)/tissue wet weight (g)). The aortic rings were brought to a basal tension of
5.5-6.5 grams of tension and then contracted three times with 47 mM KCl with wash-out
periods using Krebs solution between each contraction. Tissues were allowed to return to
baseline tension during wash-outs. A dose response curve to norepinephrine (NE) was
constructed with concentrations of 10⁻⁹ M to 10⁻⁴ M. After the final dose of NE, the
tissues were washed-out with 37°C Krebs Henseleit solution and allowed to return to
baseline tension. To test the ability of the tissue to relax after pre-contraction with NE, a
second dose of 10⁻⁶ M NE was administered to the bath and the tissues were allowed to
reach a steady state of contraction. Acetylcholine (ACh) was then administered without
wash-out at concentrations of 10⁻⁸ M to 10⁻⁵ M to develop a relaxation response curve to
ACh. Following wash-out and a third dose of 10⁻⁶ M NE, sodium nitroprusside (SNP)
was administered in selected experiments at concentrations of 10⁻⁸ M to 10⁻⁵ M to
generate a relaxation response curve.
Statistical Analyses

Data are expressed as the mean ± standard error (SEM). Statistical comparisons were made using the one-way analysis of variance, followed by the Fisher’s least significant difference test for multiple parametric comparisons. Differences between means were considered significant when P < 0.05.
RESULTS

Diet composition and animal weights

Animals in all four treatment groups consumed 125g of chow daily. Animal body weights did not differ significantly amongst the four groups prior to feeding (0 weeks) or at the end of the feeding trials (6, 8, or 16 weeks) (data not shown), which suggests that the energy content of the experimental diets did not differ significantly. The data measuring the nutritional composition of the experimental diets supports this contention and is reported elsewhere (4). The addition of 10% flaxseed notably elevated the total fat and ALA content of the FX and CF diets. The n-6/n-3 PUFA ratios were 9-fold less in the flaxseed supplemented diets as compared to the RG and CH diets (4).

Effects on Lipid Concentrations

Initial plasma cholesterol and triglyceride levels were not significantly different amongst the four groups (Figure 1). Following 6, 8 and 16 weeks of dietary treatment, animals fed a pro-atherogenic diet (CH and CF groups) had up to a 15-fold increase in plasma cholesterol levels as compared to the RG and FX groups (Figure 1a). The cholesterol levels exhibited by these cholesterol-fed rabbits are similar to those found in hypercholesterolemic patients. There was no significant difference in plasma cholesterol levels between the CH and CF groups at any point in the trials. Plasma triglyceride levels were also elevated in the CH group at all time points (Figure 1b). The addition of 10% flaxseed to the cholesterol diet significantly attenuated this rise. A general decrease in plasma triglyceride levels was noted in the cholesterol fed groups with an increasing length of feeding trial.
Plasma total fatty acid (TFA) levels were measured in all of the groups (Table 1). The total fatty acid content was elevated in the plasma of the cholesterol fed groups following all end points. Notable differences in plasma fatty acid content are as follows: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2 n-6), and arachadonic acid (C20:4 n-6) levels were all significantly higher in the CH and CF groups versus the RG and FX groups following 6, 8, and 16 weeks, with lower levels of the fatty acids in the CF group as compared to the CH group at 16 weeks. Alpha-linolenic acid (C18:3 n-3) levels were elevated in animals fed both flaxseed and cholesterol following all feeding durations. A small rise was noted in plasma ALA levels of the cholesterol fed animals following 16 weeks. The long chain omega-3 fatty acid, eicosapentaenoic acid (EPA; C20:5 n-3) was only detected in the cholesterol fed groups following 16 weeks, with greater levels observed in the CF group. Docosahexaenoic acid (DHA; C22:6 n-3) levels in the plasma were only detected in trace amounts. The ratios of (n-6)/(n-3) PUFAs were significantly lower in the flaxseed supplemented groups (as much as 91-fold) as compared to the RG and CH groups with the difference diminishing with the length of feeding trial.

The dietary interventions also had an effect on the levels of lipid found in the vascular tissue following 8 and 16 weeks of treatment (Table 2). The total fatty acid (TFA) content was elevated in the aortic tissue of the cholesterol fed groups, with the highest aortic TFA levels in the CF group. Notable differences in the aortic fatty acid content are as follows: levels of the longer chain fatty acids (C20:1, C20:2 n-6, C20:3 n-6, C22:1, and C24:1) were elevated in the cholesterol fed groups. Linoleic acid levels were elevated in the cholesterol fed groups at 8 and 16 weeks, and were highest in the CF
group at 16 weeks. The long chain PUFA, arachadonic acid decreased significantly in the FX, CH and CF groups versus the RG group at 16 weeks. Aortic alpha-linolenic acid levels were elevated with flaxseed supplementation as well as in cholesterol fed animals at 8 weeks, however by 16 weeks, ALA levels were elevated only in the flaxseed fed groups, with the highest levels seen in the CF group. The long chain omega-3 fatty acid, eicosapentaenoic acid was detected only in the aortic tissue of the cholesterol fed groups at 8 weeks, and at 16 weeks was only observed in the CF group. Docosahexaenoic acid levels were also detected in the FX, CH, and CF groups following 8 weeks but only in the CF group following 16 weeks of feeding. At both time points, the highest values were detected in the CF group. The addition of dietary flaxseed diminished the (n-6)/(n-3) PUFA ratio in the aortic tissues of the FX and CF groups as compared to the RG and CH groups.

*Effects of dietary interventions on atherosclerosis*

Tissue weights of the 3 mm aortic sections that were used to assess vascular response were not significantly different amongst the four groups following the 6 and 8 week trials. However, aortic tissue weights were significantly greater, due to large atherosclerotic lesions, in the CH and CF groups following the 16 week dietary intervention, as compared to the RG and FX groups (CH: 35.4 ± 2.3, CF: 39.0 ± 4.7, RG: 21.5 ± 0.8, and FX: 20.9 ± 1.5 mg).

Aortae were cut longitudinally and the luminal surface was digitally photographed to measure the atherosclerotic lesion area. Plaque formation was not visible in the aortae of the control and flax-fed animals at any timepoint. However, extensive atherosclerotic plaques were apparent in the cholesterol-fed rabbits (*Figure 2*). Both the cholesterol and
cholesterol-flax fed groups had significantly greater plaque formation than the control and flax fed groups following all trials. There was a statistically significant inhibition of atherosclerotic plaque formation in the cholesterol-flax group in comparison to the animals fed cholesterol alone at the 6 and 8 week timepoints. In contrast, the CF group developed more atherosclerotic plaques than the CH group following 16 weeks of hypercholesterolemic conditions.

A similar qualitative effect on plaque formation was observed in cross sectional analysis (Figure 3). Plaques were only present in cholesterol fed animals and were more severe in animals that did not receive flaxseed supplementation. However, at 16 weeks, the protective effects on plaque thickness were not observed.

Similar results with respect to atherosclerotic plaque formation were observed in carotid vessels although the extent of the atherosclerosis was not as severe (Figure 4). Carotids were not collected after the 6 week trial. After 8 weeks of dietary supplementation, atherosclerotic plaque formation in the carotids was inhibited by including flaxseed in the cholesterol diet. This protective effect was lost after 16 weeks of dietary intervention.

Effects of Dietary Flaxseed and Cholesterol on Vascular Contractile Response

The response of aortic rings from animals fed the different dietary regimens was investigated first as a function of the contractile agonists. No differences in KCl-induced vasoconstriction were observed in any groups following 6 and 8 weeks of dietary treatment (Figures 5a, 5b). However, aortic rings from both of the cholesterol-supplemented groups exhibited an attenuated contractile response to KCl in comparison to the RG and FX groups after 16 weeks of dietary treatment (Figure 5c).
A slight depression in the contractile response to NE was observed after 6 weeks of dietary intervention, as aortic preparations from both cholesterol-supplemented groups contracted significantly less in response to $10^{-7}$ M NE than did the RG and FX groups (Figure 6a). No difference in NE-induced contraction was observed between the groups following 8 weeks of dietary treatment (Figure 6b). However, the CH and CF groups contracted significantly less in response to a range of NE concentrations ($10^{-6}$ M, $10^{-5}$ M and $10^{-4}$ M NE) as compared to the RG and FX groups after 16 weeks of dietary treatment (Figure 6c). There was also a small decline in the overall contractile response to NE observed as a function of the age of all of the animals (Figures 6a-c).

Aortic relaxation responses were also monitored after pre-contraction with $10^{-6}$ NE as a function of the dietary interventions. Aortic rings from the CH group exhibited significantly less endothelium-dependent relaxation in response to higher doses of ACh ($10^{-6}$ M and $10^{-5}$ M) than the RG group after 6 weeks of dietary interventions (Figure 7a). Flaxseed added to the diet effectively prevented these cholesterol-induced defects. After 8 weeks of cholesterol feeding, the CH group relaxed less in response to $10^{-6}$ M ACh than the RG and FX groups (Figure 7b). Again, this was prevented by including flaxseed in the diet. Following the 16 week trial, the CH group again demonstrated a significant defect in endothelial-dependent relaxation to ACh. The addition of flaxseed partially prevented these cholesterol-induced defects in vascular relaxation, however the protective effect no longer achieved statistical significance at concentrations of $10^{-5}$ M ACh (Figure 7c).

Endothelium-independent vasorelaxation was also investigated using sodium nitroprusside (SNP). The results from the 16 week dietary intervention are shown in
**Figure 8.** There were no significant differences in the extent of endothelium-independent relaxation to SNP amongst the four groups following 16 weeks of dietary intervention (Figure 8a). Furthermore, the rate of SNP-induced relaxation was also unaltered by the choice of dietary interventions (Figure 8b)
DISCUSSION

Flaxseed supplementation in the diet resulted in a significant elevation of ALA levels in the plasma and aortic tissue (Tables 1 & 2). This elevation occurred in the absence or presence of additional dietary cholesterol. Although the plasma concentration of the longer chain omega-3 fatty acids EPA and DHA were only detected in low levels with cholesterol feeding following the extended feeding trials, EPA and DHA were detected in the aortic tissue of rabbits consuming both flaxseed and cholesterol following 8 weeks of feeding. The highest levels were seen in animals consuming a combination of cholesterol and flaxseed. These results demonstrate that ALA is metabolised to a small extent to longer chain fatty acids like EPA and DHA in the rabbit. Flaxseed supplementation also reduced the ratio of n-6 to n-3 PUFAs, primarily as a result of the elevated levels of circulating n-3 PUFA. The addition of dietary flaxseed to the atherogenic diet also mitigated the cholesterol-induced rise in plasma triglyceride levels (Figure 1b). These results are consistent with previous work from our lab using this dietary intervention (4).

Our findings that dietary flaxseed can inhibit the development of atherosclerotic plaques on the aortic luminal surface are consistent with previous reports (41, 52, 54, 59, 64). Because dietary flaxseed supplementation did not alter circulating cholesterol levels, either in the presence or in the absence of additional cholesterol in the diet (Figure 1a), it is clear that its anti-atherogenic effects were not achieved through a cholesterol-lowering action. These effects are consistent with previous studies (2, 4, 24, 25, 52), but in conflict with other reports which found that flaxseed supplementation lowers circulating cholesterol levels (41, 54, 56, 59). Because the anti-atherosclerotic effects of fibre are due
to a cholesterol-lowering action, the high fibre content of flaxseed, therefore, is unlikely to be responsible for the anti-atherogenic action in the present study. Alternatively, the lignan SDG in flaxseed has been shown to possess potent anti-atherogenic properties (52, 54, 57) and is the most likely component within the flaxseed responsible for these beneficial effects. It is a potent anti-oxidant (50, 51, 55). Our results extend these findings to demonstrate that the protection afforded by dietary flaxseed was also observed in an important resistance artery, the carotid (Figure 4). Thus, the anti-atherogenic effects of flaxseed on atherosclerosis may have implications for the pathogenesis of stroke as well as heart disease. Consistent with this finding, several clinical studies have shown negative correlations between plasma ALA levels with the incidence of stroke (20, 37, 67) and coronary heart disease (17, 18, 27). However, our study has also identified limits to the anti-atherogenic capacity of flaxseed. The cholesterol and flax group also developed extensive atherosclerotic lesions after prolonged periods of hypercholesterolemia (Figure 2). The extended duration of hypercholesterolemia appeared to overwhelm the beneficial effects of flaxseed supplementation.

The present study has demonstrated that dietary cholesterol had a deleterious effect on vascular contractile function (Figures 5 & 6). These findings are consistent with the impaired vascular response of vessels exposed to a high cholesterol environment (9, 29-31, 69). The present results extend this to an attenuation of agonist-induced vascular contractility as well as an impairment of vascular relaxation. Because we observed defects in both KCl and NE-induced vascular tension generation, this suggests that there is a general defect in smooth muscle function. Cholesterol-induced changes in ion transport pathways including the regulation of Ca^{2+} homeostasis in smooth muscle
cells (SMC) may represent the mechanism for this effect (5, 6, 10, 13, 23, 65). This may explain both the contraction and relaxation defects identified in the present study. However, three additional factors may also play a role in the depressed contractile response to KCl and NE. First, tension generation was measured as a function of tissue weight. Tissue weight increased per length of each aortic ring in the cholesterol fed animals because the plaque increased the vessel thickness. This increase in tissue weight would tend to artificially decrease tension/tissue weight. Secondly, the plaque found in the aortic ring would contain non-muscular cell types such as fibroblasts, foam cells and macrophages that would contribute to tissue weight but not to total tension generation. Thirdly, atherosclerosis is known to transform the phenotype of existing SMC from a contractile to a synthetic phenotype (1, 11, 46, 66, 73). This conversion results in a loss of contractile proteins. Synthetic SMCs are no longer transcriptionally programmed to support contractile activity, resulting in an impaired vascular response in atherosclerotic vessels.

This study is the first to describe the effects of flaxseed supplementation on vascular function in atherosclerotic vessels (Figure 7). The protective action of dietary flaxseed on vascular response was selective. Flaxseed protected against the changes in vascular relaxation but did not protect against the contractile dysfunction induced by the elevation in circulating cholesterol. The protective effect of dietary flaxseed on vascular relaxation was only attenuated following prolonged hypercholesterolemic conditions, under which flaxseed no longer prevented atherosclerotic development. Omega-3 fatty acids have previously been reported to improve vascular function by limiting the progression of atherosclerosis and reducing endothelial activation and vascular
inflammation (3, 16, 40, 45). The protective effects on relaxation identified in our study clearly involved a selective, endothelium-dependent site of action. This finding is consistent with a study reporting that a high flaxseed diet can enhance endothelial vasorelaxant function in hypertensive rats, without improving blood pressure (71). The vascular response to SNP was not different in any of the groups demonstrating that the endothelial-independent routes of modulating vascular relaxation were unaltered (Figure 8). Because there were no intrinsic changes in relaxation capacity in response to nitric oxide (NO) in the form of SNP, the lesion appears to be due to the generation of NO by ACh. Because we did not observe any beneficial effects on vascular function of the flaxseed enriched diet on its own when compared to a regular diet, this would suggest that flaxseed is not altering the intrinsic characteristics of endothelial cells but instead selectively attenuates the detrimental changes induced by cholesterol. Flaxseed did not achieve this effect by altering the circulating cholesterol levels. In contrast, dietary flaxseed did increase PUFA levels in the plasma and aortic tissue. The largest changes in PUFA concentrations were in ALA levels in the tissue and plasma compartments with only minor changes in DHA and EPA levels. Therefore, it is reasonable to hypothesize that ALA induced the protective changes observed. In support of this contention, ALA has been shown to lower serum markers of vascular inflammation and endothelial activation (49, 60, 61, 72, 78), reduce platelet aggregation (74) and induce changes in intracellular Ca\(^{2+}\) movements (4, 15, 27). Furthermore, circulating ALA has been positively associated with endothelium-dependent vasodilation in normocholesterolemic and hypercholesterolemic subjects (19, 63, 70).
Oxidative stress may be another important mechanistic factor in cholesterol-induced vascular contractile dysfunction (22, 28, 33, 43) and in atherosclerosis (35, 57, 75). For example, statins can improve endothelium-dependent vascular relaxation in hypercholesterolemic patients by decreasing oxidative stress (33). It is possible, therefore, that the potent antioxidant capacity of flaxseed was responsible for its beneficial action on atherosclerosis and vascular relaxation (50, 54). The lignan SDG found in flaxseed is thought to be responsible for this antioxidant action (34, 51, 54, 55, 58) and may also have contributed to its protective effect against the cholesterol-induced defects in vascular relaxation.

In summary, our data demonstrate for the first time that flaxseed can protect against atherosclerotic plaque deposition in carotid arteries and confirms its anti-atherosclerotic effects on the aorta. We have also shown for the first time that dietary flaxseed can improve endothelium-dependent vascular relaxation in the presence of a high cholesterol diet. However, dietary flaxseed is not a panacea. If the high cholesterol diet is prolonged in duration, flaxseed eventually loses its ability to protect against cholesterol-induced lesions. Despite this limitation, our data reinforces the hypothesis that long term supplementation of the diet with ground flaxseed may be an effective, safe dietary strategy to limit the early and moderate stages of atherogenesis and vascular dysfunction associated with atherosclerosis. The flaxseed dosage used in this investigation is similar to that used previously in human studies (14, 42). Our results, therefore, suggest that dietary modification with flaxseed may have an important protective action in humans against vascular disease in both the heart and in stroke.
ACKNOWLEDGEMENTS

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TABLE 1
PLASMA FATTY ACID CONCENTRATIONS OF RABBITS
FED EXPERIMENTAL DIETS FOR 6, 8, AND 16 WEEKS\(^1,2,3\)

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<th>6 weeks</th>
<th>8 weeks</th>
<th>16 weeks</th>
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<tbody>
<tr>
<td></td>
<td>RG</td>
<td>FX</td>
<td>CH</td>
</tr>
<tr>
<td>14:0</td>
<td>t</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>16:0</td>
<td>0.11±0.01(^a)</td>
<td>0.09±0.01(^a)</td>
<td>0.71±0.08(^b)</td>
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<td>18:0</td>
<td>0.12±0.01(^a)</td>
<td>0.11±0.01(^a)</td>
<td>0.26±0.03(^b)</td>
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<td>16:1</td>
<td>t</td>
<td>t</td>
<td>0.18±0.02(^b)</td>
</tr>
<tr>
<td>18:1</td>
<td>0.12±0.01(^a)</td>
<td>0.09±0.01(^a)</td>
<td>1.22±0.12(^b)</td>
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<td>18:2(n-6)</td>
<td>0.11±0.01(^a)</td>
<td>0.09±0.01(^a)</td>
<td>0.62±0.08(^b)</td>
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<td>20:3(n-6)</td>
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<td>20:4(n-6)</td>
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<td>0.12±0.02(^a)</td>
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<td>20:5(n-3)</td>
<td>t</td>
<td>t</td>
<td>t</td>
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<td>22:6(n-3)</td>
<td>t</td>
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<td>t</td>
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<td>n-6/n-3</td>
<td>75.9±20.0(^a)</td>
<td>2.4±0.3(^b)</td>
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<td>TFA (mg/mL)</td>
<td>0.50±0.02(^a)</td>
<td>0.45±0.04(^a)</td>
<td>3.2±0.4(^b)</td>
</tr>
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</table>

\(^1\) Plasma samples obtained following 6, 8, or 16 weeks of feeding (n = 7-8).

\(^2\) Values are means ± SEM. Values in the same row for each time point not sharing a common superscript are significantly different (P < 0.05).

\(^3\) Abbreviations: RG, regular fed; FX, 10% flaxseed fed; CH, 0.5% cholesterol fed; CF, 0.5% cholesterol plus 10% flaxseed fed; t, trace amounts present (< 0.01 mg/100 mg fatty acids); TFA, total fatty acids (mg fatty acid in 1 mL of plasma).
## TABLE 2

CONCENTRATIONS OF FATTY ACIDS IN AORTIC TISSUE FROM RABBITS FED EXPERIMENTAL DIETS FOR 8 AND 16 WEEKS

<table>
<thead>
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<th>8 weeks</th>
<th></th>
<th>16 weeks</th>
<th></th>
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<td>RG</td>
<td>FX</td>
<td>CH</td>
<td>CF</td>
</tr>
<tr>
<td></td>
<td>mg/g tissue</td>
<td>mg/g tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>0.14±0.06</td>
<td>0.18±0.03</td>
<td>0.30±0.05</td>
<td>0.26±0.04</td>
</tr>
<tr>
<td>16:0</td>
<td>2.45±0.70a</td>
<td>2.57±0.40a</td>
<td>4.76±0.6b</td>
<td>4.50±0.64b</td>
</tr>
<tr>
<td>18:0</td>
<td>1.14±0.18a</td>
<td>1.16±0.08a</td>
<td>1.76±0.22b</td>
<td>1.70±0.16b</td>
</tr>
<tr>
<td>20:0</td>
<td>t</td>
<td>t</td>
<td>0.07±0.01b</td>
<td>0.06±0.01b</td>
</tr>
<tr>
<td>22:0</td>
<td>0.04±0.01a</td>
<td>0.04±0.01a</td>
<td>0.07±0.00b</td>
<td>0.06±0.00ab</td>
</tr>
<tr>
<td>24:0</td>
<td>t</td>
<td>t</td>
<td>0.03±0.01b</td>
<td>0.03±0.01b</td>
</tr>
<tr>
<td>16:1</td>
<td>0.27±0.11a</td>
<td>0.26±0.06a</td>
<td>0.65±0.07b</td>
<td>0.58±0.10b</td>
</tr>
<tr>
<td>18:1</td>
<td>2.49±0.76a</td>
<td>2.55±0.26a</td>
<td>8.22±1.08b</td>
<td>6.95±1.01b</td>
</tr>
<tr>
<td>20:1</td>
<td>t</td>
<td>t</td>
<td>0.24±0.04b</td>
<td>0.22±0.05b</td>
</tr>
<tr>
<td>22:1</td>
<td>t</td>
<td>t</td>
<td>0.04±0.01b</td>
<td>0.02±0.01ab</td>
</tr>
<tr>
<td>24:1</td>
<td>t</td>
<td>t</td>
<td>0.09±0.02b</td>
<td>0.06±0.02b</td>
</tr>
<tr>
<td>18:2(n-6)</td>
<td>1.38±0.42a</td>
<td>1.44±0.11a</td>
<td>3.83±0.56b</td>
<td>3.64±0.54b</td>
</tr>
<tr>
<td>20:2(n-6)</td>
<td>t</td>
<td>t</td>
<td>0.27±0.04b</td>
<td>0.21±0.04b</td>
</tr>
<tr>
<td>20:3(n-6)</td>
<td>t</td>
<td>t</td>
<td>0.23±0.11b</td>
<td>0.28±0.06b</td>
</tr>
<tr>
<td>20:4(n-6)</td>
<td>0.90±0.05</td>
<td>0.75±0.03</td>
<td>0.90±0.04</td>
<td>0.86±0.04</td>
</tr>
<tr>
<td>18:3(n-3)</td>
<td>0.31±0.12a</td>
<td>1.27±0.15ab</td>
<td>1.49±0.52b</td>
<td>2.23±0.35b</td>
</tr>
<tr>
<td>20:5(n-3)</td>
<td>t</td>
<td>t</td>
<td>0.05±0.03ab</td>
<td>0.07±0.01b</td>
</tr>
<tr>
<td>22:6(n-3)</td>
<td>t</td>
<td>0.03±0.01b</td>
<td>0.02±0.01ab</td>
<td>0.06±0.00c</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>7.42±0.44a</td>
<td>1.67±0.12b</td>
<td>3.34±0.35c</td>
<td>2.11±0.18b</td>
</tr>
<tr>
<td>TFA</td>
<td>9.53±2.45a</td>
<td>10.66±1.05a</td>
<td>24.27±3.50b</td>
<td>22.78±3.11b</td>
</tr>
</tbody>
</table>
1 Fatty acids were extracted from aortas following 8 or 16 weeks of feeding (n = 4-5).

2 Values are means ± SEM. Values in the same row for each time point not sharing a common superscript are significantly different (P < 0.05).

3 Abbreviations: RG, regular fed; FX, 10% flaxseed fed; CH, 0.5% cholesterol fed; CF, 0.5% cholesterol plus 10% flaxseed fed; t, trace amounts present (< 0.01 mg/g of aortic tissue); TFA, total fatty acids (mg fatty acid in 1g of aortic tissue).
FIGURE LEGENDS

Figure 1. Plasma cholesterol (a) and triglyceride (b) concentrations in rabbits prior to (0 weeks) and following 6, 8 or 16 weeks of dietary interventions. RG, regular diet; FX, 10% flaxseed supplemented diet; CH, 0.5% cholesterol supplemented diet; CF, 0.5% cholesterol and 10% flaxseed supplemented diet. Values are means ± SEM, n = 4-8. *P < 0.05 versus RG and FX groups. † P < 0.05 CF versus CH group.

Figure 2. Extent of aortic atherosclerotic lesions following (a) 6, (b) 8, and (c) 16 weeks of dietary treatment. The lesion area was measured as the percentage of aortic luminal area covered by atherosclerotic lesions. Values are means ± SEM, n = 3-7. *P < 0.05 versus RG and FX groups. † P < 0.05 CF versus CH group. RG, regular diet; FX, 10% flaxseed supplemented diet; CH, 0.5% cholesterol supplemented diet; CF, 0.5% cholesterol and 10% flaxseed supplemented diet.

Figure 3. Representative aortic cross sections from the four experimental groups showing Oil Red O stained lipid deposits. RG group, 6 weeks (a); FX group, 6 weeks (b). Representative pictures from the RG and FX groups at 8 and 16 weeks are not shown as atherosclerotic lesions were not apparent in the RG and FX groups at any time point; CH group, 6 (c), 8 (e), and 16 (g) weeks; CF group, 6 (d), 8 (f), and 16 (h) weeks. RG, regular diet; FX, 10% flaxseed supplemented diet; CH, 0.5% cholesterol supplemented diet; CF, 0.5% cholesterol and 10% flaxseed supplemented diet.
Figure 4. Extent of carotid atherosclerotic lesions following (a) 8 and (b) 16 weeks of dietary treatment. The lesion area was measured as the percentage of luminal area of the carotid arteries covered by atherosclerotic lesions. Values are means ± SEM, n = 4-7. *P < 0.05 versus RG and FX groups. RG, regular diet; FX, 10% flaxseed supplemented diet; CH, 0.5% cholesterol supplemented diet; CF, 0.5% cholesterol and 10% flaxseed supplemented diet.

Figure 5. Contractile response to 47 mM KCl of proximal aortic rings isolated after (a) 6, (b) 8, and (c) 16 weeks of dietary treatment. Values are means ± SEM, n = 5-8. *P < 0.05 versus RG and FX groups. RG, regular diet; FX, 10% flaxseed supplemented diet; CH, 0.5% cholesterol supplemented diet; CF, 0.5% cholesterol and 10% flaxseed supplemented diet.

Figure 6. Contractile response to increasing doses of norepinephrine (NE) of proximal aortic rings isolated after (a) 6, (b) 8, and (c) 16 weeks of dietary treatment. Values are means ± SEM, n = 6-8. *P < 0.05 versus RG and FX groups. RG, regular diet; FX, 10% flaxseed supplemented diet; CH, 0.5% cholesterol supplemented diet; CF, 0.5% cholesterol and 10% flaxseed supplemented diet.

Figure 7. Endothelium-dependent relaxation in response to acetylcholine (ACh) in aortic rings following pre-contraction with 10^{-6} M NE at (a) 6, (b) 8, and (c) 16 weeks of dietary treatment. Results are represented as percent of tension following ACh administration after pre-contraction with 10^{-6} M NE. Values are means ± SEM, n = 6-8. *P < 0.05 versus
RG group. † P < 0.05 versus CH group. RG, regular diet; FX, 10% flaxseed supplemented diet; CH, 0.5% cholesterol supplemented diet; CF, 0.5% cholesterol and 10% flaxseed supplemented diet.

**Figure 8.** (a) Endothelium-independent relaxation in response to sodium nitroprusside (SNP) in aortic rings after pre-contraction with $10^{-6}$ M NE, following 16 weeks of dietary treatment. Results are represented as percent of tension following SNP administration after pre-contraction with $10^{-6}$ M NE. (b) Rate of relaxation in response to $10^{-6}$ M SNP (g tension/sec). Results represent the loss of tension during the first minute after the administration of $10^{-6}$ M SNP. Values are means ± SEM, n = 7-8. RG, regular diet; FX, 10% flaxseed supplemented diet; CH, 0.5% cholesterol supplemented diet; CF, 0.5% cholesterol and 10% flaxseed supplemented diet.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

(a) Graph showing % relaxation from contraction to 10^{-6} M NE for different groups.

(b) Bar graph showing rate of relaxation to 10^{-6} M SNP for different groups.