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Dietary fatty acid unsaturation levels, lipoprotein oxidation and circulating chemokine in experimentally induced atherosclerotic rats

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Abstract

The dietary balance of long-chain fatty acids may influence processes involving leukocyte endothelial interactions, such as atherogenesis and inflammation. The relationship between proatherogenic lipoproteins and chemotactic motility is still controversial. However, the interaction of the former can increase recruitment of monocytes to the vessel walls and accelerate the events of atherogenesis. The current study examined the effects of unsaturated fatty acid levels on the oxidative susceptibility of lipoprotein, chemokine expressions and their relationship to atherosclerotic lesion development in experimental rats. Male Wistar rats were fed an atherogenic diet for 4 months and the diet was then supplemented with 10% v/w of virgin olive oil (OO group), sunflower oil (SO group) or fish oil (FO group) for 4 and 8 weeks. Blood samples were collected at four time points: at baseline, after feeding with the atherogenic diet and during the dietary regimen (4 and 8 weeks). Plasma lipid profile and lipoprotein oxidative susceptibility (LOS), C-reactive protein (CRP), monocyte chemoattractant protein (MCP-1), and regulated upon activation normal T-cell expressed and secreted (RANTES) were measured. The superoxide dismutase (SOD) and reduced glutathione (GSH) antioxidant activities were also studied in aortic segments. Histological assessment of the aortic segment was determined. Compared to baseline data, the high-fat and cholesterol-enriched diet increased atheroma formation, plasma LOS and inflammatory indexes (CRP, MCP-1, RANTES). However, it dramatically reduced aortic SOD and GSH contents. Dietary treatment of atherosclerotic rats with OO greatly reduced LOS and remarkably increased aortic SOD and GSH contents as compared to the SO- and FO-treated groups. The FO-supplemented diet had a more pronounced lowering effect on MCP-1 and RANTES compared to the OO and SO diets. In conclusion, this study demonstrated a strong relationship between LOS and circulating levels of chemokines. OO is a potent antioxidant and moderate anti-inflammatory, which effectively reduced aortic atherosclerotic lesions more than the SO- or FO-treated groups in male Wistar rats.

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Introduction

Atherosclerosis, formerly known as bland lipid storage disease, actually involves an ongoing inflammatory response. Over the last few decades, a plausible model linking lipids and inflammation to atherogensis has emerged (Libby et al 2002).

According to oxidation hypothesis, low density lipoprotein (LDL) retained in the intima by binding to proteoglycan undergoes oxidative modification (Berliner et al 1997). Lipid hydroperoxides, lysophospholipids, carbonyl compounds and other biologically active moieties are often localized in the lipid fraction of atheroma (Witztum & Berliner 1998). These modified lipids can induce the expression of adhesion molecules, chemokines, proinflammatory cytokines and other mediators of inflammation in macrophages and vascular wall cells (Stemme et al 1995).

The translation of leukocyte T-lymphocytes from the vascular system into the extra cellular matrix is a central event associated with information and tissue injury. Such a process is governed by proinflammatory chemoattractants (e.g. chemokines) (Franitza et al 2000). One of these chemokines is regulated upon activation normal T-cell expressed and secreted (RANTES), which is a potent chemoattractant for eosinophils, lymphocytes,

monocytes and basophils (Alam 1997). Monocyte chemoattractant protein (MCP-1) represents another candidate for this group (Gu et al 1998). Atherosclerosis and inflammation therefore share similar mechanisms in their early phases, involving increased interaction between vascular endothelial and circulating leukocytes (De Caterina 2000).

Highly unsaturated and n-3 fatty acids in particular are receiving increasing attention as potential anti-atherogenic and anti-inflammatory agents (Baro et al 2003). Recent findings have led to a new way of thinking about fatty acids and their balance in diet, and consequently in membrane phospholipids as modulators of cell response to cytokines. This concept has broad implications in human pathobiology, nutrition and therapeutics (De Caterina et al 2000).

It was logical therefore to study the relationship between atherosclerosis and different kinds of oils, whether monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (n-6 and n-3 PUFA), to throw more light on their effects as a part of therapy. Secondly, the role of these fatty acids in the modulation of LDL oxidative susceptibility with special emphasis on circulating levels of chemokines and other inflammatory factors dealt with atherosclerosis induced experimentally in a rat model. This may lead to a new understanding of the mechanism of action of these nutrients.

Materials and Methods

Experimental animals

Seventy male Wistar rats (250 ± 10 g) were supplied by the Egyptian Organization for Biological Products and Vaccines. Rats were subjected to controlled conditions of temperature ($25 \pm 2°C$) and illumination (12-h light/dark), and allowed free access to normal rat chow diet and water ad libitum. This protocol was approved by the Animal Care and Use Committee of the Biochemistry Department, Faculty of Pharmacy, Zagazig University.

Experimental protocol

One week after acclimatization, the animals were switched to the atherogenic diet, which consisted of 21% butter (Valio, Helsinki, Finland), 1% cholesterol, 0.5% sodium cholate and 19.5% casein (Sigma, St Louis, MO, USA) (Napoli et al 2002) for 4 months. Rats which achieved a plasma cholesterol level of $> 200\ mg\ dL^{-1}$ were selected for this study. Atherosclerotic rats were randomly divided into three experimental groups (16 rats in each). The first group received the atherogenic diet supplemented with 10% v/w MUFA, virgin olive oil, OO (El-Salheya Co, Egypt), as a rich source of oleic acid (cis-18:1 n-9). The second received the atherogenic diet enriched with 10% v/w of n-6 PUFA sunflower oil, SO (Arma Food, Cairo, Egypt), which contains about 70% of linoleic acid (cis-18:2 n-6). The atherogenic diet of the last group was supplemented with 10% v/w of n-3 PUFA as fish oil, FO, which contains eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3). This dietary regimen was followed for 8 weeks. Rats from each group were anaesthetized with urethane ($1.3\ mg\ kg^{-1}$) and blood samples were collected via retro-orbital bleeding at four time points: at baseline, after feeding with the atherogenic diet and the remaining during dietary treatment (4 and 8 weeks). Serum and plasma were stored at $-20°C$ until analysis. Animals (eight rats of each) were then killed by decapitation and abdominal aortas were excised, cleaned of adherent fat and connective tissue and cut into ring segments. Part of them was frozen in liquid nitrogen and analysed immediately to determine aortic oxidative stress markers (SOD, GSH). The remaining parts of the aortas were kept in neutral buffered formal saline and used for histological examination.

Methods of analysis

Plasma levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol (LDLC), and triacylglycerol (TAG) were estimated enzymatically using commercially available kits (Spinreact, Santa Coloma, Spain). Atherogenic indexes were calculated from the ratios of TC:HDLC and LDLC:HDLC.

Lipoprotein oxidation susceptibility (LOS) was measured according to the principle of Dujovne et al (1994). Briefly, very-low-density lipoprotein cholesterol (VLDLC) and LDLC were precipitated from 500 μL of plasma by 50 μL of dextran sulfate/magnesium chloride. The pellet was dissolved in 4% saline solution. A volume of redissolved precipitate containing 100 μg non HDLC was mixed with 4% sodium chloride to give 500 μL of total solution. Copper solution (0.5 μM $CuCl_2$) was added and incubated at 37°C for 5 h in a shaking water bath. The solution was assayed for thiobarbituric acid reactive substances as an index for oxidation. The number of nanomoles of malondialdehyde (MDA) present in the samples was estimated from a standard curve prepared using 1,1,3,3-tetraethoxypropane.

Aortic rings (8 mm in length) were placed in an ice-cold 50 mM Tris HCl buffer (pH 7.4) containing 1.15% KCl, 1 mM EDTA, 5 mM D-glucose and 0.1 mM DL-dithiothreitol, and homogenized with a glass–glass homogenizer. The crude homogenate was centrifuged twice at 750 *g* for 5 min to remove the bigger fragments. The protein content of the supernatant was measured according to Chromy & Fischer (1979) using the diagnostic kit provided by Biocon (Germany). Aliquots (150 μg total protein) were used for the determination of superoxide dismutase (SOD) activity spectrophotometry as previously described (Siveski-Iliskovic et al 1995). Reduced glutathione (GSH) contents were measured following its reaction with 5,5-nitrobenzoic acid in phosphate buffer (pH 8) (Ahmed et al 1991). SOD and GSH contents were expressed per milligram of protein.

Serum C-reactive protein (CRP), MCP-1 and RANTES were evaluated by ELISA techniques using a kit purchased from Biosource (USA).

Histological study

The aorta specimens were processed in ascending grades of alcohol, cleared in xylol and embedded in soft paraffin.

Slices of about 4 μm thickness were stained with haematoxylin and eosin for light microscopical examination (Drury & Wallington 1980).

Statistical analysis

The parameter between treated and control groups were evaluated for significance using Student's *t*-test (SPSS program, Chicago, IL, USA). Comparisons of three or more groups were conducted by one-way analysis of variance followed by Tukey's test. The statistical associations between functional parameters were assayed using Spearman's nonparametric correlation analysis. Results are expressed as mean ± s.d. Significance was set at $P < 0.05$.

Results

The present study demonstrated that feeding the atherogenic diet for 4 months significantly ($P < 0.0001$) induced a marked dyslipidaemia, represented by elevated TC, LDLC, TAG and an approximate two-fold increase in atherogenic indexes compared to baseline values. Atherogenic indexes were reduced more by following FO diets than OO-enriched diets, whereas SO diets had no significant effect after 4 and 8 weeks of dietary treatment (Table 1).

In atherosclerotic rats there was a marked increase in oxidative stress markers. Such dysfunction was evidenced by increased LDL oxidation susceptibility and decreased aortic antioxidant enzymes (SOD and GSH) in comparison with baseline data (Table 2). Inflammatory markers (CRP, MCP-1, RANTES) were significantly ($P < 0.01$) and greatly exaggerated following feeding with the atherogenic diet (Figure 1A, B & C).

It is noteworthy that the OO diet significantly ($P < 0.05$) inhibited LOS more than PUFAs (n-3 and n-6) after 4 and 8 weeks of treatment (75 ± 8 and 59 ± 7 vs 92 ± 11 and 100 ± 16, 70.5 ± 9 and 92.8 ± 10). Among the striking results is the level of aortic antioxidant enzymes (SOD and GSH) that were dramatically increased in the OO group, more than in the others after 8 weeks of the dietary regimen (Table 2).

OO- and FO-supplemented diets successively reduced CRP, whereas the SO-enriched diet failed to affect these parameters. MCP-1 and RANTES were significantly reduced following the FO-enriched diet compared to OO- and SO-supplemented diets after 8 weeks of dietary intake (Figure 1A,B).

Using the combined results from all animals, there were significant positive correlations between lipoprotein oxi-

Table 1 Effect of MUFAs (OO) and PUFAs (SO, FO) on lipid profile of atherosclerotic rats

	Baseline (70)	Atherosclerotic rats (54)	4 weeks			8 weeks		
			OO (8)	SO (8)	FO (8)	OO (7)	SO (6)	FO (7)
TC ($mg\,dL^{-1}$)	88 ± 9	$211 \pm 21^{\#}$	165 ± 11*	193 ± 13	139 ± 11*	153 ± 12*	185 ± 13	131 ± 9*
LDLC ($mg\,dL^{-1}$)	54 ± 9	$155 \pm 13^{\#}$	121 ± 16*	146 ± 13	80 ± 9*	104 ± 10*	138 ± 15	72 ± 7*
HDLC ($mg\,dL^{-1}$)	33 ± 4	37 ± 4.8	36 ± 4.8	37 ± 5	38 ± 4.9	35 ± 5	35 ± 4	37 ± 4
TC:HDLC	2.67 ± 0.34	$5.7 \pm 0.93^{\#}$	4.6 ± 0.62*	5.2 ± 0.82	3.66 ± 0.38*^{bc}	4.37 ± 0.65*	4.9 ± 0.85	3.54 ± 0.40*^{bc}
LDLC:HDLC	1.64 ± 0.22	$4.91 \pm 0.72^{\#}$	3.36 ± 0.43*	3.92 ± 0.63	2.1 ± 0.33*^{bc}	2.94 ± 0.70*	3.95 ± 0.71	1.95 ± 0.34*^{bc}
TG ($mg\,dL^{-1}$)	81 ± 8	$142 \pm 13^{\#}$	125 ± 11*	130 ± 14	107 ± 14*^{bc}	119 ± 14*	137 ± 9	89 ± 9*^{bc}

All values are mean ± s.d.; number of rats per group is given in parentheses. $^{\#}P < 0.0001$ vs baseline values, $^{*}P < 0.05$ vs atherosclerotic group, $^{b}P < 0.05$ vs SO group, $^{c}P < 0.05$ vs OO group.

Table 2 Dietary OO, SO and FO modulate lipoprotein oxidation susceptibility and aortic antioxidant enzyme contents in atherosclerotic rats

	Baseline (70)	Atherosclerotic rats (54)	4 weeks			8 weeks		
			OO (8)	SO (8)	FO (8)	OO (7)	SO (6)	FO (7)
LOS (nmol MDA mg^{-1} non HDLC)	63 ± 7	134 ± 13	75 ± 8*^{ab}	100 ± 16*	92 ± 11*	59 ± 7*^{ab}	92.8 ± 10*	70.5 ± 9*
SOD ($U\,mg^{-1}$ protein)	69 ± 9 (8)	37 ± 8 (8)$^{\#}$	56 ± 7*	42 ± 8	47 ± 4	64 ± 8*^{ab}	51 ± 7*	54 ± 6*
GSH (nmol g^{-1} protein)	234 ± 27 (8)	156 ± 16 (8)$^{\#}$	204 ± 17*	182 ± 16*	172 ± 11	226 ± 13*^{ab}	198 ± 21*	190 ± 21*

All values are mean ± s.d.; number of rats per group is given in parentheses. $^{\#}P < 0.0001$ vs baseline values, $^{*}P < 0.01$ vs atherosclerotic group, $^{a}P < 0.05$ vs SO group ,$^{b}P < 0.05$ vs FO group.

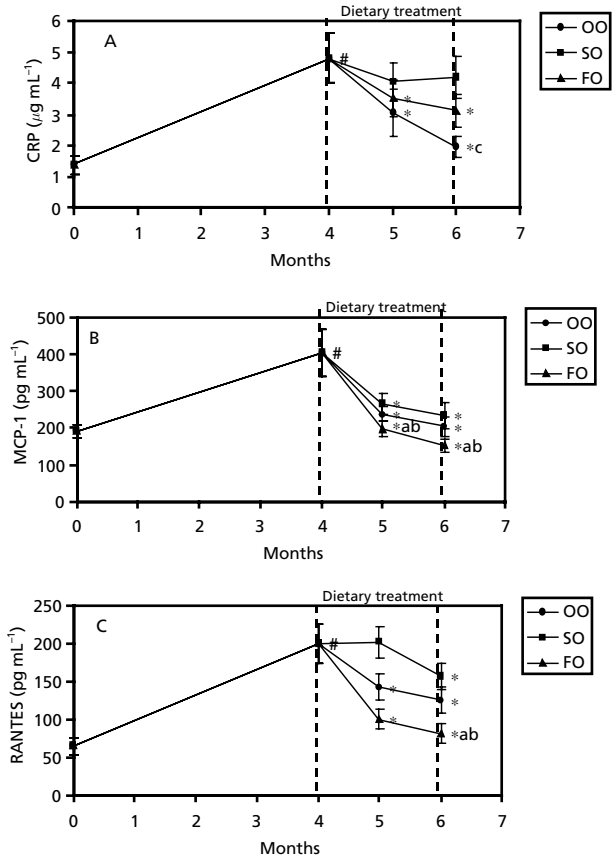

Figure 1 Changes in CRP (A), MCP-1 (B) and RANTES (C) concentrations at baseline, following feeding with the atherogenic diet for 4 months and during dietary treatment. #$P < 0.001$ vs baseline value, *$P < 0.01$ vs atherogenic rats, [a]$P < 0.05$ vs OO, [b]$P < 0.05$ vs SO and [c]$P < 0.05$ vs FO.

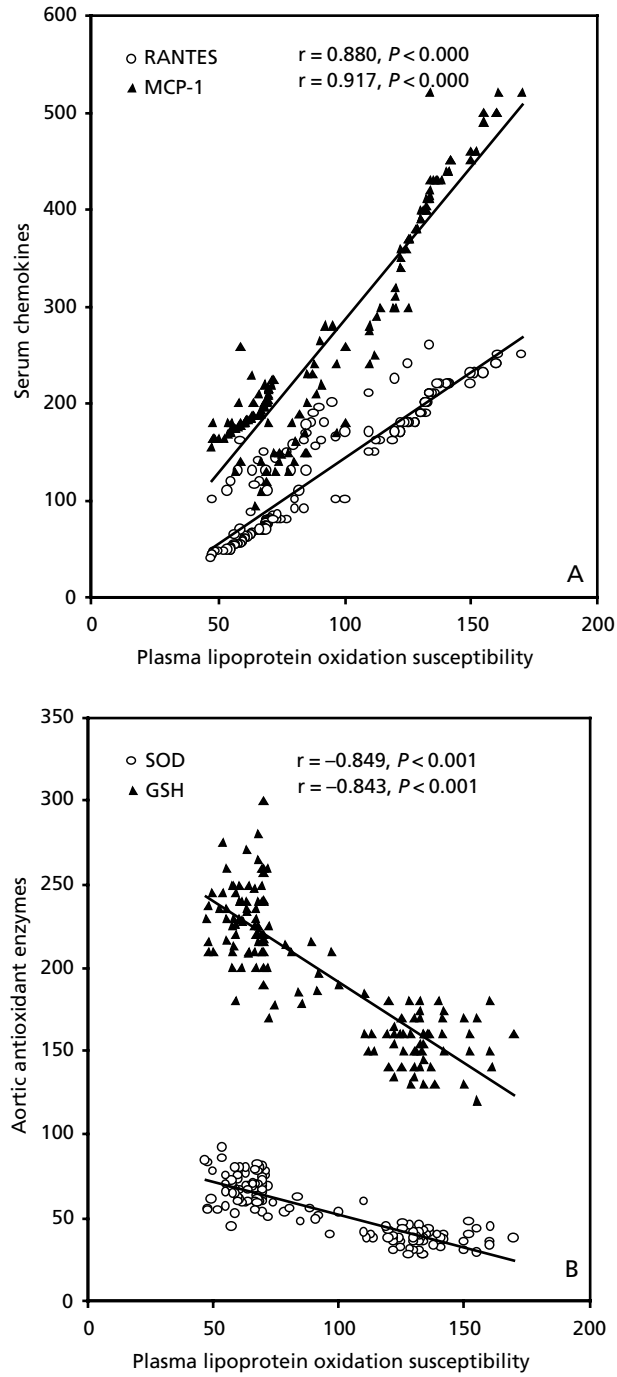

Figure 2 Correlation between plasma lipoprotein oxidation susceptibility and each of serum chemokines (A) and aortic antioxidant enzymes (B).

dation susceptibility and each of MCP-1 and RANTES (r = 0.917, 0.880, $P < 0.000$; Figure 2A). However, it negatively correlated with SOD and GSH (r = −0.849, −0.843, $P < 0.001$; Figure 2B).

Histopathological examination of the atherosclerotic aorta showed thick intima with thick and irregular endothelium sub-basal endothelial lamina (SBL), multiple cellular infiltrations and vacuoles, and white longitudinal streaks compared to normal aorta (Figure 3A,B).

Dietary treatment of atherosclerotic rats with OO, SO, and FO attenuated the above-mentioned changes compared to the atherosclerotic control group. However, the haematoxylin and eosin sections of the OO group showed thin intima and endothelium, relative reduction in cellular infiltrations, vacuolations, white longitudinal streaks of intima and media with no cystic masses compared to FO and SO groups (Figure 3C,D,E).

Discussion

As mentioned before, rats receiving the atherogenic diet demonstrated marked dyslipidaemia as evidenced by increased atherogenic indexes and plasma LDL levels, which are directly related to the incidence of coronary and cardiovascular events (Heller et al 1993), but how does elevated LDL produce the complex lesions of atherosclerosis with their hallmark features of inflammation, necrosis, cellular proliferation and lipid deposition? The answer may lie in the types of unsaturated fatty acid of

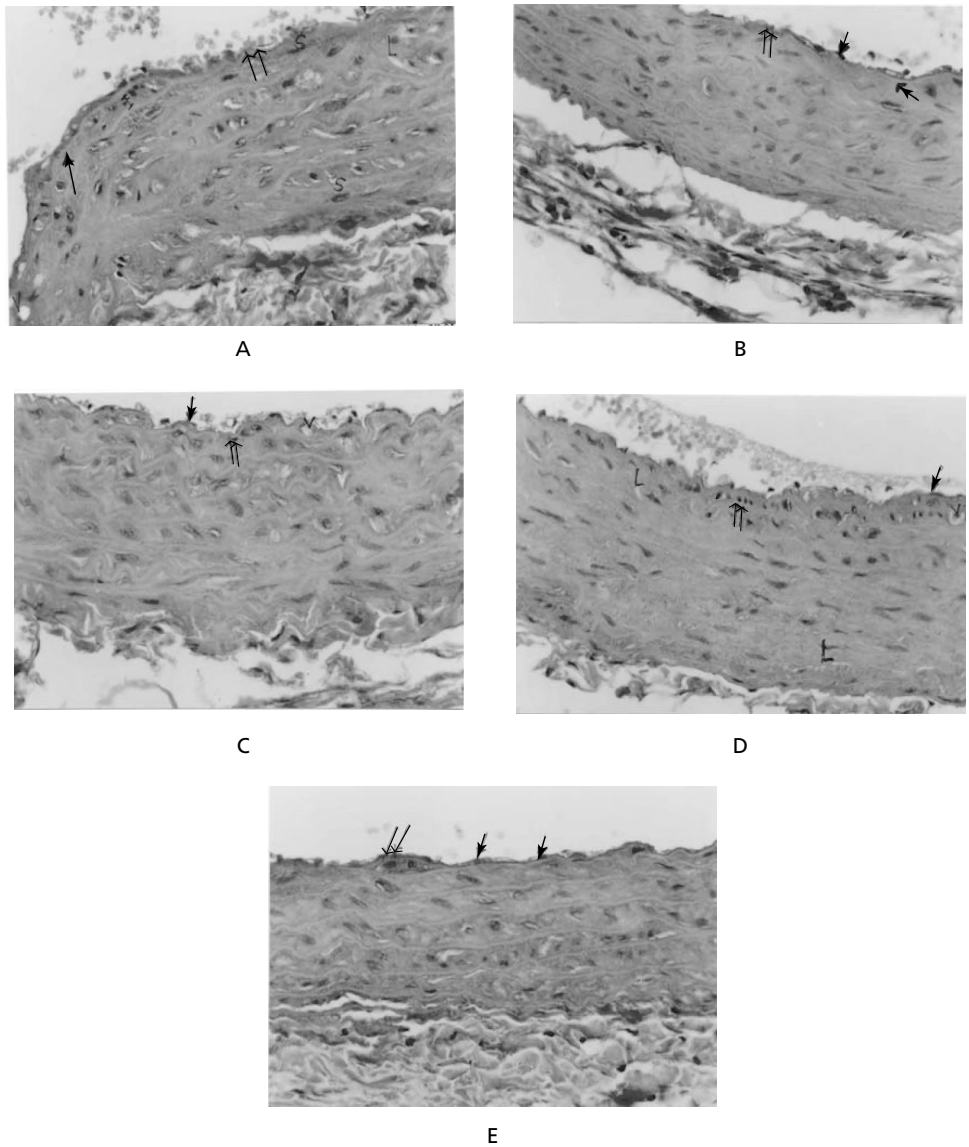

Figure 3 (A) Representative histological section of aorta at baseline showing relatively thin intima (arrow) of aorta with thin and flat endothelium (arrow head) and thin SBL (double arrows) (H & E × 400). (B) Representative histological section of aorta after feeding with atherogenic diet showing relatively thick intima (arrow) with thick and irregular endothelium (arrow head) and thick SBL (double arrows). Multiple cellular infiltrations (I), vacuoles (V), white longitudinal streaks (L) and nuclei of smooth muscle (S) are seen in intima and media (H & E × 400). (C) A photomicrograph of aorta of OO-treated group showing relatively thin intima with thin endothelium (arrow). There is a relative reduction in cellular infiltration (double arrows), vacuolations (V) and white longitudinal streaks (L) of intima and media (H & E × 400). (D) A photomicrograph of aorta of SO-treated group showing thin intima with flat and thin endothelium (arrow). Relatively few cellular infiltration (double arrows) and vacuolations (V) are seen in intima and media (H & E × 400). (E) A photomicrograph of aorta of FO-treated group showing relatively thin intima with thin and flat endothelium (arrows) and thin SBL. There are small cystic mass (double arrows) containing few cellular infiltrate. Few white longitudinal streaks are seen in media (H & E × 400).

LDL (cholesteryl esters and phospholipids) (Ross 1999). Within the artery wall, oxidation of unsaturated fatty acids of LDL can generate toxic aldehydes and epoxides, inducing inflammatory cytokine production (Steinberg 1997). Thus, the pathological consequence of its accumulation may lead to localized deposition of its fatty acids at damaged sites in the artery wall (Goldstein & Brown 2001). The observed dyslipidaemia here may therefore also represent an inducer of oxidative stress. Such findings may be manifested by increased LOS and decreased aortic SOD and GSH contents.

Oxidation of LDL generates a variety of oxidatively modified molecules, including phosphatidylcholine and phosphorylcholine (pc) oxidized phospholipids (Silva et al 2002) and other oxidation specific epitopes. These are also

formed on the surface membranes of cells undergoing apoptosis (Huber et al 2002). These particles are proinflammatory and proathergenic, and in addition are precursors of bioactive fatty acids affecting the vascular wall (Marathe et al 2001).

The coexistence of lipoprotein oxidation and inflammation dramatically enhances the atherogenic process. Accumulation of oxidized LDL (ox LDL) in monocyte, smooth muscle and macrophages plays a key role in the early stages of white streak formation and foam cell or vacuoles (Ross 1999). Daniel et al (2003) added that cells of the immune system could infiltrate intima, initiating the formation of auto antibodies against glycated LDL. CRP is a part of an innate immune response; it binds both ox LDL and apoptotic cells through recognition of PC moieties on their surface (Chang et al 2002). CRP also includes adhesion molecule expression from the endothelium and tissue factor secretion from monocytes. This may initiate coagulation, contributing to atherogenesis (Ford et al 2003).

Recently, Silva and colleagues (2002) have found that the bioactive fraction of ox LDL phospholipids has a platelet-activating factor, which leads to the accumulation of several classes of white blood cells. This effect may increase both mRNA for MCP-1 and RANTES, which have been implicated in the formation of atherosclerosis. The results of the present study clearly show positive correlations between lipoprotein oxidation and both MCP-1 and RANTES.

Dietary manipulations that increase LDL oxidative resistance could potentially delay the progression of atherosclerosis (Hargrove et al 2001). Our results found that although both MUFA- and PUFA-enriched diets lower total and LDL cholesterol concentrations, LDL oxidation susceptibility in rats fed with the MUFA diet is less than for those consuming a diet supplemented with PUFAs (n-3, n-6) for 8 weeks.

Recently a large body of research has supported the hypothesis that oxidation of LDL in vessel walls may be attenuated by the phenol content of olive oil (Moreno 2003). These oil antioxidants increase the resistance of LDL against oxidation and contribute to the health effects of the Mediterranean lifestyle (Leenen et al 2002). Additionally, it activates mRNA transcription of GSH-related enzymes (Masella et al 2004). This concern was evidenced by a significant increase in aortic SOD and GSH content in atherosclerotic rats fed with olive oil rather than the others following 8 weeks of dietary treatment.

In the same way, in the OO-treated group, the intima were relatively thin with a relative reduction in cellular infiltration, vaculoation and white longitudinal streaks of intima and media in the wall of the aorta. The improvement in histological patterns may also be related to the antioxidant effects of the biophenolic compound found in MUFA. Reduction of oxidative stress is joined to the protection of elastic fibres from breaking down and subsequent release of glycosaminoglycan (Aguilera et al 2001). Meanwhile, there was marked reduction in the cellular infiltration of intima following FO treatment; there were small cystic masses containing few cellular infiltrates. This discrepancy may be dose or duration dependent.

Recent studies that have examined the effect of consuming specific unsaturated fatty acids on the oxidative susceptibility of LDL have not resulted in clear support of the notion that the higher the degree of unsaturation of dietary polyunsaturated fatty acids, the greater the susceptibility of LDL to oxidation (Higdon et al 2001). However, studies of aqueous micelles have suggested that the presence of highly unsaturated fatty acids (n-3) might actually decrease total lipid peroxidation in vitro (Yazu et al 1998).

The LDL particles consist of an amphipathic surface monolayer and a hydrophobic core, suggesting they may be have more like the biphasic system of the aqueous micelle than homogeneous solutions of fatty esters. Moreover, fatty acids with three or more double bonds have been found to form bicycloendoperoxyl radicals (Frankel 1998). In the FO-supplemented diet therefore, highly unsaturated n-3 fatty acids might also form more polar radicals, which would be more likely to localize at the surface of LDL particles, resulting in an increased rate of termination and slower propagation rate.

Uncontrolled production of reactive oxygen species (ROS) and arachidonic acid metabolites therefore contribute to the pathogenesis of cardiovascular disease. Inflammatory cells infiltrated in the atheroma plaque are a major source of ROS and initiation of atherosclerosis (Moreno 2003). Again atherosclerosis and inflammation share a similar mechanism.

Activation of the vascular endothelium by dietary fatty acids may be among the most critical early events of atherosclerosis development (Toborek et al 2002). Our data also showed the ability of dietary unsaturated fatty acids to reduce endothelial chemokine expression. These results are in agreement with other studies (De Caterina et al 1998). One possible explanation relates to the intracellular mediators of NF-κB activation, namely ROS derived from the activation of NADH or NADPH oxidase following cytokine activation. A scavenging effect of O^{2-} and the presence of the NO radical are likely to account for the inhibition of NF-κB activation, possibly through enhanced transcription or stabilization of the inhibitor I-κB (Peng et al 1995). It is conceivable that similar oxygen-scavenging reactions occur with unsaturated fatty acids. These would lead to prevention of O_2 from generating hydrogen peroxide and by this mechanism reduction of cell activation. Moreover, it might be that PUFAs induce some hydrogen peroxide degrading enzymes, e.g. glutathione peroxides (Meskini et al 1993).

Recent studies have highlighted the role of peroxisome proliferator-activated receptors (PPARs), and α, γ and β/δ as transcription factors in the modulation of atherogenesis (Marx et al 2004). Both EPA and DHA have been shown to be ligands for PPAR γ and α, exerting an anti-inflammatory effect both in-vitro and in-vivo (Li et al 2005). This effect may be mediated through inhibition of synthesis of pro-inflammatory molecules such as IL-6 and prostaglandins, and monocyte cytokine expression subsequent to inhibition of the activation of NF-κB via the PPAR γ-dependent pathway (Poynter & Daynes 1998).

The larger number of double bonds seems to be critical for the activity of n-3 compared to n-6 fatty acids in inhibiting endothelial activation (De Caterina et al 2000); these properties are likely to be relevant to the antiatherogenic and anti-inflammatory properties of n-3 fatty acids. In addition, activated endothelium inhibition by dietary fatty acids manipulation may provide a new target for therapeutic intervention.

In conclusion, the present study demonstrates that dietary fatty acid types greatly affect the lipoprotein oxidation susceptibility as well as circulating chemokines associated with development of atherosclerosis. MUFA (OO), which combines both potent antioxidant and moderate anti-inflammatory properties, is more effective in reducing aortic atherosclerotic lesion than n-3 and/or n-6 PUFA-supplemented diets in male Wistar rats. Further studies are planned to identify the mechanism of action of olive oil concerning the reduction of atherosclerotic lesions and to clarify its potential therapeutic benefits.

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