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Original Article

Influence of fatty acids in maternal diet on atherogenesis in offspring of LDL receptor-deficient mice

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Abstract: Aims: The present study investigated the effect of a maternal diet rich in omega-6 (E6D) or omega-9 (E9D) on atherogenesis in the offspring of mice. Main methods: LDL receptor-deficient mice were fed a diet rich in either omega-6 (E6D) or omega-9 (E9D) for 45 days prior to mating and until the birth of the offspring, evaluating the effect on the offspring aorta in comparison to a standard diet (STD), by immunohistochemical analysis, morphometric analysis and electron microscopy. Key findings: Hypercholesterolemic female mice fed E6D generated offspring with high levels of total cholesterol, triglycerides (TG) and CC-chemokine ligand 2/monocyte chemoattractant protein 1 (CCL2/MCP-1) as well as a reduction in high-density lipoprotein. The ascending aorta of these animals exhibited an increase in arterial wall thickness as well as increased expression of CCL2/MCP-1 and vascular cell adhesion molecule 1. The ultrastructural analysis revealed severe alterations in endothelial cells. The offspring from mothers fed E9D exhibited a reduction in TG and an increase in low-density lipoprotein. The ultrastructural analysis revealed a well-preserved aortic endothelium in these animals. Significance: The results suggest that hypercholesterolemic mothers feed a diet rich in omega-6 predispose their offspring to endothelial dysfunction.

Keywords: Maternal nutrition, maternal hypercholesterolemia, omega-6, omega-9, offspring, MCP-1, VCAM-1, atherogenesis, blood lipids

Introduction

Omega-6 fatty acid is the precursor of the long-chain polyunsaturated fatty acid known as arachidonic acid (AA), which has considerable importance in the early months of life as a component of cell structures and a precursor of inflammatory mediators. During neonatal development, AA is one of the main components of cerebral fatty acids. The mother is the determinant factor of the supply of fatty acids in children [1].

However, an increased intake of omega-6 fatty acids, as currently found in the western diet, leads to the pathogenesis of a large number of diseases, including cardiovascular disease, cancer, osteoporosis, inflammatory diseases and autoimmune diseases. The metabolic eicosanoid products of AA, specifically prostaglandin, tromboxane, leukotriene, hydroxyl fatty acids and lipoxin are formed in large amounts due to high omega-6 intake, contributing to the formation of thrombi and atheroma. Moreover, an increase in the percentage of omega-6 in low-density lipoprotein (LDL) increases its susceptibility to oxidation and, consequently, atherogenicity [2].

Inflammatory alterations in the arterial wall play a central role in the development of atherosclerosis. A number of mediators, such as adhesion molecules, cytokines and chemokines, are involved in the onset and progression of atherosclerotic lesions [3]. Iiyama et al [4] assessed the expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) and demonstrated that the expression of these cytokines appears to be proportional to the extent of the atherosclerotic lesion. In turn, monocyte chemoattractant protein 1 (MCP-1), which is a member of the CC

chemokine family, is a potent chemotactic factor for monocytes and has been demonstrated to play a fundamental role in the onset and progression of atherosclerotic lesions in hyperlipidemic mice [5].

Hypercholesterolemia is a concern that should be carefully monitored and managed during pregnancy, considering its association with an important increase in the formation of fatty streaks in the fetal aorta and rapid development of atherosclerosis [6, 7]. Palinski et al [8] found a reduction in atherosclerosis in the offspring of rabbits treated with cholestyramine, vitamin E or a combination of the two. However, the interruption of the synthesis of total cholesterol in the first trimester of pregnancy is potentially dangerous to the growing embryo [9].

Olive oil is the main component of the Mediterranean diet and is rich in monounsaturated fatty acids and antioxidants. Llorente-Cortés et al [10] submitted patients at high risk for cardiovascular disease to a diet rich in olive oil, the benefits of which were a reduction in total cholesterol and LDL, along with an increase in high-density lipoprotein (HDL) and a reduction in the expression of MCP-1. The inhibition of MCP-1 indicates the anti-inflammatory effect of the Mediterranean diet.

In a previous study, our group demonstrated that hypercholesterolemic mothers fed a diet rich in omega-6 predispose their offspring to hepatic steatosis, whereas a diet rich in omega-9 has a protective effect [11]. The aim of the present study was to analyze the influence of a diet rich in omega-6 or omega-9 on the aortic endothelium of offspring from hypercholesterolemic mothers.

Materials and methods

Animals and diet

The local ethics committee approved all procedures carried out in the present study (process no. 0397-07/CEUA-FIOCRUZ). Twenty-three homozygous mice with the absence of the LDL receptor gene ($LDLR^{-/-}$) generated on a C57BL/6 background, aged 30 days and weighing 13.75 ± 1.32 g were divided into three groups and kept in cages with free access to food and water. LDLR-/- mice are typically used as a model for familial hypercholesterolemia, as these animals have a lipoprotein abnormality, with an increase in both LDL and very low density lipoprotein, and do not develop atherosclerosis when fed a standard diet [12]. The animals were examined for health status and kept under a controlled light cycle (12/12 h light/dark) and constant temperature (22 ± 1 ºC). Throughout the eight-day adaptation period, the mice were fed a standard laboratory diet with a 4% fat content. The animals were then submitted to one of the following diets: Control diet - mice (n = 7) fed the standard laboratory diet (STD) for 45 days prior to copulation and during gestation and lactation; Experimental Diet 1 - mice (n = 8) fed a diet rich in omega-6 polyunsaturated fatty acids [E6D - standard diet supplemented with 20% (w/w) soybean oil] for the same period as in the control group, but ending at birth; and Experimental Diet 2 - mice (n = 8) fed a diet rich in omega-9 monounsaturated fatty acids [E9D - standard diet supplemented with 20% (w/w) extra virgin olive oil] for the same period as in the E6D group. The diets were confected based on the recommendations of the American Institute of Nutrition [13]. Throughout the initial 45 days, the mice were weighed on a weekly basis for the assessment of weight gain.

After copulation with healthy males ($LDLR^{-/-}$), the females were lodged individually. Following the birth of the offspring, the maternal diet was changed to the standard ration in order to reduce the effect of diet during lactation [7]. Due to the large number of mice, approximately 10 offspring in each group were sacrificed per day. Offspring at 6.06 ± 2.10 days of life were sacrificed by decapitation. Blood was collected in a tube without anticoagulant and centrifuged. The serum was separated and stored at -80 ºC for subsequent biochemical analysis. The mothers were anesthetized; cardiac blood was collected in a tube without anticoagulant and processed as described above. The aorta attached to the heart of the offspring was quickly dissected and processed for immunohistochemical analysis, morphometric analysis and electron microscopy.

Biochemical determinations

Blood levels of total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and triglycerides (TG) were measured photometrically in an automatic analyzer (Cobas Integra 400, Roche, Mannheim, Germany), us-

ing Roche kits. Serum levels of monocyte chemoattractant protein 1 (MCP)-1 were determined by enzyme immunoassay (eBioscience). The biochemical analyses were carried out in triplicate on a serum pool from 20 offspring per group. For the mothers, blood levels of TC and TG were analyzed using the same method.

Immunohistochemical analysis and morphometry of cross sections of ascending aorta

The aorta was removed together with the heart, fixed in a 10% formalin solution for 24, processed and embedded in paraffin. Cross-sectional cuts (5 μm) of the ascending aorta were adhered to slides treated with 3-aminopropyl triethoxy silane (Sigma, USA). Antigen recovery was performed in a vapor chamber for 30 minutes in 0.01 M sodium citrate buffer, pH 6.0. Monoclonal anti-MCP-1 (dilution 1:50; Clone 2H5; eBioscience) and monoclonal anti-VCAM-1 (dilution 1:50; Clone 429; eBioscience) were used as the primary antibodies. The antigen-antibody reaction was viewed with an avidin-biotin-peroxidase system (Universal Dako LSAB ® + Kit, Peroxidase), using 3,3-diaminobenzidine as the chromogen. The cuts were counterstained with hematoxylin.

Morphometric measurements of the thickness of the aortic wall from three to four mice per group were performed with the aid of an inverted microscope (Observer Z1, Zeiss MicroImaging GmbH) equipped with a camera (AxioCam MRm Zeiss) and coupled to an image analysis program (Axio Release 4.7.4, Zeiss). Eight sequential histological cuts (5 μm) beginning at the origin of the aorta were used to determine the thickness of the aortic wall based on the mean measurement of four diametrically opposed points (0°, 90°, 180°, 270°), following a modified version of the method described by Aguila and Mandarim-de-Lacerda [14].

Transmission electron microscopy

Three to four mice from each group were analyzed. The aorta attached to the heart was fixed overnight in a solution containing 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. After fixation, the samples were washed twice in the same buffer, post-fixed in a solution containing 1% osmium tetroxide, 2 mM calcium chloride and 0.8% potassium ferricyanide in 0.1 M sodium cacodylate buffer, pH 7.2., then dehydrated in acetone and embedded in Epon 812 resin (Sigma Company, USA). Polymerization was carried out at 60 ºC for two days. Ultrathin cuts (60 to 70 nm) were obtained on an ultramicrotome, collected on a 300-mesh nickel grid and contrasted with an aqueous solution of 5% uranyl acetate for one hour and 1% lead citrate for three minutes. The samples were viewed in an electron microscope (Morgani FEI 268D).

Statistical analysis

The data were analyzed using ANOVA, followed by Tukey's test or LSD, with the level of significance set at 5% ($p < 0.05$) [15]. The chi-square test was used to compare MCP-1 values in the pools of offspring serum. The Kruskal-Wallis test and Dunn's a posteriori test were used for the morphometric analysis of the cross-sectional cuts of the aorta.

Results

Effect of diets rich in omega-6 and omega-9 on body weight and blood lipids in mothers

In all groups, every female became pregnant and the offspring was born healthy. Mean birth weight was 2.79 ± 0.76 g. No statistically significant differences in weight gain were detected between groups for 45 days prior to gestation and through to the birth of the offspring ($n = 23$, $p > 0.05$). Regarding serum lipid levels, TC was significantly higher in the E6D group (339.58 ± 38.83) in comparison to the E9D (267.41 ± 46.87) and STD (270.40 ± 57.23) groups (post-hoc LSD, $n = 23$, $p < 0.05$). No significant differences in TG were detected between groups: STD: 116.43 ± 48.60; E6D: 112.58 ± 32.70; and E9D: 78.18 ± 6.97.

Effect of maternal diet on biochemical blood parameters in offspring

The serum concentration of TG (496.26 ± 28.72) was significantly lower ($F_{(2,6)} = 17.551$; $p = 0.003$) and the LDL (125.30 ± 2.75) level was significantly higher ($F_{(2,6)} = 29.463$; $p = 0.00079$) among the offspring of mothers in the E9D group in comparison to those of mothers in the STD (TG: 574.61 ± 14.12; LDL: 104.45 ± 4.74) and E6D (TG: 587.95 ± 15.29; LDL:

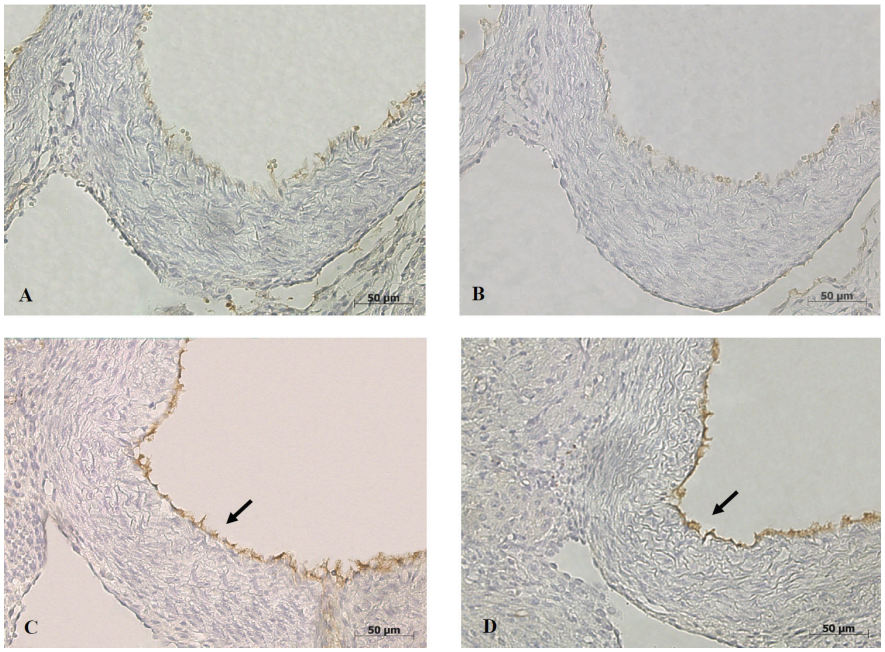

Figure 1. Immunohistochemistry for CCL2/MCP-1 and VCAM-1 in endothelial cells of ascending aorta in offspring; (1 A-B) labeling absent on surface of endothelial cells of offspring of mothers fed standard diet and diet rich in omega-9; (1 C-D) intense labeling on surface of aortic endothelial cells of offspring of mothers fed diet rich in omega-6; bar = 50 µm.

101.11 ± 4.75) groups. The concentration of HDL was significantly lower in the offspring of mothers in the E6D (69.67 ± 1.53) group in comparison to those in the E9D (78.00 ± 1.73) group ($F_{(2,6)}$ = 9.9787; p ≤ 0.0123) and significantly high in the offspring of mothers in the E9D group; STD: HDL = 73.67 ± 3.21. Serum TC was significantly higher ($F_{(2,6)}$ = 13.300; p = 0.00623) in the offspring of mothers in the E6D (345.76 ± 5.49) and E9D (353.05 ± 5.29) groups (4 and 6%, respectively) in comparison to those of mothers in the STD (332.82 ± 3.58) group. A significant increase in the blood concentration of MCP-1 was detected in the offspring of mothers in the E6D (233.334) group (p < 0.05); STD: MCP-1 = 130.206 and E9D: MCP-1 = 147.898.

Immunohistochemical analysis and morphometry of cross sections of ascending aorta

Among the offspring of mothers in the STD and E9D groups, the endothelial cells of the ascending aorta did not express CCL2/MCP-1 or VCAM-1 (**Figure 1A-B**), whereas those of mothers in the E6D group exhibited endothelial cells with intense labeling for CCL2/MCP-1 and VCAM-1 on the surface (**Figure 1C-D**). The thickness of the wall of the ascending aorta differed significantly between groups ($H_{(2;69)}$ = 29.50; p < 0.0001). A significant increase in thickness of the aortic wall was observed among the offspring of mothers in the E6D group (76.57 ± 16.78 µm) in comparison to those of mothers in the E9D group (61.04 ± 19.59 µm) (p < 0.005). Moreover, the offspring of mothers in both the E6D and E9D groups exhibited a significant increase in thickness of the aortic wall (69% and 35%, respectively) in comparison to those of mothers in the STD group (45.23 ± 9.72 µm) (p < 0.005) (**Table 1**).

Table 1. Effect of maternal diet on thickness of aorta in offspring

Parameter	STD	E6D	E9D
Thickness (µm)	45.23±9.72[c]	76.57±16.78[a]	61.04±19.59[b]
Increase in % of thickness		69	35

STD – offspring of mothers fed standard laboratory diet; E6D – offspring of mothers fed diet enriched with omega-6 polyunsaturated fatty acids [20% (w/w) soybean oil]; E9D – offspring of mothers fed diet enriched with omega-9 monounsaturated fatty acids [20% (w/w) extra-virgin olive oil]; Values expressed as mean ± standard error; Mean values with different letters are significantly different (Kruskal-Wallis/Dunn, p < 0.005)

Transmission electron microscopy

The ultrastructural analysis of the ascending aorta in the offspring of mothers in the STD group revealed an intima layer with regular endothelial cells and continuous elastic lamina (**Figure 2A**). The same pattern was found in the offspring of mothers in the E9D group (**Figure 2B**), in which the endothelial cells exhibited cytoplasm with well-preserved mitochondria (**Figure 2C**). Among the offspring of mothers in the E6D group, severe alterations in the endothelial cells indicative of cell degeneration were found, such as the presence of vacuoles and condensed nuclei (**Figure 2D**), with some cells in the process of cell death (**Figure 2D**). In this group, the elastic lamina was discontinuous, with sites of detachment of endothelial cells, the cytoplasm of which exhibited a number of mitochondria with a loss of organization and cristae (**Figure 2E** and **2F**).

Discussion

Disturbances in the uterine environment through maternal malnutrition may predispose offspring to cardiovascular disease. In studies with animal models, Napoli et al [16] demonstrated that maternal hypercholesterolemia and lipid peroxidation cause fetal atherogenesis. Ghosh et al [17] demonstrated that the adult offspring of rats fed a diet rich in saturated fat during pregnancy exhibited vascular dysfunction and disorders in plasma lipids and the composition of fatty acids in the vascular wall. Elahi et al [18] found that the treatment of hypercholesterolemic mice with pravastatin during gestation both reduces cardiovascular risk in the offspring and has a beneficial effect on the health of the mother, improving blood pressure and locomotion. Hypolipidemic drugs reduce TG and LDL and increase the level of HDL, but have a number of significant side effects. Therefore, diet quality may be improved through a change in the amount and type of fat ingested without causing side effects and offering lower costs in comparison the use of drugs [19].

Omega-6 and omega-9 fatty acids are important to normal growth and development and appear to have important functions in the modulation of cardiovascular disease. The present study investigated the consequences of feeding hypercholesterolemic LDL receptor-deficient female mice a diet rich in either omega-6 or omega-9 with regard to lipid profile, inflammatory markers in the blood and the wall of the ascending aorta as well as ultrastructural alterations in the aortic endothelium of the offspring. The mothers in the E6D group exhibited a significant increase in circulating TC in comparison to those in the STD and E9D groups. This result may explain the possible metabolic alterations observed in the offspring of this group. Moreover, the serum lipid profile of the offspring of mothers in the E6D group exhibited an increase in both TG and TC as well as a reduction in HDL, whereas the offspring of the mothers in the E9D group had higher levels of LDL and HDL and a lower concentration of TG. Epidemiological studies demonstrate that high levels of HDL potentially contribute more toward the antiatherogenic properties of this lipoprotein, including its capacity to inhibit the oxidation of LDL and protect endothelial cells from the cytotoxic effect of oxidized LDL [20]. Thus, the increase in HDL in the offspring of mothers fed a diet rich in omega-9 suggests a protective effect from atherosclerosis.

Members of the superfamily of endothelial adhesion molecule immunoglobulins, VCAM-1 and ICAM-1, have a strong participation in the adhesion of leukocytes in the endothelium [21]. Iiyama et al [4] evaluated the expression of VCAM-1 and ICAM-1 in rabbit and mice models for atherosclerosis and demonstrated that the expression of VCAM-1 and ICAM-1 was high in the aorta of hypercholesterolemic animals and proportional to the extent of the atherosclerotic lesion. Likewise, in the present study, the offspring of mothers fed a diet rich in omega-6 were hypercholesterolemic and their aortic endothelial cells exhibited a high expression of VCAM-1, thereby suggesting a predisposition toward atherosclerosis in these offspring.

MCP-1 is a member of the CC chemokine family and a potent chemotactic factor for monocytes. MCP-1 has been demonstrated to play a fundamental role in the onset and progression of atherosclerotic lesions in hyperlipidemic mice [5]. MCP-1-mediated inflammation in the arterial wall activates a positive feedback mechanism, thereby increasing the inflammation and proliferation of the lesion in the arterial wall [22]. In the present study, the offspring of mothers fed a diet rich in omega-6 exhibited a significant increase in serum levels of MCP-1 associated to a high expression of this chemokine in

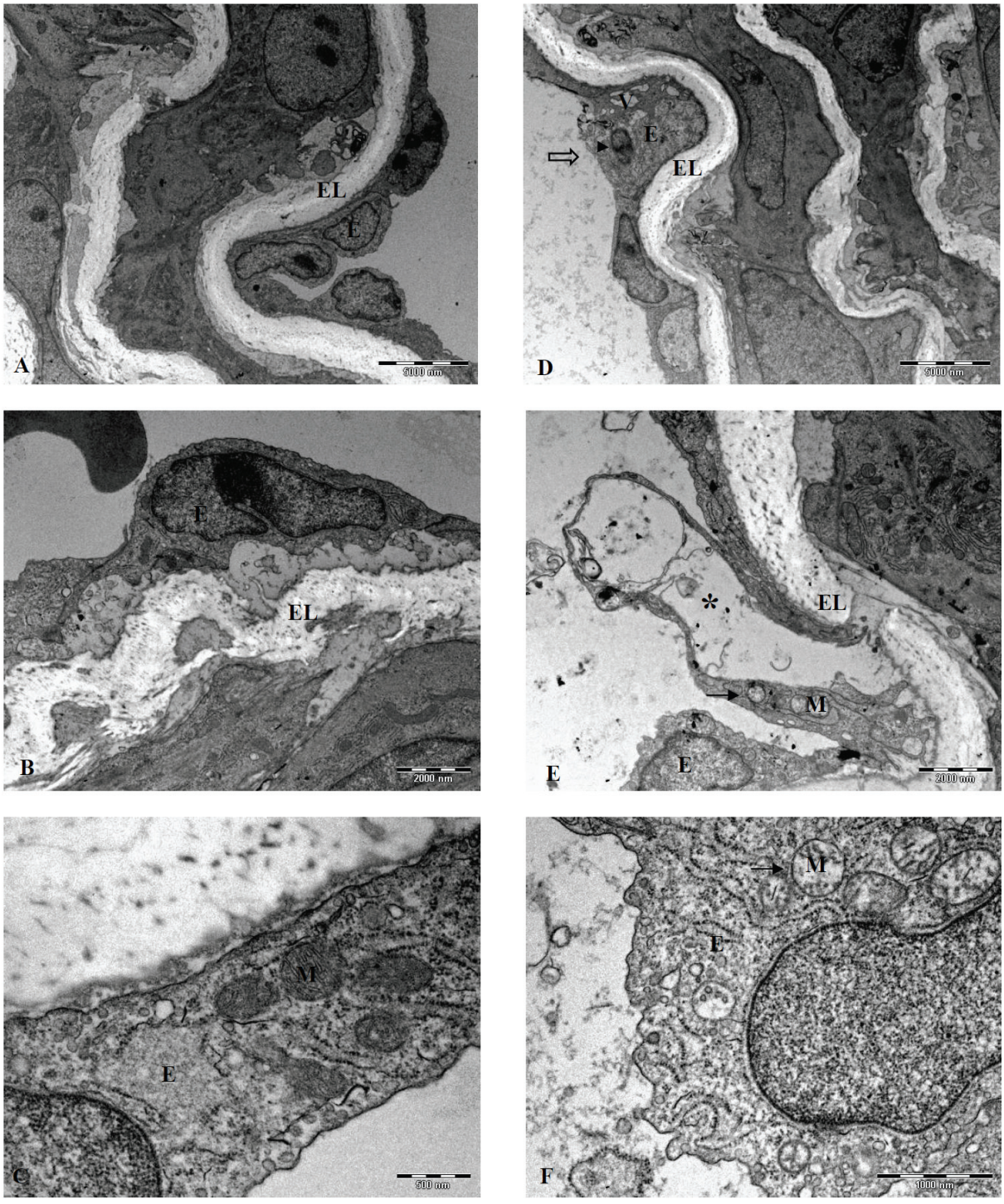

Figure 2. Ultrastructure of ascending aorta of offspring of mothers in STD group, showing intima layer with regular endothelial cells (E) and continuous elastic lamina (EL) (2 A); same pattern in offspring of mothers in E9D group (2 B), with endothelial cells (E) exhibiting well-preserved mitochondria (M) (2 C); severe alterations in endothelial cells (E) of offspring of mothers in E6D group, with presence of vacuoles (V), condensed nucleus (arrow head), cells in process of cell death (arrow outline) (2 D), discontinuous elastic lamina (EL), with sites of detachment (asterisk) of endothelial cells (E) (2 E), the cytoplasm of which showing several degenerated mitochondria (arrows) (2 E, F).

the arterial endothelium. As a consequence, an increase in the thickness of the arterial wall was observed in these offspring, along with severe morphological alterations in the endothelium, such as a discontinuous elastic lamina, with sites of detachment of endothelial cells. Moreover, the endothelial cells exhibited vacuoles, a condensed nucleus and a number of degenerated mitochondria, thereby denoting cell damage. According to previous studies, an intact endothelium is critical to maintaining adequate functioning of the blood vessel and endothelial alterations may lead to endothelial dysfunction, thereby causing the development of atherosclerosis [23].

Maternal hypercholesterolemia during pregnancy initiates pathogenic events in the fetus [6]. Llorente-Cortés et al [10] submitted patients at high risk for cardiovascular disease to a diet rich in omega-9, the beneficial effects of which were a reduction in TC, increase in HDL and reduction in the expression of MCP-1, indicating the anti-inflammatory effect of this diet. In the present study, the offspring of hypercholesterolemic mothers fed a diet rich in omega-9 exhibited a reduction in TC and an increase in HDL. A reduction in blood MCP-1 associated to the absence of an endothelial expression of VCAM-1 and MCP-1 indicated an anti-inflammatory action of this diet on the offspring, accompanied by a reduction in the thickness of the aorta. Moreover, the morphological analysis of endothelial cells revealed that the maternal diet rich in omega-9 did not cause endothelial damage. Taken together, the data indicate a protective effect from this diet.

In conclusion, the results of the present study suggest that hypercholesterolemic mothers fed a diet rich in omega-6 predispose their offspring to atherosclerosis, whereas a diet rich in omega -9 confers protection to the endothelium.

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