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Tetramethylpyrazine and paeoniflorin combination (TMP-PF) alleviates atherosclerosis progress by reducing hyperlipemia and inhibiting plaque angiogenesis via the NR4A1/VEGFR2 pathway

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ABSTRACT

Atherosclerosis remains a great threat to human health worldwide. Previous studies found that tetramethylpyrazine (TMP) and paeoniflorin (PF) combination (TMP-PF) exerts anti-atherosclerotic effects *in vitro*. However, whether TMP-PF improves atherosclerosis *in vivo* needs further exploration. The present study aims to assess the anti-atherosclerotic properties of TMP-PF in ApoE^{-/-} mice and explore the related molecule mechanisms. Results showed that TMP and high-dose TMP-PF decreased serum triglyceride and low-density lipoprotein cholesterol levels, suppressed vascular endothelial growth factor receptor 2 (VEGFR2) and nuclear receptor subfamily 4 group A member 1 (NR4A1) expression in aortic tissues, inhibited plaque angiogenesis, reduced plaque areas, and alleviated atherosclerosis in ApoE^{-/-} mice. Also, TMP-PF exhibited a better modulation effect than TMP or PF alone. However, NR4A1 agonist abolished the anti-atherosclerotic effects of TMP-PF. In conclusion, TMP-PF was first found to alleviate atherosclerosis progression by reducing hyperlipemia and inhibiting plaque angiogenesis via the NR4A1/VEGFR2 pathway, indicating that TMP-PF had a positive effect on reducing hyperlipemia and attenuating atherosclerosis development.

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1. Introduction

Atherosclerosis commonly causes cardio-cerebrovascular diseases and denotes a major global health problem^[1-2]. Dyslipidemia promotes the development and progression of atherosclerosis, thus leading to high morbidity and mortality worldwide^[3]. Although current therapeutic strategies for reducing atherosclerosis mainly focus on

lipid, angiogenesis is also an important factor for atherosclerotic plaque instability^[4]. In the atherosclerotic pathological process, oxidized low-density lipoprotein (ox-LDL) is prone to leading to endothelial cell proliferation, migration, tube formation and promoting angiogenesis, thus inducing plaque instability and accelerating atherosclerosis^[5-6]. Patients with plaque angiogenesis generally feature high plaque volumes and plaque burden, and thus plaque angiogenesis could predict a high risk of plaque progression^[7]. Plaque neovessels and intraplaque haemorrhage colocalize with vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR2), while VEGFR2 blockade could result in a significant 44% decrease in intraplaque haemorrhage, and therefore reducing the risk of cardio-cerebrovascular events^[8-9].

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Rhizoma Chuanxiong and *Radix Paeoniae Rubra* are traditional Chinese herbal medicines and are also used as dietary supplements. They are commonly used as a combination with lipid-lowering and anti-inflammatory effects in China with a long history^[10–11]. Tetramethylpyrazine (TMP) and paeoniflorin (PF) are the representative active ingredients of *Rhizoma Chuanxiong* and *Radix Paeoniae Rubra*. TMP is a kind of pyrazines found in Chinese black vinegar^[12] and Baijiu^[13] and PF is the predominant monoterpene glycoside in tree peony seed traditionally used for edible oil production^[14]. Our previous studies have found that the combination of TMP and PF (TMP-PF) suppressed ox-LDL-induced angiogenesis by inhibiting the VEGF/VEGFR2 and Jagged1/Notch1 signaling pathways *in vitro*, which is more effective than either alone^[15]. However, whether TMP-PF alleviates the development of atherosclerosis, reduces hyperlipemia and inhibits plaque angiogenesis *in vivo* is not yet known.

Nuclear receptor subfamily 4 group A member 1 (NR4A1) is a transcription factor that regulates many physiological and pathological processes such as cell cycle and apoptosis, lipid metabolism and inflammation^[16]. NR4A1 is also an important transcription factor in VEGF pathway-mediated angiogenesis, and its silence could significantly inhibit VEGF-induced angiogenesis^[17]. Previous studies have primarily focused on the bidirectional role of NR4A1 in the inflammatory response of atherosclerosis^[18] or NR4A1-mediated regulation of angiogenesis in tumour growth and skin wound healing^[19–20]. However, very few studies have focused on the role of NR4A1 in atherosclerotic plaque angiogenesis.

This study aimed to determine the anti-atherosclerotic properties of TMP-PF in ApoE^{-/-} mice *in vivo*, and subsequently explore the molecular mechanisms of TMP-PF on atherosclerosis based on the role of NR4A1.

2. Materials and methods

2.1 Materials and reagents

Hydrochloride TMP (KM0513CA14) and PF (X12A8C33672) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Simvastatin (M035962) was obtained from Hangzhou MSD Pharma Pte. Ltd. (Hangzhou, China), and cytosporone B (CsnB; HY-N2148) was from MedChemExpress (Shanghai, China). High-cholesterol diet (21% fat and 0.15% cholesterol) was brought from Beijing Ke'ao Xieli Feed Co., Ltd. (Beijing, China). CD31 antibody (GB12063), vWF antibody (GB11020), Alexa Fluor 488-labelled secondary antibody (GB25303), CY3-labelled secondary antibody (GB21301) and DAPI (G1012) were gathered from Servicebio Technology Co., Ltd. (Wuhan, China). Enzyme-linked immunosorbent assays (ELISA) kit (28380334) of VEGF was purchased from MultiSciences Biotech Co., Ltd. (Hangzhou, China). VEGFR2 antibody (9698s) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (5174) were gathered from CST (MA, USA). NR4A1 antibodies (Ab109180 and 25851-1-AP) were purchased from Abcam (Cambridge, UK) and Proteintech (Chicago, USA). Immunoglobulin (Ig)G (H+L) horseradish peroxidase (HRP) antibody (111-035-003) was obtained from Jackson Laboratories (ME, USA).

2.2 Atherosclerosis mouse model establishment and grouping

All animal experiments were performed in accordance with the institutional guidelines and approved by the Ethics Committee of Xiyuan Hospital of China Academy of Chinese Medical Sciences. Eight-week-old male ApoE^{-/-} mice and C57BL/6J background mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). C57BL/6J mice were used as the controls, while ApoE^{-/-} mice were divided randomly into the model, 4 different doses of TMP-PF and simvastatin groups ($n = 6–8$ in each group). C57BL/6J mice were given an ordinary diet, while ApoE^{-/-} mice were fed with a high-cholesterol diet for 3 months to establish the mouse model of atherosclerosis as described and verified previously^[21]. Control and model mice were treated with normal saline, while ApoE^{-/-} mice were administered with different doses of TMP-PF (low dose: 1 mg/(kg·day) TMP + 10 mg/(kg·day) PF, medium dose: 2 mg/(kg·day) TMP + 20 mg/(kg·day) PF, high dose: 5 mg/(kg·day) TMP + 50 mg/(kg·day) PF, very high dose: 10 mg/(kg·day) TMP + 100 mg/(kg·day) PF) or simvastatin (2.5 mg/(kg·day)) for one month. The ratio of TMP and PF (1:10) was reported in our previous studies^[15], and the concentrations refer to previous studies^[22–23]. Then TMP and PF were studied individually using the same doses as in the high-dose TMP-PF group. After comparing the effects of TMP-PF and either alone, the determined optimal dose of TMP-PF was adopted for subsequent mechanism studies with or without NR4A1 agonist (CsnB), and the dose of CsnB is 13 mg/(kg·day).

2.3 Biochemical assays

After 1% pentobarbital sodium anesthesia, blood serum was collected by the abdominal aortic method and centrifuged at 3 000 r/min for 10 min at room temperature. The levels of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein-cholesterol (HDL-C) in serum were detected using an automatic biochemical analyser (Roche, Basel, Switzerland).

2.4 Haematoxylin and eosin (H&E) staining

The plaque areas were detected by H&E staining. The aortic root tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin blocks were cut into 5 μ m slices and stained with H&E. The pathological morphology of atherosclerotic plaque was observed using an Eclipse E100 microscope (Nikon, Tokyo, Japan). The plaque areas were quantified with Image-Pro Plus 6.0 and calculated as plaque area (PA)/vascular lumen area (LA).

2.5 Immunofluorescence assay

Angiogenesis markers (CD31 and vWF) in aortic plaques were detected by immunofluorescence. The aortic root tissues were fixed in 4% paraformaldehyde and embedded in paraffin. After antigen retrieval and protein blocking, slides were incubated with primary antibodies against CD31 (1:200) and vWF (1:500) at 4 °C overnight and then incubated with Alexa Fluor 488-labelled secondary antibody (1:400) and CY3-labelled secondary antibody (1:300). Nuclei were stained with DAPI. Slides were visualized under a fluorescence

Eclipse C1 microscope (Nikon, Tokyo, Japan), and the integral optical density (IOD) was analysed by ImageJ 1.49p.

2.6 Immunohistochemistry assay

After antigen retrieval and protein blocking, aortic root and heart tissues paraffin slides were incubated with primary antibodies against CD31 (1:100), NR4A1 (1:500) and VEGFR2 (1:1 000) at 4 °C overnight and then incubated with HRP labeled secondary antibody. After 3,3'-diaminobenzidine chromogenic reaction, slides were counterstained with hematoxylin stain solution to counterstained nucleus, which finally were visualized under an Eclipse E100 microscope (Nikon, Tokyo, Japan), and the IOD was analysed by Image-Pro Plus 6.0.

2.7 ELISAs

VEGF levels in serum were determined using ELISA kits according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microtiter plate reader (Thermo, Waltham, MA, USA).

2.8 Western blot

Mouse aorta samples were harvested and lysed, and protein contents were measured. Cell lysates were boiled and loaded on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The membranes were blocked and incubated with specific primary antibodies against VEGFR2 (1:1 000), NR4A1 (1:1 000) and GAPDH (1:1 000) overnight at 4 °C. Following incubation with secondary antibody IgG (H+L) HRP (1:10 000), protein blots were

visualized, the gray analysis was performed on Gel Image System 4.00 (Tanon, Shanghai, China), and the relative expression was calculated.

2.9 Statistical analysis

Statistical analysis was carried out using SPSS 17.0. Data are expressed as the means $\pm$ standard deviation, and the graphs and diagrams were generated by GraphPad Prism 6.07. Comparisons between 2 groups were performed using Student's *t*-test, and comparisons among multiple groups were performed using one-way analysis of variance (ANOVA). $P \leq 0.05$ was considered statistically significant.

3. Results

3.1 TMP-PF reduced hyperlipemia and attenuated atherosclerosis progress in ApoE^{-/-} mice

Since hyperlipidemia is one of the major risk factors of atherosclerosis, serum lipid profiles and plaque areas were first evaluated to observe the effects of different doses of TMP-PF on atherosclerosis. The results showed that serum TC, TG and LDL-C levels were significantly increased, and HDL-C levels were significantly decreased in the models compared with the controls ($P < 0.05$, Figs. 1A-D). Whereas, compared with the model group, the TG levels significantly decreased in mice of the high-dose, very high-dose TMP-PF, and simvastatin groups ($P < 0.05$, Fig. 1B); LDL-C levels significantly decreased in the high-dose TMP-PF-treated mice ($P < 0.05$, Fig. 1C), indicating that high-dose TMP-PF might have better effects than other doses on lipid. Meanwhile, TMP and PF were studied individually using the same doses as in

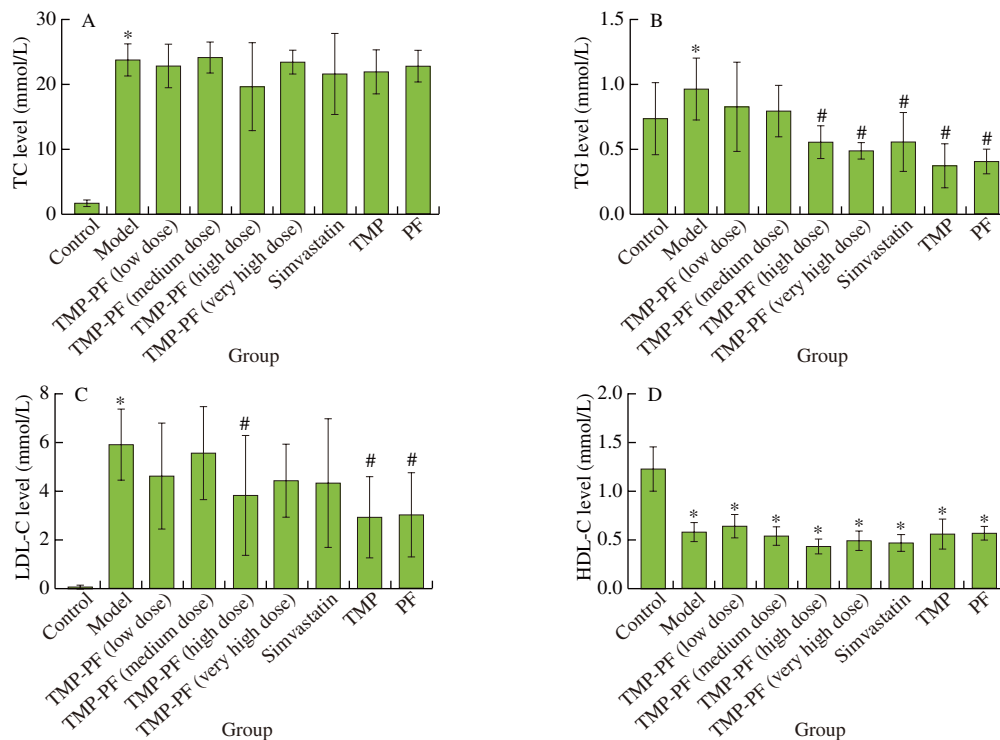


Fig. 1 TMP-PF improved serum lipid levels and plaque areas in ApoE^{-/-} mice. The effects of TMP-PF on serum (A) TC; (B) TG; (C) LDL-C; (D) HDL-C in ApoE^{-/-} mice. (E) Photomicrographs of plaque areas with H&E staining (scale bar: 200 μ m). (F) Quantification of plaque area (PA/LA). Data are means $\pm$ SD; * $P < 0.05$ versus the control group, # $P < 0.05$ versus the model group.

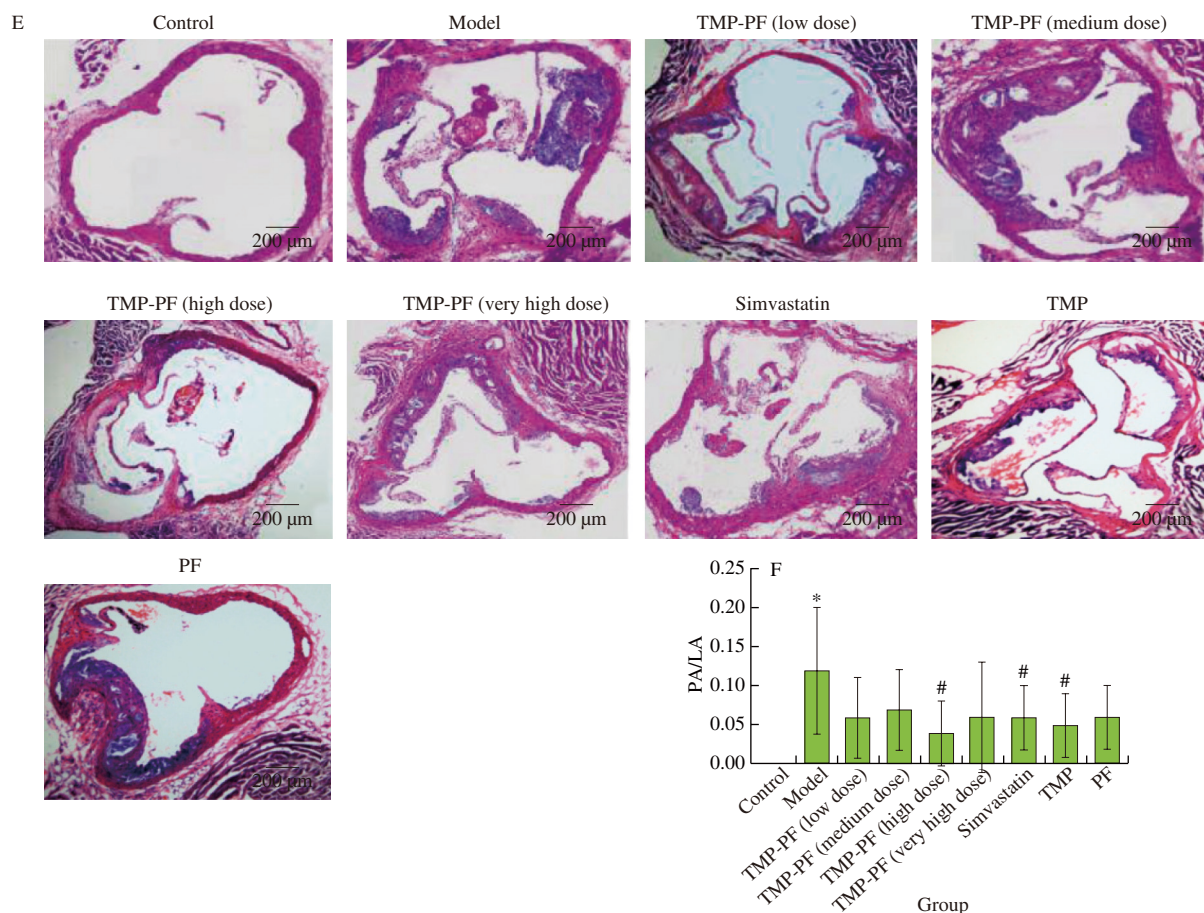


Fig. 1 (Continued)

the high-dose TMP-PF group, and results showed that either TMP or PF could lower TG and LDL-C levels ($P < 0.05$, Figs. 1B-C). In addition, H&E staining showed that the control mice had even aortic wall thickness without plaques, and the aortic root of the model mice varied in thickness with obvious plaques. TMP, high-dose TMP-PF, and simvastatin significantly lowered the PA/LA ratio and reduced plaque areas, thus attenuating atherosclerosis progress ($P < 0.05$, Figs. 1E-F). These results demonstrated that TMP-PF might attenuate the development of atherosclerosis in ApoE^{-/-} mice.

3.2 TMP-PF inhibited plaque angiogenesis in ApoE^{-/-} mice

To investigate whether TMP-PF regulates plaque angiogenesis, angiogenic factors including CD31, vWF, VEGF, VEGFR2 and NR4A1 were analyzed. The expression of angiogenesis markers (CD31 and vWF) in aortic plaques was significantly increased in the models compared with the controls ($P < 0.05$, Figs. 2A-C); compared with the model group, TMP, PF, high-dose TMP-PF and simvastatin significantly suppressed CD31 expression ($P < 0.05$, Fig. 2B), while no significant change was observed in vWF expression ($P > 0.05$, Fig. 2C). Since high-dose TMP-PF showed better effects on angiogenesis, the effects of both high-dose TMP-PF and equivalent doses of TMP and PF on angiogenesis-related factors were further assessed. There was no significant difference in serum VEGF among groups ($P > 0.05$, Fig. 2D). In addition, the expression of VEGFR2 and NR4A1 in aortic tissue was significantly increased in the models compared with

that in the controls ($P < 0.05$, Figs. 2E-G), while compared with the model group, the expression of VEGFR2 in aortic tissue was significantly decreased after TMP or TMP-PF administration ($P < 0.05$, Figs. 2E-G), and the expression of NR4A1 in aortic tissue was significantly reduced after TMP, PF, TMP-PF or simvastatin administration ($P < 0.05$, Figs. 2E-G). These results indicated that TMP-PF might ameliorate plaque angiogenesis and inhibit the expression of NR4A1 and VEGFR2 in ApoE^{-/-} mice.

3.3 TMP-PF reduced hyperlipemia and attenuated atherosclerosis progress by inhibiting NR4A1

Since TMP-PF decreased NR4A1 expression in aortic tissue of ApoE^{-/-} mice, NR4A1 might play an important role in the anti-atherosclerotic effects of TMP-PF. Therefore, the effects of TMP-PF with or without NR4A1 agonist (CsnB) on atherosclerosis were observed in ApoE^{-/-} mice. The results showed observably increased TC, TG and LDL-C levels and decreased HDL-C levels along with enlarged plaque areas in the models compared with the controls ($P < 0.05$, Figs. 3A-F); and compared with the models, TG and LDL-C levels significantly decreased, with reduced plaque areas and alleviated atherosclerosis progress after TMP-PF administration ($P < 0.05$, Figs. 3B, C, E, F), while these effects were rescued by NR4A1 agonist (CsnB; $P < 0.05$, Figs. 3B, C, E, F). These results indicated that TMP-PF might reduce hyperlipemia and attenuate atherosclerosis progress by inhibiting NR4A1 expression.

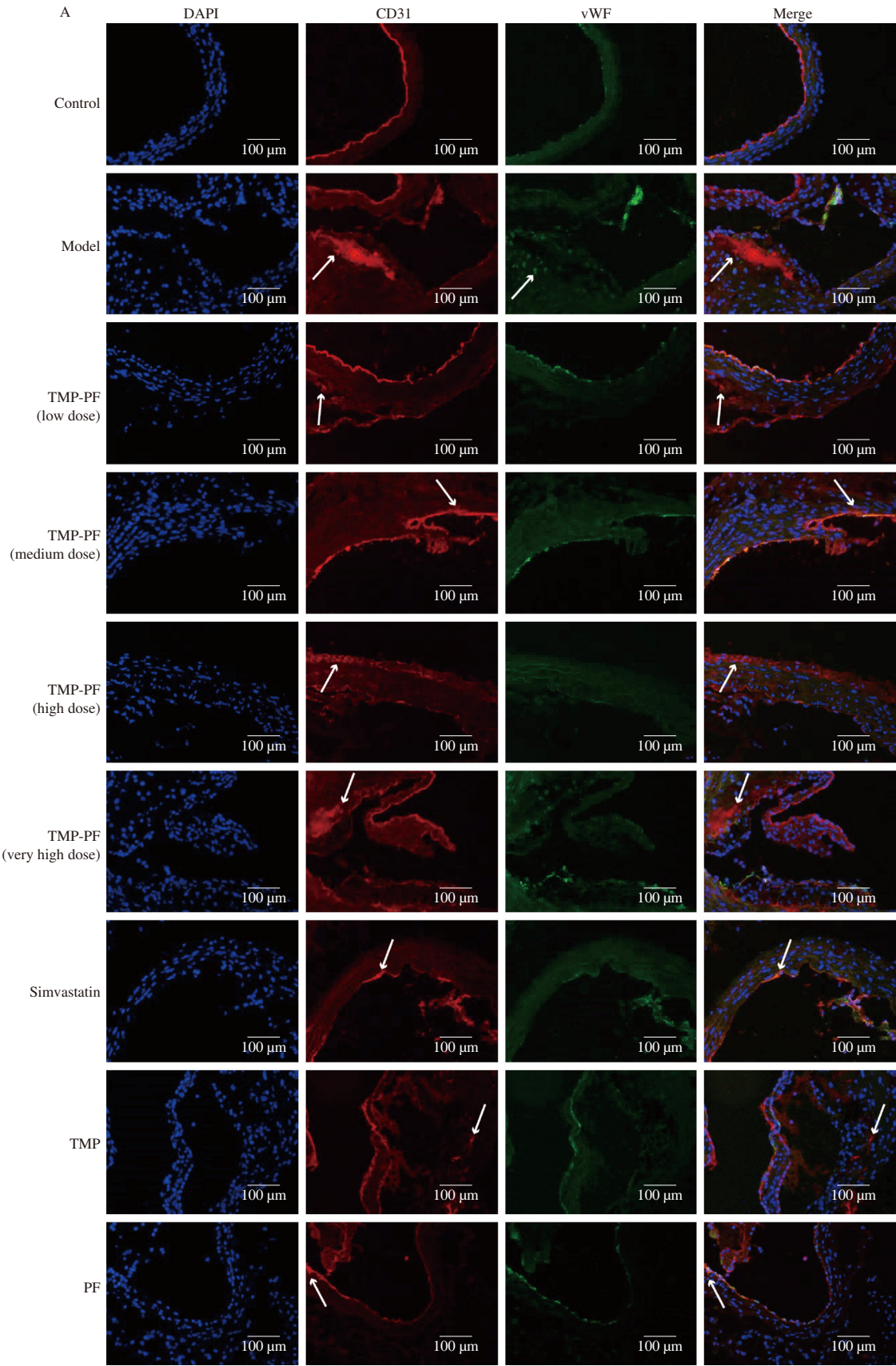


Fig. 2 TMP-PF inhibited plaque angiogenesis and angiogenesis-related factors in ApoE^{-/-} mice. (A) Representative images of immunofluorescence staining of CD31 and vWF in aortic root (scale bar: 100 μm). (B-C) Quantitative analysis of the IOD value of CD31 and vWF in plaques. (D) VEGF levels in serum were evaluated by ELISA. (E-F) Relative expression of VEGFR2 and NR4A1 in aortic tissue was evaluated by Western blot. (G) Representative Western blot images of VEGFR2 and NR4A1. Data are means ± SD; **P* < 0.05 versus the control group, #*P* < 0.05 versus the model group.

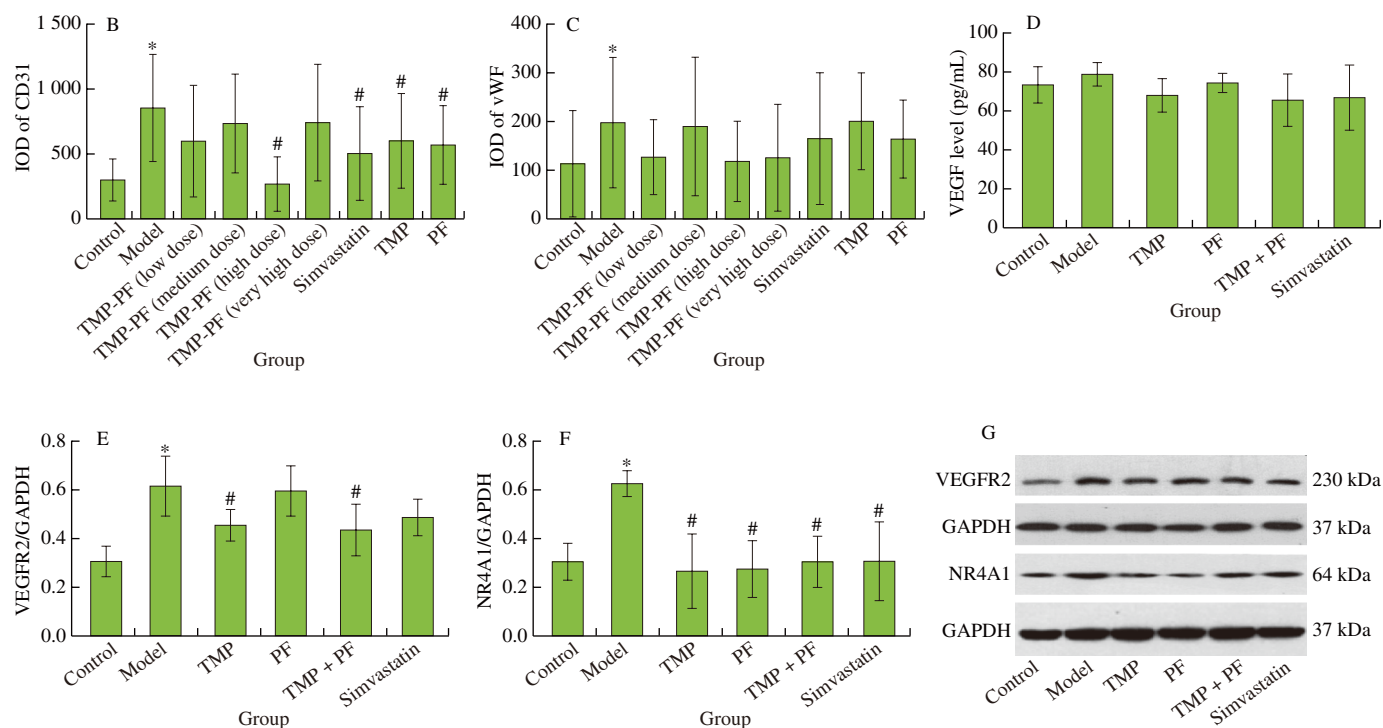


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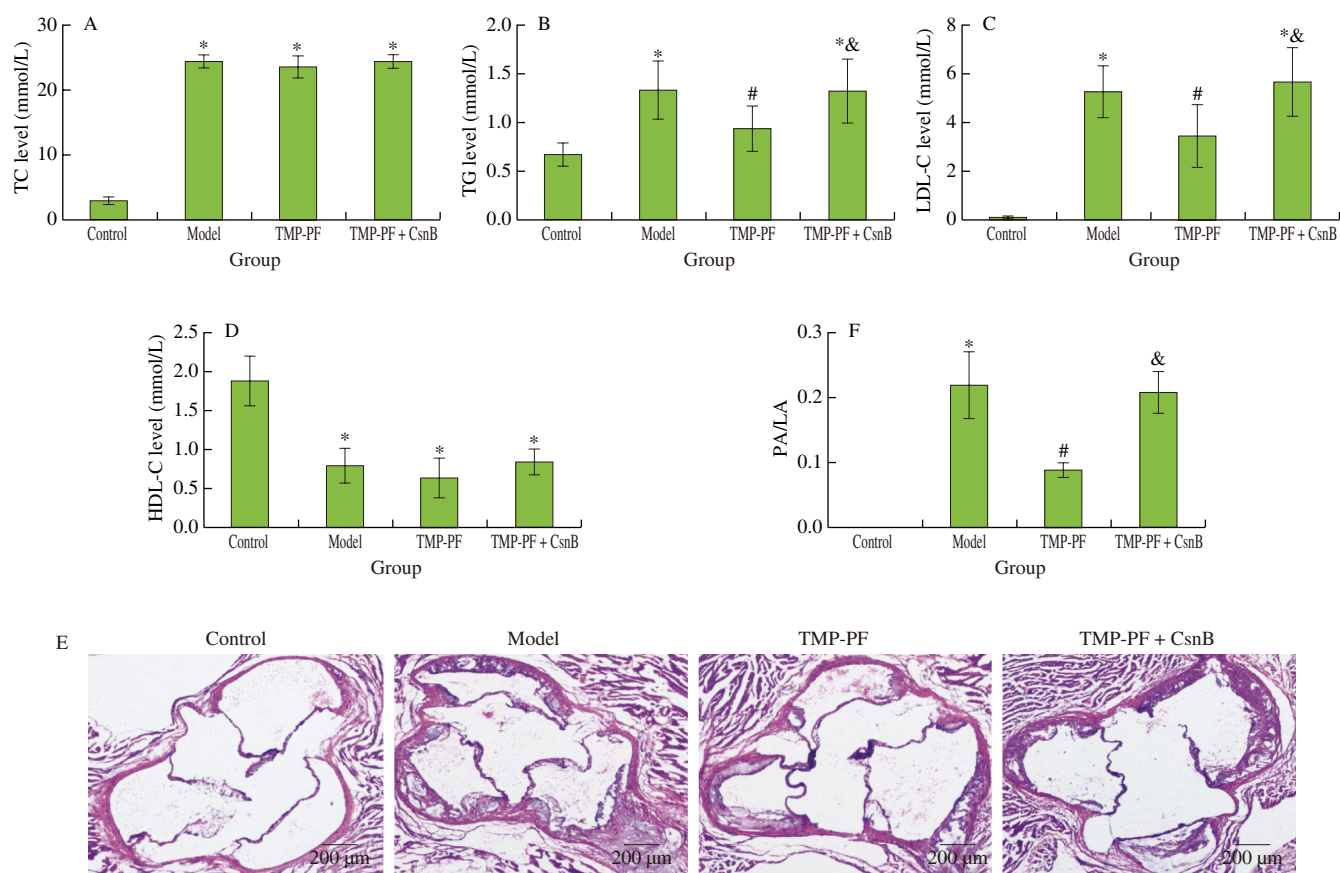


Fig. 3 TMP-PF ameliorated atherosclerosis by inhibiting NR4A1 expression in ApoE^{-/-} mice. The effects of TMP-PF on serum (A) TC; (B) TG; (C) LDL-C; (D) HDL-C levels in ApoE^{-/-} mice. (E) Photomicrographs of plaque areas with H&E staining (scale bar: 200 μ m). (F) Quantification of plaque area (PA/LA). Data are means $\pm$ SD; * P < 0.05 versus the control group, # P < 0.05 versus the model group, & P < 0.05 versus the TMP-PF group.

3.4 TMP-PF inhibited plaque angiogenesis through the NR4A1/VEGFR2 pathway

Several angiogenesis-related factors were analyzed to explore the underlying mechanism of TMP-PF in inhibiting angiogenesis and suppressing atherosclerotic development. Increased microvessel density (CD31) was found in atherosclerotic plaques, as well as increased expression of NR4A1 and VEGFR2 in the models compared with that in the controls ($P < 0.05$, Figs. 4A-F); compared with the models, TMP-PF significantly inhibited the expression of CD31, NR4A1 and VEGFR2 ($P < 0.05$, Figs. 4A-F), while these effects were abrogated by NR4A1 agonist (CsnB; Figs. 4A-F). To further verify the potential mechanism of TMP-PF on angiogenesis in atherosclerotic animals, the expression of NR4A1 and VEGFR2 in aortic tissue was confirmed by Western blot analysis. The expression of NR4A1 and VEGFR2 in aortic tissue were significantly increased in the models compared with that in the controls ($P < 0.05$, Figs. 4G-I); compared with the models, the expression of NR4A1 and VEGFR2 in aortic tissue were significantly decreased after TMP-PF treatment

($P < 0.05$, Figs. 4G-I), while these effects were abolished by NR4A1 agonist (CsnB; Figs. 4G-I). These results indicated that TMP-PF might inhibit plaque angiogenesis through the NR4A1/VEGFR2 pathway.

3.5 TMP-PF did not exhibit significant side effects on myocardial histology or angiogenesis in myocardium of ApoE^{-/-} mice

Since anti-angiogenic therapy might have an increased risk of heart injury, the effects of TMP-PF on myocardial histology and angiogenesis in myocardium were further determined, and angiogenesis marker (CD31) was used to evaluate the microvascular density (MVD). In this study, inflammatory infiltration was observed in the models, while no significant change was found in other groups (Fig. 5A). CD31 expression in myocardium had no statistical differences among groups ($P > 0.05$, Figs. 5B-C), indicating that there was no significant change in myocardial MVD after TMP-PF treatment. These results indicated that TMP-PF might have no significant side effects on myocardial histology or angiogenesis in myocardium of ApoE^{-/-} mice.

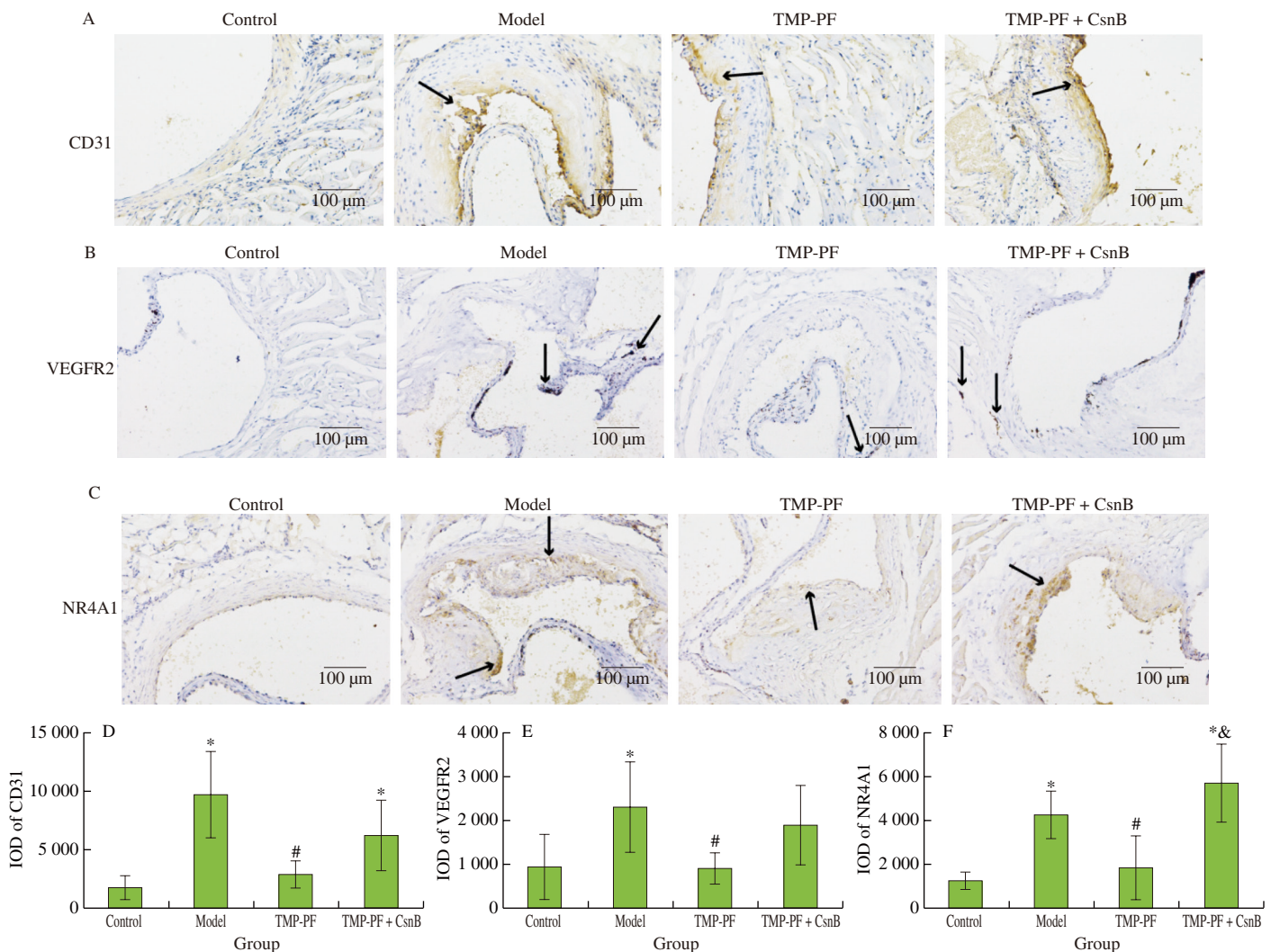


Fig. 4 TMP-PF inhibited plaque angiogenesis by inhibiting the NR4A1/VEGFR2 pathway in ApoE^{-/-} mice. (A) Photomicrographs of plaque angiogenesis (CD31 expression) with immunohistochemical staining (scale bar: 100 μm). (B-C) Photomicrographs of VEGFR2 and NR4A1 expression with immunohistochemical staining (scale bar: 100 μm). (D) Quantification of plaque angiogenesis with CD31 expression. (E-F) The expression of VEGFR2 and NR4A1 was evaluated by the IOD. (G-H) Quantification of the relative expression of VEGFR2 and NR4A1 normalized to GAPDH in aortic tissue. (I) Representative western blot images of VEGFR2 and NR4A1 in aortic tissue. Data are means ± SD; * $P < 0.05$ versus the control group, # $P < 0.05$ versus the model group, & $P < 0.05$ versus the TMP-PF group.

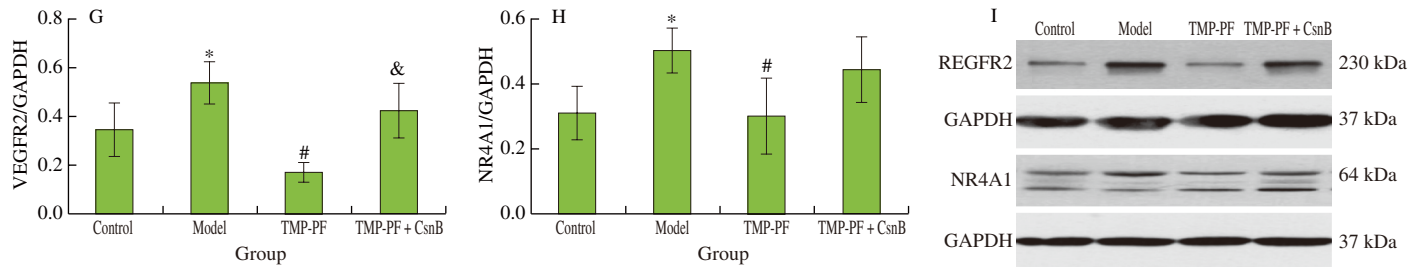


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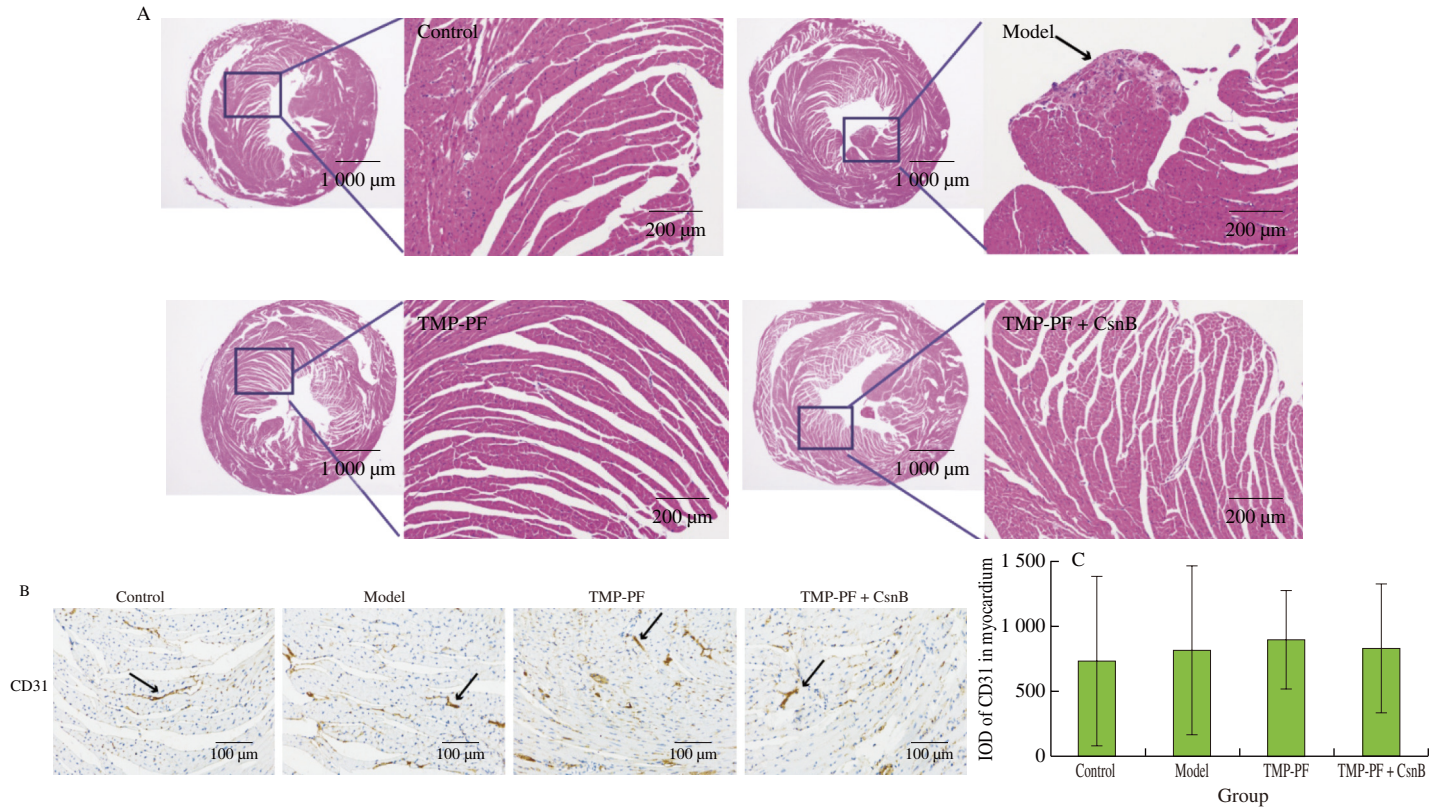


Fig. 5 TMP-PF had no significant side effects on myocardial histology or angiogenesis in myocardium of ApoE^{-/-} mice. (A) Photomicrographs of myocardial histology with H&E staining (scale bar: 1 000 μ m, 200 μ m). (B) Photomicrographs of microvascular density (MVD) (CD31 levels) in myocardium with immunohistochemical staining (scale bar: 100 μ m). (C) Quantification of CD31 expression in the myocardium.

4. Discussion

Our previous studies have reported that TMP-PF exerted anti-angiogenesis effects in ox-LDL-induced endothelial cells by regulating VEGF and Notch pathways or circSCRG1 signalling^[15,24]. So far, many studies have focused on the anti-atherosclerosis effects of TMP and PF *in vitro*. However, there are a few studies on the biological functions and underlying mechanisms of TMP-PF *in vivo*. The present study preliminarily confirmed that 1) TMP-PF alleviated atherosclerosis progress by reducing hyperlipemia and plaque areas, and inhibiting plaque angiogenesis in ApoE^{-/-} mice; 2) TMP-PF might have a better modulation effect on atherosclerosis than TMP or PF alone; 3) TMP-PF might inhibit plaque angiogenesis by regulating the NR4A1/VEGFR2 pathway, finally exert effects of anti-atherosclerosis (Fig. 6). Taken together, our results confirmed from different perspectives that TMP-PF had a positive effect on reducing hyperlipemia and attenuating atherosclerosis development, which might provide a novel strategy for atherosclerosis.

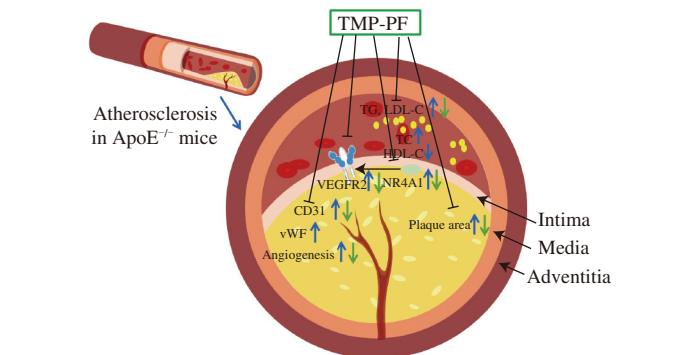


Fig. 6 Schematic diagram showing the mechanisms associated with the effects of TMP-PF on the development of atherosclerosis by reducing hyperlipemia and inhibiting plaque angiogenesis via the NR4A1/VEGFR2 pathway. The blue arrow represents the pathological state, and the green arrow represents TMP-PF-related effects.

Increasing evidence demonstrates that the *Rhizoma Chuanxiong-Radix Paeoniae Rubra* herb pair and its active constituents (Xiongshao

capsule) have beneficial effects on atherosclerosis and ischemic heart disease, and the mechanisms include antiplatelet aggregation, anti-inflammation, and improving endothelial function and myocardial ischemia^[10,25]. In the present study, TMP-PF alleviated atherosclerosis progress by reducing hyperlipemia and inhibiting plaque angiogenesis, which partially clarified the anti-atherosclerotic effects of the *Rhizoma Chuanxiong-Radix Paeoniae Rubra* herb pair. Previous studies showed that TMP had anti-inflammatory, anti-apoptotic, anti-oxidative, anti-platelet and endothelial cells protection effects in the treatment of cardio-cerebrovascular diseases, such as atherosclerosis, myocardial ischemia and hypertension^[26], it was capable of regulating lipid levels and attenuating oxidative stress, inflammatory responses and endoplasmic reticulum stress in gestational diabetes mellitus model mice^[27]; TMP suppressed endothelial glycocalyx degradation and protected integrity of glycocalyx structure during inflammation, which might be considered a novel strategy in the treatment of atherosclerosis^[28]. Previous studies also showed that PF had anti-hyperglycaemic, anti-inflammatory, anti-oxidative and anti-apoptotic effects, and it attenuated ox-LDL-induced injury in endothelial cells by inhibiting apoptosis and activating autophagy^[29], PF also attenuated dyslipidemia in mice by regulating *N*-acetylgalactosaminyltransferase 2-angiopoietin-like protein 3-lipoprotein lipase (GALNT2-ANGPTL3-LPL) pathway^[30] and exerted multiple anti-inflammatory effects by modulating toll-like receptor-mediated signaling in immune and parenchymal cells (such as macrophages and dendritic cells)^[31]. In this study, TMP alone reduced TG and LDL-C levels, inhibited the expression of VEGFR2 and NR4A1, alleviated plaque angiogenesis, and reduced plaque areas, while PF alone reduced TG and LDL-C levels, inhibited NR4A1 expression, and alleviated plaque angiogenesis. Although lipid regulatory effects of TMP and PF were partly consistent with previous research, the joint effects of TMP and PF on hyperlipemia and plaque angiogenesis were explored for the first time in the present study. Additionally, TMP-PF reduced TG, LDL-C, VEGFR2 and NR4A1 levels, alleviated plaque angiogenesis, and reduced plaque areas, all of which were not studied before. Furthermore, the effects of TMP-PF on plaque angiogenesis and plaque areas were more significant than TMP or PF alone, thus providing more evidence for the compatibility and synergistic effects of TMP and PF.

Dyslipidemia plays a critical role in atherosclerosis and cardiovascular disease and lipid lowering therapies are essential for the prevention of these diseases^[32]. A study demonstrated that higher serum TG levels tended to be associated with an increased risk of cardiovascular disease^[33]. Lipid-lowering therapy targeting LDL-C by statins was shown to reduce the mortality and morbidity of cardiovascular disease in the primary and secondary preventions^[34]. As the results of the present study, the reductions of TG and LDL-C after TMP-PF treatment are likely to be clinically significant in reducing hyperlipemia, alleviating atherosclerosis and then reducing cardiovascular disease risk. However, HDL-C and TC were not significantly improved after TMP-PF treatment, which indicated that TMP-PF might be more suitable for the treatment of hypertriglyceridemia.

Except for the lipid profiles, plaque angiogenesis also leads to the development of atherosclerosis. VEGF is a secreted mitogen involved in angiogenesis and is closely associated with atherosclerosis^[35]. VEGFR2 is a key receptor in VEGF-induced angiogenesis and has

been associated with plaque instability, and VEGFR2 antagonism could reduce vascular sprouting in aortic rings of mice^[36]. NR4A1 is a subfamily of nuclear receptors, which mainly mediate growth factor and cytokine signaling to regulate angiogenesis, change the activation status of dendritic cells, regulate mesenchymal stromal cells functions, promote the maintenance of functional regulatory T-cells, and influence the generation of peripheral myeloid and T-lymphocyte lineages^[37]. In chronic inflammatory conditions, NR4A1 has been shown to regulate pathological angiogenesis^[38]. A study demonstrated that ginsenosides F1 and Rh1 suppressed the expression of NR4A1 and inhibited VEGF-induced vascular leakage, suggesting that NR4A1 might be the key target in inhibiting vascular leakage^[39]. Therefore, NR4A1 is regarded to play a critical role in inflammation and angiogenesis-related disease. In the present study, VEGFR2 and NR4A1 were highly expressed in aortic tissue of ApoE^{-/-} mice; TMP-PF downregulated VEGFR2 and NR4A1 expression and inhibited angiogenesis, while these effects were abolished by NR4A1 agonist. Therefore, TMP-PF might inhibit plaque angiogenesis through the NR4A1/VEGFR2 pathway, which is likely to be clinically significant in impeding atherosclerosis progression.

Since VEGF is a secreted glycoprotein in regulating vascular development and tube formation^[40], serum VEGF levels were explored in this study. The results showed that TMP-PF had no significant effect on serum VEGF levels, which indicates that TMP-PF might have no side effect on angiogenesis in other tissues and thus may avoid side effects of anti-angiogenesis therapy. Moreover, CD31 and vWF are both specific markers of blood vessels, while vWF is more correlated with mature blood vessels than CD31^[41]. The present results showed that TMP-PF decreased CD31 expression while exerting an insignificant effect on vWF expression, suggesting that TMP-PF might inhibit plaque angiogenesis while it has a faint effect on mature blood vessels. Accordingly, the present study suggested that TMP-PF might have no significant side effects in other tissues of ApoE^{-/-} mice and no significant inhibitory effects on mature blood vessels.

Furthermore, since angiogenesis has been widely reported to play a double-edged role in ischemic myocardium and atherosclerotic plaques: promoting angiogenesis improved reperfusion to the ischemic myocardium in coronary heart disease, while inhibiting plaque angiogenesis was a potential therapeutic target for coronary atherosclerotic heart disease^[42-43], the myocardial MVD was initially detected in a mouse model of atherosclerosis to observe whether TMP-PF showed an effect on angiogenesis in myocardium in this study. The results showed that myocardial MVD showed no significant difference among groups, which indicates that TMP-PF has no significant side effect on myocardium. It could be an interesting topic for further investigation whether multi-target drugs could further effectively inhibit angiogenesis in atherosclerosis and concurrently avoid potential side effects possibly caused by single-target anti-angiogenesis therapies.

Our previous studies indicated that TMP-PF enhanced the vessel sprouting in chemical-induced vascular insufficiency zebrafish^[44] while the present studies indicated that TMP-PF alleviated atherosclerosis progress by inhibiting plaque angiogenesis, which suggested that TMP-PF might promote angiogenesis in the vascular insufficiency state while inhibiting angiogenesis in the pathological vascular excessive state, thus playing a holistic regulatory role. Therefore, the holistic regulatory role of TMP-PF in ischemic

myocardium and atherosclerotic plaques could be further explored in the future, and this would be expected to provide a new option for coronary atherosclerotic heart disease.

The limitation is that this study did not observe the NR4A1-mediated inflammation, future studies could be performed to investigate the effects and molecular mechanisms of TMP-PF on regulating inflammation through the NR4A1-mediated pathway. Additionally, the effects of TMP-PF should be further studied in atherosclerosis patients, so as to provide more reliable evidence for TMP-PF on hyperlipemia and atherosclerosis progress.

5. Conclusion

Food preferences and poor eating habits influence the occurrence of dyslipidemia and atherosclerosis, which may possibly lead to more dangerous conditions such as cardio-cerebrovascular diseases. This study found that TMP-PF ameliorated atherosclerosis by reducing hyperlipemia and countering plaque angiogenesis in ApoE^{-/-} mice via the NR4A1/VEGFR2 pathway, indicating that TMP-PF possesses lipid-lowering and anti-atherosclerotic effects, which have a positive joint effect on attenuating atherosclerosis development and preventing cardio-cerebrovascular diseases. This also raises the possibility that TMP-PF and their herb extracts could be used as functional ingredients in food, drinks and pharmaceutical products.

Conflict of interests

Weihong Cong is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors declare no competing interests.

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