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Dehydroabietic Acid Attenuates Atherosclerosis in Apolipoprotein E-Deficient Mice and VCAM-1 Expressions *in-vitro*

(Asid Dehidroabietik Melemahkan Aterosklerosis dalam Tikus Apolipoprotein E-Kurang dan Pengekspresan VCAM-1 *in-vitro*)

Xiao Liang & Yan Wu*

Department of General Medicine, The First People's Hospital of Lianyungang, Lianyungang, Jiangsu, China, 222000

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ABSTRACT

Dehydroabietic acid (DHA) is an analog of abietic acid (AA) for cardiovascular disease prevention, known to act pharmacologically against aging, inflammation, bacterial infections and cancer. The current research investigated the molecular mechanisms of DHA on the adhesiveness of endothelial leukocytes and activation of NF-κB in TNF-α treated HAEC and ApoE−/− mice in experimental atherosclerosis. The HAECs were tested for toxicity using MTT assay at various DHA concentrations. The cell-surface expression of CAM against endothelial leukocyte adhesion molecule-1, ICAM -1, or human VCAM-1 was determined by the ELISA test, followed by western blot analysis. Endothelial cell-leukocyte adhesion assay involving U937 cells was carried out followed by the determination of NF-κB p65 expression. ApoE−/− mice were fed with a high cholesterol diet every day followed by oral administration of DHA (10 and 20 mg/kg/day). The DHA (5 and 10 mM) substantially reduced ($p < 0.05$) human (U937) cell lines binding to TNF-α activated human endothelial aortic cells. The assays involving the 32P-labelled NF-kB as a probe demonstrated that DHA pre-treatment decreased the shifted bands density following the stimulation of TNF-α. Nuclear extracts immune blot assessment and immune fluorescence staining showed a significant decrease ($p < 0.05$) in the NF-κB p65 concentration in the nuclei with DHA treated human endothelial aortic cells. Together these findings indicate that DHA inhibits nuclear trans localization of TNF-α-induced NF-κB p65, and thus considerably suppresses ($p < 0.05$) the VCAM-1 expression, which contributes to lower leukocyte adherence suggesting that DHA helps in preventing inflammatory atherosclerosis *in vivo.*

Keywords: ApoE−/−; atherosclerosis; dehydroabietic acid; TNF-α

ABSTRAK

Asid dehidroabietik (DHA) ialah analog asid abietik (AA) untuk pencegahan penyakit kardiovaskular yang diketahui bertindak secara farmakologi terhadap penuaan, keradangan, jangkitan bakteria dan kanser. Penyelidikan semasa mengkaji mekanisme molekul DHA pada kelekatan leukosit endotelium dan pengaktifan NF-κB dalam TNF-α yang dirawat HAEC dan ApoE-/- tikus dalam aterosklerosis uji kaji. HAEC telah diuji untuk ketoksikan menggunakan ujian MTT pada pelbagai kepekatan DHA. Pengekspresan permukaan sel CAM terhadap molekul lekatan leukosit endotelium-1, ICAM -1, atau VCAM-1 manusia ditentukan melalui ujian ELISA, diikuti oleh analisis pemblotan western. Ujian lekatan sel-leukosit endotelium yang melibatkan sel U937 telah dijalankan diikuti dengan penentuan pengekspresan NF-κB p65. ApoE−/− tikus diberi makan dengan diet kolesterol tinggi setiap hari diikuti dengan pemberian oral DHA (10 dan 20 mg/kg/hari). DHA (5 dan 10 mM) mengurangkan dengan ketara ($p <0.05$) garisan sel manusia (U937) yang mengikat kepada sel aorta endotelium manusia yang diaktifkan TNF-α. Ujian yang melibatkan NF-kB berlabel 32P sebagai prob menunjukkan bahawa pra-rawatan DHA mengurangkan ketumpatan jalur beralih berikutan rangsangan TNF-α. Ekstrak nuklear penilaian pemblotan imun dan pewarnaan pendarfluor imun menunjukkan penurunan ketara ($p <0.05$) dalam kepekatan NF-κB p65 dalam nukleus dengan sel aorta endotelium manusia yang dirawat DHA. Bersama-sama penemuan ini menunjukkan bahawa DHA menghalang penyetempatan trans nuklear NF-κB p65 yang disebabkan oleh TNF-α dan dengan itu menekan dengan ketara ($p <0.05$) pengekspresan VCAM-1, yang menyumbang kepada pematuhan leukosit yang lebih rendah yang menunjukkan bahawa DHA membantu dalam mencegah aterosklerosis radang secara *in vivo.*

Kata kunci: ApoE−/−; asid dehidroabietik; aterosklerosis; TNF-α

Introduction

The key pinewood derivative for abietic acid (AA) (Gonçalves et al. 2018) is dehydroabietic acid (DHA). DHA was known to act pharmacologically (Luo et al. 2016) against ageing (Kim et al. 2015), inflammation (Kim et al. 2019; Zhang et al. 2019b), bacterial infections (Moreti et al. 2017), and cancer (Wang et al. 2018). DHA supplements in the chow showed substantially lower blood sugar levels in genetically obese KK-Ay mice (Zhang et al. 2019b). DHA increased development of PPAR-γ and-α (Luchnikova et al. 2019) and facilitated *in-vitro* glucose absorption into adipocyte 3T3-L (Kim et al. 2019). It was reasonable to assume that DHA can boost some deleterious effects of thiazolidinediones (TZDs) as it proposes a PPAR-γ/α dual-action. Furthermore, anti-inflammatory effects by inhibiting the NF-κB signal pathway are frequently suggested pathways to boost insulin sensitivity (Kim et al. 2019; Zhang et al. 2019b) and to increase adiponectin and glucose absorption of Glut-4 (Zhang et al. 2019b). The DHA therapy was shown to suppress the production of pro-inflammatory cytokines such as monocyte chemo attractant protein-1 and TNF-α (Zhang et al. 2019b) to inhibit the NF-κB signaling pathway (Kim et al. 2019) to enhance adiponectin in expression anti-inflammatory cytokines (Zhang et al. 2019b). Thus, the mechanism underlying DHA's action in endothelial cells was evaluated to determine if it improves the adhesion molecules expression or blocks it.

The binding of leukocytes to vascular endothelium in circulation was a significant instant growth of atherosclerosis (Shimizu & Minamino 2020). Specific adhesion molecules like Vascular CAM, endothelial leukocyte adhesion molecule-1 and ICAM-1 were regulated by endothelial expression by localized leukocyte deposition (Kumboyono et al. 2021). Increased adhesion molecules of ECs can result in increased recruitment of leukocytes into atherosclerotic sites in human atherosclerotic lesions (Gaul et al. 2021). Adhesion expression regulation was made at the level of transcription and regulated partially through the transcription factor of NF-κB redox-sensitive (Shah et al. 2021). Oxidative stress is caused by NF-κB inducers activation such as TNF-α or UV radiation, indicating that the ROS induction was a signal that activates the NF-κB response. Although considerable attention was given to these inducible molecules, the effects of DHA on the adhesion molecule expression is scarcely known. Hence a deeper understanding of the effect of DHA can provide valuable insights into atherogenesis prevention.

Cardiovascular diseases are caused by lipid deposition in the artery walls, oxidation of lipids, inflammation and dysfunction of immune responses (Poursaleh et al. 2021), which are the significant causes of early deaths in advanced and developing countries (Ammirati et al. 2015). The adhesion of leucocytes to the elicited endothelium and their subsequent invasion of the vessel wall was a critical event for atherosclerosis.

Through the mediation of these cell processes endothelial cell (EC), adhesion molecules were regulated upwardly, and leukocyte-derived chemical factors on the vascular wall were increasingly expressed (Wolf & Ley 2019). This transition can be affected by oxidative stress. To promote atherosclerosis, modified LDL, especially oxidized form of LDL, and offers many atherogenic causative effects (Wolf & Ley 2019). Plasma oxLDL levels were gradually elevated formerly to the formation of atherosclerotic lesions in ApoE-/- mice (Kato et al. 2009), signifying that oxLDL plays an important role in atherosclerotic lesions at the initial developmental stages of atherosclerosis. Earlier reports demonstrated that the lectin-like LOX-1 receptor (Hofmann et al. 2017) was the main receptor expressed in ECs for experimental animal and human atherosclerotic lesions (Liu et al. 2021). LOX-1 fosters the absorption of LDL by macrophages and endothelial cells (ECs), thus facilitating its outcomes (Chen et al. 2016; Singh & Gautam 2019). oxLDL promotes a) Endothelial cellular apoptosis and apoptotic cell phagocytosis; (b) monocyte adhesion to active endothelium; and (c) foam cell production resulting from macrophages. More recently, LOX-1 is the primary suspect of signaling adverse effects of defective HDL in patients with coronary artery disease, such as oxidized HDL and HDL, followed by up-regulation through a variety of atherogenic causative factors, such also LDL, stress, homocysteine, TNF-α and increased glucose levels (Li et al. 2015). The latest proposal for LOX-1 was also an appealing targeted atherosclerosis therapy (Li et al. 2015).

Possible DHA effects on adherent molecules may play a significant part in the suppression or management of disorders (cardiovascular-related). Therefore, our research was planned to study the ability of DHA to modify the NF-κB expression on adherence molecules and the transcription factor of the HAECs (human aortic endothelial cells) along with the assessment of ROS modulation. DHA also significantly prevents the human myeloid leukaemia U937 from adhering to human aortic endothelial cell. However, DHA's prospective *in-vivo* anti-atherosclerotic effects are still not known. Hence,

the current investigation was intended to examine the potential benefits of DHA on the *in-vitro* and *in-vivo* expression of VCAM-1 mediated by NF-κB expression suppression and the development of plaque related atherosclerosis in ApoE-/-mice fed with diet containing high cholesterol.

MATERIALS AND METHODS

HAEC cells (human aortic endothelial cells) growth medium 200; Trypsin neutralizer solution; EDTA or trypsin solution; LSGS (low serum growth supplement) are procured from Sigma Aldrich, China. FBS, RPMI-1640 medium and antimycotic-antibacterial mixture were procured from Shanghai XP Biomed Ltd., China. DHA (Dehydroabietic acid); DCFH-DA (dichlorofluoresceindiacetate) and BCECF-AM were procured from Sigma Aldrich, Beijing China. Primary antibodies of the mouse (NF-κB) and goat antibodies for Vascular CAM, endothelial leukocyte adhesion molecule-1 and ICAM-1(Invitrogen, USA).

CULTURE OF HAECS

Cryopreserved, tertiary cultures were collected from the Research Center (China), growing in endothelial cell growth media flasks added with 3 % BSA, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione (1.5 mg/mL), human EGF (15 ng/mL) FGF (4 ng/mL), unfractionated heparin (12 mg/mL), streptomycin (100 pg/mL) and amphotericin B (1.25 mg/mL). In a humidified atmosphere of 95% air, 5% CO_2, the cells were developed at room temperature. The purity of culture was tested with a monoclonal antibody to stain the human factor von Willebrand (vWF) (Zhang et al. 2019a).

CELL VIABILITY ASSAY USING MTT

To test cell viability, an MTT assay was used. The assay principle in viable cells reduces MTT into blue formazan-crystals with mitochondrial dehydrogenase. In short, cells were developed in 96-well plates and allowed for incubation at room temperatures with different DHA concentrations. Then 0.5 mg/mL of MTT were applied to each well and incubated at room temperature for 4 h. Later, the solvent medium was separated cautiously, so that the crystals formed by formazan were not disturbed. For each well, 100 mL of dimethyl-sulfoxide (DMSO), which dissolves crystallized formazan, was applied and micro-plate readings at 530 nm were reported. As standardized cell viability tests for cells incubated in the control medium, cytokine and DA based optical density reduction were found 100% viable.

ELISA ASSAY

HAECs were placed in 96-well plates and pre-treated with defined DHA concentrations to test cell-surface expression of adhesion molecules for 20 h and then activated for 8 h at room temperature of TNF-α (2 ng/mL) with DHA (Fong et al. 2016). The reaction was prevented by incorporating specific goat's antibodies for a 30-minute duration to the E-select in, ICAM-1 or VCAM-1wells (500 µg/mL in HBSS containing 2% of skim milk), and then washed in HBSS at room temperature of 1 h in 0.1 % in Tween-20, in combination with HRP and anti-goat IgG (500 µg/mL in HBSS comprising 2% skim milk). By incorporating 1 M H_2SO_4 (40 mL), the reaction was prevented. Using an ELISA plate reader at 510 nm, the plates were read.

WESTERN BLOT ANALYSIS

Cells were plated in 96-wells and pretreated at 37 °C with 10 mM DHA for 18 h, then stimulated at room temperature with 2 ng/mL of tumor necrosis factor-α for 6 h with DHA. Cells were subsequently washed with PBS (pH of 7.2), centrifuged at 2000 × g for 15 min at 50 °C and lysed with lysis buffer containing 1 M NaCl, 40 mM Tris, 0.5 mm Ethylenediaminetetraacetic acid, 0.1% SDA, 0.1% Triton X-100, 0.5 nM phenylmethylsulphonyl fluoride (pH 7.2) for one hour at 50 °C. Using a kit from Bio-Rad, the protein levels in the supernatants were measured (Fong et al. 2016). The supernatant was exposed to SDS ± PAGE at 10%, then transmitted to the polyvinylidene difluoride (PVDF) membrane for 1 h at room temperature, which was incubated with 1% Tween 20 to 1% skimmed milk for one h at room temperature. After washing at room temperature for 1 h, membranes were incubated with HRP conjugated rabbit anti-goat IgG. Immunodetection was carried out using Chemisorptions method.

ENDOTHELIAL CELL-LEUKOCYTE ADHESION ASSAY

In the 24-well plates, the HAECs were dispersed and allowed for confluence. Cells were further incubated for 20 h at room temperature with a growth medium accompanied by DHA at specified concentrations, and then for 7 h at room temperature by 2 ng/mL of tumor

necrosis factor-α in the existence of DHA. Through the culture set, the U937 cells, cultivated in RPMI-1640 medium consisting of 4% fetal bovine serum and sub cultured three times a week at 1:6 ratio, were labeled with BCECF/AM (5 mM) at room temperature in serum-free RPMI-1640 medium for 1 h. The labeling of U937 cells was applied to each well containing a HAEC, and the incubation lasted for another hour. Two gentle PBS cells isolated non-adherent cells, and four different fields using a fluoresce microscope were measured for the amount of bound U937 cells (6400 ×). Random fields were selected to count adherent cells from the monolayer center at the half-radius size (Fong et al. 2016).

NUCLEAR EXTRACT PREPARATION AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear protein extracts were processed, as stated previously (Chen et al. 2016). Random fields were chosen to count the half-radius size of adherent cells from the middle of the monolayer. After washing with phosphate buffer saline, 0.5 mL of ice-cold buffer A containing N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (12 mM, pH 8), KCl (5 mM), dithiothreitol (2 mM), phenyl-methylsulfonyl-fluoride (2 mM), MgCl (2 mM) and aprotinin, pepstatin and leupeptin (3 μg/mL each) were shredded onto the plates. After centrifuging at 500 × g for 15 min at 50 °C, these cells were replaced in buffer B (75 μL containing 0.05% Triton X-100 in buffer A), put on ice for 15 min, then centrifuged at 15 000 × g at 50 °C for 15 min. Centrifugation was done at 12000 g for 40 min at 50 °C for re-suspended nuclear pellets in 70 μL of ice-cold buffer C consisting of HEPES (20 mM; pH 8.0), $MgCl_2$ (2 mM), NaCl (0.5 M), dithiothreitol (2 mM), Ethylenediaminetetraacetic acid (0.3 MM), phenyl-methyl sulfone-fluoride (2 mM), 15% glycerol and aprotinin, pepstatin and leupeptin (3 μg/mL each) and then incubated at 50 °C for 45 min (Fong et al. 2016). The subsequent supernatant was deposited at -60 °C as a nuclear extract. Protein levels were calculated with the Bio-Rad method. A Nuclear Factor-κB probe in the gel shift assay was the 31-mer double-stranded hybrid oligonucleotide with a particular κB site. Double-stranded Radio labeled DNA was produced by ICN end-labeling, using T4 polynucleotide kinase for electrophoretic mobility shift assay. Unincorporated nucleotides were isolated from gel filtration process on a Sephadex G25 column. The DNA binding reaction was performed at room temperature at a volume of 15 μL consisting of 2 μg nuclear extract, 3×10^5c.p.m/ng ^{32}P-labelled-kB (1 ng), 10 μg salmon sperm DNA and 15 μL binding buffer consisting of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (15 mM; pH 8), glycerol (15%), Potassium Chloride (0.2 M), Ethylenediamine tetra-acetic acid (0.3 mM), phenylmethyl sulfonyl fluoride (0.4 mM), dithiothreitol (0.4 mM) and leupeptin (4 μg/mL). Nuclear extract-oligonucleotide mixtures were extracted by 5% polyacrylamide gel (30:1) from unbound DNA by electrophoresis in the TBE buffer, pH 7.4. Gels were vacuum-dried before being exposed to auto-radiographed using UMAX scanner to scan films.

DETERMINATION OF NF-kB p65 EXPRESSION

The protein expression of Nuclear Factor -kB p65 was established in nuclear extracts in accordance to the procedure described by the Chen et al. (2016). A 10% SDS ± PAGE was used to extract a 20 μg protein sample and then placed onto PVDF membranes. The primary anti-murine (HAMA) Nuclear Factor-kB p65 antibody and Horseradish per oxidase-conjugated goat anti-mouse IgG was then incubated for 1 h at room temperature (0.5 μg/mL). Chemiluminescence Reagent Plus (NEN) and Biomax MR® lm Exposure was observed to detect bound antibody (Kodak). Confluent HAECs (control and DHA-treated cells for 20 h) were placed on slides with2 ng/mL of TNF-α for 45 min to monitor NF-kB expression *in situ*, then fixed for 20 min at 50 °C to 5% paraformaldehyde in phosphate buffer saline (pH 7.2), rinsed with phosphate buffer, blocked for one hour at 37 °C with 5% bovine serum albumin in phosphate buffer saline, and then allowed for reaction with HAMA NF-kB p65 antibody for one h at 37 °C (1:400 dilutions in Phosphate-buffered saline). After washing, the slides were allowed for incubation with FITC-conjugated goat anti-mouse IgG at room temperature and examined using a fluorescent microscope (Fong et al. 2016).

ASSESSMENT OF HYDROGEN PEROXIDE GENERATION

The DHA effect on hydrogen peroxide generation in human aortic endothelial cells was determined using ELISA involving 2',7'-Dichlorofluorescin Diacetate as a probe (Fong et al. 2016). The principle involves the oxidation of non-fluorescent DCFH-DA by H_2O_2 to fluorescent DCF (2',7'-dichlorofluorescein). A confluent cell density of HAECs at 10^5 cells per well were pre-treated with different DHA concentrations in 48-well plates for 18 h. After extracting DHA, the HBSS was used

to rinse the cells, followed by the addition of DCFH-DA prepared in HBSS (10 mM) and incubated for one hour at room temperature (Fong et al. 2016). The intensity of fluorescence was determined at an excitation (490 nm) and an emission wave length (520 nm) using the microplate reader (fluorescence). To assess the influence of in the production of hydrogen peroxide under oxidative stress, 2 ng/mL of tumor necrosis factor-α was incorporated into the medium to measure fluorescence intensity for 60 min at an interval of 15 min each. The cells were viewed using fluorescence microscopy and photographed.

ANIMAL EXPERIMENT

The University Committee on Animal Ethics approved all experimental protocols (China). The ARRIVE guide to animal experiments was documented all the procedures for the studies. Male ApoE-/-mice with a history of C57BL/6J and wild-type controls (C57BL/6J) were obtained from the animal research center. During a light/dark cycle of 12 h, the mice were housed in the animal room. After six weeks over 16 weeks, the mice were fed with high cholesterol diet (1.25% cholesterol, 0.5% cholic acid and 16% fat) (Liu et al. 2015; Vinué et al. 2018). The ApoE-/-mice were administered everyday with DHA (10 or 20 mg/kg) solubilized in 1% CMC-Na through oral gavage or received 1% of a vehicle suspension containing CMC-Na alone (n = 8 in each group). In this study, the DHA dose was based on previous studies of its effectiveness in animals with dual activation of PPAR-α and PPAR-α by providing diet containing high fat for inducing hepatic steatosis and insulin resistances. As a control group, ten male C57BL/6J age-matched mice were used and treated with vehicle control.

HISTOLOGICAL AND IMMUNOHISTOCHEMISTRY ANALYSIS

As detailed previously (Guo et al. 2020), the aortic sinus was examined morphometrically and immune-histochemically. Slices 8 μm thick were used for immune-histochemical staining of CD68, LOX-1 and Nuclear Factor-κB p65. The color was formed by diaminobenzidine (Sigma-Aldrich, USA). The Matrix Metalloproteinase-9 and α-SMA (smooth muscle actin) were used to stain the portions of aortic sinus for immunohistochemical analysis. The remaining portions were used for hematoxylin-eosin (H&E) staining to investigate simple lesion microstructure and collagen staining of Masson trichomes.

EVALUATION OF SERUM LIPIDS AND PRO-INFLAMMATORY CYTOKINES

The blood samples were obtained over a 42-day period as a baseline. Peri-orbital plexus technique was used after overnight fasting for assessing the lipid levels after the dietary treatment period for 22 weeks. As previously mentioned, colorimetric tests were used to measure TSL, HDL, LDL, and TG (Guo et al. 2020). Cytokine Assay Kit was used to identify serum pro-inflammatory cytokines as specifications indicated by the manufacturer.

MORPHOMETRIC EVALUATION OF ATHEROSCLEROTIC LESIONS

The cryosection techniques for en-face and aortic sinus were employed to measure the progression of atherosclerosis in the aorta and its vessels. The Oil Red-O staining was employed to analyze the en-face and aortic sinus sustained area. A proportion of Oil Red O-positive stained area was quantified with computer-aided morphometry for plaque areas in the aorta using Image J software. The degree of the lesions in the aorta and the en-face arterial tree was determined by the investigators masked to the study groups.

in-situ PRODUCTION OF ROS (REACTIVE OXYGEN SPECIES)

The ROS development *in situ* within the cryosections of aortic root was evaluated using stained sections with dihydroethidium (DHE) through fluorescence microscopy as stated earlier. With NIH Image J software, the fluorescence was quantified.

REAL-TIME PCR

As stated earlier (Liu et al. 2015), real-time PCR was performed. For the analysis, the oligonucleotide primers were used. Target gene levels of mRNA concerning the GAPDH were evaluated and quantified by qRT-PCR. The resulting increase/reduction against control cells was determined using the ($2^{-\Delta\Delta Ct}$).

WESTERN BLOT ANALYSIS

As mentioned previously, Western blot analysis was performed. The concentration of Matrix Metallopeptidase-9, Lectin-type oxidised LDL receptor-1, ICAM-1, VAM-1 and Nicotinamide adenine dinucleotide-oxidase subunit 4 were tested using antibodies such as rabbit anti- Metallopeptidase-9,

Lectin-type oxidised LDL receptor-1, ICAM-1, VAM-1 and Nicotinamide adenine dinucleotide-oxidase subunit 4antibodies.MouseIgG1 isotype antibodies were procured from Sigma-Aldrich. The protein band's densitometry was calculated using BioRad Quantity-One software and provided data as the band intensity ratio relative to the α-tubulin.

GELATINZYMOGRAPHY

DHA was pretreated for 3 h with rat aortic smooth muscles cells (SMC) and stimulated for 24 h with oxLDL (80 μg/mL). As previously reported in conditioned media, gelatinolytic effects of MMP-9 were evaluated (Guo et al. 2020). The intensity of the band was measured using the software Quantity One (Bio-Rad).

STATISTICAL ANALYSIS

For multivariate comparisons, a One-way ANOVA and Bonferroni post-hoc were used, or if appropriate, an unpaired t-test for comparing two groups. The statistical significance of the difference was determined. The GraphPad Prism was used to analyze the tests with P value less than 0.05 as statistically significant.

RESULTS AND DISCUSSION

TOXIC EFFECTS OF DHA ON HAECS

The cell viability was determined utilizing the MTT method. The cytotoxic effects for HAECs with TNF-α (2 ng/mL) at 6 h were not achieved. Cell viability was 87.27+3.1, 112.7+9.3, 102.7+6.7, 88.3+6.2, 67.5+ 4.3, 21.5+2.1, or 0+0% of control values, the three highest doses showing the substantial decline in the cell viability after 24 h incubation of 1, 10, 20, 30, 40, 50 or 60 μM DHA. Current research on DHA treatment had shown no toxic effect on HAECs and commendably suppressed Vascular CAM-1 expression both after *in-vitro* TNF-α stimulation of human aortic endothelial cells and *in-vivo* high fed cholesterol-fed ApoE-/−mice.

DHA REDUCED CELL SURFACE EXPRESSION OF VCAM-1 INDUCED BY TNF-α IN HAECS

Treatment with TNF-α (2 ng/mL) was found to considerably upregulate the cell surface expression of Vascular CAM, endothelial leukocyte adhesion molecule-1 and ICAM-1 at 6 h of treatment. The DHA effects on HAEC expression of Vascular CAM, endothelial leukocyte adhesion molecule-1 and ICAM-1 induced by TNF-α were investigated by pretreating human aortic endothelial cells with DHA (5 mM) for 20 h before adding TNF-α (2 ng/mL), which caused decrease in VCAM-1 cell surface expression (65.3+2.2% expression relative to Tumor Necrosis Factor-α-treated human aortic endothelial cells), with no action on ICAM-1 and E-selectin-1 cell surface expression (Figure 1(A)). The 24 h expression of DHA adhesion molecules was nearly similar relative to 18-hour therapeutic effects of DHA on CAM expression. The Vascular CAM-1 expression declined to 65.3+2.2% with TNF-α treated human aortic endothelial cell. There was, however, no important difference in the expression of Intercellular Adhesion Molecule-1and E-Select in between DHA-treated and untreated groups. Our findings observed that DHA pretreatment considerably reduced the expression of TNF-α induced Vascular CAM-1 in human aortic endothelial cells and DHA treatment also decreased the expression of VCAM-1 and caused decrease thickness of the intima in ApoE-/−mice fed with cholesterol. Moreover, the U937 was inhibited to bind with TNF-α activated HAECs and regulated by lowering H_2O_2 in control and TNF-α treated human aortic endothelial cells. Our findings are evident that endothelial dysfunction, like adhesion molecule disruptions, plays a significant role in atherogenesis. The current study shows that TNF-α induced Vascular Cell Adhesion Molecule-1 expression was substantially diminished in human aortic endothelial cells pretreated with DHA, while ICAM-1 and E-selectin expression was not affected (Fong et al. 2016).

THE EFFECTS OF DHA AND TNF-A ON THE CYTOPLASMIC EXPRESSION OF VASCULAR CAM, ENDOTHELIAL LEUKOCYTE ADHESION MOLECULE-1 AND ICAM-1 IN HAECS

Western cell lysate blot analysis was carried out to assess the cytosolic adhesion molecules expression. The cellular lysates western blot analysis was conducted to examine cytoplasmic adhesion molecules (CAM) expression. As Figure 1(B) shows that the amount of Intercellular Adhesion Molecule-1and Vascular Cell Adhesion Molecule-1 in control human aortic endothelial cells was very low, but TNF-α treatment enhanced their expression as expected. Interestingly, HAECs found E-selectin to be expressed constitutively. TNF-α mediated Vascular Cell Adhesion Molecule-1 expression activation was significantly inhibited by

DHA pretreatment but did not affect TNF-α-mediated activation by Intercellular Adhesion Molecule-1. TNF-α did not affect E-selectin expression, with or without DHA. This was the first paper used by HAECs as a model to estimate the DHA effect on the CAM expression. In the presence of receptor-mediated signals such as TNF-α, DHA was unlikely to affect the early intercellular signals generated by ligand's interaction with the receptor. This indicates that Vascular CAM, endothelial leukocyte adhesion molecule-1 and ICAM-1 instantaneously exchange similar regulatory signals after the stimulation of receptor involving mechanisms of gene-specific signaling pathways. Based on the possible Vascular CAM-1 involvement in the monocyte recruitment to initial atherosclerotic lesions, our results indicate the mechanism of DHA in the prevention of atherosclerosis. However, exact fundamental mechanisms for preventing inhibition of DHA treatment with gene expression of ICAM-1 and E-select in was a major unanswered question.

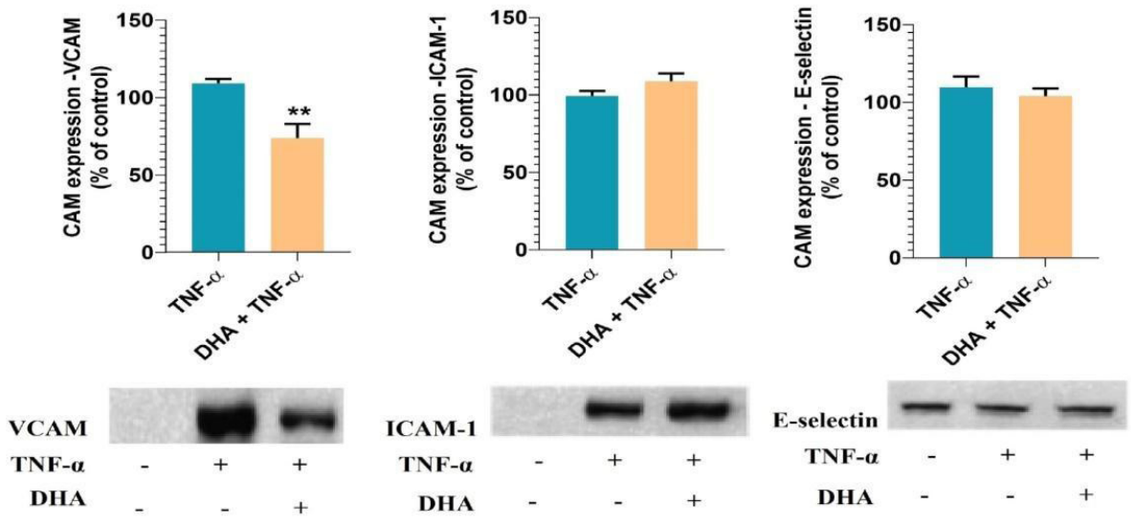

FIGURE 1. Effect of DHA on adhesion molecule expression in HAECs. (A) Control cells or cells pretreated for 18 h with DA were treated for 6 h with TNF-α (2 ng/ml), and expression of the adhesion molecules, VCAM-1, ICAM-1, and E-selectin, was measured by cell-ELISA. Data are expressed as the mean + SEM of three experiments performed in triplicate. **P < 0.01 compared to TNF-α-treated HAECs. (B) Western blot analysis of VCAM-1, ICAM-1, and E-selectin protein levels in cultured HAECs. Three independent experiments gave similar results.

DHA REDUCES U937 CELL-ADHESION TO TNF-A-STIMULATED HAECS

To examine DHA's impact on endothelial interactions, we investigated U937 cell adhesion to human aortic endothelial cells that are activated by cytokines under standard conditions. The confluent human aortic endothelial cells controls showed that U937 cell adhesion was negligible, but binding by TNF-α treatment of HAECs was considerably enhanced (Figure 2(A)). HAEC confluence with DHA (5 mM) suppressed U937 cell adhesion to the HAECs before treatment with tumor necrosis factor-α (Figure 2(A)). The amount of U937 cells attached to TNF-α activated human aortic endothelial cells was reduced by HAECs pretreatment with 0.625, 1.25, 2.5, 5 or 10 mM DHA (Figure 2(B)) with TNF-α-treated HAECs (5- or 10-mM DA).

DHA DECREASES NUCLEAR FACTOR-KB EXPRESSION ACTIVATION AND NUCLEAR FACTOR-KB P65 TRANSLOCATION IN TUMOR NECROSIS FACTOR-A STIMULATED HAECS

A Gel-shift assay analysis was done using a ^{32}P-labeled oligonucleotide consisting of a consensus NF-κB binding sequence to investigate if DHA inhibited Nuclear Factor-κB activation. In the adhesion molecule

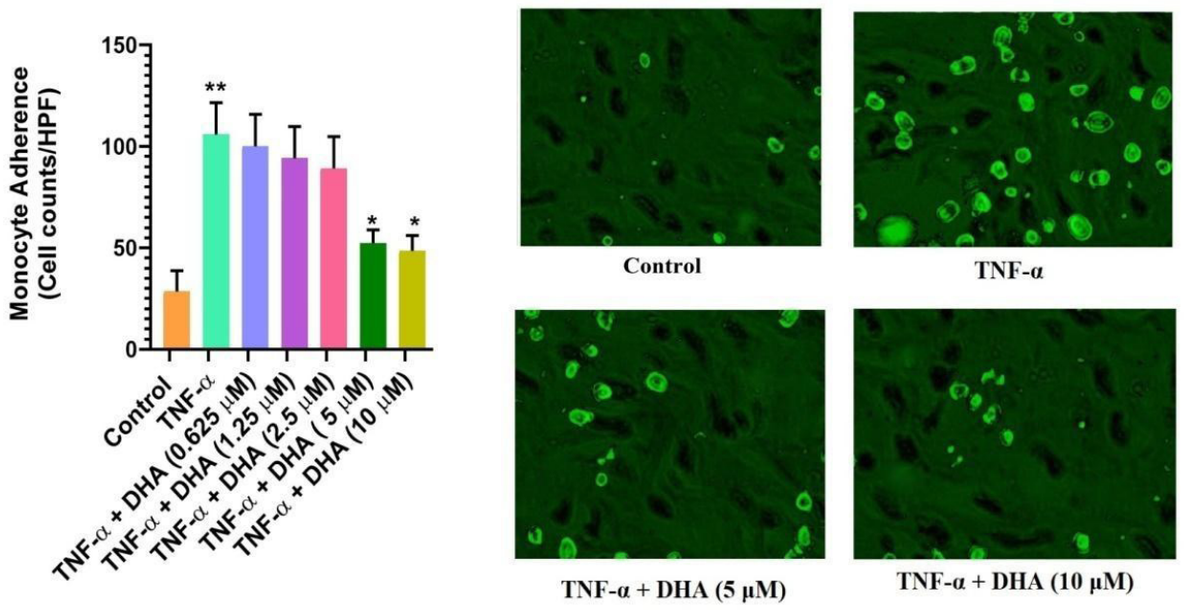

FIGURE 2. Effect of DHA on adhesion of U937 cells to TNF-α stimulated HAECs. Control or HAEC cells pre-treated for 18 h with DHA were incubated with TNF-α (2 ng/ml for 6 h). A) Representative fluorescent photo-micrographs showing the effect of pretreatment with 5 mM DA on the TNF-α-induced adhesion of fluorescein-labelled U937 cells to HAECs. (B) Confluent HAECs were pre-incubated with the indicated concentration of DHA, then incubated with TNF-α for 6 h. The data are representative of three experiments and the value are reported as the mean number of U937 cells bound per high power field (HPF) ± SEM. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to control and TNF-α alone respectively

expressions caused by cytokines, transcriptional modulation, including NF-κB activation, was involved. At 37 °C, pre-incubated HAECs with 5mM DHA were then stimulated for around 30 min with TNF-α. The TNF-α treatment showed that there were shifted bands (Figure 3(A)) that are unique to NF-κB binding. DHA pretreatment decreased the Nuclear Factor-kB shift bands density enhanced by Tumor Necrosis Factor-α (Figure 3). In the nuclei of Tumor Necrosis Factor-α treated human aortic endothelial cells via immune-fluorescence and Western blots, NF-κB P65 protein concentrations were also considered to define if activation of NF-κB included in the pre-translation behavior of DHA on the expression of adhesion molecules. In the nucleus, TNF-α activated HAECs demonstrated pronounced staining of the NF-κB p65, whereas DHA-pretreated cells indicated decreased expression of NF-κB but increased cytoplasmic staining (Figure 3(B)). The western blots (Figure 3(C)) showed increased Nuclear Factor-κB p65 protein levels in Tumor Necrosis Factor-α stimulated human aortic endothelial cells nuclei relative to the HAECs as control. However, NF-κB expression significantly reduced after pretreatment with DHA. β-actin. The transcription factor (NF-κB) is the main element that is the reason behind TNF-alpha induced adhesion molecule expression. In this study, we were shown that stimulation of TNF-α causes the endothelial expression of NF-κB and suggests that the up-regulation of expression of VCAM-1 triggered by TNF-α mediated through transcriptional factor (NF-κB). However, NF-κB was ubiquitous transcription factor stimulated by distinct signals, through the IκB phosphorylation, followed by the dissociation from the inactive complex of the cytoplasm, thus causing translocation of active dimmers (p50 and p65) onto the nucleus.

The Nuclear Factor-κB drove viral replication of HIV-1 is prevented by antioxidants, especially N-Acetyl Cysteine and other cysteine derivatives (Poles et al. 2021). The Nuclear Factor-kB is inhibited with antioxidants, PDTC and NAC in many immortalized cell lines through several stimulating agents such as LPS, Inter Leukin-1β, PIC, and TNF-α. Our analysis showed a similar

trend of antioxidant (DHA) - the reactive expression of VCAM-1 inactivation and behavior of NF-κB in HAECs. Besides, our findings offer the concept that NF-κB factors are essential for endothelial cell activation of VCAM-1 gene expression (Kleinholz et al. 2021), they do raise significant issues regarding the functioning of Nuclear Factor-kB in endothelial leukocyte adhesion molecule-1 and ICAM-1. Based on DNA evaluation of the transfection from the deleted E-select in gene promoting structures, an NF-κB consensus portion of the cis-acting promoter was found to be crucial for the transcriptional IL-1β action (Gal-Ben-Ari et al. 2018). We observed, however, that DHA was not inhibited by induction of E-select in. DHA suppressed the NF-κB activation, however, the E-select in and ICAM-1 expressions were not altered implying that NF-κB transcription factors are important elements in the E-select in and ICAM-1 gene activation.

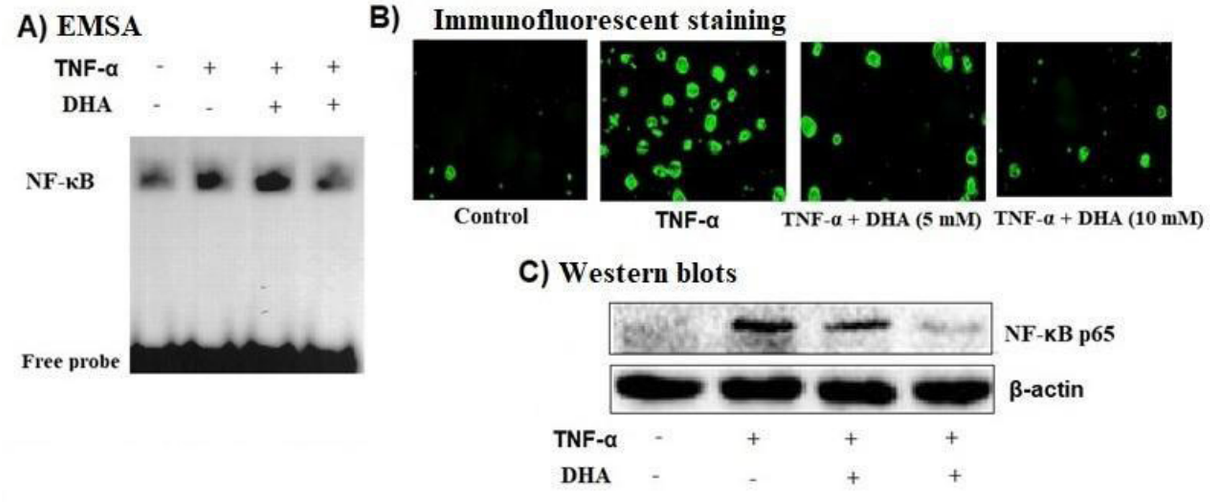

FIGURE 3. Effect of DHA on NF-κB activation in TNF-α-stimulated HAECs. Control cells or cells were pretreated for 18 h with 5mM DA, then stimulated with 2 ng/ml TNF-α for 30 min at 37°C. (A) EMSA, showing the effect of DA on TNF-α-stimulated HAECs. Nuclear extracts were prepared and tested for DNA binding activity of NF-κB. A representative result from three seperate experiments is shown. (B) Immunofluorescent staining for NF-κB p65. (C) Western blotting of NF-κB in nuclear extracts oh HAECs. The results are representiva of three separate

DHA SUPPRESSES HYDROGEN PEROXIDE PRODUCTION IN CONTROL AND TNF-A TREATED HAECS

The DHA effects on the production of H_2O_2 in HAECs were analyzed. Non-activated (non-incorporated cytokine) treatment with DHA (5 or 10 mM) reduced fluorescence intensity of DCF relative to untreated DCF fluorescence control (Figure 4(A)). Pretreatment with DHA had no substantial effect on 15 min fluorescence measurement under oxidative stress conditions (TNF-α stimulation), but there had been a significant concentration-dependent decrease in the generation of H_2O_2 for all time points between 30-60 min (Figure 4(B)). Evidence of several reports suggests that Reaction Oxygen Species are involved in the Nuclear Factor-κB activation and serves as a secondary messenger for the activation of NF-κB through numerous stimulus-specific pathways. Potent antioxidants such as PDTC and NAC prevent the activation of NF-κB not only through hydrogen peroxide and ionizing radiation but also through non-oxidant stimuli. Malekmohammad et al. (2019) argued that ox-LDL or TNF-α enhances the production of endothelial CAM expression through the NF-κB activation and the presence of radical scavenging activity compounds prevent the impact on the production of endothelial CAMs expression, indicating that the NF-κB-mediated expression of adhesion molecules that follows the stimulation of several radical-producing processes. The recent research demonstrated that DHA explicitly

scavenges hydrogen peroxide and noticeably reduced the H_2O_2 levels produced by HAECs under oxidative stress. Our findings suggested the DHA's inhibitory effect could be attributed to its antioxidant activities on the expression of VCAM-1 and NF-κB stimulation and that it functions by explicitly scavenging H_2O_2. Moreover, reducing lipid peroxidation by DHA can also lead to the inactivation of NF-κB. The crucial steps of NF-kB activation by ROS in the signal transduction cascade, however, remain to be determined.

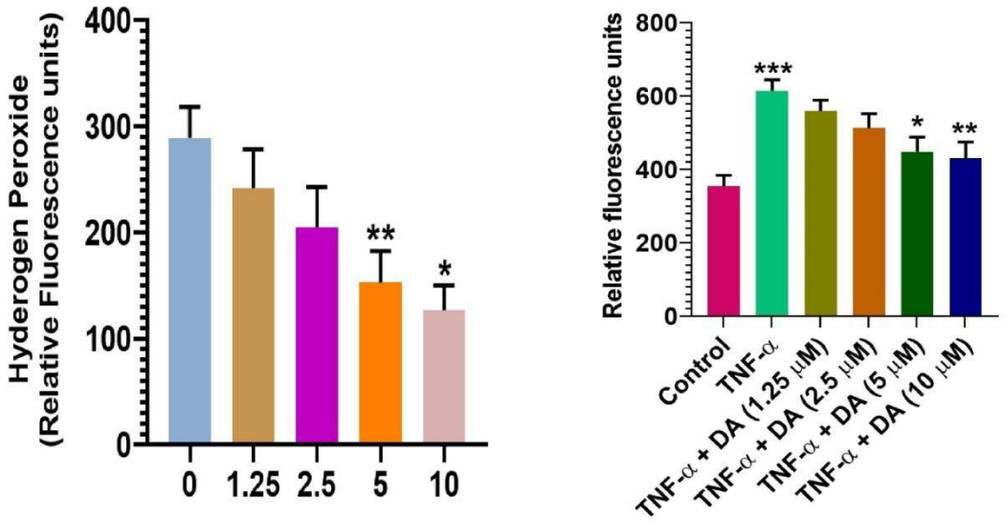

FIGURE 4. Effect of DHA on intracellular H_2O_2 production in HAECs. (A) HAECs in 48-well plates were incubated for 18 h with 0, 1.25, 2.5, 5, or 10 mM DA, washed three times with HBSS to remove DHA, then DCFH-DA was added to the wells. After 45 min incuation at 37oC, DCF fluorescence was measured. The data are the mean+ SEM of three independent experiments (with seperate endothelial cells isolates) performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ compared to the DHA-untreated control. (B) Effects of DHA on H_2O_2 generation by TNF-α-stimulated for 18 h with 0, 1.25, 2.5, 5, or 10mM DHA, then stimulated wotj ng/ml of TNF-α for 1 h. A significant difference ($^{***}P < 0.02$)compard to control samples was seen after 60 min. *$P < 0.05$ and **$P < 0.01$ compared to the TNF-α-treated HAECs

DHA SUPPRESSES PROGRESS OF ATHEROSCLEROTIC PLAQUE IN APOE−/− MICE

The effectiveness of DHA was tested in dietary atherosclerosis in ApoE /-mice. DHA therapy (15 and 45 mg/ kg/day) considerably improved the production of atherosclerotic lesions in the *en face* aorta (Figure 5(A) & 5(B)) and aortic sinus (Figure 5(C) & 5(D)) of ApoE-/mice administered with high cholesterol diet for 16 weeks relative to the vehicle control group. An evaluation of pro-inflammatory serum cytokines and lipid profile showed that DHA substantially decreased the pro-inflammatory cytokines serum levels of-1β, -6, -17A, IFN-γ and TNF-α (Figure 6). These findings show that DHA had strong anti-inflammatory and anti-atherosclerotic effects regardless of the serum lipid content in experimental atherosclerosis. Our study outcomes examined the DHA's effect on the atherosclerosis progression in high cholesterol diet-fed ApoE-/-mice, an established atherogenesis model. Our results indicated that intervention with DHA (10 or 20 mg/kg/day) significantly reduced atherosclerotic plaques in aortic sinuses and *en face* aorta and improved the plaque steadiness from further progression, as observed in necrotic core regions and macrophage aggregation and elevated α-SMC and collagen levels, supporting-*vitro* results showing DHA with vasculo-protective effects in macrophages.

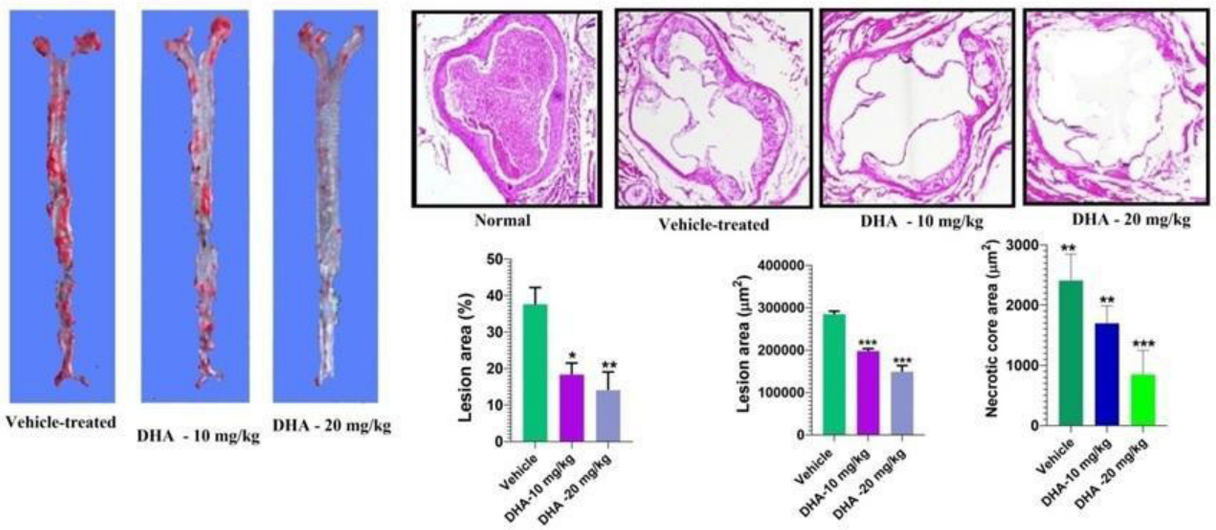

FIGURE 5. DHA attenuates atherosclerotic lesion size in ApoE-/- muce. (A) Atherosclerosis in the arterial tree was evaluated by Old Red O Stainig. (B) Quantification of Oil Red O-positive areas in en face aorta by NIH ImageJ software. n = 6 for each group. (C) Representative photomicrographs of Oil Red O staining of the athresclerotic lesions in the arotice sinus. Original magnification, x100.(D) Morphometri analysis of atherosclerotic lesion size and necrotic core area in aortic sinus. Avearage sizes of atherosclerotic lesions were calculated from eight sections in ApoE-/- mice feed an atherogenic diet. n = 10 for each group. *P < 0.05, **P < 0.01, **P < 0.001 compared with vehicle group respectively. Scale bar = 200 μm

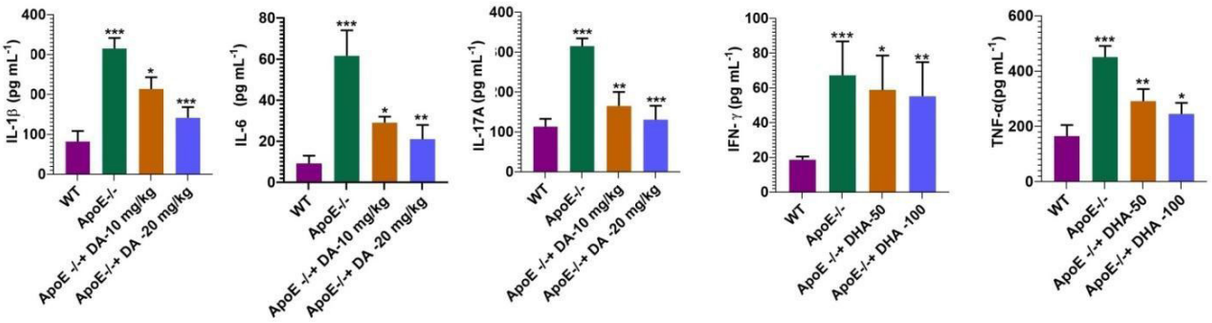

FIGURE 6. Serum levels of pro-inflammatory mediators in ApoE-/- mice and treatment groups. Data are represented as mean ± SEM. ***P < 0.001 compared with Control group (n = 10 per group). *P < 0.05. ** P < 0.01. ***P < 0.001 compared with ApoE-/- group (treated with vehicle control)

DHA IMPROVES PLAQUE STABILITY CHARACTERISTICS IN APOE−/− MICE

In ApoE-/- mice, we examined plaque composition to further analyze the DHA impact on plaque morphology. In DHA-treated mice, atherosclerotic plaques formed reduced necrotic core regions, as shown in Figure 7(A) pronounced reduction in macrophage levels was seen in the CD68immunostaining in DHA-treated plaques related atherosclerosis compared to control mice treated with vehicle. In contrast, in atherosclerotic lesions induced by DHA-treated mice, as described by Masson's

staining trichomes, an intense surge in collagen levels was observed (Figure 7(B)). MMP-9 is a key factor that decides the malfunction of atherosclerotic plaque. The effect of DHA on the expression of MMP-9 was investigated. Surprisingly, no noticeable change in Matrix Metallo-proteinase-9 expressions in oxidized low-density lipoprotein treated macrophages was observed. However, DHA interventions in SMCs significantly reduced the MMP-9 expression and action of oxLDL in a dose-dependent approach (Figure 8). Following this result, the immunohistochemistry analysis found that MMP-9 expression in the SMC lesion in DHA-treated ApoE−/− plaques were decreased relative to control mice treated with vehicle (Figure 9). The α-SMA stained area of the atherosclerotic plague was substantially improved in the group treated with DHA. Our findings were shown that DHA suppression of the expression of NF-κB in plaques of atherosclerosis and decreases serum pro-inflammatory cytokines levels in ApoE-/-mice. For the first time, this research demonstrates the efficacy of DHA in the suppression of LOX-1 expression in lesions of atherosclerosis, signifying the anti-atherosclerotic effect with a decline in the expression of LOX-1. Further, we found that DHA treatments were mitigated ROS development and activation in the arterial wall of redox-sensitive NF-κB indicating that the anti-atherosclerotic effect of DHA is partially controlled by an inhibitory effect on oxidative stress.

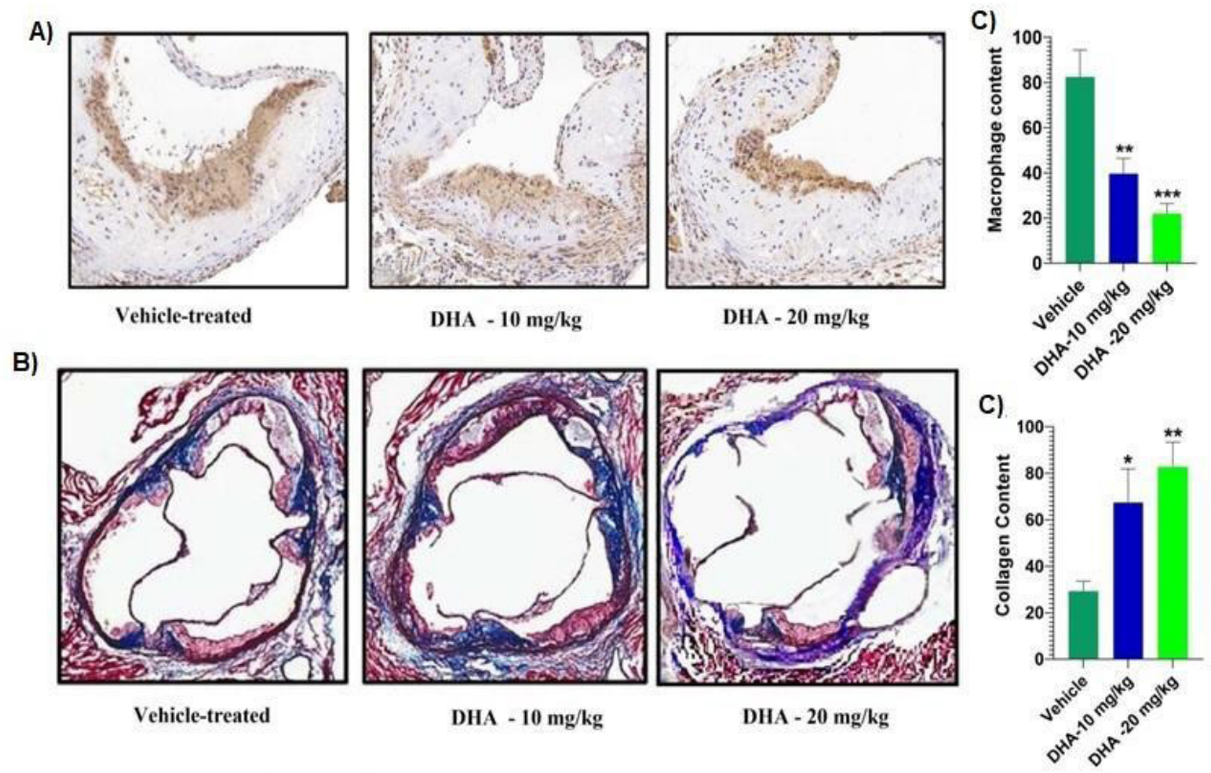

FIGURE 7. DHA induces features of atherosclerotic plaque stability in ApoE-/- mice, Photomicrographs of sectione aortic sinus were immunohistochemically stained for macrophage depositin (DC68 positive, panel B, original magnification, x100), and collagen content (indicated in blue colour with Masson's trichrome staining, panel C, original magnification, x100). Corresponding positive areas (CD68 and collagen content) were analyzed and quantified using Image-Pro Plus 6.0 software and the percentages of the above plaque components in the entire plaque were evaluated. n = 6 for each group. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$ compared with vehicle group respectively. Scale bar = 200 μm

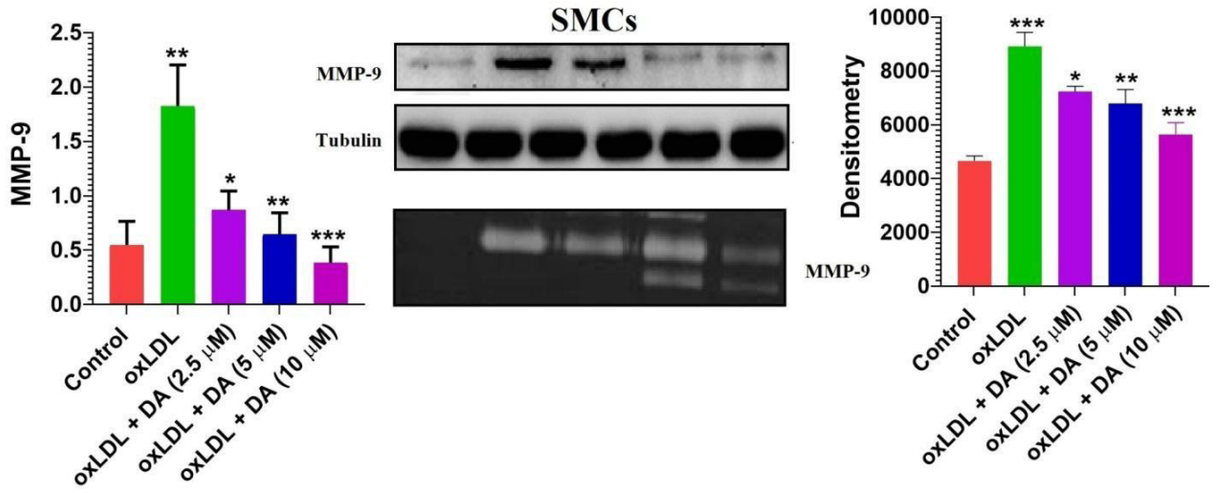

FIGURE 8. -DA reduces MMP-9 expression and activity. Effect of DHA on oxLDL-stimulated MMP-9 expression and activity in SMCs. Rat aortic SMCs were pretreated with DHA for 3 h then stimulated with oxLDL (80 μg/mL) for 24 h. (A) Whole cell lysates were subjected to MMP-9 protein expression. (B) The conditioned medium from each treatment group was collected and subject to gelatin zymography n= 6. $P < 0.01$. ** $P < 0.01$ and $^{***}P < 0.001$ compared with untreated control group; $^{*}P < 0.05$, ** $P < 0.01$ & $^{***}P < 0.001$ compared with oxLDL-treated group respectively

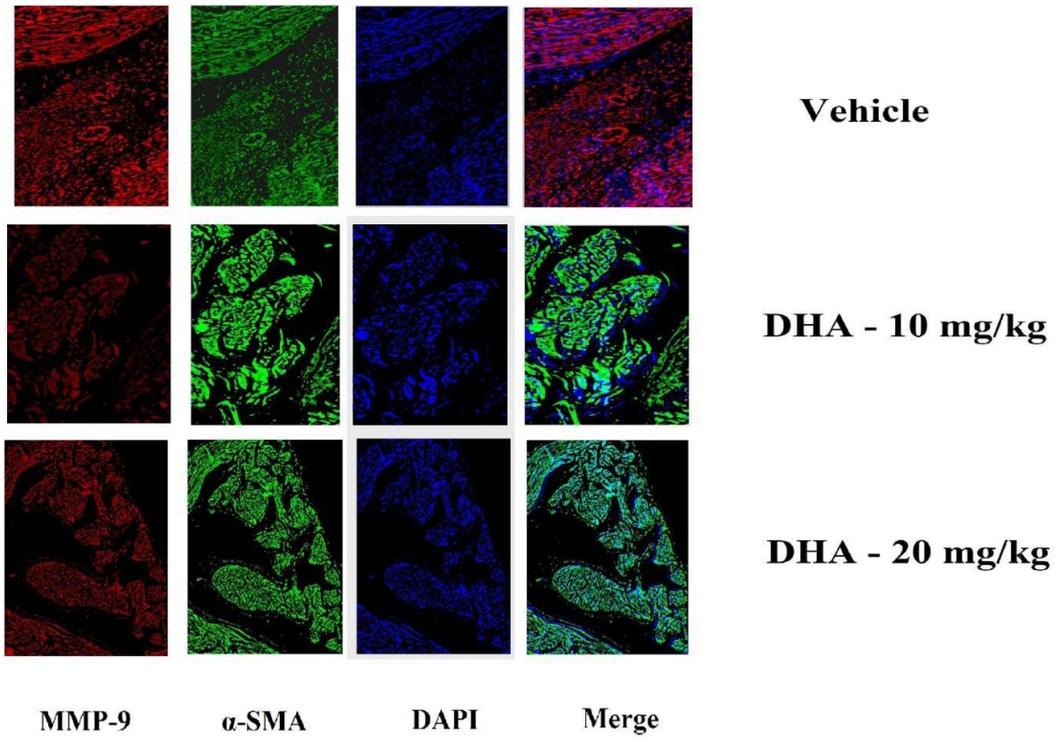

FIGURE 9. DHA reduces MMP-9 expression and activity showing MMP-9 immunohistochemical staining (red) or aortic sinus from ApoE-/- mice receiving DHA or vehicle treatment. SMCs were stained with α-SMA (green) and nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (blue). Scale bar, 50 μm. n = 8 for each group

DHA suppresses ROS production, LOX-1 expression and activation of NF-kB in APOE−/− mice

Lectin-type oxidized LDL receptor 1 is a crucial scavenging receptor involved in the instigation and development of atherosclerosis. We assessed if DHA inhibits Lectin-type oxidized LDL receptor-1 mediated signaling pathway in ECs to defend against atherosclerosis. Immunohistochemistry findings of the vehicle and DHA-treated ApoE-/-mice in the aortic sinus indicate that DHA suppressed the dose-related expression of aortic LOX-1 (Figure 10). Additional investigation of ROS production by DHA staining *in-situ* showed that DHA suppressed ROS production substantially (Figure 10). Besides, immune staining evaluation showed that NF-κB activation was prominently augmented in the aortic regions of ApoE-/-mice fed with high cholesterol diet and that activation was substantially dose-dependently inhibited by DHA (Figure 10). Previous studies have shown that by controlling multiple signaling pathways, DHA provides cytoprotective actions on neurodegenerative and cardiovascular diseases, cerebrovascular disorders and tumors. The potential benefits of DHA on atherosclerosis progression might therefore be a cumulative product of different interventions and LOX-1 could be the main regulators. It is important to decide if the beneficial effects of DHA on the progression of the atherosclerotic plaque were eliminated in EC-specific LOX-1 and systemic LOX-1 knock-out mice, which needs further studies to be performed in future. The limitations of the study are 1) Effect of DHA on macrophage secretion of CCL2 (MCP-1) and monocytic cell migration induced by 27-hydroxycholesterol was studied. 2) The effect of DHA on M1 polarization induced by 27-hydroxycholesterol have not been investigated. 3) In order to understand that DHA reduced MMP-9 expression of smooth muscle cells, but not of macrophages, immunoreactivity of

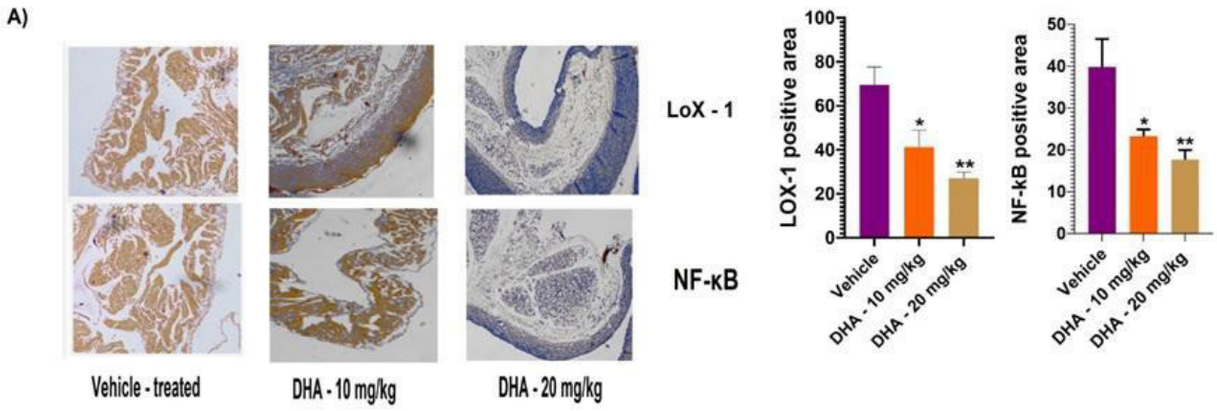

FIGURE 10. DHA decreases the expression of LOX-1 and NF-κB p65 within the atherosclerotic lesions. (A) Effect of DHA treatment on the expression of LOX-1 and NF-κB p65 in the aorta by immunohistochemistry. (B) Quantitative morphometric analysis of the expression of LOX-1 and NF-κB p65 relative to total plaque size. n = 10 for each group. $^{*}P < 0.05$, $^{**}P < 0.01$ & $^{***}P < 0.001$ compared with vehicle group respectively. Scale bar = 200 μm

macrophage marker (e.g. MOMA-2) needs to be studied.

Conclusions

The study findings showed that DHA suppresses ROS generation in OxLDL-exposed ECs. In the preliminary process of atherosclerosis, OxLDL-mediated endothelial cell stimulation by ROS-triggered signal transduction was essential for the dysfunction of the endothelium It was shown that LOX-1 expression can be induced by ROS and LOX-1 stimulation, in turn, promotes ROS production that produces a major cycle of disturbance between ROS and LOX-1.The adhesion of oxLDL to LOX-1 improves the development of ROS and the activation of NF-κB that regulates the LOX-1expression and activity, thus amplifying the oxLDL vicious cycle. Thus, the blocking

of the signaling trajectory mediated by LOX-1 by CTS's binding to LOX-1 was a possible mechanism for protection of endothelial dysfunction by oxLDL, which helps to generate excessive Intercellular ROS and to activate the signaling pathway NF-κB. DHA's effects on LOX-1 and MMP-9 expression were further examined, and results suggest that DHA was active in reducing LOX-1 expression and MMP-9 activities.

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*Corresponding author; email: tezhenwuyan@163.com