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The effects of Eicosapentaenoic acid on the endothelium of the carotid artery of rabbits on a high-cholesterol diet

Sevil Cayli[1,*], Leyla Sati[1,*], Yasemin Seval-Celik[1], M. Altug Tuncer[2], Bengi Yaymaci[2], Zafer Berkman[3], Tuncay Altug[4] and Ramazan Demir[1]

[1]Department of Histology and Embryology, Faculty of Medicine, Akdeniz University, Antalya, Turkey, [2]Departments of Cardiovascular Surgery and Cardiology, Kosuyolu State Hospital Kartal, Istanbul, [3]Department of Neurosurgery, Faculty of Medicine, Namik Kemal University, Tekirdag, Turkey and [4]Department of Medical Biology, Faculty of Medicine, Bilim University, Istanbul, Turkey

* These authors contributed equally to this work

Summary. The preventive and therapeutic effects of Eicosapentaenoic acid (EPA) on diet-induced hyperlipidemia in rabbits have been investigated. Eighteen New Zealand rabbits were randomly divided into three groups of 6 subjects each; experimental group-I (EG-I) was administered a cholesterol rich diet, experimental group-II (EG-II) was treated with EPA (300 mg/kg/d) following a cholesterol-rich diet and the control group (CG) had a standard diet. Blood samples were collected at day 0 and at the 4th and 12th weeks of EG-II to obtain serum levels of total cholesterol (TC), high density lipid-cholesterol (HDL-C), low density lipid-cholesterol (LDL-C) and triglyceride (TG). From each group tissue samples were collected from the carotid artery for immunohistochemistry and electron microscopy. Our results showed that EPA could significantly lower ($p<0.001$) serum TC, LDL-C, HDL-C and TG levels with a reduction of 35%; 55%; 44% and 51%, respectively. Scanning and transmission electron microscopy results revealed that endothelial damage was more prominent in EG-I when compared to EG-II. The ruptured endothelial lining and damaged cellular surface was increased in EG-I when compared to EG-II. Ultrastructural observations showed that after EPA treatment, the degeneration and cellular surface damage on the endothelium were also decreased.

These biochemical and ultrastructural results suggest that EPA is a potential drug which significantly lowers the serum lipid profile and partially repairs endothelial dysfunction due to hyperlipidemia.

Key words: EPA, Cholesterol, Atherosclerosis, Endothelium, Ultrastructure, Rabbit

Offprint requests to: Prof. Dr. Ramazan Demir, Department of Histology and Embryology, Faculty of Medicine, Akdeniz University, Antalya Turkey. E-mail: rdemir@akdeniz.edu.tr

This study is dedicated to our generous and compassionate mentor, Prof. Dr. Turkan Erbengi

Introduction

The vascular endothelium is a dynamic structure that plays important roles in the regulation of vascular tone, homeostasis, immune and inflammatory responses (Vane et al., 1990). Various vasoactive and vasoconstructive substances regulate the biological activity of the vascular endothelium, such as prostaglandine 12 (PG-12), nitric oxide (NO), endothelin-1 (ET-1), angiotensin II (Ang-II), tromboxane A2, vascular endothelial growth factor (VEGF) and free oxygen radicals (ROS) (Demir et al., 2006, 2007; Hartge et al., 2006, 2007). An imbalance between vasodilatation and vasoconstriction causes endothelial dysfunction, which is an early event in the development of cardiovascular diseases and coronary risk factors, such as hypertension, dyslipidemia, diabetes and atherosclerosis.

Atherosclerosis is a chronic inflammatory disease characterized by enzymatic destruction of the normal arterial skeleton (largely elastin, collagen and smooth muscle), and replacement by disorganized collagen and elastin, cholesterol, and foam cells (Devaraj et al., 2007). One of the main reasons for atherosclerosis is hyperlipidemia, the increase of cholesterol and fat in the blood. The present studies indicate that the effects of chronic hyperlipidemia are complex, in that the condition results not only in the deposition of lipids in

the atherosclerotic regions, but also may produce the primary endothelial injury that initiates the process of atherosclerosis as well (Micallef and Garg, 2008; Roberts, 2008). In order to comprehend the cause and optimize treatment of hyperlipidemia, researchers focus on the nutritional, genetic, biochemical, pharmacological and molecular aspects of the disease. Therefore, a particular emphasis is placed on the studies of various medical agents and some medical herbs involved in hyperlipidemia, and of new factors that promote or prevent the disease (Dehmer et al., 1988; Zamble et al., 2006; Rocha et al., 2007; Tuncer et al., 2009).

The cardiovascular benefits of fish oil enriched in the n-3 long-chain polyunsaturated fatty acids, including eicosapentaenoic acid (EPA), have been demonstrated in humans and in experimental animals (Von Au et al., 1988; Yokoyama et al., 2007). Long-term treatment with omega-3 fatty acids is known to improve impaired endothelial-dependent relaxation of blood vessels in animals and humans (Yin et al., 1991; Chin and Dart, 1994, 1995). Omega-3 fatty acids cause endothelial relaxation and increase arterial compliance (Nestel et al., 2002). In addition, they lower blood pressure in people with both normal and high blood pressure (Geleijnse et al., 2002). In diabetic rats, long term oral administration of EPA may stimulate nitric oxide (NO) production, and increased NO likely inhibits enhanced cardiac sympathetic activity (Nishimura et al., 2000). The n-3 fatty acids promote the synthesis of beneficial NO in the endothelium (Connor and Connor, 1997). Impaired endothelial function reflects reduced production of NO, which can be regarded as an important marker of endothelial cell dysfunction. Overcoming endothelial dysfunction by EPA contributes to suppression of atherogenic processes and of ischemic heart disease.

It has been shown that EPA reduces myocardial infarct size, mainly by inducing calcium channel mediated and partially NO-mediated mechanisms in rabbits (Ogita et al., 2003). Considerable effort has been made to clarify the possible pharmacological function(s) of EPA. Administration of EPA has been reported to have a number of actions, including suppression of platelet aggregability (Siess et al., 1980; Goodnight et al., 1981), decrease in blood pressure (Norris et al., 1986; Knapp, 1989) and reduction in restenosis after coronary angioplasty (Dehmer et al., 1988). EPA has also been suggested to have a hypolipidemic effect (Von Lossonczy et al., 1978; Harris et al., 1983; Phillipson et al., 1985) and protect endothelial cells against anoikis, a phenomenon by which reactive oxygen species (ROS) lead to the detachment of endothelial cells.

Although it is obvious that EPA has some beneficial effects on the cardiovascular system, the ultrastructural changes in vascular endothelium of the carotid artery in response to EPA administration have not been elucidated. Therefore, the aim of this study is to investigate the effects of EPA on vascular endothelium in rabbits fed with a cholesterol-rich diet. The results suggest that EPA reduces and stabilizes atherosclerotic regions in the endothelium of the carotid artery.

Materials and methods

Research design

Eighteen New Zealand albino rabbits (7-8 months old and mean body weight 3500-4000g) were obtained from The Experimental Animal Center of Cerrahpasa Medical Faculty, and randomly divided into three groups: Experimental group-I (EG-I) (n=6) was fed with a cholesterol-rich diet composed of standard diet with an additional 1% cholesterol (Merc103672, Germany) and tapwater for 12 weeks, experimental group-II (EG-II) (n=6) was fed with a cholesterol-rich diet for 4 weeks and then treated with Eicosapentaenoic acid (EPA) (300 mg/kg/day, p.o.) (Sato et al., 1993; Yano et al., 1995) for eight weeks in addition to a cholesterol-rich diet. The control group (CG) (n=6) was fed with only standard fabrication diet composed mainly of vegetables together with 25% saturated and 75% unsaturated fat and tapwater; all groups were experimented for 12 weeks.

The animals were housed individually in stainless steel cages under standard conditions (21±2°C and 50±5% humidity) with a 12 hour light-dark cycle as described previously (Demir et al., 2005). The animals were kept in the laboratory for two weeks prior to the experiments to allow for acclimatization. The experimental protocol was reviewed and approved by the Committee for Ethics in Animal Care and Experiments, Istanbul University, Cerrahpasa Medical Faculty.

Transcardiac perfusion was performed with Ringer's solution (NaCl, 111.87 mM; KCl, 2.47 mM; $CaCl_2$, 1.08 mM; $NaHCO_3$, 2.38 mM, pH 7.2) for each rabbit (Demir et al., 2005). A. Carotis communis samples were fixed with 10% formalin for 8 h and prepared using routine techniques and collected for immunohistochemical analysis and histological evaluation.

Tissue sections from each experimental group were also stained with periodic acid-Schiff (PAS) and orcein, a strong marker for elastic lamellae identification used in basic histological examination.

Biochemical analysis

Blood samples were collected at day 0 and at weeks 4 and 12 from all groups, and total serum cholesterol (TC), high density lipid-cholesterol (HDL-C), low density lipid cholesterol (LDL-C) and triglyceride (TG) levels in serum were measured using a Beckman-Coulter Synchron LX20 PRO (Beckman-Coulter Inc., Fullerton, CA) using Syncron system reagents.

Immunohistochemical analysis

Procedural details have been described previously (Sati et al., 2007). Briefly, serial paraffin wax embedded sections were cut and collected on poly-L-lysine-coated

slides (Sigma-Aldrich, St. Louis, MO), dewaxed and rehydrated by routine protocol and placed in citrate buffer. To unmask antigens, an antigen-retrieval procedure was performed by heating the samples twice in a microwave oven at 750W for 5 min each time. After cooling for 20 min at room temperature, the sections were washed in phosphate buffered saline (PBS) and then soaked in 3% H_2O_2 for 15 min to quench endogenous peroxidase activity. After blocking with Ultra V blocking reagent (Lab Vision, Fremont, CA) for 10 min to reduce non-specific binding, sections were incubated overnight at 4°C with a rabbit polyclonal anti-vimentin antibody (sc-6260; 1:300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a cytoskeleton component responsible for maintaining cell integrity and structure, and a rabbit polyclonal anti-α-SMA (sc-53142; 1:200; Santa Cruz Biotechnology, Inc.) as a smooth muscle cell marker. The binding of the primary antibody was detected using a LSAB2 streptavidin-biotin horseradish peroxidase (HRP) kit (Dako, Glastrup, Denmark), employed according to the manufacturer's instructions, for 30 min at room temperature. In the LSAB2 system, a biotinylated secondary antibody forms a complex with HRP-conjugated streptavidin molecules. Antibody complexes were visualized by incubation with diaminobenzidine (DAB) chromogen (Lab Vision, Fremont, CA) prepared according to the manufacturer's instructions as 1-2 drops (40-100 ml, approximately 0.9 mg) of DAB chromogen to each 1ml of hydrogen peroxide substrate in buffer, for 20 s Sections were counterstained with Mayer's hematoxylin (Dako, Denmark) for 10 s, dehydrated, and mounted. All dilutions and thorough washes between steps were performed using PBS and all steps were at room temperature unless otherwise specified. Photographic images were captured using a digital camera (Canon PowerShot G5) attached to an Axioplan microscope (Zeiss, Oberkochen, Germany). Negative controls were performed by replacing the primary antibody with normal rabbit serum (Dako, Denmark).

Table 1. The lipid profiles of EG-II measured in the different periods of the experiment (mg/dL).

	Day 0	After 4 weeks	After 12 weeks
CG			
TC	30.3± 12.3	32.2± 14.1	33.1± 13.1
LDL-C	12.2±3.9	10.2±2.1	14.1±10.0
HDL-C	28.5±11.4	32.4±15.2	30.5±15.3
TG	56.6±29.4	53.4±17.2	57.5±23.4
EG-I			
TC	32.3± 13.3	869.1± 17.2	849.1± 165.1
LDL-C	11.4±6.4	414.5±237.3	433.7±268.6
HDL-C	31.3±14.3	287.7±14.3	294.8±17.2
TG	61.6±38.6	159.4±43.4	179.3±72.3
EG-II			
TC	33.3± 2.5	820.3±41.1	554.6±38.1
LDL-C	12.4±1.1	303.6±19.3	196.7±15.0
HDL-C	25.1±2.5	274.6±12.9	165.4±10.5
TG	57.7±4.1	142.3±7.8	88.4±3.2

TC: total cholesterol, LDL-C: low density lipoprotein-cholesterol, HDL-C: high density lipoprotein-cholesterol, TG: triglyceride. CG and EG-I lipid profile results were published recently by our group (Tuncer et al., 2009).

Transmission electron microscopy (TEM) analysis

Tissue samples were fixed with phosphate buffered (pH 7.4) 2.5% glutaraldehyde solution followed by 1 h at 40°C with 2% osmium tetroxide. An additional glutaraldehyde-osmium tetroxide double immersion fixation method was applied in order to perform TEM analysis (Acar et al., 2004). Samples were dehydrated through a gradually increasing series of ethanol and embedded in Araldite resin (Araldite CY 212, 91 ml+dodecenyl succinic anhydride (DDSA) 84 ml+N,N-dimethylbenzylamine (BDMA) 3.5 ml, TAAB Company, England). Semi-thin (1 µm) and thin (40-60 nm) sections were cut using diamond knives on a Leica ultramicrotome (Leica ultracut, UCT, Leica MZ6, England). Ultra-thin sections were collected on copper grids (Mesh 100, TAAB, England) coated with Formvar 15/95 Resin polyvinyl formol (EMS-Electron Microscopy Sciences, Fort Washington, PA, USA). In semi-thin sections, routine cresyl violet and toluidine blue staining methods were applied for orientation. In thin sections, double-contrast staining was applied with uranyl acetate (100 ml methanol and 5 g uranyl acetate) and Reynold's lead nitrate solution (1.76 g sodium citrate, 1.33 g lead nitrate, 50 ml distilled water and 8 ml 1N.NaOH) (Demir et al., 2005). Thin sections were examined using a LEO 906 transmission electron microscope (Leo 906E, Zeiss, Germany).

Scanning electron microscopy (SEM) analysis

Tissue samples from the carotid artery were also prepared for SEM analysis. Following the same fixation procedure used for TEM, samples were dehydrated through a gradually increasing acetone series. The tissues were placed on bottle corks and kept in amyl acetate (Demir et al., 1995) for critical point drying (E300, Polaron, ThermoVG Micro Tech, England). Tissues were coated with gold-palladium particles (Demir, 1979) and examined using a Zeiss 1430 scanning electron microscope (Leo 1430, Zeiss, Germany).

Statistical analysis

Data analysis for the lipid profile was carried out using Sigmastat for Windows, version 2.0 (Jandel Scientific Corporation Corporation, San Rafael, CA, USA). Differences among groups were compared using one-way ANOVA analysis on normally distributed data and the Kruskal-Wallis One-Way ANOVA on ranks on data that were not normally distributed. All data are

presented as mean ±SEM.

Results

Effects of EPA on serum lipid profile

The biochemical analyses of control group and experimental group-I were presented in our recent study (Tuncer et al., 2009). These results are summarized as follows: at the end of week 4 with the cholesterol-rich diet there was a significant increase in the lipid profiles in EG-I. Following EPA treatment TC, LDL-C, HDL-C and TG levels decreased significantly, 32%, 35%, 40% and 38% respectively ($p<0.001$) (Table 1). However, serum lipid levels were still higher compared to CG even though there was a decrease in the levels compared to

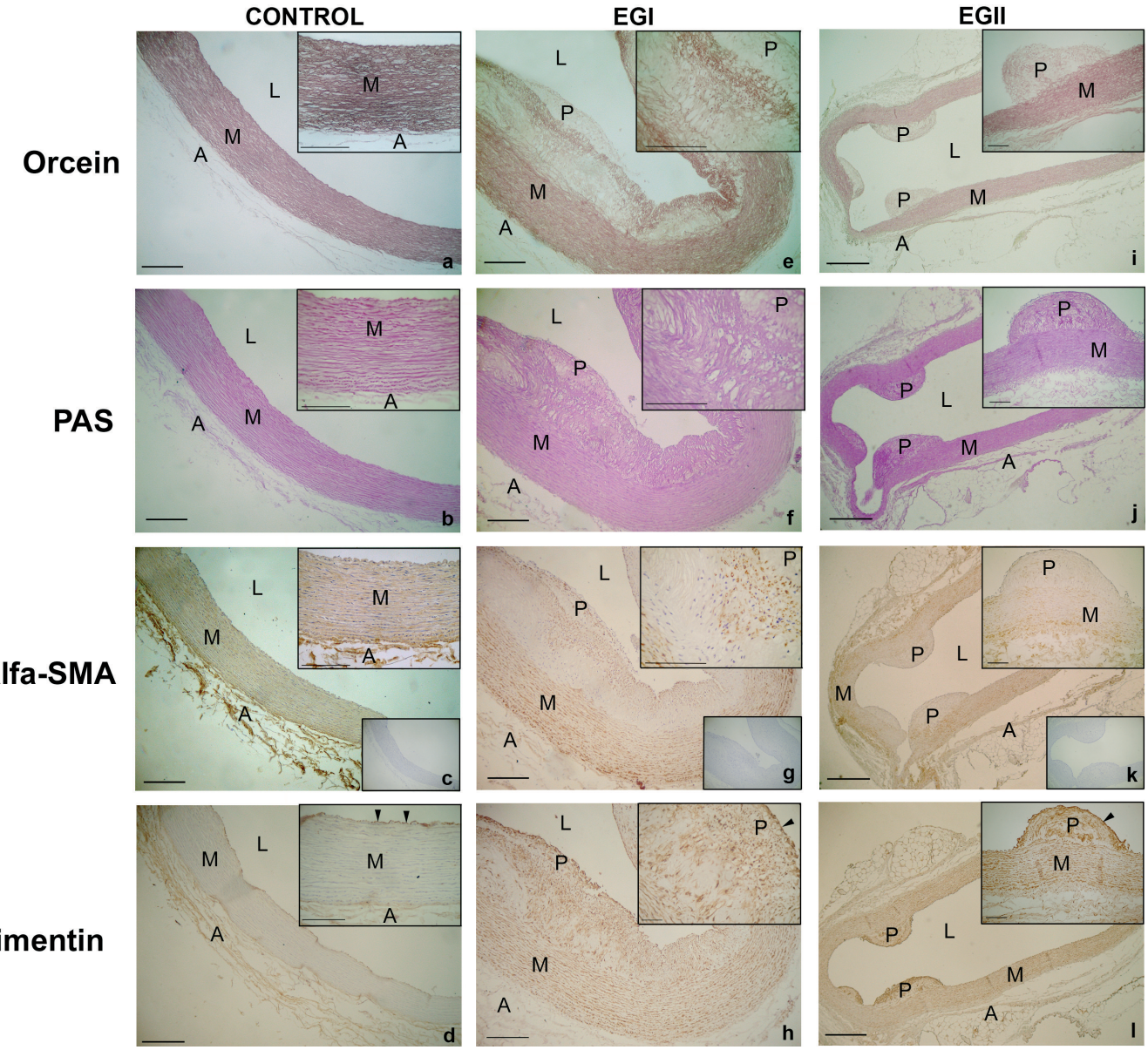

Fig. 1. Orcein (**a, e, i**), PAS (**b, f, j**), α-SMA (**c, g, k**) and vimentin (**d, h, l**) stained parallel sections of carotid artery. The size of the atherosclerotic plaques and the damage in the tunica intima are more prominent in EG-I compared to EG-II (**f, j**). The smooth muscle cells of the tunica media showed positive immunoreactivity with α-SMA (**c, g, k**). The immunohistochemical distribution of vimentin in the endothelium (arrow heads) and vascular layers of carotid artery is presented (**d, h, l**). Negative controls showed no immunoreactivity (small insets c, g and k). L: lumen; M: Tunica media; A: Tunica adventitia; P: plaque. Scale bars: a-h, 200 μm; i-l, 400 μm; insets, 100 μm.

EG-I after 12 weeks (Tuncer et al., 2009).

EPA reduces atherosclerotic regions and changes plaque composition

As shown in Fig. 1a-d, vascular endothelium, *tunica intima* and *tunica media* layers in control group showed normal vascular organization. However, tunica intima and lumen of carotid artery were partly occupied with atherosclerotic plaques in experimental groups (EG-I

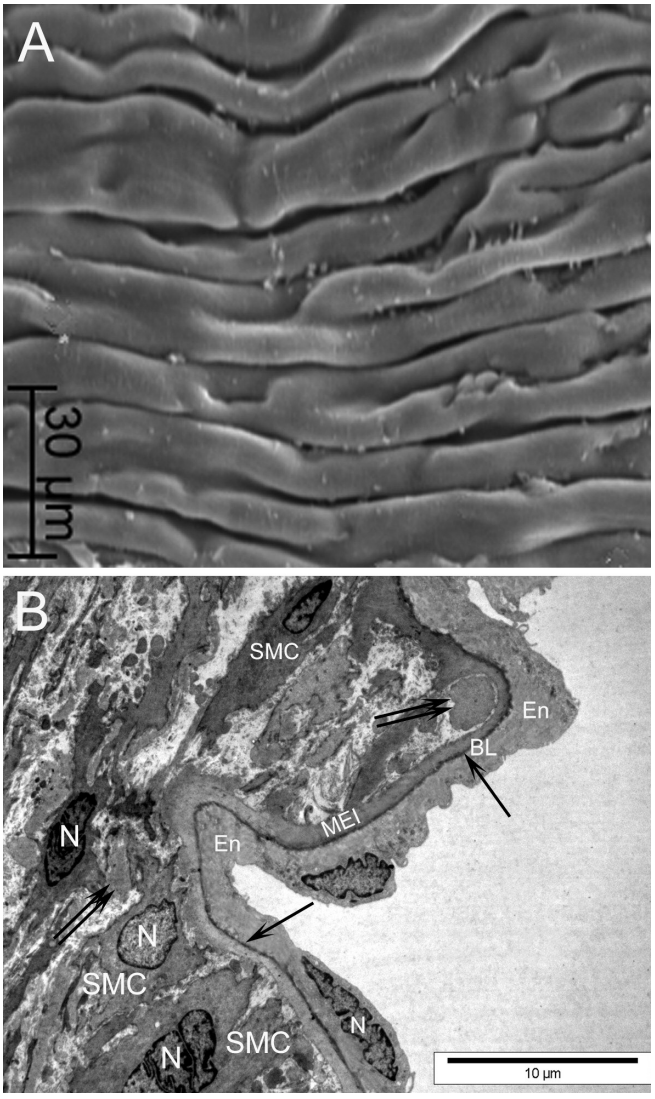

Fig. 2. Three dimensional **(A)** and ultrastructural **(B)** features of the endothelium (En) in control group are seen in normal condition. Endothelial cells (En) with heterochromatic nuclei (N) are lined on basal lamina (BL, with single arrows), which is situated on the definitive membrana elastica interna (MEI). Smooth muscle cells (SMC) with specific ultrastructural features were observed. Moderate or higher electron dense substance accumulations (double arrows) are observed near MEI.

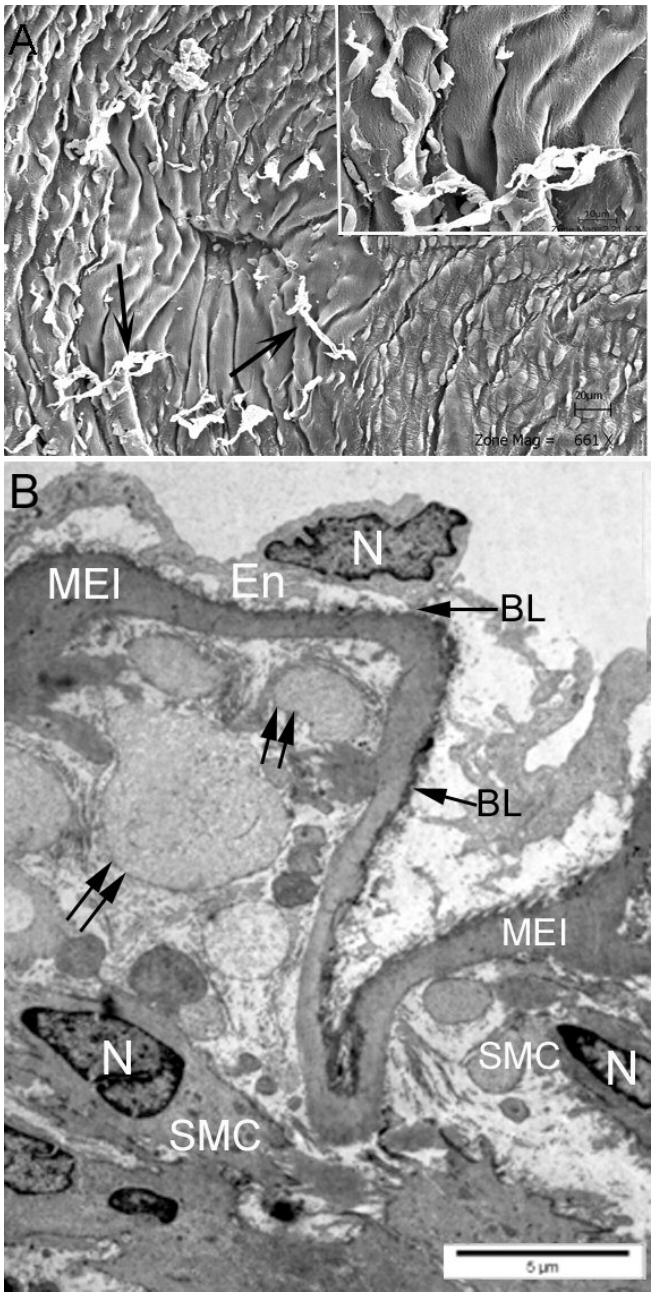

Fig 3. A. Three dimensional structure of the endothelial lining of carotid artery from EG-I. A local endothelial wound (**A** and inset) is formed on the surface of the endothelial cover. Cytoplasmic ruptures of endothelial lining and some cytoplasmic protrusions due to endothelial damage (arrows) are seen. **B.** In a parallel section of SEM observation **(A)**, ultrastructural damage of carotid artery endothelium is clearly seen. Deformed endothelium (En) with heterochromatic nucleus (N) detached from the basal lamina (BL, single arrows) shows local rupture and many vacuoles. Deformed basal lamina shows no connection to the endothelium. Accumulation of large vesicular bodies and electron dense substances are seen under membrane elastica interna (MEI) (double arrows) SMC: smooth muscle cells. Scale bars: A, 20 µm; inset in A, 10 µm; B, 5 µm

and EG-II) (Fig. 1e-l). There was an increase in the number and size of the atherosclerotic plaques in EG-I in comparison to control and EG-II groups. EPA significantly suppressed the development of atherosclerotic lesions in the carotid artery (Fig. 1i-l). The elastic lamellae of the tunica media were positively stained with orcein (Fig. 1a,e,i, inset). The atherosclerotic lesions were stained PAS positive due to the proteoglycans present (Fig. b,f,j). Moreover, the smooth muscle cells were positive for α-SMA (Fig. c,g,k). Additionally, *tunica intima* and *tunica media* layers of carotid artery showed positive immunoreactivity for vimentin (Fig. 1d,h,l). However, no significant differences were found between control and experimental groups by means of immunopositive cellular components and the intensity of immunolabelling based on H-score analysis. No immunoreactivity was seen in sections where the primary antibodies were replaced with isotype rabbit antibodies (Fig. 1c,g,k, small insets).

SEM, TEM Results

A summary of some of the ultrastructural changes (TEM and SEM) observed in experimental groups are indicated in Table 2.

Control group

Ultrastructural features of A. Carotis communis in control groups showed normal 3-dimensional (Fig. 2A) and ultra structural organization (Fig. 2B). The endothelial surface of the carotid artery appeared smooth and no pathological damage such as ruptures or protrusions were observed (Fig. 2B). The endothelial layer lining on the basal lamina and the internal elastic membrane under the basal lamina was observed in normal condition. A few cellular and fibrillar substances were also seen in the tunica intima layers (Fig. 2B).

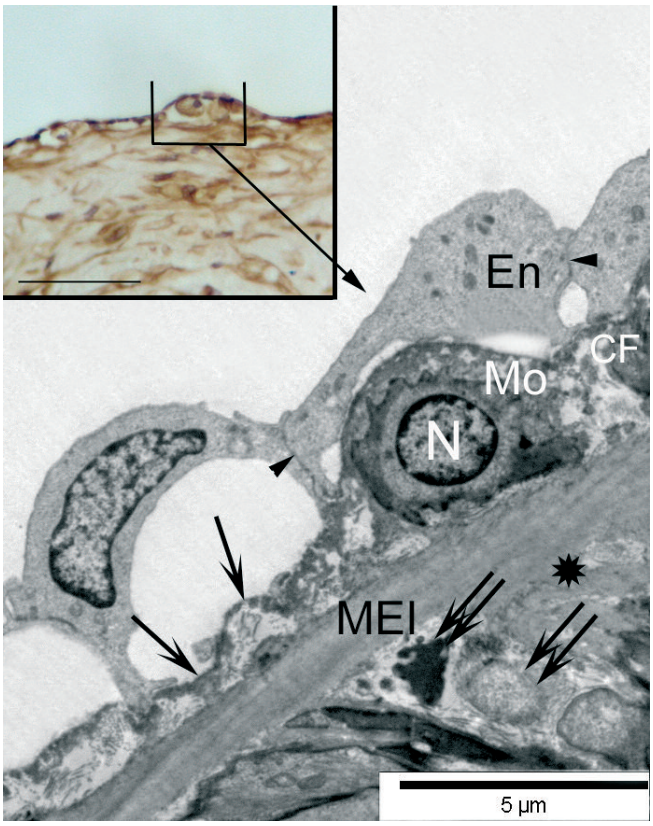

Fig. 4. Subendothelial region in the atherosclerotic plaque stained with vimentin in carotid artery wall of the EG-I (inset). Ultrastructural deformation of endothelium with heterochromatic nucleus and subcellular components are seen. Monocyte-like cell (Mo) and cell fragments (CF) in the tunica intima are shown. MEI shows local mixing with extracellular components (star) and accumulation of some electron dense substances in various sizes are seen under MEI (double arrows). Large vacuoles in the endothelium, detachment of the endothelium from the basal lamina (arrows) and connection complexes between endothelial cells (arrow heads) are shown. Scale bars: 5 µm, inset: 50 µm.

Table 2. Semiquantitative analysis of TEM and SEM results of experimental groups.

	CG	EG-I	EG-II
TEM Analysis			
Damage to endothelium (cytoplasmic protrusions, ruptured luminal lining, partial detachment etc.)	-	+++	+
Rupture of the internal elastic membrane	-	-	-
Foam cells	-	+++	++
Cell fragments	-	+++	++
Atherosclerotic plaques	-	+++	++
Multivesicular bodies in intima and media	-	+++	+++
SEM Analysis			
Damage to endothelium (rupture, endothelial protrusions and shrinkages)	-	+++	+
Atherosclerotic lesions	-	+++	+

-: None; +: Rarely; ++: Moderately; +++: Frequently

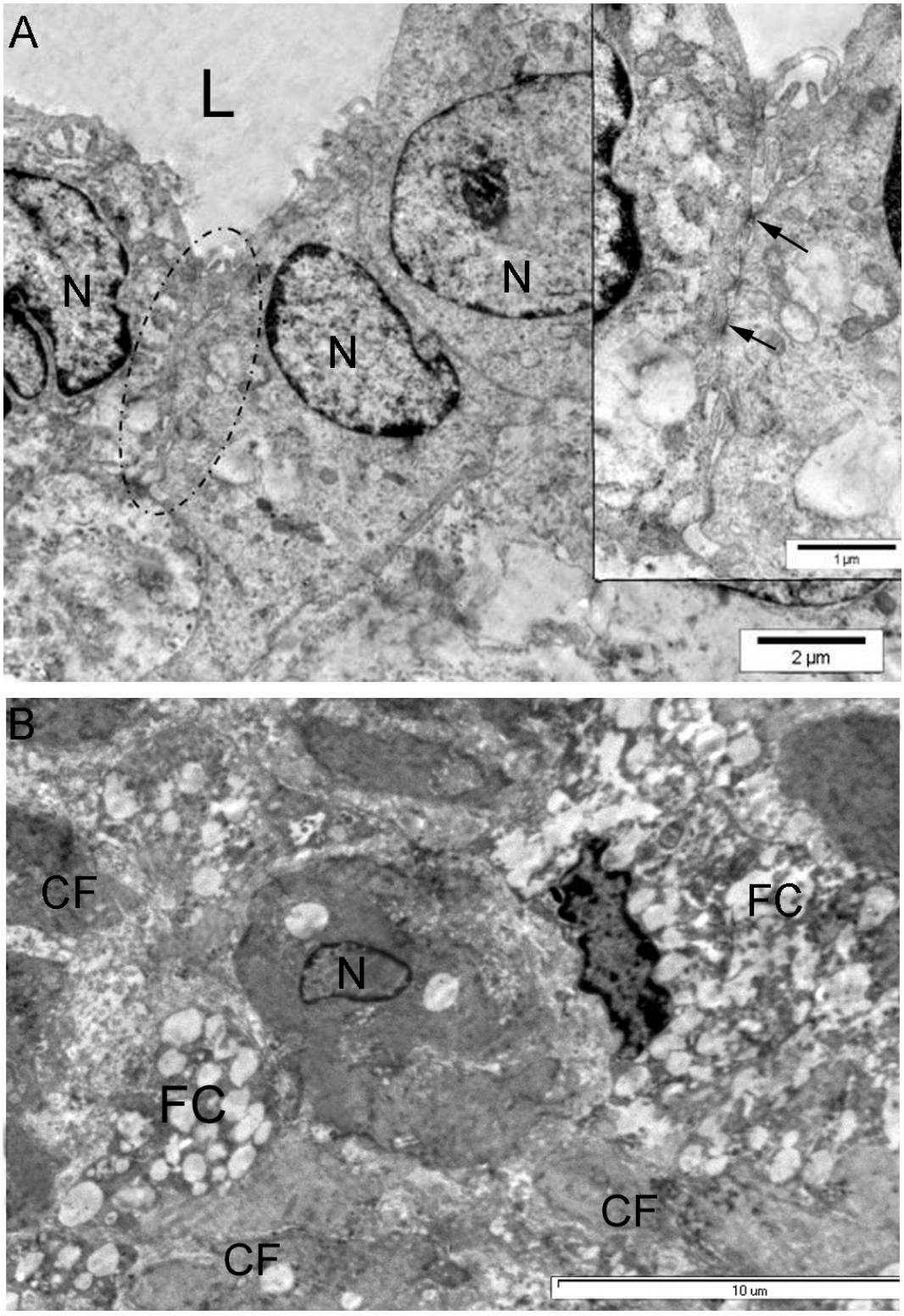

Fig. 5. A. Fine structure of the atherosclerotic plaque. Ultrastructure of the endothelial cells (En) of the plaque with heterochromatic nucleus (N) and foamy features are seen. The connection complexes (circled) between these cells are notable in EG-I. High magnification of the connection complexes (inset, arrows) between the endothelial cells is shown. **B.** Foam cells (FC), cell fragments (CF) with extracellular substance accumulation are seen. N: nucleus.

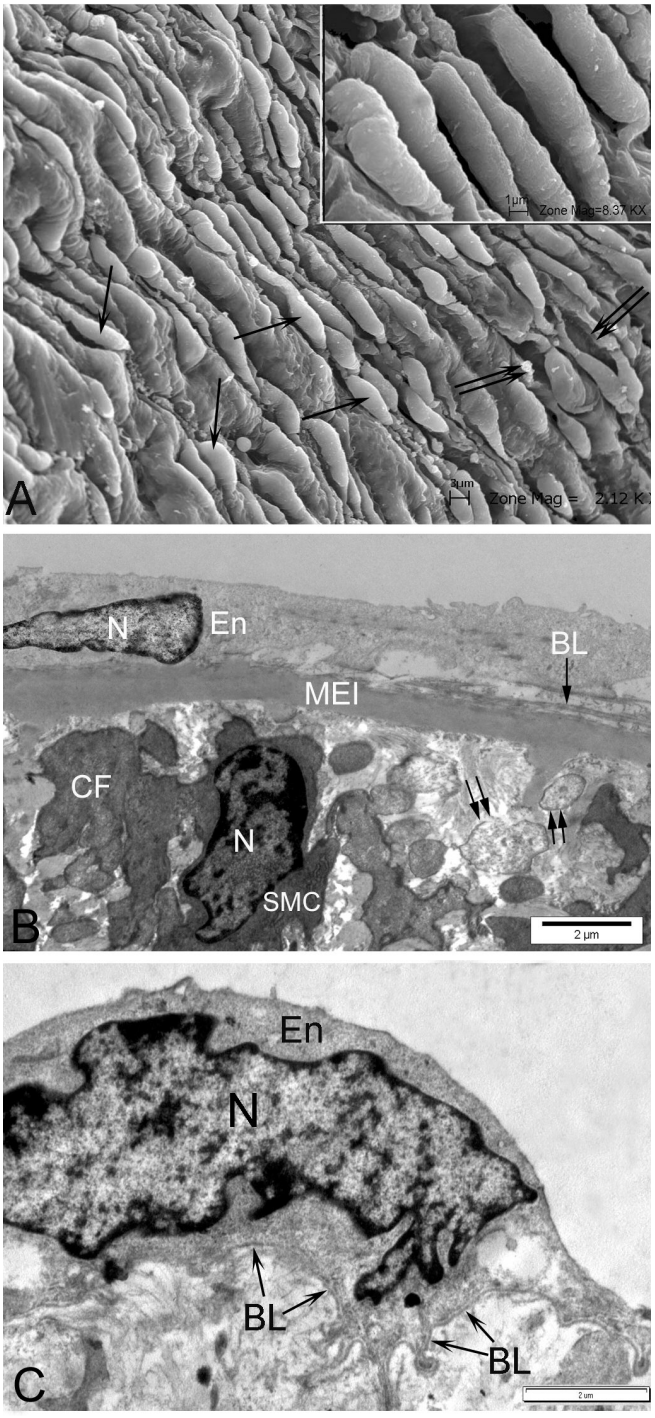

Fig. 6. A. Three dimensional surface of the endothelial lining of the carotid artery in EG-II. The endothelial surface appears improved and bulkings (with arrows), small ruptures (with double arrows) are rarely seen. Higher magnification of endothelial surface appears moderately healthy (inset). **B.** The ultrastructure of the carotid artery wall in the EG-II group is seen. The improved components of the artery wall confirm the SEM micrograph **(A)**. The endothelium (En), basal lamina (BL, single arrow), membrane elastica interna (MEI), cell fractions (CF), smooth muscle cells (SMC) and nuclei (N) are seen. Accumulations of some electron dense substances in various sizes (double arrows) are seen in tunica media. **C.** In a parallel section of the SEM, it can be seen that, the ultrastructure of endothelial cells (En) is partially repaired when compared to EG-I. A slight detachment between the basal lamina (BL, single arrows) and endothelial cells is noted. The basal lamina was found to be well protected. N: nucleus. Scale bars: A, 3 µm; inset in A, 1 µm; B, C, 2 µm

Experimental group I (EG-I)

SEM analysis of vascular endothelium revealed severe damage in this cholesterol-rich diet fed group. Some cytoplasmic protrusions, ruptured endothelial lining and detachment of the endothelial surface were the most important pathological observations. SEM observations revealed that there was a local endothelial wound that was involved in the formation of atherosclerotic plaques (Fig. 3A). On the other hand, the endothelial layer and basal lamina showed severe damage in this group as shown by TEM analysis. Detachment of the endothelium from the basal lamina with irregular heterochromatic nuclei and the accumulation of electron dense substances in *tunica intima* were clearly observed (Fig. 3B). The internal lamina of the vascular wall was obvious and mixed with fibrils and cellular components. Besides, moderate electron-dense substances, edematous regions, fibrillar and cellular fragments were observed under membrane elastica interna (Fig. 3B). Some segments of the tunica intima layer were occupied with atherosclerotic plaques consisting of different cell types, such as macrophages, foam cells, fibroblast-like cells, extracellular matrix components and fibrillar elements. We also observed monocyte-like cells in the sub-endothelial region (Fig. 4). In addition, epithelial-like cells located among the cells of atherosclerotic plaques with euchromatic nuclei and active cellular components were clearly observed. Connection complexes (Fig. 5A, inset) were noted in the deformed endothelium (Fig. 5). Moreover, inside the atherosclerotic plaques we predominantly observed cell fragments and foam cells (Fig. 5B).

Experimental group II (EG-II)

Following EPA treatment, the ultrastructural appearance of the carotid artery layers was different from EG-I and showed less pathological features. Endothelial ruptures, protrusions and shrinkages were less prominent in this group. SEM analysis revealed that the endothelium of the carotid artery had an almost normal appearance compared to EG-I (Fig. 6A, inset). However, it was noticeable that there were protrusions without ruptures on the endothelial surface, which was different from control group.

In this group, endothelium, sub-endothelium and tunica media seemed to be protected and had less

damage (Fig. 6B). TEM observation revealed that endothelial cells with irregular heterochromatic nuclei and elongated cytoplasmic bodies lining on basal lamina revealed normal ultrastructure compared to EG-I. The connection of endothelium to basal lamina was intact in this group (Fig. 6C).

Discussion

The present study has shown that EPA treatment following a cholesterol rich diet is capable of decreasing serum lipid levels in rabbits. Moreover, EPA administration was shown to affect histological and ultrastructural features of the endothelium by modulating atherosclerotic plaque formation.

In accordance with our data, it has been demonstrated that EPA is effective in lowering the lipids in all conditions tested, under normolipidemic and hyperlipidemic conditions, induced either exogenously or endogenously (Mizuguchi et al., 1992). We also observed that EPA possesses the ability to decrease lipids in cholesterol fed rabbits. Abbey et al. reported that highly purified EPA improved the increment of plasma TC induced by an atherogenic diet in marmosets (Abbey et al., 1990). There have been several reports to support our results that EPA significantly attenuated the elevated level of plasma lipids (Hevia et al., 1980; Terpstra et al., 1981; Wada et al., 1981; Abbey et al., 1990). These findings indicate that EPA appears to be a promising drug for the treatment of human hyperlipidemia and related diseases. The precise mechanism of the hypolipidemic action of EPA is still under investigation, but the mode of action of EPA could be suggested as follows: One of the primary actions of EPA might be at the step of cholesterol absorption, which could reduce the serum lipids in different animals. Rustan et al. (1988) reported that EPA can also inhibit acyl-CoA cholesterol acyltransferase (ACAT), which is the most important enzyme in the regulation of intestinal cholesterol absorption. Another inhibitory action of EPA might present at the level of intestinal absorption of fatty acids or enzymatic degradation of lipids via lipases. However, it is still not clear how EPA influences these steps.

On the other hand, we also demonstrated the effect of EPA treatment following a cholesterol rich diet on the structural changes of the carotid artery using immunohistochemical and ultrastructural techniques. Immunohistochemical and ultrastructural analysis showed severe damage in the endothelium due to the cholesterol rich diet. Cholesterol-rich fed groups had endothelial and subendothelial vacuoles in different densities and endothelial shrinkages. Our findings provide evidence that EPA can effectively improve the structure of the endothelium by preventing the progression of atherosclerotic lesions. However, endothelial damage in some segments was partially repaired, suggesting a limited healing effect of EPA. The degree of healing with EPA supplementation could be associated with the quantity and the duration of treatment. Long-chain n-3 polyunsaturated fatty acids (PUFAs) such as EPA and DHA are usually consumed in small quantities (Kromhout et al., 1995). Another study assessing the effect of EPA supplementation on atherosclerotic plaque is that of Rapp et al. (1991), which showed substantial incorporation of EPA and DHA into plaque lipids after consumption of a very high level of fish oils. Even at the modest level of dietary supplementation used in our study, some endothelial regions still showed disruptions.

Additionally, histological studies showed that more atherosclerotic plaque formation was seen in EG-I than in either of the other groups. Furthermore, plaques from EG-I were bigger and infiltrated with different cells, such as macrophages and foam cells. After treatment with EPA, plaques were less heavily infiltrated with cells and they were smaller in size. The observations regarding plaques and their composition in EG-II might be linked, since macrophages are major contributors to plaque inflammation and instability (Libby et al., 1996). Therefore, one of the important effects of EPA might cause modulation of cell numbers, like macrophages. Whether the reduction in macrophage numbers within the plaque is attributable to fewer monocytes and macrophages entering the plaque, or to a greater number leaving the plaque, is unclear.

Results of cell-culture and animal-feeding studies have shown that EPA can reduce adhesion molecule expression on endothelial cells, monocytes, and macrophages (Calder, 2002). A lower expression of adhesion molecules in the plaque might be related to a decrease in entry of monocytes and macrophages. On the other hand, dietary fish-oil lowers production of chemoattractants, including leukotriene B4 (Calder, 2002), platelet-derived growth factor (Wallace et al., 1995) and monocyte chemoattractant protein-1 (Baumann et al., 1999). This is, therefore, another possible mechanism by which entry of monocytes and macrophages into the plaque might be reduced through decreased generation of chemoattractants.

In accordance with our study, a recent study also showed the rapid effect and response of EPA treatment on atherosclerotic plaque morphology (Thies et al., 2003). The characteristics of the plaque that make it vulnerable to the rupture are the determinant of atherosclerosis (Plutzky, 1999). If the carotid artery plaques can be stabilized by EPA, the risk of advanced carotid atherosclerosis could be reduced. According to our study, following EPA treatment the atheroma formation was decreased and edematous areas were reduced, with partial restoration in endothelium.

As a conclusion, our results demonstrate that EPA prevents the progression of atherosclerotic regions and improves the ultrastructure of the damaged endothelium. This may provide a basis for the use of EPA as a treatment of patients in clinical applications.

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