

All-trans retinoic acid reduces endothelin-1 expression and increases endothelial nitric oxide synthase phosphorylation in rabbits with atherosclerosis

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Abstract. All-trans retinoic acid (ATRA) is a natural derivative of vitamin A that ameliorates atherosclerosis (AS) by regulating inflammatory factors. However, studies concerning the role of retinoic acid in artery endothelial function are rare. Therefore, the present study investigated its role in regulating the production of endothelin-1 (ET-1) and nitric oxide (NO) in rabbits with AS. The rabbits were randomly divided into 3 groups: The control group was administered an ordinary diet, while the high fat group and the ATRA drug intervention group were administered a high fat diet. After 12 weeks, the blood lipid levels of rabbits, the morphological structure of the arterial wall, the arterial intimal permeability, the activity of blood endothelial nitric oxide synthase (eNOS) and the level of plasma NO were investigated. Western blot analysis was used to detect the levels of ET-1, eNOS and eNOS phosphorylation at Ser-1177 (p-eNOS), and a radioimmunoassay was performed to detect the level of ET-1 in the plasma. It was identified that plaque formation was alleviated in the ATRA group compared with the high fat group, as revealed by hematoxylin and eosin and oil red O staining, and a similar trend was reflected in the immunofluorescence results for endothelial permeability. Western blotting demonstrated significantly decreased ET-1 expression levels in the arterial tissue of rabbits in the ATRA group compared with the high fat

group, together with increased p-eNOS level ($P < 0.05$), however, no difference was observed in the expression of eNOS ($P > 0.05$). The trends observed for ET-1 and the activity of eNOS in plasma were similar to those for arterial tissue. Therefore, the present study demonstrated that ATRA may regulate the grade of AS by the reduction of ET-1 secretion and increased NO formation via increased phosphorylation of eNOS. ATRA provides a potential novel method for the treatment of atherosclerosis.

Introduction

Atherosclerosis (AS) is an important factor leading to cardiovascular and cerebrovascular diseases, which have a high morbidity and mortality. The formation mechanisms of AS are complicated and diverse (1,2), while the structural and functional injury of endothelial cells (ECs) is regarded as the initial step of AS and influences the grade of AS (3,4). Vasomotor dysfunction is the primary presentation of AS, therefore, the imbalance of factors that affect vascular contraction and relaxation serve important roles in its pathogenesis (5,6). Several types of cells, particularly ECs, secrete factors involved in autoregulation, including nitric oxide (NO) and endothelin (ET), thus regulating vasomotor function (7-9).

The major functions of NO are to sustain vascular tension, regulate blood pressure, inhibit the migration and hyperplasia of vascular smooth muscle cells, and influence the vasomotor function (10). The formation of NO is regulated primarily by endothelial NO synthase (eNOS) (11). ET, which is associated with various human diseases, is comprised of three isomers (ET-1, ET-2 and ET-3). At present, ET-1 is regarded as the most potent endothelial-independent vasoconstrictor peptide (12,13). The levels of NO and ET-1 that regulate vascular endothelial function are important in the AS process.

All-trans retinoic acid (ATRA), a natural derivative of vitamin A, exerts extensive biological effects (14). As indicated in previous studies, it inhibits the migration and hyperplasia of smooth muscle cells, in addition to possessing anti-inflammatory and antifibrotic functions (15-17). However, studies investigating the effects of ATRA on the secretion of NO and ET-1 are rare. The present study replicated an AS model to analyze the effects of ATRA on AS rabbits, and compared the

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expression of ET-1, NO, eNOS and phosphorylated (p)-eNOS (Ser-1177) levels in the plasma and arterial tissue, in addition to determining the permeability of the arterial wall, in order to investigate the potential mechanism of ATRA on AS and to provide a novel basis for the treatment of AS in the clinic.

Materials and methods

Duplication of AS model. A total of 24 male New Zealand pure breed white rabbits (age, 10 weeks; weight 1.8 ± 0.2 kg) were provided by Qingdao Shandong Kangda group. Animals were housed at 22°C, 50% relative humidity, 0.03% CO₂ and 12 h light/dark cycle. The rabbits were fed standard diet to acclimate for 1 week prior to being randomly divided into three groups (n=8): Group A was the control group, which was administered an ordinary diet; group B was the high fat group; and group C was the ATRA (provided by the Institute of Pharmacology, Anhui Medical University, Hefei, China) intervention group. Groups B and C were administered high fat feed (94% ordinary feed, 1% cholesterol and 5% lard), and all feed was purchased from the Laboratory Animal Center of Anhui Medical University (Hefei, China). The ATRA group received gavage (10 mg/kg/day) from week 2 (18). At the end of week 12, the animals were sacrificed to obtain blood and arterial specimens for fixation and analysis. All procedures were approved by the Internal Animal Care and Use Committee of Anhui Medical University.

Specimen preparation. For anesthesia, 3% pentobarbital sodium was administered through the auricular vein and the carotid arterial blood collected. After standing for 2 h, the blood was centrifuged for 10 min at 1,006 x g, and the upper serum was removed and stored at -80°C. The animals were dissected through the abdomen, the blood was drained and the aorta was separated as soon as possible, sections of which were embedded with optimal cutting temperature compound (OCT) following the above procedures, other sections were frozen with liquid nitrogen and stored at -80°C until use, prepared for oil red O staining or were fixed with 4% paraformaldehyde at room temperature for 24 h followed by embedding in paraffin and sectioning at 4 μ m.

Reagents. Triglyceride (TG; cat. no. 0949-2008) and total cholesterol (TC; cat. no. 1568-2003) assay kits were provided by Zhejiang Dong'ou Diagnostic Products Co., Ltd. (Wenzhou, China). The plasma radioimmunoassay ET-1 kit (cat. no. D11PJA) was purchased from the Beijing North Institute of Biological Technology (Beijing, China). Low-density lipoprotein (LDL; cat. no. A113-2), high-density lipoprotein (HDL; cat. no. A112-2) assay kits, endothelial nitric oxide synthase assay kit (eNOS; cat. no. H195) and a nitrate reductase NO kit (cat. no. A012) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Sulfo-NHS-LC-biotin was purchased from Pierce (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Anti- β -actin (cat. no. BM0627) antibody was obtained from Wuhan Boster Biological Technology, Ltd. (Wuhan, China), and ET-1 (cat. no. sc-517436), eNOS (cat. no. sc-136977) and horseradish peroxidase-conjugated anti-mouse IgG (cat. no. sc-2005) and anti-rabbit IgG (cat. no. sc-2004) antibodies were provided by Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-p-eNOS (Ser-1177; cat. no. 9571s) antibody was

obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Stripping buffer was purchased from Beyotime Institute of Biotechnology (Haimen, China).

Blood lipid detection. An enzyme coupling colorimetric method was utilized to detect the serum TC and TG, while a precipitation method was used to detect the HDL and LDL in model rabbits. All were performed in strict accordance with the manufacturer's instructions for the assay and ELISA kits.

Hematoxylin and eosin and oil red O staining. The paraffin sections from each group were dewaxed and hydrated in a graded series and stained at room temperature with hematoxylin for 10 min and eosin for 5 min, which was followed by mounting in neutral resin. Sections were observed under a light microscope and images were captured. For oil red O staining, the harvested whole specimen of arterial wall was placed in the stock solution (oil red powder 0.5 g dissolved in 100 ml IPA), rinsed with distilled water at a ratio of 6:4 respectively, placed in 40% formaldehyde solution and observed under a light microscope to capture images.

Western blot analysis. A total of 1 ml PBS was added to the frozen aorta, which was cut it into pieces on ice. Subsequently, protein extraction buffer (0.15 mol/l NaCl, 1.5 mmol/l MgCl₂, 10 mmol/l KCl, 10 μ g/ml aprotinin, 0.5 μ g/ml leupeptin, 3 mmol/l phenylmethylsulfonyl fluoride, 3 mmol/l dithiothreitol, 1 mmol/l Na₃VO₄, 10 mmol/l hydroxymethyl aminomethane, 5 mmol/l ethylenediaminetetraacetic acid, pH 7.5) was added to prepare the homogenate, followed by freeze-thawing three times at -80°C and room temperature, and centrifugation for 15 min at 1,006 x g and 4°C. The supernatant was collected and the Lowry *et al* (19) was applied to quantitatively detect the protein concentration. The protein samples (15 μ l/lane) were subjected to 10% SDS/PAGE and electrophoretically transferred onto a polyvinylidene fluoride membrane. Non-specific binding was blocked by 5% skimmed milk at room temperature for 2 h. Western blotting was performed using β -actin (1:2,000 dilution), ET-1 (1:1,000 dilution), eNOS (1:2,000 dilution) and p-eNOS (Ser-1177; 1:400 dilution) primary antibodies. All primary antibodies were incubated at 4°C overnight and the secondary antibodies (1:8,000 dilution) were incubated with membranes at room temperature for 1 h. Enhanced Chemiluminescence reagent (Beyotime Institute of Biotechnology) was used for visualization. All the values were normalized to the levels of β -actin. ImageJ 2 software (National Institutes of Health, Bethesda, MD, USA) was utilized in the quantitative analysis of western blot images.

Detection of ET-1 and NO levels, and eNOS activity in plasma. The whole blood (5 ml, 3.8% sodium citrate anticoagulation) was centrifuged for 10 min at 1,006 x g, and the upper plasma was removed and stored at -80°C and assayed within 2 weeks. ET-1 was measured by using an ET-1 radioimmunoassay kit. Assay kits were used to detect the contents of NO and the activity of eNOS. All the testing steps were performed in accordance with the manufacturers' instructions.

Immunofluorescence for detecting endothelial permeability. The permeability assay using the surface biotinylation

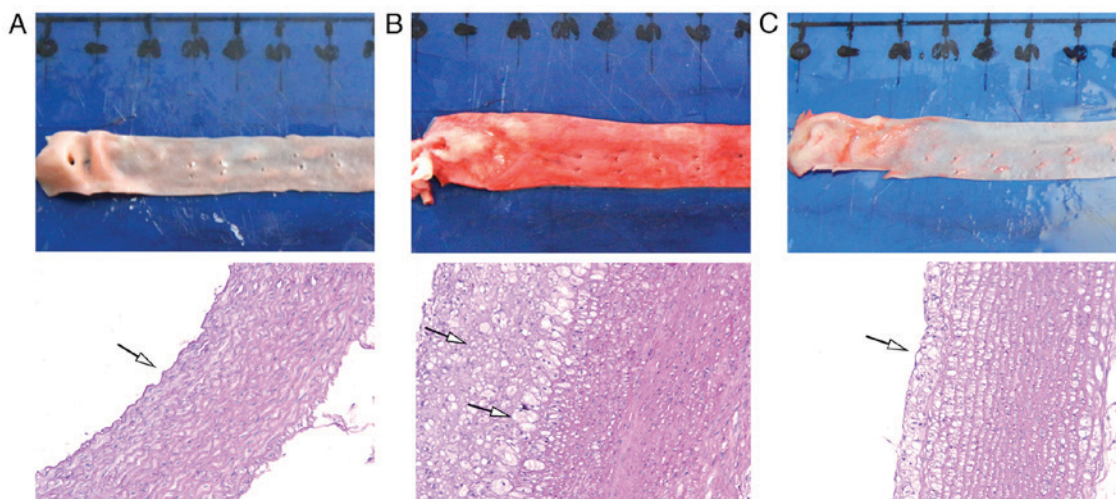


Figure 1. Oil red O and hematoxylin and eosin-stained sections of aorta. Oil red O and hematoxylin and eosin-stained sections (magnification, x200) of aorta from (A) control, (B) high fat and (C) ATRA groups. Atheromatous plaques and foam cells (indicated by the arrows) were evident in the high fat group compared with the control. Following treatment with ATRA, the areas of plaques and the amount of foam cells were reduced. ATRA, all-trans retinoic acid.

technique was performed for the aorta intima as described by Zhu *et al* (20) with certain modifications. The arterial tissue was filled with a freshly made Sulfo-NHS-LC-biotin solution in Hanks Balance Salt Solution (HBS; 137 mM NaCl, 0.4 mM $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 5.3 mM KCl, 0.5 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 1 mM CaCl_2 , 0.3 mM $\text{Na}_2\text{HPO}_4 \times 7\text{H}_2\text{O}$, 0.45 mM KH_2PO_4 , 5.5 mM glucose) containing 1 mM CaCl_2 and 1 mM MgCl_2 at 1 mg/ml for 30 min at room temperature. The aortas were rinsed with PBS three times, 5 min each time embedded in OCT and cryosectioned. Frozen sections (6 μm) were incubated with 5% skimmed milk powder (pH 7.4) at 4°C overnight. Subsequently, the milk was removed and 100 μl Rhodamine600 Avidin D (XRITC-avidin, 1:200, cat. no. A-2005; Vector Laboratories, Inc., Burlingame, CA, USA) was added, followed by incubation at 4°C for 2 h. Rhodamine was then removed, and the sections were rinsed with PBS for 10 min, PBS containing 0.05% Tween-20 for 10 min and with pure PBS twice. The sections were dried at room temperature, mounted and sealed. DAPI staining for 10 min at room temperature was used as a histological control. A fluorescence microscope (Nikon E800; Nikon Corporation, Tokyo, Japan) was used for observation and CCD-SPOT digital camera series (Diagnostic Instruments, Inc., Sterling Heights, MI, USA) was used to capture images. ImageJ 2 software (National Institutes of Health) was used to quantitatively analyze the fluorescence intensity of images.

Statistical analysis. Data are presented as the mean \pm standard deviation, and SPSS statistical software version 16.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Comparisons between each group were performed using one-way analysis of variance and the Student-Newman-Keuls method. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ATRA alleviates atherosclerotic plaque formation in rabbits. Following hematoxylin and eosin and oil red O staining,

the artery intima of the control group was smooth and continuous without intimal thickening, the smooth muscle cells arranged in neat rows and the elastic fibers only slightly disordered. Compared with the control group, the intima of the artery wall in the high fat group was visibly thickened with numerous elastic plate fractures and a large number of plaques; inflammatory and foam cells were identified in the plaques. In the ATRA group, a small amount of plaque formation was observed in the arterial wall, and compared with the high fat group, the foam cells and inflammatory cells were reduced (Fig. 1).

ATRA improves blood lipid levels in atherosclerotic rabbits.

Due to the close association between lipid levels and AS, the serum levels of TC, TG, HDL and LDL in each group were examined (Fig. 2). Compared with the control group, the levels of TC, TG, LDL and HDL were significantly higher in the high fat group ($P < 0.05$). By contrast, the levels of TC, TG and LDL in the ATRA group were lower compared with the high fat group, while HDL levels were higher in the ATRA group ($P < 0.05$).

ATRA reduces artery endothelial permeability of atherosclerotic rabbits.

To determine the effect of ATRA treatment on endothelial permeability, the transport of NHS-LC-biotin across the aortic intima into the media was assessed. The NHS-LC-Biotin concentration profiles were obtained as a function of the radial distance through the aortic wall media layer. Only the endothelial surface of the aorta intima was biotinylated in rabbits fed a normal diet, indicating no paracellular leakage of the NHS-LC-biotin (Fig. 3A), and NHS-LC-biotin leakage into the aortic intima layers was increased in the high fat group (Fig. 3B). However, aortic endothelial permeability was clearly attenuated in the ATRA group compared with the high fat group (Fig. 3C). The permeability in each group was quantified (Fig. 3D).

ATRA affects the expression of ET-1, eNOS and p-eNOS in atherosclerotic rabbit aortas. Western blotting results

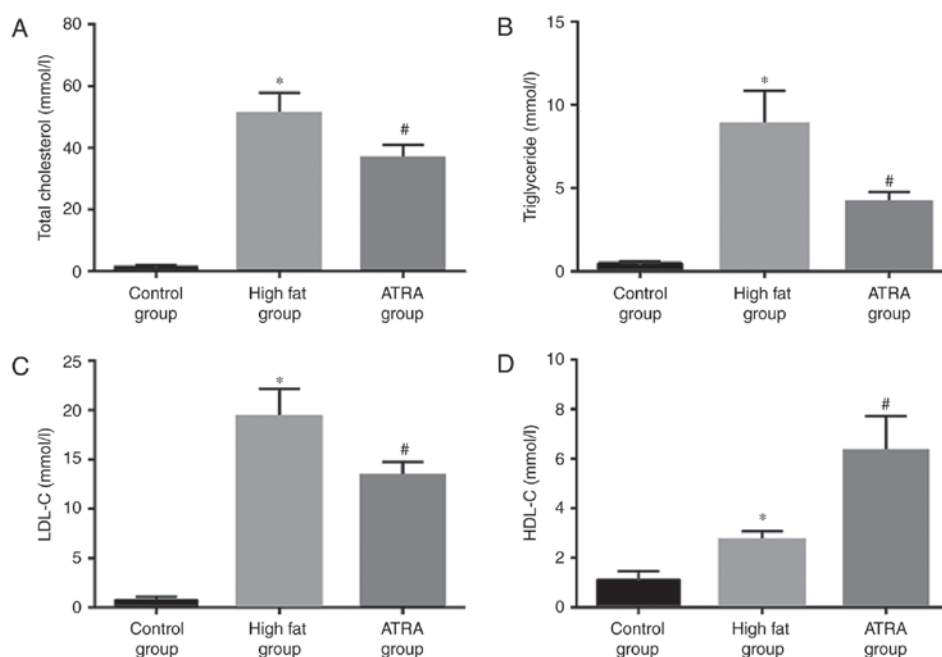


Figure 2. Effect of ATRA on serum lipid levels in atherosclerotic rabbits (n=8). Data are presented as the mean \pm standard deviation. The serum levels of (A) total cholesterol, (B) triglyceride, (C) LDL-C and (D) HDL-C were determined in control, high fat and ATRA groups following 12 weeks of treatment. *P<0.05 vs. control group; #P<0.05 vs. high fat group. ATRA, all-trans retinoic acid; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.

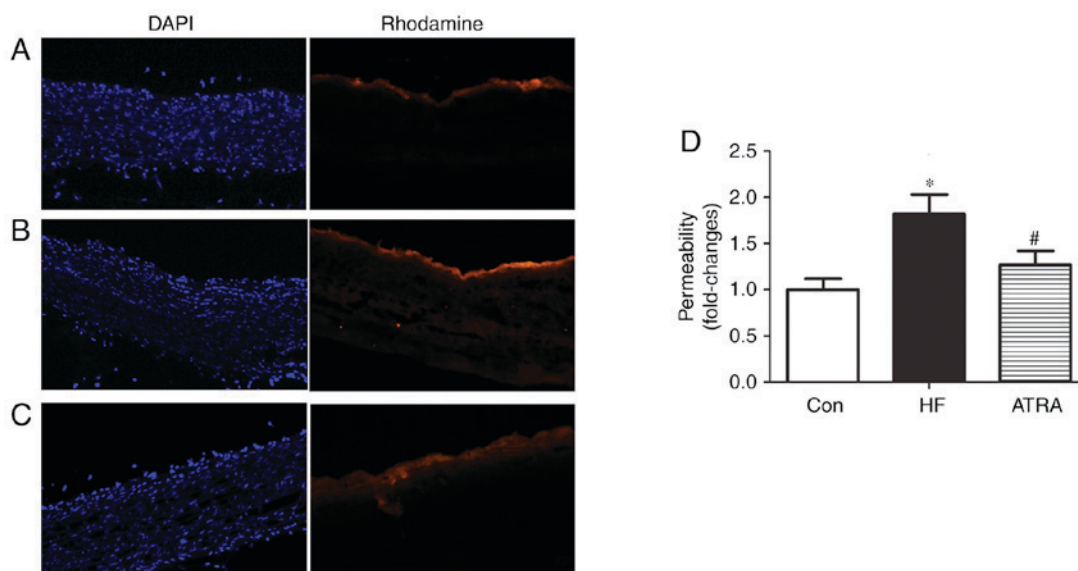


Figure 3. Permeability assay of rabbit aorta intima by surface biotinylation. (A) Control group appears as a thick, continuous line at the surface of the aorta intima. By contrast, it was observed that the biotin molecules penetrated into subendothelial spaces, which demonstrate the disruption of the tight junction seal of the aorta intima, in (B) high fat and (C) ATRA groups. However, the hyperpermeability of atherosclerotic rabbits was reduced following the treatment with ATRA. Magnification, x200. (D) Graph of Rhodamine fluorescence intensity plotted from three independent experiments. Data are presented as the mean \pm standard deviation. *P<0.05 vs. control group; #P<0.05 vs. high fat group. ATRA, all-trans retinoic acid; Con, control group; HF, high fat group.

demonstrated that the expression of ET-1 in rabbit arterial tissue in the high fat group was increased compared with the control group, however, its expression was decreased in the ATRA group. ATRA significantly increased the eNOS phosphorylation at Ser-1177 (p-eNOS); the expression of p-eNOS in the high fat group was lower compared with the control group and increased in the rabbits fed with ATRA. ATRA did not affect the expression of eNOS in each group (Fig. 4).

Effect of ATRA on eNOS activity, and ET-1 and NO levels, in the plasma of atherosclerotic rabbits. The results demonstrated that the content of NO and eNOS activity in the high fat group was significantly lower compared with the control group (P<0.05), while the level of NO and eNOS activity were increased following ATRA treatment (P<0.05; Fig. 5A and B). The detection results of ET-1 were in contrast to the levels of NO, and the expression of ET-1 in the ATRA group

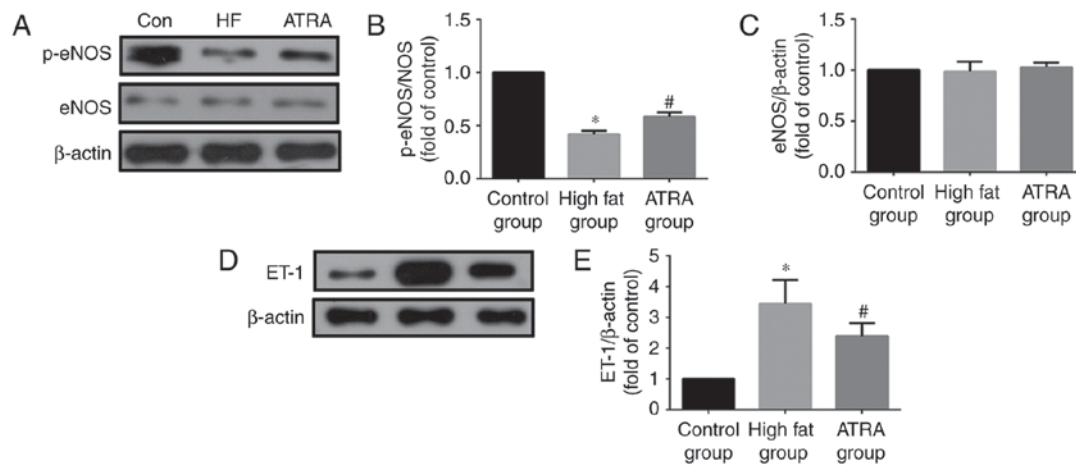


Figure 4. The protein expression of ET-1, eNOS and p-eNOS in arterial tissue. (A) Representative western blot for p-eNOS and eNOS protein expression. Quantified protein expression of (B) p-eNOS and (C) eNOS. (D) Representative western blot analysis for ET-1 protein expression. (E) Quantified protein expression of ET-1. Graphs for protein quantification were plotted from three independent experiments. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. high fat group. ET-1, endothelin-1; eNOS, endothelial nitric oxide synthase; p-, phosphorylated; Con, control group; HF, high fat group; ATRA, all-trans retinoic acid.

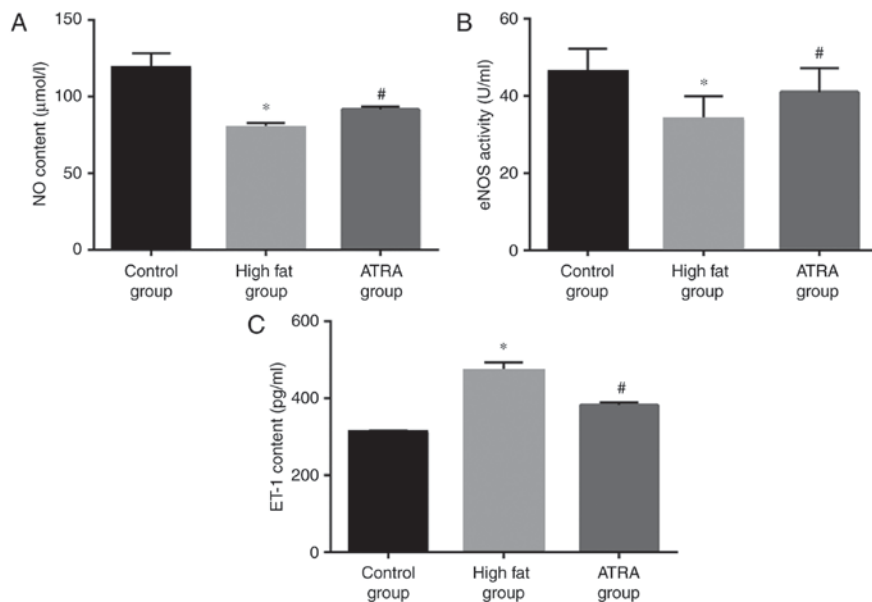


Figure 5. The levels of ET-1 and NO, and the activity of eNOS, in the plasma in the three treatment groups (n=8). (A) NO levels, (B) eNOS activity and (C) ET-1 expression in the plasma of control, high fat and ATRA groups. ATRA increased the activity of eNOS and the levels of NO, and decreased the levels of ET-1, in the plasma compared with rabbits in the high fat group. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. high fat group. ET-1, endothelin-1; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; ATRA, all-trans retinoic acid.

was significantly lower compared with the high fat group ($P < 0.05$; Fig. 5C).

Discussion

The formation mechanisms of AS are complex and numerous, and the structural and functional damage of the vascular endothelium is regarded as the initial step for the genesis of AS (21,22), which influences the AS lesion degree (23,24). It is established that under the action of numerous AS risk factors, including hyperlipidemia, hypertension, diabetes and smoking, the endothelial function is damaged and may be aggravated gradually with AS development (25). Vascular relaxation and

contraction is regulated by various vasoactive substances *in vivo*, including NO, ET, prostaglandins and angiotensin. The levels of ET and NO are important in the regulation of endothelial function (26).

ET-1 is a type of polypeptide consisting of 21 amino acids that was extracted from cultured swine aortic EC supernatant by Yanagisawa and Lefer (27), and it is the strongest endogenous vasoconstrictor substance to be identified thus far. ET has three isomers, ET-1, ET-2 and ET-3. ECs primarily secrete ET-1, which is the major ET type in blood circulation (28,29). Increased ET-1 may induce vascular endothelial dysfunction, which manifests as vasomotor dysfunction due to various stimuli, including acetylcholine, catecholamine,

mechanical pressure, extension and cold stimulation (30). It may also promote platelet aggregation and platelet-leukocyte adhesion, and accelerate the proliferation of smooth muscle cells and fibroblasts. ET-1 is a chemoattractant for monocytes and initiates endothelial lesions, while the uptake of lipid by macrophages may cause ECs to synthesize ET-1 (31). It was demonstrated in previous studies that the application of an ET-1 receptor antagonist restored the NO-mediated control of endothelial function and inhibited AS genesis (32,33), while NO inhibitors weakened such effects; therefore, it was considered that ET-1 may promote AS. In the present study, the plasma ET-1 levels in the high fat group were notably increased compared with the control group, and these levels were reduced when comparing the ATRA group with the high fat group, indicating that the anti-AS function of ATRA may, at least partially, occur through protecting the endothelial function and acting against the synthesis, release and function of ET-1.

Previous studies have demonstrated that in vascular ECs, the expression and activity of eNOS may contribute to NO synthesis and release (11,34). Asymmetric dimethylarginine (ADMA), as an endogenous eNOS inhibitor, can reduce the production of NO by inhibiting eNOS function (35) and, as a result, the expression and activity of eNOS determine the synthesis and secretion of NO, and influence the endothelial-dependent relaxation reaction. NO serves an important role in sustaining basic vascular tension and inhibiting the proliferation of vascular smooth muscle cells (36,37). NO also exerts an anti-AS function through mechanisms that include the combination of ET-1 and the ET-B receptor, which induces the release of NO and thus leads to the feedback inhibition of ET-1 synthesis and release, in addition to free radical scavenging (38). In the present study, the activity of eNOS in the high fat group was reduced in plasma when compared with the control group, and notably increased in the ATRA group. ATRA was able to increase the expression of p-eNOS in arterial tissue, which had been decreased in the high fat group, indicating that the anti-AS function of ATRA may be exercised by protecting the endothelial function, and at least partially by acting against the synthesis, release and the functioning of ET-1.

ATRA is a natural derivative of vitamin A that possesses extensive biological effects. As indicated in previous studies, ATRA inhibits the migration and proliferation of smooth muscle cells, and also exerts anti-inflammatory and antifibrosis effects (39,40). With regard to the association between ATRA and AS, on the one hand, as was reported in a previous study, ATRA may alter the levels of the endogenous NO synthase inhibitor ADMA, thus increasing the synthesis of NO (41). On the other hand, ATRA was reported to inhibit the expression of ET-1 mRNA in ECs (40,42), thus leading to anti-AS effects. The present study indicated that ATRA may alleviate AS by reducing ET-1 expression and increasing eNOS phosphorylation in AS rabbits.

Regulation of the balance between NO and ET is an important approach to prevent and treat AS. It was demonstrated in the present study that ATRA may reduce the levels of ET-1 in the plasma, increase NO content through increasing eNOS phosphorylation and regulate the balance between the two, thus improving vascular relaxation, alleviating atherosclerotic

plaque formation and exerting an anti-AS function. ATRA may provide a novel direction for future clinical treatment of AS.

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