Effect of Altering Dietary N-6:N-3 PUFA Ratio on Vascular Responsiveness in the Rat Thoracic Aorta

Diyetteki N-6:N-3 PUFA Oranındaki Değişimin Sıçan Torasik Aortasında Vasküler Yanıtlar Üzerine Etkileri

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ABSTRACT Objective: It is known that high fat diets can cause endothelial dysfunction, mediated by oxidative stress. In the present study, we aimed to evaluate the vascular response of polyunsaturated fatty acids (PUFA) and alpha lipoic acid (ALA) on rat thoracic arteries. **Material and Methods:** The present study was conducted on male Wistar albino rats. The n-6 and n-3 groups were created from the rats that were fed with polyunsaturated fatty acid-rich diet, and 60% of the total calorie content comprised of fat. Furthermore, n-6/ALA and n-3/ALA groups were created by the addition of strong antioxidant alphalipoic acid into the diet of n-6 and n-3 groups. The effects of dietary n-6 and n-3 fatty acids and ALA on vascular response of the rat thoracic arteries were evaluated in the isolated organ bath. In addition, the effects of n-6 and n-3 fatty acids and ALA on the vascular morphology were evaluated using histochemical methods. On the other hand, the glutathione levels in the red blood cells were compared between n-6, n-3, n-6/ALA, and n-3/ALA groups. **Results:** We found decreased endothelium dependent relaxation and increased oxidative stress in n-6 and n-3 groups. Treatment with ALA improved endothelium-dependent relaxation and oxidative stres, and prevented the worsening of vascular histopathology. **Conclusion:** The results indicate that a high PUFA diet caused endothelial dysfunction by increasing oxidative stress. Besides, dietary total amount of PUFA seems to be more important than n-6/n-3 ratio for cardiovascular protection.

Key Words: Vasodilation; fatty acids, omega-3; fatty acids, omega-6; alpha-lipoic acid, 4-aminobenzoic acid, aniline, benfotiamine, thioctic acid, vitamin e drug combination; oxidative stress

ÖZET Amaç: Yüksek yağlı diyetlerin oksidatif stres aracılı endotel disfonksiyonuna neden olduğu bilinmektedir. Bizim çalışmamızda çoklu doymamış yağ asitleri (PUFA) ve alfa lipoik asitin (ALA) sıçan torasik arterinde vasküler yanıt üzerine etkilerinin değerlendirilmesi amaçlandı. **Gereç ve Yöntemler:** Çalışmamızda erkek cinsiyette Wistar albino sıçanlar kullanıldı. Toplam kalori içeriklerinin %60'ı yağdan oluşan ve poliansature yağ asitlerinden zengin diyetle beslenerek ratlarda, n-6 ve n-3 grupları oluşturuldu. Ayrıca n-6 ve n-3 gruplarının diyetlerine güçlü antioksidan özelliği bulunan alpha-lipoik asid (ALA) eklenerek, n-6/ALA ve n-3/ALA grupları da oluşturuldu. Diyete eklenen N-6, N-3 yağ asitlerinin ve ALA'nın sıçan torasik arterlerindeki vasküler cevaba etkileri, izole organ banyosunda değerlendirildi. Ayrıca n-6, n-3 yağ asitlerinin ve ALA'nın vasküler morfoloji üzerindeki etkileri histokimyasal olarak değerlendirildi. Diğer taraftan; n-6, n-3, n-6/ALA ve n-3/ALA grupları arasında kırmızı küre hücrelerinde glutatyon düzeyleri karşılaştırıldı. **Bulgular:** Çalışmamızda n-6 ve n-3 gruplarında endotel bağımlı gevşemenin azaldığını ve oksidatif stresin iki gruptada arttığını bulduk. Ayrıca, ALA tedavisinin endotel bağımlı gevşeme ile birlikte oksidatif stresi düzeltiğini ve vasküler hisptopatolojide bozulmayı engellediğini tespti ettik. **Sonuç:** Bizim çalışmamızda yüksek PUFA diyetinin oksidatif strese neden olarak endotel disfonksiyonuna neden olduğu sonucuna ulaşıldı. Ayrıca diyetteki n-6/n-3 oranından ziyade toplam PUFA miktarı kardiyovasküler koruma için daha önemli gözükmektedir.

Anahtar Kelimeler: Vazodilasyon; yağ asidleri, omega-3; yağ asitleri, omega-6; alfa-lipoik asid, 4-aminobenzoik asit, anilin, benfotiamin, tioktik asit, vitamin e ilaç kombinasyonu; oksidatif stres

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Vascular endothelium plays an important role in the regulation of vascular tone by releasing relaxing factors such as nitric oxide, endothelium-derived hyperpolarizing factor and prostacyclin.[1] Endothelial dysfunction is an early and crucial event in the pathogenesis of cardiovascular disease, which is associated with atherogenesis.

Dietary fatty acids (FFAs) have an important role in cardiovascular diseases which are known to modulate vascular function.[2] It has been suggested that decreasing the amount of saturated fatty acids in diet, and increasing polyunsaturated fatty acids (PUFA) as to give more than 10% of the energy in the diet decreases the risk of cardiovascular diseases. However, the n-6, n-3 PUFA amount and n-6/n-3 PUFA ratio in diet is are debated.[3] In order to decrease the risk of cardiovascular diseases, it is recommended to decrease the amount of saturated fatty acids in the diet and to increase the polyunsaturated fatty acids so as to constitute more than 10% of the energy in the diet. However, the amount of n-6, n-3 PUFA and n-6/n-3 PUFA ratio in the diet are still debated.[3]

PUFAs can beclassified into two types: n-3 fatty acids and n-6 fatty acids. Linoleic acid is the major n-6 fatty acid, and alpha-linolenic acid is the major n-3 fatty acid. In the body, linoleic acid is metabolized to arachidonic acid (AA), and alpha-linolenic acid is metabolized to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These two classes of PUFA are important components of the cell membranes. They are metabolically and functionally distinct, and have opposing physiological functions. Their balance is important for homeostasis and normal development.

AA and EPA are parent compounds for eicosanoid production. As the amount of omega-6 fatty acids increases in diet, the amount of prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids, and lipoxins that are formed by AA increase.

The eicosanoids from AA are biologically active in very small quantities, and if formed in large amounts, can contribute to the pathogenesis of cardiovascular diseases. When added to the diet, EPA and DHA partially replace the omega-6 fatty acids, especially in cell membranes. As a result of EPA metabolism, the concentration of vasodilatory eicosanoids increases in the vascular bed.[4] Due to this, to show the positive clinical effects of the amount of omega-3 fatty acids and omega-6/omega-3 ratio becomes important. Although suggested omega-6/omega-3 ratio is 1:1 or 1:2, nowadays they usually change around 10:1 to 30:1. For this reason, in order to demonstrate positive beneficial clinical effects, the amount of omega-3 fatty acids in the diet and the omega-6/omega-3 ratio gain importance. In the past, the recommended ratio of omega-6/omega-3 was 1:1 or 2.1, but nowadays this ratio changes between 10:1 and 30:1.

It is known that high-fat diets reduce the endothelial function, increase the fat-mediated oxidative stress and decrease the antioxidative enzyme activity.[5-7]

Several antioxidants have been proven to be beneficial for decreasing oxidative stress and improving endothelium-dependent vascular relaxation caused by high fat diet (MUFA, n-6 PUFA).[8,9]

Alpha-lipoic acid (ALA) is a nutritional dithiol compound, and an essential cofactor in oxidative metabolism in the mitochondria.[10] ALA acts with its reduced form, dihydrolipoate, as a potent antioxidant to scavenge free radicals, chelate metal ions, and recycle antioxidants.[11] Potent antioxidant activity of ALA, which reduces oxidative stress both at systemic and local levels, is responsible for its positive effects on atherosclerosis and vascular relaxation.[8]

In this study, we aimed to investigate the endothelial dysfunction of with an n-6 rich PUFA diet (60% of the calory content from fats, and n-6/n-3 ratio 15:1) and an n-3 rich PUFA diet (60% of the calory content from fats, and n-6/n-3 ratio 2:1), and the effect of ALA in the rat aorta.

MATERIAL AND METHODS

ANIMALS AND DIETS

The study was initiated after obtaining the approval of the Experimental Animals Ethics Committee of

Istanbul University Cerrahpaşa Faculty of Medicine. For the care and use of the animals used in this study, our institution's guidelines for the care and use of laboratory animals were strictly followed. Five-week-old Wistar Albino Outbred male rats were housed in the temperature-controlled rooms (21-24 °C) under 12-h light/12-h dark cycle. Standart rat chow (65% kcal carbohydrate, 12% kcal fat and 23% kcal protein), n-6 high PUFA diet, (20% kcal carbohydrate, 60% kcal safflower and 20% kcal protein, n-6/n-3 15:1), and n-3 high PUFA diet (20% kcal carbohydrate, 40% kcal safflower oil, 20% kcal fish oil, 20% kcal protein, n-6/n-3 2:1) were used (Chowlab, Kocaeli, Turkey) (Table 1).

STUDY DESIGN

The control group was fed by standart rat chow diet for 8 weeks, control/ALA group was fed by standart rat chow diet for 8 weeks, and treated with ALA for the last 4 weeks. n-6/ALA group was fed by n-6 rich PUFA diet for 8 weeks, and treated with ALA for last 4 weeks. n-3 group was fed by n-3 rich PUFA diet, and n-3/ALA group was fed by n-3 rich PUFA diet for 8 weeks and treated with ALA for last 4 weeks. DL-alpha-lipoic acid (Fluca) was administered at a dose of 35 mg/kg intraperitoneally (i.p.), for 4 weeks. On the other hand, 0.9 % NaCl was administered i.p. to the control, n-6 and n-3 groups, for 4 weeks.

TABLE 1: Fatty acid composition of the diets (as the percentage of total fatty acid).

Fatty acid	Control	n-6	n-3
14:00	0.6	0.6	1.8
16:00	12.1	19.6	17.1
18:00	12.8	3.1	2.6
ΣSaturated	25.5	23.3	21.5
16:1n-9	1.0	0.4	2.4
18:1n-9	30.2	21.9	23.8
Σn-9	31.2	22.4	26.2
18:2 n-6	34.3	51.2	32.3
20:2 n-6			
20:3 n-6			
20:4n-6	4.8		2.0
22:4 n-6			
22:5 n-6			
Σn-6	39.1	51.2	34.3
18:3n-3	1.5	2.9	1.5
18:4n-3		0.2	
20:5n-3	1.1		6.2
22:5n-3	1.6		2.7
22:6n-3		0.2	7.6
Σn-3	4.2	3.3	18.0
n6/n3	9.3	15.4	1.9

FATTY ACID ANALYSIS

Fatty acids profiles were prepared by a slight modification of a method previously described by Yazıcı et al.[12] An accurately weighted portion of each sample was homogenized in cold 154 mM NaCl. Total lipids and added internal standard (100 µg nonadecanoic acid in chloroform, Sigma Chemical Co., St Louis) were extracted with chloroform/methanol (2:1). The chloroform phase was removed and evaporated to dryness under a stream of nitrogen. Total lipids were saponified with 2% KOH in methanol, and the fatty acids were methylated with 14% BF_3 in methanol. The resulting fatty acid methyl esters (FAMEs) were extracted with hexane, and analysed by capillary gas chromatography (Perkin-Elmer 8420 Capillary Gas Chromatography, Gouda, The Netherlands). Column: 50x0.25 mm WCOT fused silica, CP-sil 88; flame-ionization detector (FID) temperature 300°C; oven temperature program from 150 to 230°C at 2 °C min^{-1}; carrier gas N_2. The mass spectra of FAME from representative samples were obtained using a Hewlett-Packard (HP) 6890 capillary GC interfaced with a HP mass selective detector, and controlled by a HP Chem. Station. Column: 25x0.25 mm ID, QC2xBPx70; detector temperature 280°C; oven temperature program from 100°C to 290°C at 3°C min^{-1}; carrier gas helium.

FAMEs were identified by their retention time, and compared to those of authentic standards (Sigma Chemical Co., St Louis), and by GC-Mass Spectrometry. The detector response factors were determined by injection of equal weights of fatty acids and internal standard methyl esters on to the column. Their amounts were estimated by calculating the corresponding areas of fatty acid and internal standard.[12]

PREPARATION OF THORACIC AORTIC RINGS

At the end of the study, thoracic aortas were removed carefully, and placed in freshly prepared Krebs-Ringer Solution (KRS) (NaCl 118 mmol/l, KCl 4.7 mmol/l, $CaCl_2$ 2.5 mmol/l, $MgSO_4.7H_2O$ 1.2 mmol/l, KH_2PO_4 1.2 mmol/l, $NaHCO_3$ 25 mmol/l, and glucose 11.1 mmol/l). After the removal of fat and connective tissue, the thoracic aortas were cut into 3 mm-wide rings. The rings were opened by cutting the vessels longitudinally. Subsequently, they were fixed with stainless steel clips at both ends, and then they were placed in 20 mL organ bath containing KRS, gassed with carbogen (95% O_2+5% CO_2) at a pH of 7.4 at 37°C. The preparations were connected to isometric force displacement transducer (Grass Ins. FTO3), connected to a recorder (Grass Model 5) and were equilibrated for 90 min at optimal resting tension of 2 g. During this period, the KRS in the organ bath was exchanged every 15 minutes.

CONCENTRATION-RESPONSE CURVES

Concentration-response curves were obtained with noradrenaline (NA). NA (10^{-9}-10^{-4} mol/l) were added in a cumulative manner until a maximal response was achieved. After the addition of each dose, a plateau response was obtained before addition of a subsequent dose. Cumulative relaxation curves in response to acetylcholine (ACh) (10^{-9}-10^{-4} mol/l) and sodium nitroprusside (SNP) (10^{-9}-10^{-4}mol/l) were obtained in each strip precontracted submaximally (approximately EC90, 10^{-7} mol/l) by addition of NA. At the end of each experiment, tissue was blotted dry, measured and weighed.

Contractile responses to NA and relaxant responses to ACh and SNP were calculated as the increases for NA and decreases for ACh and SNP in tension (mg) in response to the agonist per mg of aorta. Agonist pD_2 value (=-Log EC50) was calculated from each agonist concentration-response curve by linear regression analysis of the linear portion of the curve, and taken as a measure of the sensitivity of the tissues to each agonist.

ASSAY OF RED BLOOD CELL (RBC) GLUTATHIONE (GSH) CONCENTRATIONS

Blood samples were drawn into Li-heparin containing tubes via intracardiac puncture. Plasma was separated for further analysis, erythrocytes were washed in a cold 9 g/l sodium chloride solution three times, following five dilutions. For the analysis of GSH concentrations, 0.2 mL of washed erythrocytes were obtained. The samples were kept in -80°C until the analysis.

The erythrocyte GSH concentrations were measured by the Beutler method. After the preparation of the erythrocyte (1:10) with distilled water, it was deproteinized and then centrifuged at 600 G for 20 min. Afterwards, 2 mL of sodium phosphate (0.3 M; w/v) was added into 0.5 mL of supernatant. Following the addition of 0.2 mL of dithiobis-nitrobenzoate (0.4 mg/mL, w/v) into the samples, the absorbency at 412 nm was measured immediately. GSH concentrations were calculated by using $1.36x10^{-4}$ $Mxcm^{-1}$ as the molar absorption coefficient. Intra- and interassay coefficients of variation for GSH were 3.8% and 3.9%, respectively. Results were expressed as μmol of GSH per gram of hemoglobin.

HISTOLOGICAL STUDY

Thoracic aortas were obtained for light microscopic studies, fixed in 10% neutral buffered formalin, and then embedded in paraffin before sectioning. Hematoxyln-eosin (HE) and Masson staining were used for histological examinations. Light microscopic examination was performed by Leica DM 2500.

STATISTICAL ANALYSIS

Data were presented as mean±standard error of the mean (SEM). SPSS 10.0 statistical program was used for comparisons among groups. Data were analyzed by using the one-way analysis of variance (ANOVA), followed by Tukey multiple comparisons test, and $p<0.05$ was considered as statistically significant.

RESULTS

BASAL PARAMETERS

Rats consuming a hyperlipidemic diet (n-6 rats or n-3 rats) showed a significant ($p<0.05$) weight gain compared to the rats that had the control diet (control rats). The n-6 and n-3 rats consumed less feed after the second week of the diet (expressed as

g/day). The consumed energy calculated from these values showed that n-6, n-3, n-6/ALA and n-3/ALA rats consumed 122.6, 126.8, 110,6 and 99,02 kcal/day, whereas control and control/ALA rats consumed 103.6 and 99.0 kcal/day, respectively.

VASCULAR RESPONSES IN RAT AORTIC RINGS

ACh-Induced Endothelium-Dependent Vascular Relaxation In Rat Aortic Rings

ACh (10^{-9}M-10^{-4}M) produced concentration-dependent relaxing responses in thoracic aortic rings (Figure 1A, 1B). The maximal responses (Emax) and pD_2 for ACh were significantly reduced in n-6 high PUFA diet group (Emax control=89.98±3.60 mg, Emax n-6=54.67±9.10 mg, $p<0.05$) (pD_2 control=8.02±0.22, pD_2 n-6=5.69±0.29 $p<0.05$). When ALA was added to n-6 group, the Emax and pD_2 for ACh were returned to baseline levels.

The Emax for ACh was significantly reduced in n-3 rich PUFA diet group (Emax control= 89.98±3.60mg, Emax n-3=55.36±4.85mg, $p<0.05$). When ALA was added to n-3 group, the Emax returned to baseline levels. However, no changes were seen at pD_2 values in the n-3 rich PUFA group (Table 2).

However, no changes were seen in Emax of ACh between the n-6 and n-3 groups.

SNP-Induced Endothelium-Independent Vascular Relaxation In Rat Aortic Rings

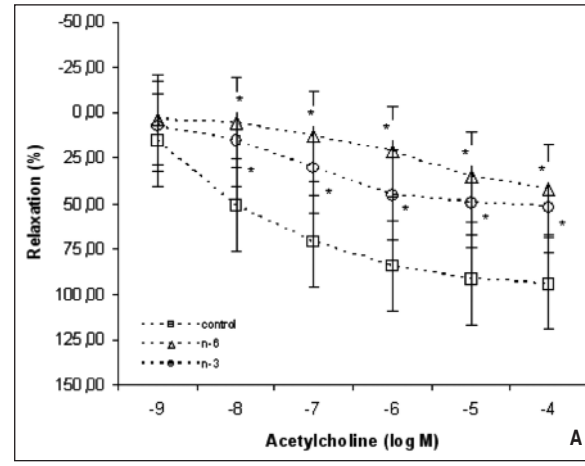
No changes were seen in maximal responses and pD_2 levels for SNP in all groups. (data not shown).

NA-Induced Vascular Contraction In Rat Aortic Rings

NA (10^{-9}M-10^{-4}M) produced concentration-dependent contractile responses in thoracic aortic rings (Figure 2). No changes were seen in Emax for NA in all groups. The pD_2 for NA was significantly increased in n-6 rich PUFA diet group (pD_2 control=7.41±0.18, pD_2 n-6=8.27±0.08, $p<0.05$), but not changed in n-3 group (Table 3).

RESULTS OF RBC GSH ANALYSIS

The GSH levels significantly decreased in n-6 rich PUFA group, and n-3 rich PUFA group compared



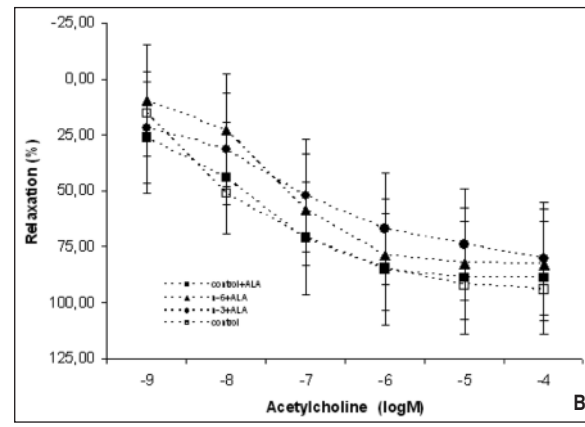


FIGURE 1: (A) Cumulative dose-response curves to asetylcholine in rat thoracic aorta rings of control, n-6, n-3 groups. *p<0.05: Significantly different from the control group. **(B)** Cumulative dose-response curves to asetylcholine in rat thoracic aorta rings of control/ALA, n-6/ALA , n-3/ALA groups. * p<0.05: Significantly different from the control group.

TABLE 2: Maximum relaxation (E_{max}) and potency (pD_2) values obtained in response to acetylcholine in aortic rings of each group.

ACh	E_{max}	pD_2
Control	89.98±3.60	8.02±0.22
Control/ALA	98.17±6.89	8.13±0.32
n-6	54.67±9.10*	5.69±0.29*
n-6/ALA	76.99±8.15	7.74±0.25
n-3	55.36±4.85*	7.11±0.22
n-3/ALA	80.24±6.30	7.70±0.27

ACh: Acetyl choline; ALA: Alpha lipoic acid.

p		
Pairwise comparisons	<0.001	<0.001
Control-Control/ALA	0.823	0.993
Control-n-6	0.032	0.042
Control-n-6/ALA	0.871	0.987
Control-n-3	0.042	0.910
Control- n-3/ALA	0.962	0.982

* p<0.05 significantly different from the control group.

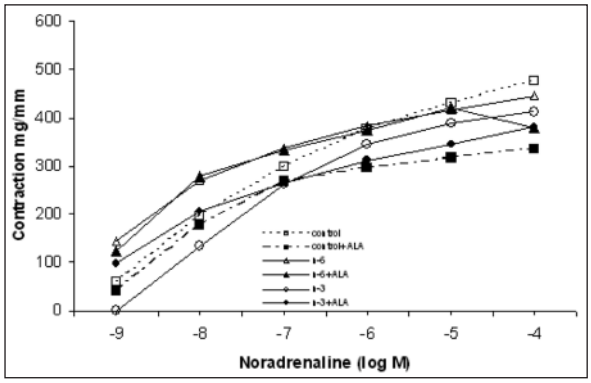


FIGURE 2: Cumulative dose-response curves to noradrenaline (NA) in rat thoracic aorta rings, from control control/ALA n-6, n-6/ALA, n-3, n-3/ALA groups. * p<0.05: Significantly different from the control group.

to the control group (control=5.45±0.36μg/mg, n-6=2.77±0.07 μg/mg, n-3=3.12±0.20 μg/mg p<0.05). When ALA was added to n-6 rich PUFA and n-3 rich PUFA groups, GSH levels returned to the levels of the normal group (control/ ALA=5.81±0.27μg/mg, n-6/ALA= 4.37±0.31μg/mg, n-3/ALA=4.44±0.26μg/mg). There were no significant differences for RBC GSH levels between the control and the control/ALA groups (Table 4).

VASCULAR HISTOLOGICAL FINDINGS

In n-6 rich PUFA group, a mononuclear cell infiltration was detected in the endothelial and subendothelial layers, in addition to an injury in the endothelium, an increase in the subendothelial layer thickness, a smoothness and disorganisation in elastic fibers of the tunica media, and degeneration in several parts of the smooth muscle cells (Figures 3C-3F). The vessel sections of the control group showed a lumen coated by smooth endothelial cells, tortuous elastic fibers and normal smooth muscle cells (Figures 3A, 3B). Furthermore, apoptotic endothelial cells were also observed. In n-6/ALA group, a similar morphology was observed with the control group including scarce degeneration in the tunica media and the endothelial cells. Although the morphology of the elastic fibers was similar to that of the control group, it was observed that there was smoothness in some areas, and an increase in thickness within the subendothelial area (Figures 3G-3I). In the n-3 rich PUFA group, tunica media and tunica intima were partially thickened, and a mononuclear cell infiltration was observed. A partial degeneration and apoptotic cells were determined on the endothelial cell surface. In some areas, smoothness of the elastic fibers and degenerative muscle cells were observed between the fibers (Figures 4A-4E). Despite this, the elastic fiber morphology of n-3/ALA group was similar to that of the control group, and muscle cell degeneration was seen in some parts of the tunica media, and a

TABLE 3: Maximum relaxation (E_{max}) and potency (pD_2) values obtained in response to noradrenaline in aortic rings of each group.

Ach	E_{max}	pD_2
Control	479.48±19.48	7.41±0.18
Control/ALA	350.65±23.77	8.20±0.14*
n-6	452.84±36.55	8.27±0.08*
n-6/ALA	467.77±28.77	8.02±0.20*
n-3	399.00±90.55	7.41±0.13
n-3/ALA	382.60±30.04	8.04±0.16

NA: Noradrenaline; ALA: Alpha lipoic acid.

p		
Pairwise comparisons	p=0.051	<0.001
Control-Control/ALA	0.511	0.001
Control-n-6	0.825	0.001
Control-n-6/ALA	0.934	0.722
Control-n-3	0.690	1.000
Control- n-3/ALA	0.671	0.124

* p<0.05 significantly different from the control group.

TABLE 4: Plasma glutathione levels of each group.

Groups	GSH mg/g Hb
Control	5.45±0.36
Control/ALA	5.81±0.27
n-6	2.77±0.07*
n-6/ALA	4.37±0.31
n-3	3.12±0.20*
n-3/ALA	4.44±0.26

GSH: Glutathione; ALA: Alpha lipoic acid; Hb: Hemoglobin.

p	
Pairwise comparisons	<0.036
Control-Control/ALA	0,921
Control-n-6	0,031
Control-n-6/ALA	0,096
Control-n-3	0,043
Control-n-3/ALA	0,107

Values are expressed as mean±standard error of the mean (SEM), the statistical analyses of the data were performed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. p<0.05 was considered as statistically significant. GSH:Glutathione.

* p<0.05 significantly different from the control group.

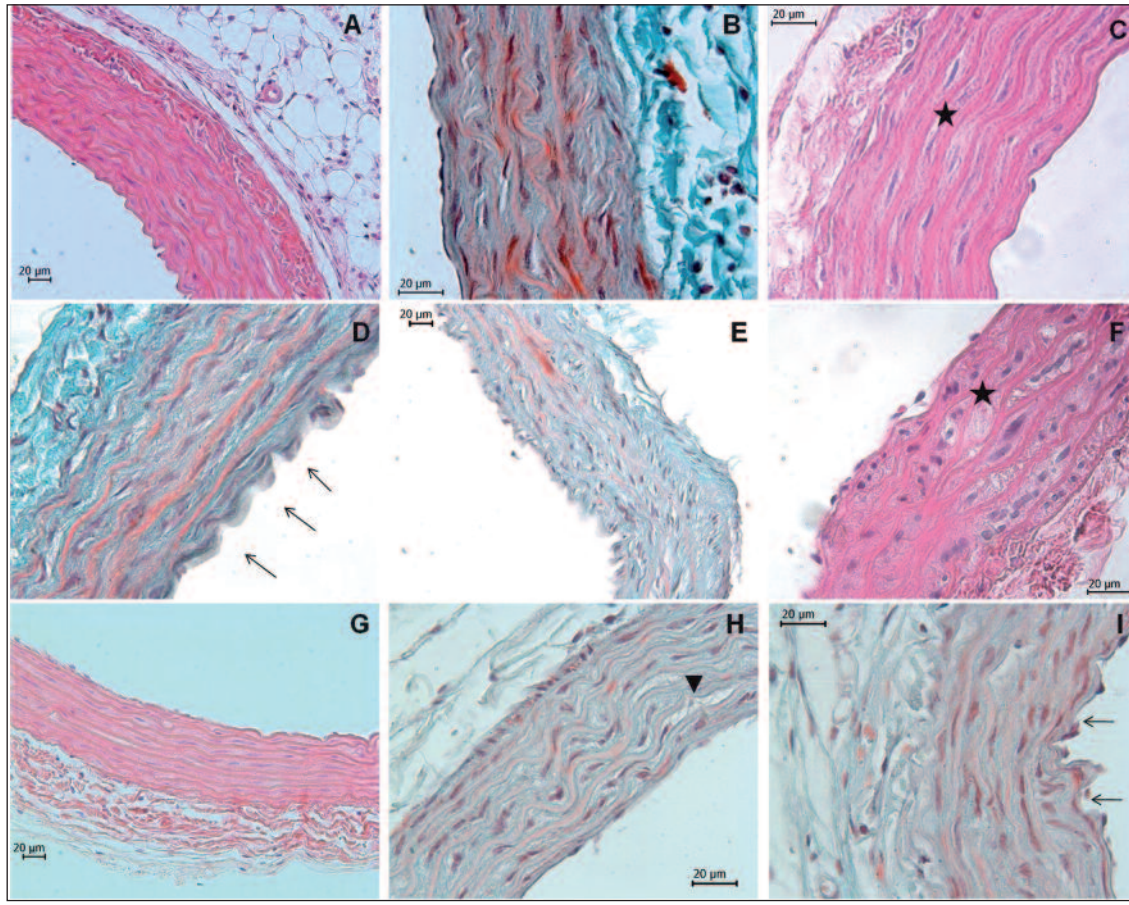


FIGURE 3: The vessel sections of the control group include a lumen coated by smooth endothelial cells, tortuous elastic fibers and normal smooth muscle cells **(A-B)**. In n-6 group, the elastic fibers in tunica media are shown to become smoothen completely (*)**(C)**, subendothelial layer is shown to become thick and a mononuclear cell infiltration is seen (↑) **(D)**. Significant elastic fiber and smooth muscle cell damage is observed in both tunica intima and media **(E)**. Degenerative smooth muscle cells, and elastic fiber disorganization are observed (*) **(F)**. Besides a similar morphology **(G)** to that of the control group in the vessel sections belonging to n-6/ALA treated group, an ongoing degeneration in tunica media (▲) **(H)** and endothelial cells (↑) **(I)** are partially observed. Hematoxylin-eosin stain: **(A,C,F,G)**, Masson's trichrome stain: **(B;D,E,H,I)**. Bar:20 µm.
(See color figure at Bkz. http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

partially thickened subendothelial intimal layer was observed. Mononuclear cell infiltration and a constant damage on the endothelial cells were detected (Figures 4F, 4G). In the control/ALA group, a similar morphology to the control group was observed in the elastic fibers, smooth muscle cells, and the vessel structure (Figures 4H, 4I). Mononuclear cell infiltration was detected in tunica adventitia in vessel sections obtained from this group.

DISCUSSION

The present study showed decreased ACh induced endothelium-dependent vasorelaxation responses in n-6 rich PUFA (n-6/n-3 15:1) and n-3 rich PUFA (n-6/n-3 2:1) diet groups, and it was also shown that antioxidant ALA treatment improved ACh induced endothelium-dependent vasorelaxation in rat aorta.

A number of studies showed that saturated and unsaturated high-fat diets (HFD) reduced endothelial function, increased fat-mediated oxidative stress, and decreased antioxidative enzyme activity.[5,13-15]

ALA and its reduced form, dihydrolipoic acid (DHLA), which are essential for reactions catalyzed by dehydrogenases in the mitochondria, react with reactive oxygen species such as superoxide radicals, hydroxyl radicals, hypochlorous acid, per-

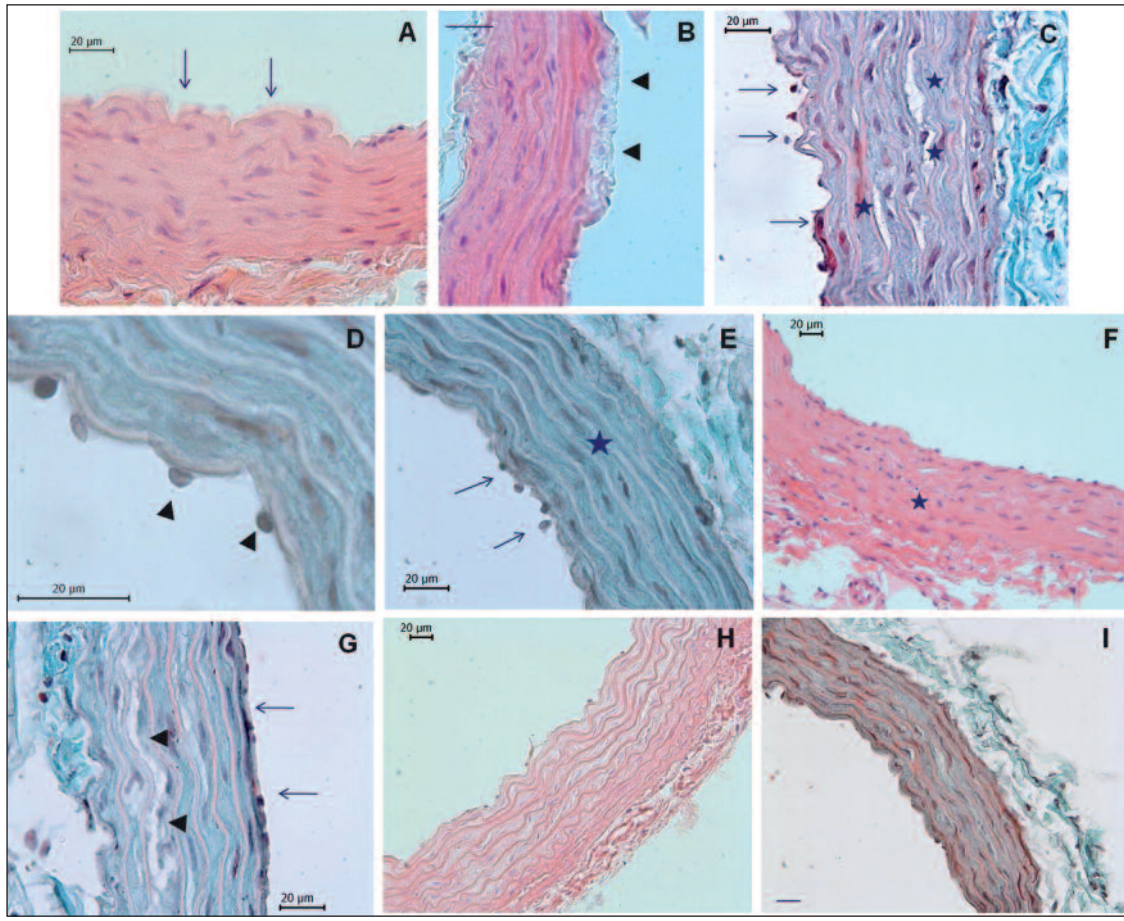


FIGURE 4: In the n-3 group, a partial thickness (↓) in tunica intima, **(A)** a mononuclear cell infiltration, (▲) **(B)** a disarrangment in endothelial layer (→) and degenerative muscle cells between the elastic fibers (*) **(C)** are observed. Apoptotic endothelial cells (▲) **(D)**, a degeneration of the endothelium surface (↑) and an extensive smoothness in elastic fibers in comparison to the control group are shown (*) **(E)**. In the n-3/ALA treatment group, degenerative muscle cells between the elastic fibers in some parts of the tunica media are shown (*) **(F)**. Just one part of the vessel (↑) is observed to increase in thickness (↑) and the damage (▲) **(G)**. The vessel sections belonging to control ALA group including a similar morphology to that of control group **(H,I)**. Hematoxylin-eosin stain: **(A,B,F,H)**, trichrome stain: **(C;D,E,G,I)**. Bar:20 µm.

(See color figure at Bkz. http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

oxyl radicals, and singlet oxygen. They also protect membranes by interacting with glutathione.[16,17]

Reactive oxygen radicals that increase with high fatty acid diet have been shown to cause endothelial dysfunction via decreasing nitric oxide (NO) bioavailability.[18] In our study, adding ALA to diet recovered the endothelium-dependent relaxing responses and GSH levels, and this supported the idea that n-6 rich PUFA and n-3 rich PUFA diets caused endothelial dysfunction via oxidative stress. It has been demonstrated that reactive oxygen radicals that increase with high fatty acid diets cause endothelial dysfunction via decreasing NO bioavailability.[18] It may be considered that decrease in NO might have resulted from NO destruction, which was due to the presence of reactive oxygen radicals.

In this study, Ach-induced endothelium-dependent vasorelaxation responses found to be decreased, but SNP-induced endothelium-independent vasorelaxation was unaffected in n-6 rich PUFA and n-3 rich PUFA diet groups.

Decreased endothelium-dependent relaxation in response to ACh in n-6 and n-3 rich PUFA diets are unlikely to be due to NO insensitivity, because the relaxation induced by SNP, which is a NO donor, was unaffected. This situation was interpreted as a reduction in endothelial NO release.[19]

In this study, ACh Emax responses were significantly reduced both in n-6 rich PUFA diet group and n-3 rich PUFA diet group, and when ALA was added to diet, it was improved.

Maximum ACh relaxation responses and GSH levels were significantly reduced in n-6 rich PUFA group and n-3 rich PUFA group, and when ALA was added to diet, GSH levels returned to the levels of the normal group. These results show that endothelial dysfunction is caused by oxidative stress. These results suggest that oxidative stress results in endothelial dysfunction.

PUFAs are the key constituents of plasma membranes in vascular smooth muscles and endothelium, and they may modulate the physical properties of biological membranes via alteration of membrane lipid composition affecting numerous physiological processes. In this study, ACh sensitivity (pD_2) was shown to be reduced in n-6 rich PUFA diet group, and it did not change in n-3 rich PUFA diet group. In the current study, it was observed that the ACh sensitivity (pD_2) decreased in high n-6 PUFA diet group, and it did not change in high n-3 PUFA diet group. The effects of n-6 rich PUFA and n-3 rich PUFA diets on endothelial cell mebrane lipid profiles have not been investigated. In n-6 rich PUFA diet, decreasing effect on ACh sensitivity might be the result of increased n-6 fatty acids in endothelial cell mebranes. Although the effects of feeding with high n-6 PUFA and high n-3 PUFA diets on the endothelial cell membrane lipid profiles were not investigated, the decrease in ACh sensitivity (pD_2) caused by high n-6 PUFA diet might be due to the increase in n-6 fatty acids in the endothelial cell membranes.

Our study demonstrated that NA pD_2 was significantly increased in n-6 rich PUFA diet group, but it was not changed in n-3 rich PUFA diet group. In n-6 rich PUFA group, decreased ACh sensitivity and increased NA sensitivity may be suggestive of increased n-6 fatty acid ratio in endothelial cell membranes.

The effect of n-3 fatty acids on endothelial function has also been evaluated in the following experiment. In vitro studies suggest that n-3 fatty acids can decrease the expression of adhesion molecules on endothelium, and decrease leukocyte-endothelium interactions.[20] n-3 augments endothelium-dependent relaxation, and may induce this effect by increasing the production and release of nitric oxide by an activation of nitric oxide synthase (NOS).[21-23]

Although in vitro studies showed that n-3 PUFA improved endothelial function in a different manner, our results did not show correlation with the previous studies. Since we used n-3 rich PUFA diet with an n-6/n-3 ratio of 2:1, that destroyed the endothelium dependent relaxation responses. n-3 PUFA's destroying effect on endothelium relaxation responses might be the result of high fat diet. The results of the current study are not in agreement with the previous studies.

Western type diet which has an n-6/n-3 ratio changing between 10:1-25:1, is known to cause cardiovascular diseases, cancer, inflammatory and otoimmune diseases. Although there is not a clear consensus, decreasing the ratio to 2:1 and increasing n-3 amount in diet is a known fact. In our study, we did not see positive effects of n-3 fatty acids, like vasorelaxation in vascular bed, even n-6/n-3 ratio was 2:1 in the diet.

ALA is known to have atheroprotective effects, including substantial decreases in oxidative stress leading to decreased LDL oxidation, and protection from diabetes-induced increases in plasma cholesterol in apolipoprotein E-deficient mice fed by a high-fat diet.[24] Besides being a potent antioxidant, ALA also protects the vascular wall against oxidative injury.[25] The supplementation of diet with ALA in diabetic rats for 8 weeks reduces the alteration in vascular morphology of the aorta. The mechanism by which ALA protects the vascular morphology probably depends upon its antioxidant properties, whereby it effectively prevents the increase of oxidative stress in plasma and the thoracic aorta. Besides, ALA is capable of scavenging reactive oxygen species generated during the lipid peroxidation, and protects the cell structure against damage.[11] Furthermore, it is functionally efficient in helping cells to recover from oxidative

damage. Supplementation with ALA effectively inhibits dyslipidemia. Therefore, it is possible that, in addition to its antioxidant properties, its effects on carbohydrate and lipid metabolisms may also contribute to its protective effects on vascular morphology.

Furthermore, in the present study, it was detected that n-6 rich PUFA diet and n-3 rich PUFA diet caused a significant vascular degeneration, such as endothelial apoptosis, mononuclear cell infiltration in the endothelial and subendothelial layers, increased subendothelial layer thickness, smoothness and disorganisation in elastic fibers, and degeneration in smooth muscle cells.

It was observed that the vascular morphology was protected in n-6/ALA and n-3/ALA groups. Our findings showed that the ALA supplementation was protective against vascular degeneration, to some extent.

Even though adding ALA to diets correct the endothelial dysfunction in vascular bed, histopathologic examination showed too much damage in vessels. Our study duration is not long enough to give an idea on this issue. Whether the damage in vessels is reversible or it can be cured, as well as the type and the duration of therapies have to be enlightened in further studies.

These data demonstrate that n-6 and n-3 polyunsaturated high-fat diet impairs the endothelial vasodilator responses, and degenerates the arterial structure, most probably being mediated by oxidative stress in rats. ALA restores the endothelial function and improves the structural damage by improving the oxidative stress. Based on our findings, it may also be speculated that, if the amounts of polyunsaturated fatty acids were above the critical levels, they would not be able to exert their benefical effects on the vascular system.

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