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Apocynin exhibits an ameliorative effect on endothelial dysfunction/ atherosclerosis-related factors in high-fat diet-induced obesity in rats

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ABSTRACT

Objective: The aim of this study was to reveal the effect of apocynin (APO) on the factors involved in obesity-related endothelial dysfunction (ED) and atherosclerosis (AS).

Materials and Methods: Male Wistar albino rats were divided into control (CNT), high-fat diet (HFD) and HFD+APO groups. HFD and HFD+APO groups were fed HFD for sixteen weeks. APO (25 mg/kg) was administered to the HFD+APO group for the last four weeks. The effects of APO on: AS-related metabolic parameters (triglyceride, total cholesterol, high-density lipoprotein-cholesterol, insulin and leptin), oxidative stress (OS), [malondialdehyde, glutathione, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase-2, oxidised-low-density lipoprotein (ox-LDL) and 8-hydroxy-2-deoxyguanosine], low-density lipoprotein and ox-LDL uptake potential (activin receptor-like kinase-1 and lectin-like oxidized low-density lipoprotein receptor-1, respectively), tissue inflammation (myeloperoxidase, monocyte-chemoattractant-protein-1, tumor necrosis factor-alpha), ED (endothelial-nitric oxide synthase, inducible-nitric oxide synthase, nitric oxide), programmed cell death (terminal deoxynucleotidyl-transferase-dUTP-nick-end labeling, cleaved-poly-ADP-ribose-polymerase, gasdermin-D N-terminal fragment, caspase-1), smooth muscle cell transformation (alpha-smooth muscle actin), histology and ultrastructure of thoracic aorta were evaluated.

Results: In obesity, APO had an ameliorative effect on metabolic parameters, OS, inflammation, ED, programmed cell death and ox-LDL uptake potential, but not on foam cell formation and LDL uptake potential.

Conclusion: Apocynin may improve ED and AS in obesity by suppressing OS-linked factors involved in the early stage of AS.

Keywords: Apocynin, Atherosclerosis, Endothelial dysfunction, Oxidative stress, Obesity, High-fat diet

1. INTRODUCTION

Obesity is a disease characterized by a chronic low-grade inflammation causing a vicious cycle consisting of insulin resistance, oxidative stress (OS) and endothelial dysfunction (ED). It forms the basis of early and accelerated atherosclerosis (AS) [1]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) enzyme family is one of the most prominent producers of reactive oxygen species (ROS), which has been linked to atherogenesis [2]. NOX-2 in the endothelial cells (EC) is a major producer of ROS, giving rise to ED which predisposes cardiovascular complications in obesity [3].

ED, defined as the deterioration of endothelium-dependent vasodilatation due to decreased nitric oxide (NO) bioactivity in the vessel wall [4], has been associated with AS, hypercholesterolemia [5] and obesity [6]. Obesity is closely linked to ED via the presence of ROS in addition to reduced NO production [4] and/or NO bioavailability leading to impaired vasodilatation. The main cause of the onset of ED is the reduced NO bioavailability that may be a result (i.) of decreased endothelial NO synthase (e-NOS) protein expression [5,7] and (ii.) increased inducible NOS (i-NOS) activity.

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Because, decreased e-NOS expression results in decreased NO production and increased i-NOS activity reduces NO bioavailability by causing peroxynitrite formation from NO and e-NOS uncoupling that lead to the production of superoxide instead of NO [7]. It has been shown that a high-fat diet (HFD) increases i-NOS expression and decreases e-NOS expression in the aorta [8].

Atherosclerosis is the fundamental pathophysiological process underlying cardiovascular diseases. It begins with the retention of low-density lipoprotein cholesterol (LDL-c) in the subendothelial space, followed by oxidative modification of LDL to oxidized-LDL (ox-LDL) [9]. Activin receptor like-kinase-1 (ALK-1) is a receptor that functions in the uptake and transcytosis of LDL into the cell [10]. Ox-LDL plays a key role in AS, mostly through the scavenger receptor lectin-like oxidised low density lipoprotein receptor-1 (LOX-1), which enables its uptake into the cells [11]. It has been demonstrated that AS is associated with increased LOX-1 activity and gene knock-out of LOX-1 lead to a reduction in atherogenesis in LDL receptor deficient mice fed with a high-cholesterol diet [12].

Apocynin (APO) is an acetophenone that has an ameliorating effect on cardiovascular disorders. It acts as an inhibitor of NOX-2, in addition, as an antioxidant and scavenger of non-radical oxidant species [13]. Previous studies have reported that APO decreases blood pressure, increases e-NOS activity and resulting NO levels in the aorta by elevating e-NOS expression in spontaneously hypertensive rats [14]; decreases i-NOS expression in the aorta of diabetic rats [15] and descends the progression of AS in apoE-deficient mice [2].

Apocynin may ameliorate ED and AS in a wild – type obesity animal model through its anti-inflammatory and antioxidant effects. To address this issue, this study aimed to reveal the effect of APO on the factors involved in the development of ED and subsequent AS as a result of OS associated with HFD-induced obesity in wild – type rats. Meanwhile, this is the first study in the literature to address ALK-1 involvement regarding LDL uptake, and the effect of APO on pyroptosis in HFD-induced obesity-related ED/AS.

2. MATERIALS and METHODS

Animals and Experimental Design for an Obesity Model

Twenty-one adult male Wistar albino rats (aged two months, 250-300g) obtained from the Marmara University Experimental Animal Implementation and Research Center (DEHAMER), were used for this study. The animals were randomly assigned to three groups (7 animals per group). Standard control (CNT) group was fed with a standard laboratory chow (6% fat, 24% protein,70% carbohydrate) diet while HFD and HFD+APO groups were fed with HFD (45% saturated fat, 20% protein, 35% carbohydrate) for 16 weeks to obtain an obesity model. All animals were kept in 12/12 hours light/dark conditions. Access to water for the animals was ad libitum. Based on previous studies, solvent [16], APO administration method, dosage [16-18] and timing [19] were determined. APO (TRC, Toronto, Canada, 25 mg/kg dose dissolved in 8% dimethyl sulfoxide) and vehicle dimethyl sulfoxide were administered by orogastric gavage to the HFD+APO and HFD groups, respectively, five days a week for the last four weeks. Since, it has been shown in previous studies that the administration of APO (at the dose applied and above) alone to the standard control group does not have a significant effect on the tissues [16, 20, 21], we excluded the use of APO treated standard control group in this study to avoid unnecessary animal sacrifice. The rats were weighed once a week. The applied animal design was approved by the Marmara University Local Ethical Committee for Experimental Animals (07.05.2018-50.2018.mar). None of the rats died during the 6-week study. Finally, after 12 hours fasting by removal of chow, the rats were decapitated under light ether anaesthesia, then trunk blood and thoracic aorta tissue samples were collected. All the experiments and analyses were performed in blinded study groups by investigators.

Biochemical Analysis

The collected thoracic aorta tissue samples and serum were biochemically analysed by the ELISA method using commercial ELISA kits. The blood samples were centrifuged at 15000 rpm for 15-20 minutes. The aorta tissue samples were homogenized as 10% homogenates in phosphate buffered saline (PBS) solution (pH: 7.4). The homogenates were centrifuged at 15000 rpm for 15 minutes and supernatants were collected. Leptin (E-EL-M0039, Elabscience Biotechnology, Houston, USA), insulin, total cholesterol (TC), triglyceride (TG) and high-density lipoprotein cholesterol (HDL-c) (YLA0037RA, YLA0388RA, YLA0770RA and YLA0444RA, respectively, YL Biont, Shanghai, China) levels were measured in the serum. Malondialdehyde (MDA), glutathione (GSH) (MBS728071 and MBS265966, respectively, MyBioSource, San Diego, USA), tumor necrosis factor-alpha (TNF-α), monocyte-chemoattractant-protein-1 (MCP-1), ox-LDL (E0764RA, E0193RA and E0699RA, respectively, BTLab, Shanghai, China), NO (ADI-917-020, Enzo Life Sciences, Lausen, Switzerland), 8-hydroxy-2-deoxyguanosine (8-OHdG) levels and, myeloperoxidase (MPO) activity (SL2044Hu and SL1230Hu, respectively, Sunlong Biotech, Hangzhou, China) were measured in aorta tissue homogenates.

All procedures applied in the ELISA method were performed according to the manufacturer's instructions. Metabolic disorders caused by HFD were assessed by serum TG, TC, HDL-c, insulin and leptin levels. Lipid peroxidation and antioxidant capacity in the aorta were assessed by MDA and GSH levels, respectively. Inflammation was assessed by MPO activity, MCP-1 and TNF-α levels. Oxidative damage was assessed by ox-LDL and 8-OHdG (a marker for oxidative DNA damage) levels. Endothelial function was assessed by NO levels.

Histological Analysis

The aorta tissues were fixed in 10% formalin for 24 hours and routinely processed for light microscopic examination. Processed tissues were embedded and blocked in paraffin wax. Approximately, 4-µm thick paraffin sections were stained with haematoxylin-eosin (HE) and Verhoeff–Van Gieson stain. Stained aorta sections were observed under a light microscope

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(Olympus BX51, Tokyo, Japan) and photographed. Medial elastic fiber degeneration (MEFD) and medial extracellular matrix accumulation (MEMA) were scored from 0 to 3 (0: none, 1: mild, 2: moderate and 3: severe). Smooth muscle cell nucleus (SMCN) number in the tunica media area was counted for each sample and expressed as SMCN / area (mm^2). Tunica intima + tunica media thickness (IMT) was measured in equally spaced 15 randomized parts of each specimen slide using Image J-Fiji 1.53c Software. Mean of measurements was evaluated as IMT value. Histopathological analyses were scored by two investigators blinded to the groups and the investigators' assessments concurred.

Immunohistochemical Analysis

Immunohistochemical staining was performed according to a previous protocol [16]. Assessed primary antibodies (all incubated at 4 °C) were: (i) NOX-2 as a marker of oxidative stress (1:200, bs-3889R, Bioss Antibodies, Woburn, Massachusetts, USA), (ii) e-NOS, (iii.) i-NOS as markers of ED (1:300 and 1:50, NB300-500 and NB300-605, Novus Biologicals, Centennial, USA), (iv) LOX-1 as a marker of ox-LDL uptake (1:200, BS-2044R, Bioss Antibodies) and (v) α-SMA as a marker of foam cell formation potential (1:2000, ab7817, Abcam, Cambridge, UK). Randomized five parts of each field were evaluated to calculate the density of immunoreactivity (ir), and density was expressed as the ratio of the DAB-stained area to the total related vessel wall area (%). To detect DNA strand breaks resulting in programmed cell death, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) method was performed using a commercial kit (EMD-Millipore, Darmstadt, Germany) according to the manufacturer's protocol. To evaluate TUNEL staining in tunica media, positive vascular smooth muscle cell (VSMC) nuclei were counted per 5 optical field (40× objective) at random locations in the vessel wall of the sections of each rat. TUNEL positive cell index was calculated by dividing the total number of TUNEL positive cell nuclei to the total cell nuclei number in the counted areas.

Transmission Electron Microscopy

Approximately 3 mm^3 pieces of aorta samples were fixed with 2.5% glutaraldehyde in PBS (pH 7.2, 0.1 M) for 12 hours at 4 °C. Then, the samples were post-fixed in 1% osmium tetroxide in PBS and dehydrated by passing through an ascending series of alcohols. Dehydrated samples were cleared in propylene oxide and embedded in Epon 812 (45359, Sigma–Aldrich Chemical, Schaffhausen, Switzerland).1-µm semi-thin sections were stained with toluidine blue. Ultrathin sections (60-80 nm-thick) were contrasted using uranyl acetate and lead citrate then were observed using a JEOL 120 EXII transmission electron microscope (Tokyo, Japan).

Western Blot Analyses

Tissue samples from the aorta were homogenized in 1x Radioimmunoprecipitation assay buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Complete, EDTA-free Protease Inhibitor Cocktail Tablets, Roche, Rotkreuz, Switzerland) using homogenizer. The tissue homogenates were centrifuged at 14,000 g for 15 min and the supernatant was collected. Total protein amounts were quantified using Take3™ Multi-Volume Plate (BioTek Instruments Inc., Vermont, USA). Proteins of 30 µg were separated by 10% SDS-PAGE electrophoresis and transferred to the nitrocellulose membranes. Membranes were incubated with primary and secondary antibodies. Primary antibodies used in this study were Beta-actin, i-NOS, e-NOS, cleaved-poly-ADP-ribose-polymerase (c-PARP) as a marker of apoptosis (1:500, 1:400, 1:500 and 1:500, NB600-501, NB300-605, NB300-500, and NBP2-27335; respectively, Novus Biologicals), caspase-1 and GSDMDC1 as markers of pyroptosis (1:200, sc-56036 and sc-393581; Santa Cruz Biotechnology, Dallas, USA) and ALK-1 as a marker of LDL uptake (1:200, sc-101556; Santa Cruz Biotechnology). All antibodies were HRP-conjugated. The bands were visualized using chemiluminescent HRP substrates (Western Bright ECL-Advansta, K-12045-050). Quantification was performed using Chemiluminescence Imaging System (Celvin S, BioStep, Burkhardtsdorf, Germany).

Ribonucleic Acid Isolation and Quantitative Real-Time Polymerase Chain Reaction Analysis

Ribonucleic acid (RNA) was isolated from fresh frozen tissues with PureLink™ RNA Mini Kit (12183018A, Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. All RNA lysates were stored at – 80 °C until use. High-Capacity cDNA Reverse Transcription kit (4368814, Thermo Fisher Scientific) was used for cDNA synthesis in a reaction volume of 20 µL. Taqman expression assays (4453320, Thermo Fisher Scientific) were used for gene expression analysis. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in 20 µL of final reaction volume according to manufacturer's recommendations for cycling conditions. All reactions were performed in duplicate for reference housekeeping gene beta actin, oxidized low-density lipoprotein receptor1 (OLR1), activin receptor like kinase 1 (ACVRL1) and nitric oxide synthase 3 (NOS3) by using Light Cycler 480 instrument (Roche, Roche Diagnostics International AG, Rotkreuz, Switzerland). Relative expression was quantified by delta-delta Ct method after normalization of target gene expressions relative to the house keeping gene expression.

Statistical Analyses

Graph-Pad Prism 8.0.1 (GraphPad Software, San Diego, USA) program was used for statistical analysis. One-Way ANOVA method and post – hoc Tukey test for multiple comparisons were used for all statistical analyses. $P < 0.05$ was considered significant. All data are expressed as the mean ± standard error of the mean (SEM).

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3. RESULTS

Body Weight Gain

At the end of the 16-week period, animals in the HFD group had increased (P < 0.0001) body weight gain compared to the CNT group. When the two groups fed a high-fat diet were compared within themselves, weight gain was less (P < 0.0001) in the APO-administered group (Figure 1a).

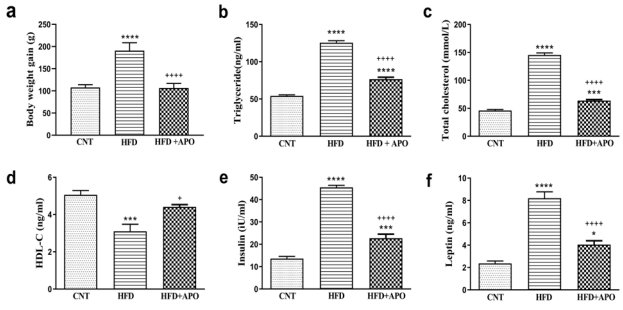

Figure 1. *The effect of HFD and APO on body weight gain and serum fasting metabolic parameters. *P < 0.05, ***P < 0.001 and ****P < 0.0001 vs CNT group, ⁺P < 0.05 and ⁺⁺⁺⁺P < 0.0001 vs HFD group. All data were expressed as mean ± SEM (n=7).*

Biochemical Serum Analysis

Serum TG and TC levels were increased significantly in the HFD (P < 0.0001 for both) and the HFD+APO (P < 0.0001 for TG and P < 0.001 for TC) groups while serum HDL-c levels were decreased significantly in the HFD group (P < 0.001) compared to the CNT group. APO decreased TG and TC levels but increased HDL-c levels significantly (P < 0.0001 for TG and TC, P < 0.05 for HDL-c) compared to the HFD group (Figure 1b-1d).

Serum insulin and leptin levels were increased significantly in the HFD (P < 0.0001 for both) and HFD+APO groups (P < 0.001 for insulin and P < 0.05 for leptin) compared to the control group. APO administration decreased both insulin and leptin levels significantly (P < 0.0001 for both) compared to the HFD group (Figure 1e and 1f).

Biochemical Tissue Analysis

In the HFD group, aortic MDA levels increased significantly compared to the CNT group (P < 0.001) while the APO had significantly decreased MDA levels compared to the HFD group (P < 0.0001, Figure 2a). In the HFD group, GSH levels decreased significantly compared to CNT group (P < 0.0001) while APO significantly increased GSH levels compared to the HFD group (P < 0.0001, Figure 2b). In the HFD group, MPO activity (P < 0.0001), MCP-1 (P < 0.01) and TNF-α (P < 0.01) levels increased significantly compared to the CNT group. APO significantly decreased MPO activity (P < 0.0001), MCP-1 (P < 0.05) and TNF-α (P < 0.01) levels compared to the HFD group (Figure 2c-2e). In the HFD group, ox-LDL (P < 0.001) and 8-OHdG (P < 0.05) levels increased significantly compared to the CNT group. APO significantly decreased 8-OHdG levels compared to the HFD group (P < 0.05, Figure 2g). In the HFD group, NO levels decreased significantly compared to the CNT group (P < 0.0001). In the HFD+APO group, NO levels increased significantly compared to the HFD group (P < 0.0001, Figure 2h).

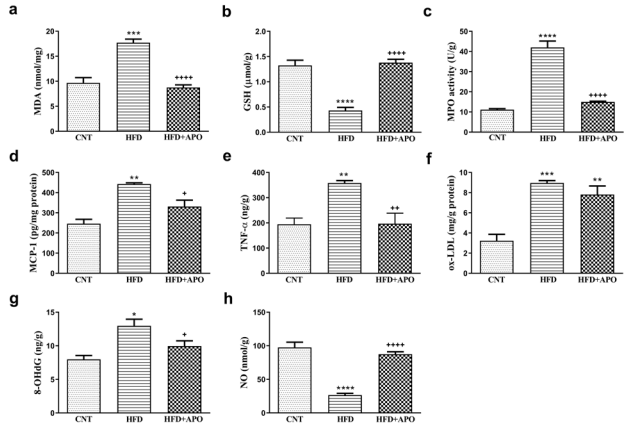

Figure 2. *The effect of HFD and APO on (a) MDA, (b) GSH, (c) MPO activity, (d) ox-LDL, (e) 8-OHdG, (f) MCP-1, (g) TNF-α and (h) NO levels in aorta tissue. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 vs CNT group, ⁺P < 0.05, ⁺⁺P < 0.01 and ⁺⁺⁺⁺P < 0.0001 vs HFD group. All data were expressed as mean ± SEM (n=7)*

Aorta Histology

Staining with HE demonstrated the structure of both the elastic lamina and the cellular density of tunica media. The CNT group had a smooth alignment of endothelium and elastic lamina (Figure 3a). The HFD group displayed prominent disintegration of elastic lamina (expressed as MEFD), accumulation of mucoid extracellular matrix (expressed as MEMA) and foam cells (Figure 3b). Treatment with APO ameliorated elastic lamina alignment and reduced MEMA (Figure 3c). Verhoeff–Van Gieson staining displayed the elastic lamina which was regularly structured in the CNT group (Figure 3d) and disintegrated in the HFD group (Figure 3e), but in the HFD+APO group the elastic lamina showed nearly regular morphology (Figure 3f). Toluidine blue stained sections displayed both elastic lamina and media structure; in the CNT group, elastic lamina aligned regularly (Figure 3g) whereas in the HFD group the medial layer showed disintegrated and expanded morphology besides in some regions the cellularity was reduced (Figure 3h). On the other hand, the HFD+APO group demonstrated regeneration in the elastic lamina and reduction of the space between the lamina besides the regular cellularity (Figure 3ı).

Medial elastic fiber disintegration and MEMA were increased in both the HFD (P < 0.0001 for both) and the HFD+APO (P < 0.01 and P < 0.0001, respectively) groups significantly , compared to the

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CNT group. APO decreased MEFD ($P < 0.01$) and MEMA ($P < 0.001$) levels significantly compared to the HFD group (Figure 3j and 3k). The value of SMCN/Area (mm^2) indicating cellular loss, significantly decreased in the HFD group compared to the CNT group ($P < 0.01$). APO reversed this reduction close to CNT values ($P < 0.05$, Figure 3l). IMT increased in both HFD and HFD+APO groups, but it was not statistically significant (Figure 3m).

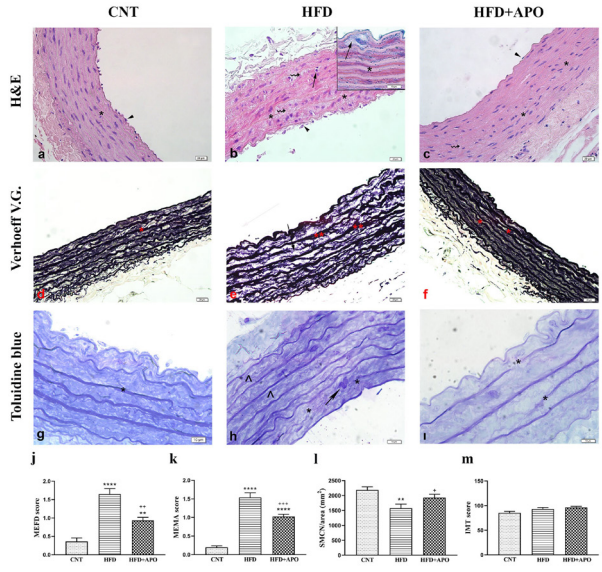

Figure 3. *Representative photomicrographs of HE (a-c), Verhoeff Van Gieson (d-f) and Toluidine blue (g-ı) – stained aorta tissue. ▶: endothelium; →: (b) foam cell formation, (e) impairment of elastic lamina, (h) twisted smooth muscle cell nucleus; broken arrows: mucoid extracellular matrix, □: elastic lamina; □□: thickness in elastic lamina; Λ: reduction in cellularity of smooth muscle cells. Histopathological score for the (j) MEFD, (k) MEMA, (l) SMCN/area (mm2) and (m) IMT. **P < 0.01 and ****P < 0.0001 vs CNT group, $^{+}P < 0.05$ $^{++}P < 0.01$, $^{+++}P < 0.001$ and $^{++++}P < 0.0001$ vs HFD group. All data were expressed as mean ± SEM (n=7). Scale bar: a-f: 20 µm; g-ı: 10µm, insert: 10µm.*

Immunohistochemistry Assays

Compared to the CNT group, TUNEL positivity ($P < 0.0001$, Figure $4a_2$ and $4a_4$), i-NOS-ir levels ($P < 0.01$, Figure $4c_2$ and $4c_4$), LOX-1-ir levels ($P < 0.0001$, Figure $4e_2$ and $4e_4$) and NOX-2-ir levels ($P < 0.01$, Figure $4f_2$ and $4f_4$) were significantly higher while e-NOS-ir levels ($P < 0.001$, Figure $4b_2$ and $4b_4$) were significantly lower in the HFD group. APO treatment reversed these levels [($P < 0.001$ for TUNEL positivity, Figure $4a_3$ and $4a_4$), ($P < 0.01$ for i-NOS-ir, Figure $4c_3$ and $4c_4$), ($P < 0.0001$ for LOX-1-ir Figure $4e_3$ and $4e_4$), ($P < 0.01$ for NOX-2-ir, Figure $4f_3$ and $4f_4$) and ($P < 0.001$ for e-NOS-ir Figure $4b_3$ and $4b_4$)] compared to the HFD group. α-SMA-ir levels were decreased in the HFD group compared to the CNT group and increased in the HFD+APO group compared to the HFD group. But the differences among the groups were not statistically significant (Figure $4d_1$-$4d_4$).

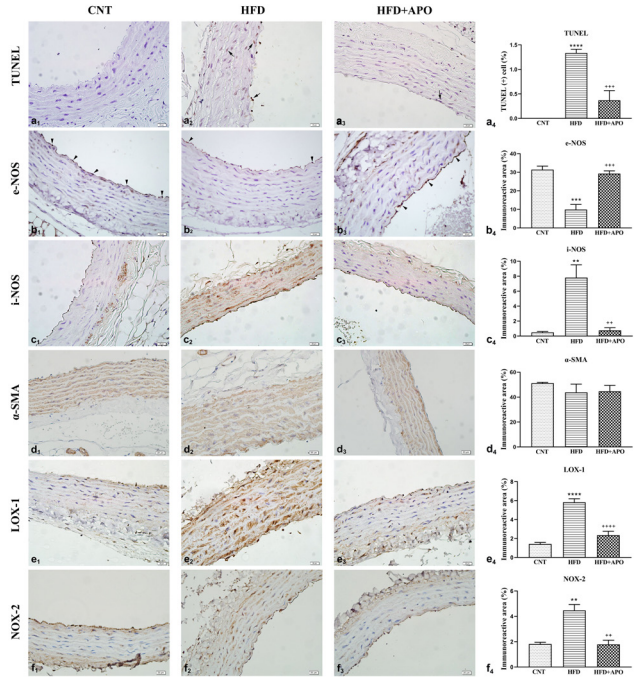

Figure 4. *Representative photomicrographs of immunohistochemically stained aorta tissue and corresponding graphs of immunoreactive area levels. **P < 0.01, ***P < 0.001 and ****P < 0.0001 vs CNT group, $^{++}P < 0.01$, $^{+++}P < 0.001$ and $^{++++}P < 0.0001$ vs HFD group. All data were expressed as mean ± SEM (n=7). Scale bar: 20 µm.*

Transmission Electron Microscopy

In CNT group, regularly arranged elastic lamina and in addition to local collagen fibers were observed (Figure 5a) while in HFD group, elastic lamina deteriorated and there was an increase in collagen between them (Figure 5b). In the HFD+APO group, improved appearance of elastic lamina and a decrease in collagen fiber density were observed (Figure 5c).

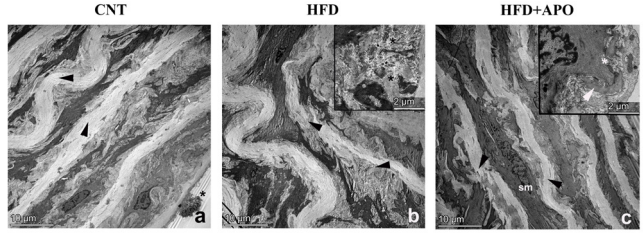

Figure 5. *Representative electron micrographs of aorta tissue. ▶: (a,c) Regular elastic lamina, (b) irregular elastic lamina; □ : (a) endothelial cell, (b-inset) increased and disorganized collagen fibers, (c) reduced density of collagen fibers, Sm: smooth muscle cell. Scale bar: 10 µm; insert: 2 µm*

Western Blot Analysis

The levels of c-PARP ($P < 0.01$), caspase-1 p10 ($P < 0.0001$) and GSDMD-N ($P < 0.01$) increased significantly in the HFD

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group compared to the CNT group, while APO reversed these levels compared to the HFD group (P < 0.01, P < 0.0001 and P < 0.05, respectively, Figure 6a-6c). The level of e-NOS (P < 0.05) decreased while the level of i-NOS (P < 0.01) increased in the HFD group compared to the CNT group, and APO reversed both levels (P < 0.05, Figure 6d and 6e) compared to the HFD group. The level of ALK-1 increased in the HFD group compared to the CNT group and reversed in the HFD+APO group, but the difference between groups was not statistically significant (Figure 6f).

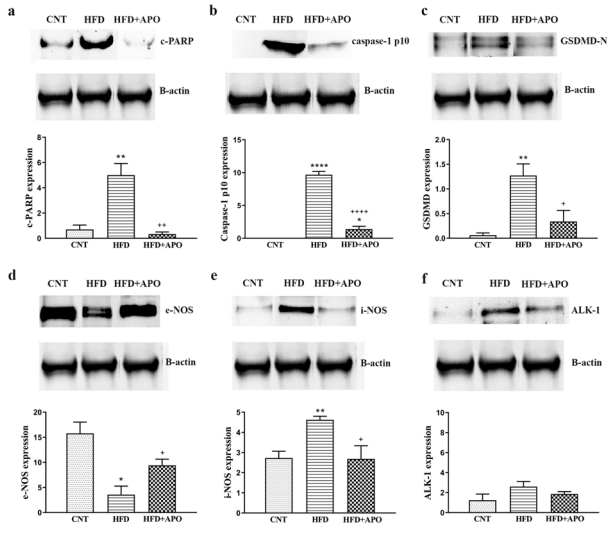

Figure 6. *Representative immunoblot images and the protein expression levels in aorta tissue. The expression levels of each band were determined using scanning densitometry and normalized to beta-actin. *P < 0.05, **P < 0.01 and ****P < 0.0001 vs CNT group, ⁺P < 0.05 ⁺⁺P < 0.01 and ⁺⁺⁺⁺P < 0.0001 vs HFD group. All data were expressed as mean ± SEM (n=7).*

qRT-PCR Analysis

The differences in mRNA expression levels of NOS-3 (e-NOS) and ACVRL1 (ALK-1) genes between groups were not statistically significant while only mRNA expression level of OLR1 (LOX-1) gene was statistically significant in the HFD+APO group compared to the HFD group (P < 0.05, Table I).

Table I. *Gene expression levels in aorta tissue.*

	CNT Group	HFD Group	HFD+APO Group
NOS3	0,9939 ± 0.3	1.197 ± 0.6	0.2593 ± 0.03
ACVRL1	1.584 ± 0.6	1.630 ± 0.4	2.388 ± 0.5
OLR1	1.772 ± 0.5	0.6144 ± 0.1	2.908 ± 0.7 *

*NOS3: Nitric oxide synthase 3, ACVRL1: Activin receptor-like kinase 1, OLR1: Oxidized low-density lipoprotein receptor 1, CNT: Standard control group, HFD: High-fat diet group, HFD+APO: High-fat diet+Apocynin group. *P < 0.05 vs HFD group. Results were expressed as fold change compared to control. All data were expressed as mean ± SEM (n=7).*

4. DISCUSSSION

Our study demonstrated that treatment of obese rats with APO for 4 weeks restored weight gain and parameters relevant to metabolic disturbance (TG, TC, HDL-c, insulin, leptin), OS (NOX-2, MDA, GSH, 8-OHdG), ED (NO, e-NOS, i-NOS), inflammation (MPO, MCP-1, TNF-α), programmed cell death (TUNEL, c-PARP, caspase-1, GSDMD-N), histologic damage and cellular ox-LDL uptake (LOX-1) which occurs in atherogenesis.

Apocynin is a natural inhibitor of the NOX-2 enzyme which is the main isoform of NOX family members up-regulated in obesity and HFD-induced vascular OS [3]. It limits the progression of AS in mice by NOX inhibition after the disease developed [22]. A previous study demonstrated a link between HFD-induced NOX-2 activity and weight gain, and administration of APO resulted in less weight gain [3]. Similar to these results, the weight gain levels in our study showed that APO significantly decreased the increased weight gain in the HFD group. Also, this decrease was paralleled by the significant decrease of NOX-2 expression, which implies oxidative stress, with APO administration in our study.

In obesity, hyperlipidaemia, a cardiovascular risk factor characterized by high TC, high TG and reduced HDL-c levels, is considered to be responsible for atherogenesis emergence [23]. In our obesity model, high fat consumption resulted in higher TC, higher TG and lower HDL-c levels in serum similar to previous studies [3, 4, 8] and APO reversed these levels similar to a previous study [24]. But our TC result did not support Gamez-Mendez's study, which showed that there was no significant difference via APO administration [4]. This difference may be caused by the use of different animal strains and dosage of APO from those used in our study.

Excess adiposity results in chronic hyperinsulinemia which occurs in atherogenesis by increasing growth and proliferation of VSMCs, monocyte/macrophage recruitment to endothelium, ROS production and inflammation, transport of LDL cholesterol into VSMCs and causing hypertension, especially ED [25]. In this study, as a result of HFD, hyperinsulinemia developed in HFD-fed rats in parallel with increased adiposity. APO reversed hyperinsulinemia similar to previous studies [3, 26]. These results indicate that APO may contribute to the suppression of ED/AS by reducing hyperinsulinemia in obesity.

In obesity, leptin is responsible for the relationship between ED and obesity, as it acts on the regulation of vascular tone / NO production and associated with proinflammatory mechanisms and ROS production leading to ED [4]. In addition, it stimulates ECs, leading to adhesion of monocytes and their migration from blood into tissue [27]. In the present study, we observed hyperleptinemia in HFD group and APO reversed this effect, similar to a previous report [4], indicating that APO may exhibit its ED-improving and AS-suppressive effects by reducing hyperleptinemia.

A high saturated fat diet over a long period of time functions as a stimulator of OS that is responsible for organ pathophysiology [28]. In our study, in addition to increased NOX-2 level, the other

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OS markers: increased MDA, decreased GSH and increased 8-OHdG levels in the HFD group were significantly reversed by APO, indicating its suppressor effect on OS, similar to previous studies [16, 26, 29, 30].

In this study the results showed that (i.) MPO activity levels, (ii.) proinflammatory TNF-α, which participates in AS via stimulating superoxide production by NOX in ECs and VSMCs [31], and (iii.) MCP-1, which has a key role in monocyte/macrophage infiltration into vessel wall in the initial stage of AS [32], levels were increased along with ox-LDL levels in obese rats. But APO reversed TNF-α and MCP-1 levels as reported in previous studies [2, 33] and to our knowledge this is the first study demonstrating the impact of APO on ED/AS in HFD-induced obesity and MPO link. It is inferred from these results that APO has a limiting effect on AS by suppressing monocyte involvement and subsequent proinflammatory cytokine production, which contributes to the progression of AS.

Obesity leads to ED via increased ROS production by NOX-2 which causes a depletion on vasoprotective NO levels [3]. NO is the key regulator of vasodilatation. It has several antiatherogenic effects acting to inhibit platelet adhesion and aggregation, decrease leukocyte adhesion to endothelium and migration into vessel, VSMC proliferation [34, 35] and inhibit proinflammatory cytokine /chemokine expression [5]. Under physiological conditions, it is generated by e-NOS. But, in pathological circumstances such as OS and inflammation, it is generated by i-NOS in excessive amounts, disturbing endothelium-dependent vasodilatation. i-NOS decreases e-NOS activity by competing with it for its cofactor [34]. Continuous NO production by i-NOS may also impair signal cascades linking endothelial receptors to e-NOS activation [35]. ED can also be a result of decreased e-NOS expression and/or e-NOS activity [5]. As described in previous reports, ED is marked with NO decrement [4, 8], e-NOS expression reduction [36] and i-NOS expression induction [8] and can be reversed by perfusion of APO in obese subjects [37]. In our study, impaired endothelial function parameters were reversed by APO as in previous studies, demonstrating elevated NO levels [4, 14] and e-NOS expression [14] in addition to lowered i-NOS expression [15], indicating that APO in this study may supress ED.

Progression of atherogenesis occurs through the response of inflammatory cells to native and modified lipids accumulating in the subendothelial region [38]. In addition, ox-LDL formation by ROS has an atherogenic effect via inducing foam cell formation and accumulation of adhesion molecules, ox-LDL, neutrophils and monocytes in the vessel lumen that result in atherogenic plaque and cell death [39]. Besides, oxidation of LDL may be carried out by MPO in the endothelium [38]. So, resulting ox-LDL increases NOX activity and decreases e-NOS activity in the vessel [7]. LOX-1 which is a scavenger receptor present in ECs, VSMCs, platelets, macrophages and fibroblasts, participates in ED and AS via mediating ox-LDL uptake into the cells. Obesity induces LOX-1 expression. In the pathogenesis of AS, monocyte adhesion to activated ECs occurs. Monocytes in the subendothelial space transform into macrophages, internalize modified lipids such as ox-LDL and form foam cells. VSMCs migrate into the subendothelial space and proliferate there, then synthesize collagen. Foam cells, VSMCs and deposited collagen form atherosclerotic plaque. Ox-LDL binding to elevated LOX-1 results in several atherogenic and/or proinflammatory effects in the vessel wall that promote this pathogenesis. Namely, it increases (i.) LOX-1 expression and ox-LDL uptake by ECs and VSMCs, resulting foam cell formation, (ii.) proinflammatory cytokine expression such as TNF-α and MCP-1, hence monocyte attachment to ECs, (iii.) NOX-2 and NOX-4 expression, hence ROS production and OS, besides (iv.) collagen synthesis, (v.) apoptosis, (vi.) VSMC migration and proliferation. On the other hand, it decreases e-NOS activity/expression and endothelial NO level [40]. In our study it was observed an increase of oxidative and inflammatory parameters and changes in NO metabolism in the HFD group, however NOX-2 inhibitor apocynin treatment reversed all these parameters except ox-LDL to the control levels by regulating the oxidant/antioxidant balance.

Antioxidants have been reported to be inhibitors of LDL oxidation [39]. In this study, as an antioxidant agent, APO decreased the markedly alleviated LOX-1 protein expression levels. But, in contrast, gene expression levels were lower in the HFD group (statistically not significant) compared with the CNT group and were significantly higher in the HFD+APO group. This may because of that high LOX-1 protein expression levels in the HFD group, increased with alleviating OS, may induce a negative feedback mechanism on LOX-1 gene expression. Hence, it may result in a decrease in gene expression. But APO by means of its strong antioxidant effect may suppress OS and prevent related LOX-1 protein expression levels from reaching the similar levels as in the HFD group. Since LOX-1 protein level was low in the HFD+APO group, the negative feedback mechanism was not activated, and LOX-1 gene expression levels may have remained high for a longer period in the HFD+APO group.

The initiation of AS is believed to occur from retention and accumulation of cholesterol-rich particles, especially LDL-c, in the subendothelial space. ALK-1 can function as a binding protein for uptake and transcytosis of LDL-c throughout vascular endothelium [10, 41]. LDL-c is not degraded when taken into cells via ALK-1 [38]. It has been demonstrated that ALK-1 activity alleviated in atherosclerotic vessel, and it is thought to have a role in VSMC proliferation in AS [41]. For the first time, this study investigated the difference in ALK-1 expression in obesity to assess LDL uptake. ALK-1 protein and mRNA expression did not differ significantly between the groups. This result may be related to the fact that increased LDL concentration in athero-prone areas in the vessel wall is caused by increased LDL retention rather than increased uptake of LDL [38].

Foam cell formation is a hallmark of AS. It may be generated from both macrophages and VSMCs [42]. Over a high cholesterol loading, VSMCs undergo dedifferentiation and downregulate some genes that is specific for contractile phenotype including α-SMA to switch to secretory phenotype in atherosclerotic lesions [43]. In our study, HFD and APO did not affect the α-SMA protein expression significantly. We demonstrated the

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presence of lipid loaden foam cell formation by VSMCs in media layer in HE stained sections. But it is concluded from α-SMA results that HFD consumption has not yet caused a phenotypic transition in VSMCs that will participate in plaque formation in the intima.

High levels of cholesterol, ox-LDL, TNF-α and ROS act as inducers of apoptosis and pyroptosis in many stages of AS. In the early stage of AS, apoptosis and pyroptosis in ECs lead to loss of endothelial function and integrity, resulting in increased permeability for lipid and monocyte which will form foam cells [44, 45]. In the progressing stages of AS, apoptosis and pyroptosis in EC, VSMCs and macrophages participate in destabilization of atheromatous plaque and thrombosis formation [44, 46]. As reported in previous studies [13, 47,48], in our study, we demonstrated the suppressor effect of APO on apoptosis marked with reduced c-PARP protein levels, which indicates apoptotic caspase-3 activity, in addition to TUNEL positivity, and on pyroptosis marked with reduced caspase-1 and GSDMD-N protein levels in addition to TUNEL positivity. Besides, TUNEL positivity was overt especially in ECs and VSMCs supporting a previous report for pyroptosis [49]. The simultaneous increase in TUNEL positivity of tunica media, ox-LDL and LOX-1 expression levels in our study supported the fact that high levels of ox-LDL cause an increment in apoptosis of VSMCs through LOX-1 [50].

Histopathologic and ultrastructural evaluations revealed a correlation with other results demonstrating metabolic disturbance and presence of factors leading to atherosclerosis. In the HFD group, we examined disintegration/disorganization in elastic lamina, VSMC nuclei loss, foam cell formation, expanded elastic lamella, twisted SMCN, mucoid extracellular matrix accumulation and increase/ disorganization of collagen fibers. APO administration significantly regressed these results, demonstrating a suppressor effect of APO on HFD-induced vessel pathology at an aorta tissue basis.

Conclusion

It is probable that APO demonstrates its improving effect on HFD-induced obesity linked ED/AS-related factors by acting on OS, programmed cell death, ox-LDL uptake and inflammation mechanisms.

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Compliance with the Ethical Standards

Ethics Committee approval: This study was approved by the Marmara University, Local Ethical Committee for Experimental Animals (Protocol Number: 50.2018.mar, Approval Date: 07.05.2018).

Conflicts of interest: Authors declare no conflict of interest.

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