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Original Article

Sarpogrelate and rosuvastatin synergistically ameliorate hyperlipidemia-inducedcardiacdamage in apolipoprotein E-deficient mice

Fan Yang[1*], Xiaoling Shou[2*], Hongyang Liu[3], Yan Sun[4], Zuowei Pei[1], Liyue Zhu[2]

[1]Department of Cardiology, Affiliated Zhongshan Hospital of Dalian University, Dalian, China; [2]Cardiac Rehabilitation Center, Zhejiang Hospital, Hangzhou, China; [3]Department of Heart Intensive Care Unit, The First Affiliated Hospital of Dalian Medical University, Dalian, China; [4]Department of Cardiology, Zhejiang Rongjun Hospital, Jiaxing, Zhejiang, China. [*]Equal contributors.

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Abstract: Hyperlipidemia is a major risk factor for cardiovascular disease. Statins are the first-line treatment for hypercholesterolemia and atherosclerosis. However, most patients receiving statins do not reach the low-density lipoprotein-cholesterol (LDL-c) goal. Sarpogrelate (SP) has been shown to reduce blood lipids and oxidative stress. Therefore, this study investigated the effects of SP plus rosuvastatin compared to rosuvastatin alone on cardiac damage in ApoE$^{-/-}$ mice with hyperlipidemia. Male ApoE$^{-/-}$ mice were randomly divided into four groups: normal diet-fed mice (control group), high-cholesterol diet-fed mice (H group), high-cholesterol diet-fed mice treated with rosuvastatin calcium (HR group), and high-cholesterol diet-fed mice treated with rosuvastatin calcium+ SP (HRS group). Total cholesterol (TC) and LDL-c levels were lower in the HR and HRS groups. SP plus rosuvastatin was more effective in reducing TC and LDL-c levels, compared to rosuvastatin alone. Morphological and immunohistochemical analyses showed that lipid deposition and macrophage infiltration were significantly suppressed in the HRS group, compared to those in the HR group. Lectin-like oxidized LDL receptor-1 (LOX-1) expression was lower in heart tissues of mice in the HR and HRS groups than in the H group. Furthermore, macrophages and proinflammatory cytokine levels were lower in the HR and HRS groups than in the H group. There were significant differences in LOX-1, macrophages, and proinflammatory cytokine levels between HR and HRS groups. Therefore, SP plus rosuvastatin might be more effective in ameliorating hyperlipidemia-induced cardiac damage in ApoE$^{-/-}$ mice, compared to rosuvastatin alone.

Keywords: Hyperlipidemia, cardiac damage, ApoE$^{-/-}$ mice, sarpogrelate, rosuvastatin

Introduction

Cardiovascular disease (CVD) is the primary cause of mortality and morbidity worldwide [1-3]. Hyperlipidemia is the primary major risk factor for CVD and may be further considered a prerequisite for CVD. Apolipoprotein E-knockout (ApoE$^{-/-}$) mice constitute the most popular animal model of atherosclerosis. In this model, mice can spontaneously develop hyperlipidemia and atherosclerotic lesions similar to those found in humans [4, 5]. Previous studies have shown that three processes are closely involved in atherosclerosis pathogenesis: lipid metabolism, oxidation, and inflammation [6, 7]. Similarly, Suciu et al. recently found that accumulation of oxidized low-density lipoprotein (oxLDL) at subendothelial sites might be one of the leading triggers of plaque formation [7]. In previous studies, it has been reported that similar pathophysiological mechanisms contribute to the progression of hyperlipidemia and chronic kidney disease [8]. However, pathogenic mechanisms underlying the relationship between hyperlipidemia and heart damage are not fully understood.

In clinical practice, statins (3-hydroxy-3-methylglutaryl coenzyme A [HMG-CoA] reductase inhibitors) are the first-line treatment for hypercholesterolemia to prevent CVD. They inhibit the key rate-limiting enzyme in hepatic cholesterol synthesis [9, 10]. Among statins, rosuvastatin is the most efficacious and is relatively

Table 1. Primers used for RT-PCR analysis

Gene	Primers
LOX-1	F: 5'-CAAAGTCTCCCAACCAACCTGCAA-3'
	R: 5'-ACATCCTGTCTTTCATGCGGCAAC-3'
LDL-r	F: 5'-TTGGGTTGATTCCAAACTCCAT-3'
	R: 5'-CCGATTGCCCCCATTGA-3'
CD36	F: 5'-CCTTAAAGGAATCCCCGTGT-3'
	R: 5'-TGCATTTGCCAATGTCTAGC-3'
ABCA1	F: 5'-AGCCAGAAGGGAGTGTCAGA-3'
	R: 5'-CATGCCATCTGGGTAAACCT-3'
IL-6	F: 5'-TACCAGTTGCCTTCTTGGGACTGA-3'
	R: 5'-TAAGCCTCCGACTTGTGAAGTGGT-3'
TNF-α	F: 5'-TCTCATGCACCACCATCAAGGACT-3'
	R: 5'-ACCACTCTCCCTTTGCAGAACTCA-3'
β-Actin	F: 5'-CGATGCCCTGAGGGTCTTT-3'
	R: 5'-TGGATGCCACAGGATTCCAT-3'

Abbreviations: LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; LDL-r, low-density lipoprotein receptor; CD36, scavenger receptor class B; ABCA1, ATP-binding cassette transporter A1; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.

safe, with low rates of severe myopathy, renal failure, and rhabdomyolysis [11, 12], due to its high hydrophilicity, hepatoselectivity, and low systemic bioavailability [12, 13]. However, most patients treated with statins do not reach the low-density lipoprotein-cholesterol (LDL-c) goal [13, 14] due to poor compliance, variability in drug response, inadequate titration of applied doses, and safety issues associated with high doses [13, 14]. Therefore, the present study investigated whether a combination of two different pharmacological drugs, statins and a non-lipid modifying agent, can achieve the LDL-c goal. Sarpogrelate (SP), a serotonin (5-HT) receptor antagonist, has been shown to reduce platelet aggregation and thrombus formation [12-16]. Recently, the ability of SP to delay atherosclerosis progression has gained much attention [11]. Several studies have shown that SP can upregulate endothelial nitric oxide synthase (eNOS) expression [17] and lower blood lipid levels and blood viscosity [18] in rabbits. SP treatment has been effective in reducing restenosis in patients with acute coronary syndrome [19]. In a study comparing the effects of sarpogrelate and placebos in patients with stable angina, restenosis rates, after coronary stenting in the SP group, were significantly reduced from 28.6 to 4.3% [20]. Another study investigating the effects of SP and aspirin treatment in patients with acute coronary syndrome showed that restenosis rates, after percutaneous balloon angioplasty, decreased in the SP group from 57 to 37% [21]. In addition, a previous study showed that SP exhibited anti-inflammatory and insulin-sensitizing effects [20]. Therefore, the present study investigated the synergistic effects of SP plus rosuvastatin, compared to rosuvastatin alone, in cardiac damage in ApoE$^{-/-}$ mice with hyperlipidemia.

Materials and methods

Animal experiments

All animal studies were approved by the Animal Studies Committee of Zhejiang Hospital. ApoE$^{-/-}$ mice were purchased from Beijing Vital River Lab Animal Technology CO., LTD. (Beijing, China). All mice were housed in a room with 12/12-hour light-dark cycles at a controlled temperature (24°C). Male ApoE$^{-/-}$ mice (8 weeks old) were randomly divided into four groups, as follows: mice fed a normal diet (control group, n = 7), mice fed a high-cholesterol diet (H group, n = 6), mice fed a high-cholesterol diet + rosuvastatin calcium (40 mg/kg/day; Mitsubishi Tanabe Pharma, Osaka, Japan) (HR group, n = 7), and mice fed a high-cholesterol diet + rosuvastatin calcium (40 mg/kg/day) + SP (50 mg/kg/day; Mitsubishi Tanabe Pharma, Osaka, Japan) (HRS group, n = 7). High-cholesterol diet contained 1.5% cholesterol and 15% fat. The experimental diet was purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). Mice in all groups were fed with the appropriate diet for 8 weeks. Blood samples were obtained from the inferior vena cava, collected in serum tubes, and stored at -80°C until use. Longitudinal sections of the hearts were fixed in 10% formalin and embedded in paraffin for histological evaluation. The remaining hearts were snap-frozen in liquid nitrogen for mRNA isolation and immunohistochemical analyses. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. This study was approved by the Ethical Committee of ZheJiang Hospital.

Biochemical measurements

Serum was obtained and stored at -80°C. Total cholesterol (TC), triglycerides (TG), and LDL-c

Table 2. Serum TC, TG, LDL-c, body weight (BW), and heart/body weight ratio (H/B) levels

	Control group (n = 7)	H group (n = 6)	HR group (n = 7)	HRS group (n = 7)
BW (g)	200.00±10.00	210.00±14.14	175.00±7.07	185.00±7.07
H/B	30.00±2.64	30.00±0.00	28.00±4.24	28.5±0.71
TC (mol/dL)	9.70±0.682	102.33±8.17*	78.28±21.25*,1	40.90±1.84*,2,a
TG (mol/dL)	2.87±0.85	3.13±0.81	2.08±0.39	1.40±0.28
LDL-c (mol/dL)	3.95±0.312	7.90±0.70*	14.70±2.12*,2	10.33±0.18*,b

Abbreviations: TC, total cholesterol; TG, triglycerides; LDL-c, low-density lipoprotein cholesterol; BW, body weight and H/B, heart weight/body weight ratio. Results are expressed as the mean ± SEM. *P<0.05, versus control group; 1P<0.05, 2P<0.01, versus H group; aP<0.05, bP<0.01, versus HR group.

levels were measured using a Hitachi 7020 automatic analyzer (Hitachi, Tokyo, Japan).

RNA isolation and real-time RT-PCR

Total RNA was isolated from the hearts using ISOGEN (Nippon Gene, Tokyo, Japan), according to manufacturer protocol. Complementary DNA (cDNA) was synthesized from total RNA using a first-strand cDNA synthesis kit (SuperScript VILO cDNA Synthesis Kit; Life Technologies Carlsbad, CA, USA), according to manufacturer protocol. Gene expression was analyzed quantitatively by real-time reverse transcription polymerase chain reaction (RT-PCR) using fluorescent SYBR Green technology (Light Cycler; Roche Molecular Biochemicals). β-Actin cDNA was amplified and quantified in each cDNA preparation to normalize relative amounts of the target genes. Primer sequences are shown in **Table 1**.

Western blot analysis

Proteins were extracted from heart tissues using radioimmunoprecipitation assay buffer (P0013B; Beyotime, Shanghai, China). Samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore, Billerica, MA, USA). Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5% skim milk, then incubated with the primary antibodies (P0023A; Beyotime) and gently shaken overnight at 4°C. Primary antibodies against lectin-like oxidized LDL receptor-1 (LOX-1) (rabbit anti-LOX-1 antibody, 1:500; Abcam) and β-actin (1:1000; Cell Signaling Technology) were used. Membranes were then incubated with a secondary antibody (anti-rabbit Ig-G, 1:1000; Cell Signaling Technology) for 1 hour. This experiment was carried out in triplicate. Protein levels were expressed as ratios to β-actin levels to minimize loading differences. Relative signal intensity was quantified using NIH ImageJ software.

Morphological analysis and immunohistochemistry

Hearts were dissected free from the surrounding connective tissue. Heart samples were collected and fixed with 4% paraformaldehyde. Samples were embedded in paraffin and then cut into slices using a microtome (Leica RM 2235 or Leica CM1850UV; Leica, Solms, Germany). Slices were then mounted onto glass slides and histological examinations were performed. Immunohistochemistry was performed using histone simple stain kit (Nichirei, Tokyo, Japan), according to manufacturer instructions. Briefly, sections were deparaffinized with xylene and then rehydrated in a descending series of ethanol. Sections were treated with 3% H_2O_2 in methanol for 15 minutes to inactivate endogenous peroxidases, then incubated with a primary antibody against LOX-1 (rabbit anti-LOX-1 antibody, 1:250; Abcam) at room temperature for 1 hour. All sections were examined under an Olympus B × 40 upright light microscope (Olympus, Tokyo, Japan).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using SPSS software, version 19.0. Inter-group variation was analyzed using one-way analysis of variance (ANOVA) and subsequent least significant difference (LSD)'s test. P<0.05 is considered statistically significant.

Results

Serum lipids

Results of serum lipids (**Table 2**) suggested that the hyperlipidemia model was successfully

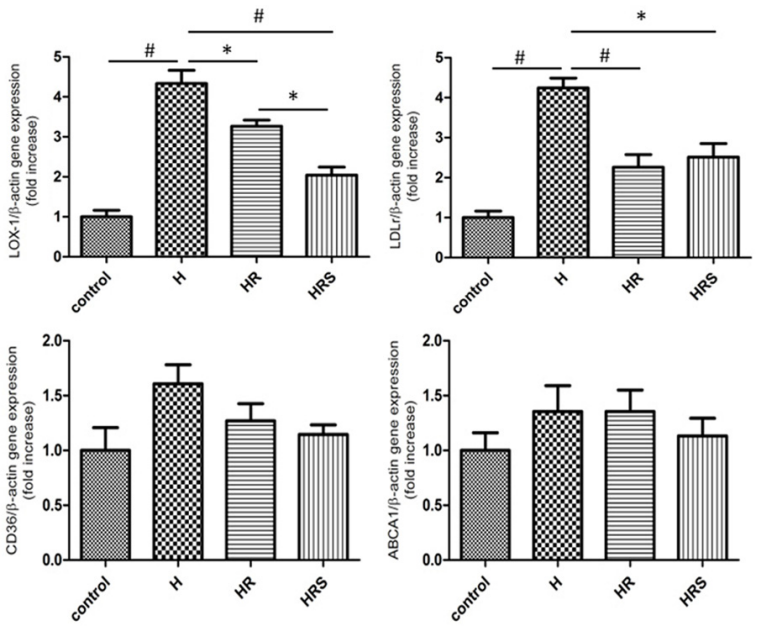

Figure 1. mRNA expression of LOX-1, LDL-r, CD36, and ABCA1 in heart tissues of four groups. LOX-1 and LDL-r gene expression significantly increased in the hearts of mice in the H group, compared to that in the control group. Increased expression of LOX-1 and LDL-r expression was suppressed in the HR and HRS groups. There was a significant difference in LOX-1 expression between the HR and HRS groups, whereas the difference in LDL-r expression between the HR and HRS groups did not reach statistical significance. CD36 expression increased in the H group, compared to that in the control group. However, it was similar in the HR and HRS groups. Expression of ABCA1 did not differ among the four groups. Results are expressed as the mean ± SEM. *P<0.05, #P<0.01.

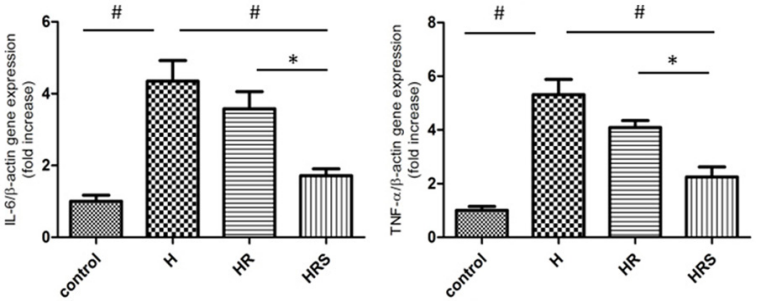

Figure 2. mRNA expression of IL-6 and TNF-α in heart tissues of four groups. Pro-inflammatory cytokines, including IL-6 and TNF-α, were upregulated in the H group, which was attenuated in the HR and HRS groups. IL-6 and TNF-α expression was significantly lower in the HRS group than that in the HR group. Results are expressed as the mean ± SEM. *P<0.05, #P<0.01.

established. ApoE$^{-/-}$ mice in the H groupshowed a marked increase in TC and LDL-c levels. TC levels decreased in HR and HRS groups, compared to those in the H group. Treatment with SP plus rosuvastatin was more effective in reducing TC and LDL-c levels, compared to treatment with rosuvastatin alone. Body weights (BW) and heart/body weight ratios (H/B) did not differ among the four groups.

mRNA expression of LOX-1, LDL-r, CD36, ABCA1, IL-6, and TNF-α in hearttissues

LOX-1 and LDL receptor (LDL-r) gene expression significantly increased in the hearts of mice in the H group, compared to that in the control group. However, this increase in LOX-1 and LDL-r expression was suppressed in HR and HRS groups. There was a significant difference in LOX-1 expression between HR and HRS groups, whereas the difference in LDL-r expression between HR and HRS groups did not reach statistical significance. Scavenger receptor class B (CD36) expression increased in the H group, compared to that in the control group. However, it was similar in HR and HRS groups. Expression of ATP-binding cassette transporter A1 (ABCA1) did not differ among the four groups (**Figure 1**). Pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), were upregulated in the H group, while attenuated in the HR and HRS groups. IL-6 and TNF-α expression was significantly lower in the HRS group than in the HR group (**Figure 2**).

Morphological analysis andimmunohistochemistry

To evaluate lipid deposition, immunohistochemical staining of LOX-1 was performed (**Figure 3**). Mice in the HR and HRS groups showed a marked reduction in LOX-1-positive staining in heart tissues, compared to that in the H group. In addition, LOX-1-positive staining was significantly lower in the HRS group than in the HR group. To examine macrophage infiltration, immunohistochemical staining of CD68 was

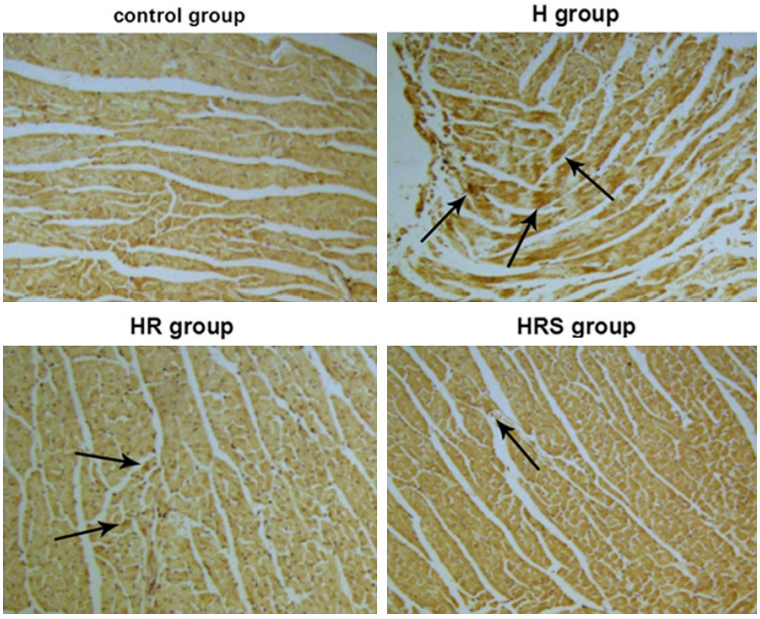

Figure 3. Immunohistochemical staining of LOX-1 in heart tissues of four groups. Mice in the HR and HRS groups showed a marked reduction in LOX-1-positive staining in the heart tissues, compared to that in the H group. LOX-1-positive staining was significantly lower in the HRS group than in the HR group. Arrows indicate positively stained cells.

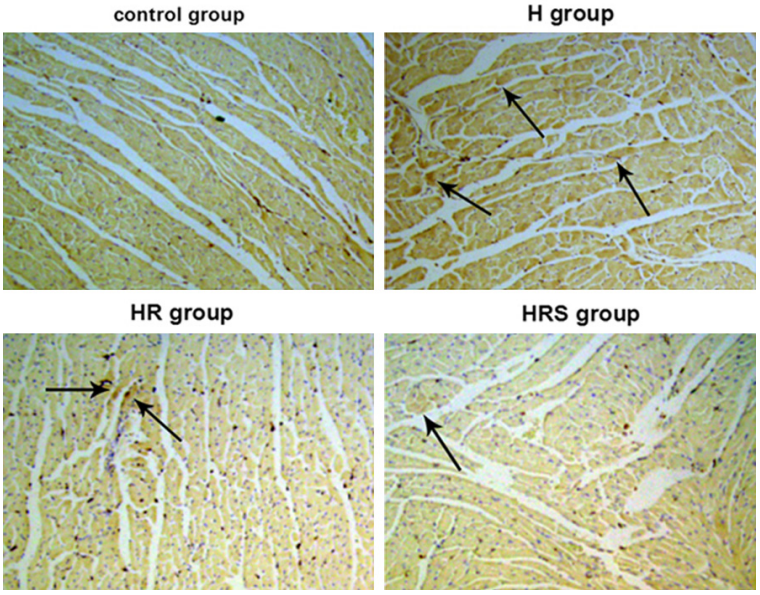

Figure 4. Immunohistochemical staining of CD68 in heart tissues of four groups. Mice in the HR and HRS groups showed an obvious decrease in CD68-positive staining in the heart tissues, compared to mice in the H group. CD68-positive staining was significantly lower in the HRS group than in the HR group.

performed (**Figure 4**). Mice in HR and HRS groups showed an obvious decrease in CD68-positive staining in heart tissues, compared to mice in the H group. In addition, CD-68-positive staining was significantly lower in the HRS group than in the HR group. Results indicate that SP plus rosuvastatin was more effective in reducing lipid deposition and macrophage infiltration in ApoE$^{-/-}$ mice with hyperlipidemia, compared to rosuvastatin alone.

LOX-1 protein expression

Immunoblotting was performed to measure LOX-1 protein expression. It was found that LOX-1 protein expression in the hearts of mice in the H group increased, compared to that in the control group. However, mice in HR and HRS groups exhibited markedly reduced LOX-1 expression in heart tissues, compared to that in the H group.In addition, LOX-1 protein expression was significantly suppressed in the HRS group, compared to that in the HR group. Results indicate that PS plus rosuvastatin was more effective in reducing LOX-1 expression in ApoE$^{-/-}$ mice with hyperlipidemia (**Figure 5**).

Discussion

Most importantly, results of the current study suggested that SP plus rosuvastatin was more effective in ameloriating cardiac damage in ApoE$^{-/-}$ mice with hyperlipidemia, compared to rosuvastatin alone. In particular, SP plus rosuvastatin significantly attenuated lipid deposition, inflammatory cytokine levels, macrophage infiltration, and hyperlipidemia-induced vascular smooth muscle cell (VSMC) migration and invasion of the heart.

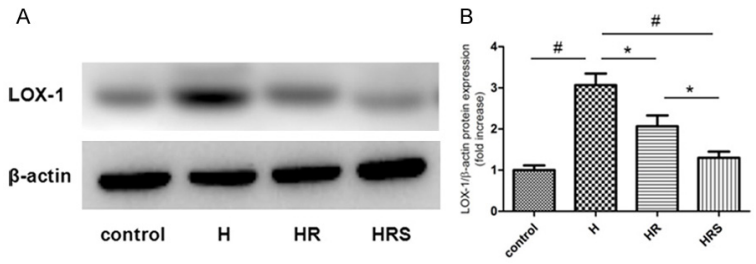

Figure 5. LOX-1 protein expression in heart tissues of four groups. A: Immunoblotting for LOX-1 in heart tissues. B: Bar graph showing quantification of LOX-1 protein expression. LOX-1 protein expression in the hearts of mice in the H group increased, compared to that in the control group. Mice in the HR and HRS groups exhibited markedly reduced LOX-1 expression in heart tissues, compared to that in the H group. And LOX-1 protein expresion was significantly suppressed in the HRS group, compared to that in the HR group. Results are expressed as the mean ± SEM. $^{*}P<0.05$, $^{\#}P<0.01$.

TC and LDL-c levels increased in the H group, compared to those in the control group. Interestingly, TC and LDL-c levels were significantly suppressed in both the HR and HRS groups, compared to that in the H group. There was a significant difference between HR and HRS groups. Results indicate that rosuvastatin plus SP synergistically lowered TC and LDL-c levels in ApoE$^{-/-}$ mice with hyperlipidemia. Present results are consistent with those of Park et al., showing that pravastatin and SP synergistically ameliorated atherosclerosis in LDLr-knockout (LDLr KO) mice [22]. However, lipid levels, except high-density lipoprotein cholesterol (HDL-c) levels, decreased significantly and similarly after 12 weeks of treatment in both the pravastatin alone and pravastatin plus SP groups. The decrease in levels of TC (pravastatin, 44%; pravastatin plus SP, 39%), TG (pravastatin, 87%; pravastatin plus SP, -72%), and LDL-c (pravastatin, 41%; pravastatin plus SP, 36%) was comparable between the two treatment groups [22]. SP did not affect lipid levels in LDLr KO mice. However, Kodama et al. revealed that SP attenuated intimal hyperplasia in rabbit vein grafts [23]. The present study did not include a group of ApoE$^{-/-}$ mice fed a high-cholesterol diet + SP alone. Thus, this study could not compare the effects of SP and rosuvastatin in hyperlipidemic ApoE$^{-/-}$ mice.

In hyperlipidemia, an imbalance of cellular lipid homeostasis can result in conversion of macrophages and migration and invasion of VSMCs. Macrophages contain massive amounts of cholesterol esters, which develop into foam cells, a hallmark of both early and late atherosclerotic lesions [24]. This process is mediated by several independent factors, including LOX-1, LDL-r, and CD36, and it regulates expression of the target gene, ABCA1 [24-26]. LOX-1 is expressed by macrophages, VSMCs, and vascular endothelial cells, which are the three most important cell types involved in the pathogenesis of atherosclerosis [27, 28]. oxLDL has been previously considered an important determining factor in the pathogenesis of atherosclerosis. LOX-1, a type II membrane protein functioning as a receptor of oxLDL, is the main receptor involved in the uptake of oxLDL. The role of LOX-1 in the pathogenesis of cardiovascular diseases, such as atherosclerosis, has been extensively studied [29, 30]. The present study measured gene expression of scavenger receptors, including LOX-1, LDL-r, CD36, and ABCA1. It was found that LOX-1 gene expression significantly increased in the hearts of mice in the H group, compared to that in the control group. This increase in expression of LOX-1 was suppressed in HR and HRS groups. There was a significant difference in LOX-1 expression between HR and HRS groups. These findings indicate that LOX-1 might be a critical factor involved in lipid accumulation in the hearts of ApoE$^{-/-}$ mice. Similarly, protein expression and immunohistochemical staining of LOX-1 in the heart tissues showed similar results. Rosuvastatin plus SP effectively ameliorated lipid deposition.

Recently, statins have been shown to exhibit immunomodulatory effects, reducing inflammatory cytokine secretion, T lymphocyte activation, mononuclear cell proliferation, and antigen-presenting capacity [31-33]. Previous studies have shown that statins reduce the production of proinflammatory cytokines, such as IL-6, IL-10, interferon-γ, and TNF-α, and stabilize vulnerable atherosclerotic plaques, which might be partially attributed to their immunomodulatory effects [22, 34-36]. Present findings are consistent with the results of a previous study. Rosuvastatin plus SP lowered lipid levels and reduced the progression of plaque lesions due to its anti-inflammatory properties, includ-

ing suppression of IL-6 and TNF-α levels. The present study showed that IL-6 and TNF-α expression was upregulated in the H group. However, this increase in expression of proinflammatory cytokines was attenuated in HR and HRS groups. In addition, IL-6 and TNF-α expression significantly decreased in the HRS group, compared to that in the HR group.

Hyperlipidemia-induced cardiac damage is usually associated with an increase in macrophages. Macrophages are major innate immune cells that play a principal role in the transition from inflammatory response to regeneration. CD68, an important macrophage biomarker, reflects macrophage burden [37-39]. Ding et al. showed that LOX-1 knockout mice exhibited less CD68 expression compared to wild-type mice [38]. In the present study, immunohistochemical staining of CD68 showed that mice in the HR and HRS groups exhibited obviously reduced CD68-positive staining in the heart tissues, compared to those in the H group. In addition, there was a significant difference between HRS and HR groups. Results suggest that SP plus rosuvastatin was more effective in reducing macrophage infiltration in ApoE$^{-/-}$ mice with hyperlipidemia, compared to rosuvastatin alone.

In conclusion, present results suggest that TC and LDL-c levels substantially decreased after rosuvastatin and SP combined therapy, compared to rosuvastatin alone treatment. Treatment with SP plus rosuvastatin demonstrated superior effects to rosuvastatin alone in terms of attenuation of lipid deposition, inflammatory cytokine expression, macrophage infiltration, and VSMC migration and invasion of the heart tissues of ApoE$^{-/-}$ mice with hyperlipidemia. The findings of this study may be beneficial in developing novel strategies for prevention and treatment of hyperlipidemia and CVD.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Liyue Zhu, Cardiac Rehabilitation Center, Zhejiang Hospital, 12 Lingyin Road, Hangzhou 310013, China. Tel: +86-571-8159517; Fax: +86-571-87980175; E-mail: liyue_zhu@163.com; Dr. Zuowei Pei, Department of Cardiology, Affiliated Zhongshan Hospital of Dalian University, 6 Jiefang Road, Dalian 116011, China. E-mail: pzw_dl@163.com

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