Cardiology Research and Practice

Endothelial Injury and Repair in Coronary Heart Disease

Lead Guest Editor: Zhen Yang
Guest Editors: Shiyue Xu and Yongping Bai

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Research Article

Effect of Chinese Medicine Xinmaitong on Blood Pressure in Spontaneously Hypertensive Rats

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Objective. To investigate the effect of traditional Chinese antihypertensive compound Xinmaitong on blood pressure and vasoactive factors of vasoconstrictor endothelin-1 (ET-1) and vasodilator calcitonin gene related peptide (CGRP) in spontaneously hypertensive rats (SHRs) with early stage hypertension. *Methods*. Twenty male SHRs were randomly divided into two groups: 10 for hypertensive control group and 10 for hypertensive treatment group. In addition, 10 Wistar rats were used as the normal control group without any intervention. SHRs of hypertensive treatment group were orally treated with Xinmaitong, while the hypertensive control group was treated with the normal saline (NS) for a total of eight weeks. The blood pressure in SHRs was examined before and after the end of the eight-week study. After treatment, the rats were killed and the blood samples were collected to measure plasma levels of ET-1 and CGRP by ELISA method, respectively. Meanwhile, the aorta rings were isolated for measuring the mRNA expression of ET-1 and CGRP by PCR. Moreover, the protein levels of ET-1 and CGRP were studied by immunohistochemical. *Results*. Daily oral administration of Xinmaitong resulted in significant fall in the SHRs' blood pressure, including systolic and diastolic blood pressures (SBP and DBP), mean blood pressure (MBP), and pulse pressure (PP). The plasma ET-1 levels were reduced and CGRP increased. In parallel, the mRNA and protein expression of ET-1 were decreased, whereas the mRNA and protein expression of CGRP were enhanced in SHRs treated with Xinmaitong. *Conclusion*. The present study demonstrated for the first time that Xinmaitong leads to the fall in blood pressure of SHRs and that this antihypertensive effect is, at least in part, due to improvement of arterial tone.

1. Introduction

Hypertension continues to be a classic worldwide problem and a major global health burden. Hypertension (HTN) or prehypertension (PreHTN) alone combined with other metabolic diseases such as obesity and diabetes is one of the major risk factors for the pathogenesis of atherosclerotic cardiovascular disease (ASCVD) [1, 2]. PreHTN, the intermediate stage between HTN and normal blood pressure, is associated with subclinical atherosclerosis and target-organ damage [3]. PreHTN and HTN pose significant clinical and public health challenges for both economically developing and developed nations. Reduced vasodilator [4] as well as increased vasoconstrictor [5] is the hall marker of hypertensive vascular injury. Therefore, effective blood pressure-lowering intervention together with the balance of

vasoactive materials towards enhanced production of vasodilator has significant clinical implication in order to prevent and treat ASCVD.

Xinmaitong is a traditional Chinese medicine compound preparation consisting of *Angelica sinensis*, *Salvia miltiorrhiza*, Uncaria Chinensis, *Panax notoginseng*, cassia seed, *Pueraria lobata*, *Sophora pubescens*, Mao Holly, *Prunella vulgaris*, and *Achyranthes bidentata*, which were usually used for antihypertension and has been also used to treat hypertension and ASCVD patients [6–13]. Clinical applications and experimental studies have shown that it has a significant effect on myocardial ischemia injury; in addition, clinical studies have also confirmed that Xinmaitong can improve the elastic index of large and small arteries and reduce the hypersensitivity CRP in patients with coronary heart disease. Another study shown that candesartan combined with Xinmaitong has a higher control rate in patients with simple diastolic hypertension. This suggests that Xinmaitong is an effective drug that can effectively protect the function of vascular endothelial cells and may play an antihypertensive effect [6, 14–18].

Calcitonin gene related peptide (CGRP) is one of the strongest vasodilators ever known, with the effects of lowering blood pressure, lowering peripheral resistance, diastolic renal arteries, and significantly increasing renal blood flow. CGRP also has a strong diastolic effect on the coronary arteries, and it is also effective on the coronary arteries of atherosclerosis. This diastolic effect does not depend on the presence of vascular endothelium, and it is not affected by serotonin receptor blockers. This indicates that CGRP binds to a specific CGRP receptor [19–22]. However, no study was performed to investigate the effects of Xinmaitong on blood pressure and vasoactive materials in spontaneous hypertensive rats (SHRs). Therefore, the present study was designed to observe the impact of Xinmaitong on blood pressure and vasoactive factors of vasoconstrictor endothelin-1 (ET-1) and vasodilator calcitonin gene related peptide (CGRP) in spontaneously hypertensive rats (SHRs).

2. Materials and Methods

Twenty male SHRs and 10 Wistar rats, aged four weeks and weighed 140–150 g, were purchased (Vital River Laboratories, Charles River Company, Beijing, China). All rats were housed in controlled temperature (23 to 25°C) and lighting (8:00 AM to 8:00 PM light; 8:00 PM to 8:00 AM dark) and had free access to standard food and drinking water. All animal experiments were approved by the Administrative Committee of Experimental Animal Care and Use of our hospital and conformed to the National Institute of Health guidelines on the ethical use of animals.

2.1. Experimental Protocol. Chinese herbal compound Xinmaitong was provided by Guizhou Yibai Pharmaceutical Co., Ltd. SHRs were bred in the Experimental Animal Center, Medical School of Sun-Yat Sen University, Guangzhou, China. After they were bred for seven days of adaptation, 20 SHRs were randomly divided into 2 groups: 10 for SHR control (SHR-C) group and 10 for SHR Xinmaitong (SHR-X) group. The 10 Wistar-Kyoto (WKY) rats were used as the normal control group. The WKY was fed without any intervention, The SHR-X were administered by gavage with 10 ml/kg body weight of 4.536% Xinmaitong suspension. The dosage of the drug was converted with the amount of the clinical routine drug (Xinmaitong 72 mg/kg) with reference to the conversion factor 6.3 between rats and humans. Furthermore, in consideration of rat generally administration volume is 1 ml/100 g, we choose dose of Xinmaitong at 10 ml/kg of body weight of 4.536% Xinmaitong suspension. In order to reduce the variability of the difference between the two groups, the SHR-C group was administered 10 ml/kg of body weight of 0.9% NS at the same time. The intervention time lasted eight weeks.

After eight weeks of intervention, all of the rats were killed and blood samples were harvested from the rats to test the plasma levels of ET-1 and CGRP. The aortas of the rats were isolated for the PCR and immunohistochemistry.

2.2. Biochemical Measurement. To observe the safety and side effects of Xinmaitong therapy, after eight weeks of intervention, all of the rats were killed and blood samples were harvested from the rats to measure hepatic and renal functions of the rats such as AST/GOT, ALT/GPT, TP, TBA, UREA, BUN, and CREA. The kits for these parameters were provided by Nanjing Jiancheng Bioengineering Institute, China.

2.3. Blood Pressure Measurement. To demonstrate the beneficial effect of Xinmaitong treatment on arterial blood pressure, we used a special sphygmomanometer called BP98A intelligent noninvasive blood pressure monitor to measure the rat's tail artery blood pressure (TABP) according to the machine manual. The first step is to open the device and software. The second step is to fix the rat so that it cannot move and fix the tail artery detector to the rat's tail. The third step is to keep the rat in a calm state and judge it to be in a stable state according to the software waveform. If the waveform is unstable, the measurement is delayed until the waveform is stable. The fourth step is to start the measurement, repeat the measurement three times, and take the average value. In order to ensure the accuracy of the measurement and to handle stress of animals, the measurement is guaranteed to be performed by the same operator in the same time period and environment. In addition, all rats had a two to three days' adaption measurement test before each measurement was performed. Furthermore, we take the performance three times only when the software shows the blood flow of the rat is stable; if the blood flow is unstable, the stabilization time can be appropriately extended and measured after the blood flow is stable.

Before and at the termination of eight-week treatment, all rat's TABP were measured by the same researcher. The systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), and pulse pressure (PP) were recorded.

2.4. ET-1 and CGRP Measurement. To evaluate the impact of Xinmaitong on vascular function, the plasma levels of vasoconstrictor ET-1 and vasodilator CGRP were examined. The ELISA kits were provided by Uscn Life Science Inc. Wuhan and we did the test according to the manufacturer's instructions.

2.5. PCR of ET-1 and CGRP. The thoracic aortas of the rats were isolated, and the isolated blood vessel was cut into three pieces of 3–4 mm wide vascular rings. Determination of thoracic aorta ET-1and CGRP mRNA were detected by PCR while the protein expression of them were examined by immunohistochemistry. A semiquantitative determination was carried out with a gelatin image analyzer, and the relative density grey value of ET-1and CGRP was used to stand for the relative expression quantity of ET-1, CGRP mRNA, and protein.

2.6. Immunohistochemistry of ET-1 and CGRP. Tissue sections were prepared of the thoracic aortas after fixation in 4% paraformaldehyde, dehydration, and embedding in paraffin. The expression of ET-1 and CGRP in the aorta tissue was examined using the SP immunohistochemistry kit according to the manufacturer's instructions. Densitometric analysis of immunocytochemical staining of ET-1and CGRP was carried out, and ET-1and CGRP staining intensity was expressed in optical density (OD) units.

2.7. Statistical Analysis. All the data were expressed as mean ± standard deviation and analyzed with the Statistical Package for the Social Sciences version 12.0 (SPSS 12.0). Comparisons between groups or between pre- and post-treatment were performed by t -test. The persons who analyzed the data were blinded to treatment-group assignment. For the graph made, Graphpad Prism 5 software was used. Throughout this study, a P-value less than 0.05 was considered statistically different.

3. Results

3.1. Safety Evaluation. The body weight and food intake of three groups were recorded before and after intervention. After eight weeks of treatment, the hearts of the rats were harvested. Weight of rats were measured and compared. For further safety and side effects evaluation, AST/GOT, ALT/GPT, TP, TBA, UREA, BUN, CREA, and γ-GT/γ-GTT were tested as the indicators for hepatic and renal function. Results show there were no significant differences in all the previously mentioned parameters in rats between the two groups ($P > 0.05$, Table 1).

3.2. Effects on SBP, DBP, MABP, and PP. SBP, DBP, MABP, and PP had no significant differences before treatment between SHR-C and SHR-X groups ($P > 0.05$) while they showed statistically significant differences after treatment (SBP lowering 46 mmHg, DBP lowering 41 mmHg, MABP lowering 42 mmHg, PP lowering 6 mmHg, $P < 0.05$, Figure 1). This indicates that Xinmaitong had an antihypertensive effect, including SBP, DBP, MABP, and PP.

Table 1: The hepatic and renal functions in experimental groups.

	Experimental groups		
	WKY	SHR-C	SHR-X
TP (mg/ml)	59.2 ± 11.6	51.0 ± 15.4	46.5 ± 20.3
CREA (μmol/l)	1.6 ± 0.3	1.5 ± 0.4	1.7 ± 0.2
BUN (mmol/L)	7.7 ± 1.1	9.9 ± 3.0	9.0 ± 2.3
TBA (μmol/L)	37.6 ± 5.5	43.6 ± 14.0	35.5 ± 18.6
ALT/GPT (IU/L)	106.9 ± 20.2	103.1 ± 8.9	92.7 ± 7.8
AST/GOT (U/L)	7.7 ± 2.7	7.4 ± 3.1	6.4 ± 6.1

Values are means ± SD. TP: total protein; CREA: creatinine; BUN: blood urea nitrogen; TBA: total bile acid; ALT: alanine transaminase; GPT: glutamate pyruvate transaminase; AST: aspartate aminotransferase; GOT: glutamic oxalo acetic transaminase. WKY: Wistar-Kyoto rats; SHR: spontaneously hypertensive rats; SHR-C: SHR control; SHR-X: SHR Xinmaitong treatment.

3.3. Impact on Vasoconstrictor ET-1 and Vasodilator CGRP. After eight weeks of treatment, the content of vasoconstrictor endothelin-1 (ET-1) in the SHR-C group was higher than that of WKY and SHR-X groups, while the content of vasodilator calcitonin gene related peptide (CGRP) in SHR-C group was lower than that of WKY and SHR-X groups, showing a statistically significant difference ($P < 0.05$, Figure 2).

3.4. Comparison of the mRNA and Protein Expression Levels of ET-1 and CGRP. In SHR-X group, the mRNA expression level of ET-1 was decreased and CGRP was increased significantly compared with the SHR-C ($P < 0.05$, Figure 3), which were consistent with protein expression results. The aorta immunohistochemistry shows the protein expression of ET-1 was decreased and CGRP was increased in SHR-X ($P < 0.05$, Figure 4).

4. Discussion

The major findings of the present study are the following. 1. Xinmaitong treatment markedly reduces the arterial blood pressure in SHRs. 2. Meanwhile, the increase in plasma CGRP levels together with upregulation of CGRP mRNA and protein are associated with the decline in ET-1 levels and ET-1 mRNA and protein expression. The present study demonstrates for the first time that Xinmaitong leads to the fall in blood pressure of SHRs and that this antihypertensive effect is, at least in part, due to improvement of arterial tone.

Xinmaitong is a traditional Chinese herbal medicine, which is extracted, concentrated, freeze-dried, and standardized from a mixture of 10 medicinal constituents and has been widely used in the treatment of ASCVD over the recent years [23]. Here, we found that Xinmaitong clearly results in the fall in arterial blood pressure in SHRs, suggesting that compared with western medication therapy traditional Chinese herbal intervention might also probably have a salutary effect on the blood pressure reduction in patient with hypertension.

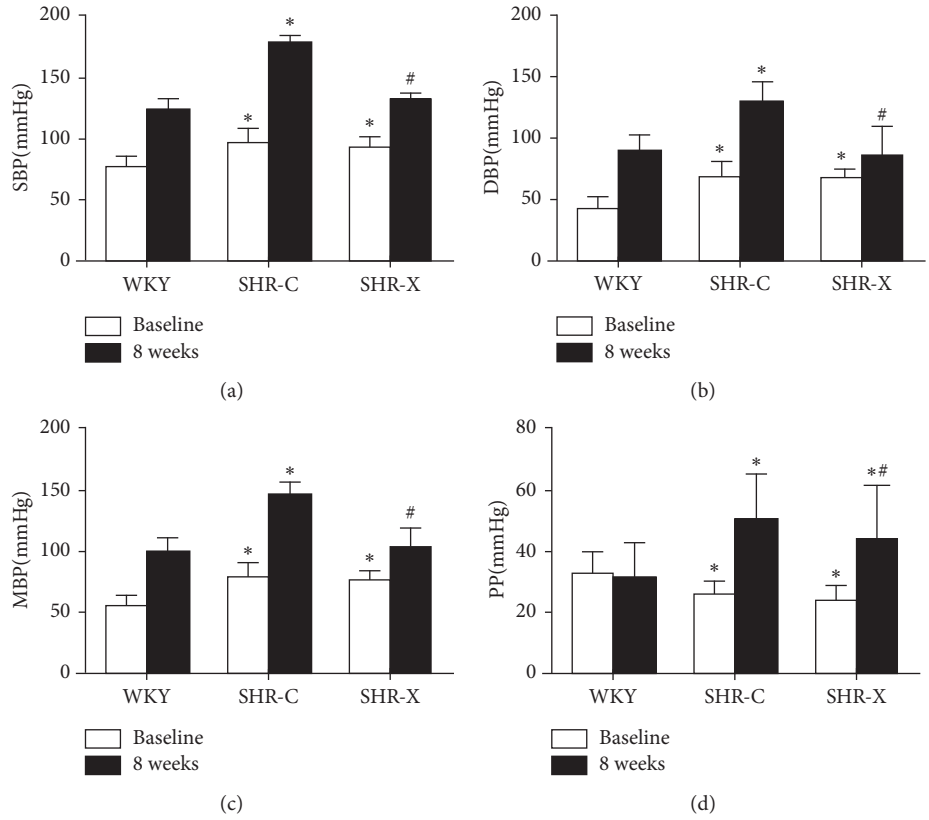

Figure 1: The effect of Xinmaitong on the blood pressure. Values are means ± SD. WKY: Wistar-Kyoto rats; SHR: spontaneously hypertensive rats; SHR-C: SHR control; SHR-X: SHR Xinmaitong treatment. $^{*}P < 0.05$ versus WKY. $^{\#}P < 0.05$ versus SHR-C.

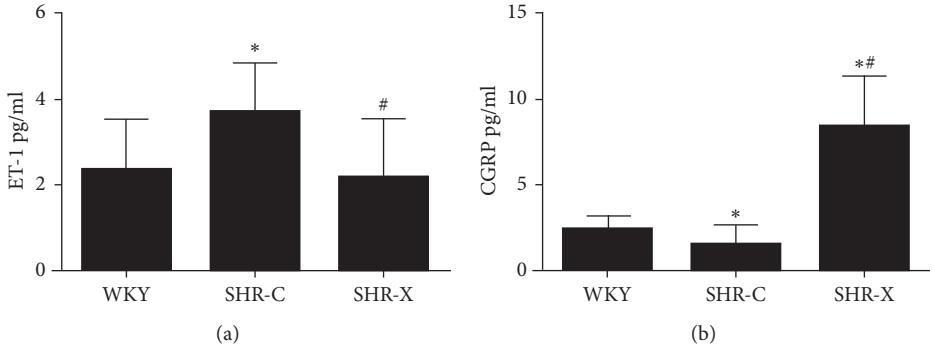

Figure 2: The effect of Xinmaitong on the blood vasoactive materials ET-1 and CGRP. Values are means ± SD. WKY: Wistar-Kyoto rats; SHR: spontaneously hypertensive rats; SHR-C: SHR control; SHR-X: SHR Xinmaitong treatment; ET-1: endothelin-1; CGRP: calcitonin gene-related peptide. $^{*}P < 0.05$ versus WKY. $^{\#}P < 0.05$ versus SHR-C.

Accumulating evidence indicates that patients with hypertension are characterized by endothelial dysfunction [24]. ET-1and CGRP are vascular endothelium-derived vasoactive factors. ET-1 has a strong endogenous biological vasoconstrictive effects. Endothelial cell damage is an important mechanism to increase the release of ET-1 [25]. CGRP is a strong endogenous vasodilatory neuropeptides, which has a strong dilation effect on blood vessels. In this study, we investigated the effects of Xinmaitong on ET-1 and CGRP. We found that Xinmaitong can not only improve the blood pressure but also reduce the secretion of ET-1 and promoting release of CGRP. Furthermore, mRNA and protein expression of CGRP and ET-1 were modulated after Xinmaitong treatment in SHRs. We supposed that these alterations are responsible for the vasoactive factors regulation of plasma CGRP and ET-1. The data reported here provide the preliminary evidence to show that Xinmaitong may protect endothelial function by maintaining the balance of vasoconstrictor ET-1and vasodilator CGRP thus helping blood pressure control.

There are some limitations in the present study. Firstly, the exact mechanism underlying Xinmaitong-mediated

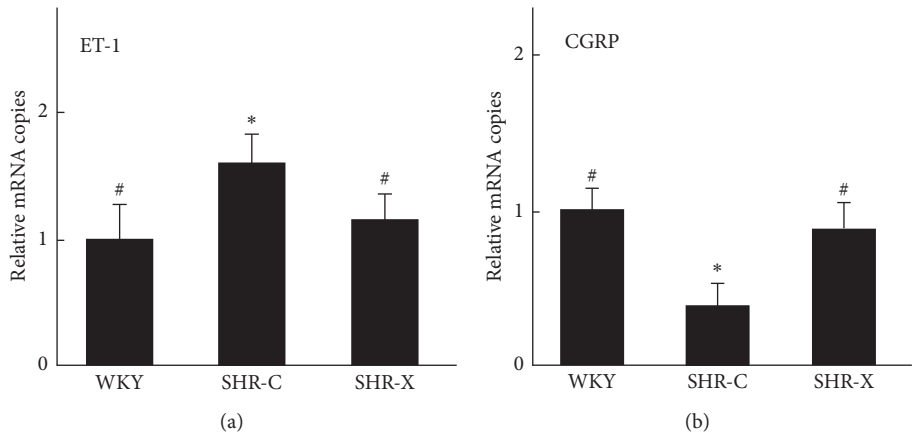

Figure 3: The level of ET-1 and CGRP mRNA. Values are means ± SD. WKY: Wistar-Kyoto rats; SHR: spontaneously hypertensive rats; SHR-C: SHR control; SHR-X: SHR Xinmaitong treatment; ET-1: endothelin-1; CGRP: calcitonin gene-related peptide. $^{*}P < 0.05$ versus WKY. $^{\#}P < 0.05$ versus SHR-C.

reduction in blood pressure of SHRs is not clear and beyond the present investigation, which remains to be further elucidated. Second, although our current data suggest that Xinmaitong treatment contributes to the improvement of vasoactive factors, the effect of Xinmaitong on endothelial function also needs to be investigated. Finally, in clinical practice, it is necessary to confirm whether patients with hypertension displayed the fall in blood pressure with Xinmaitong intervention alone.

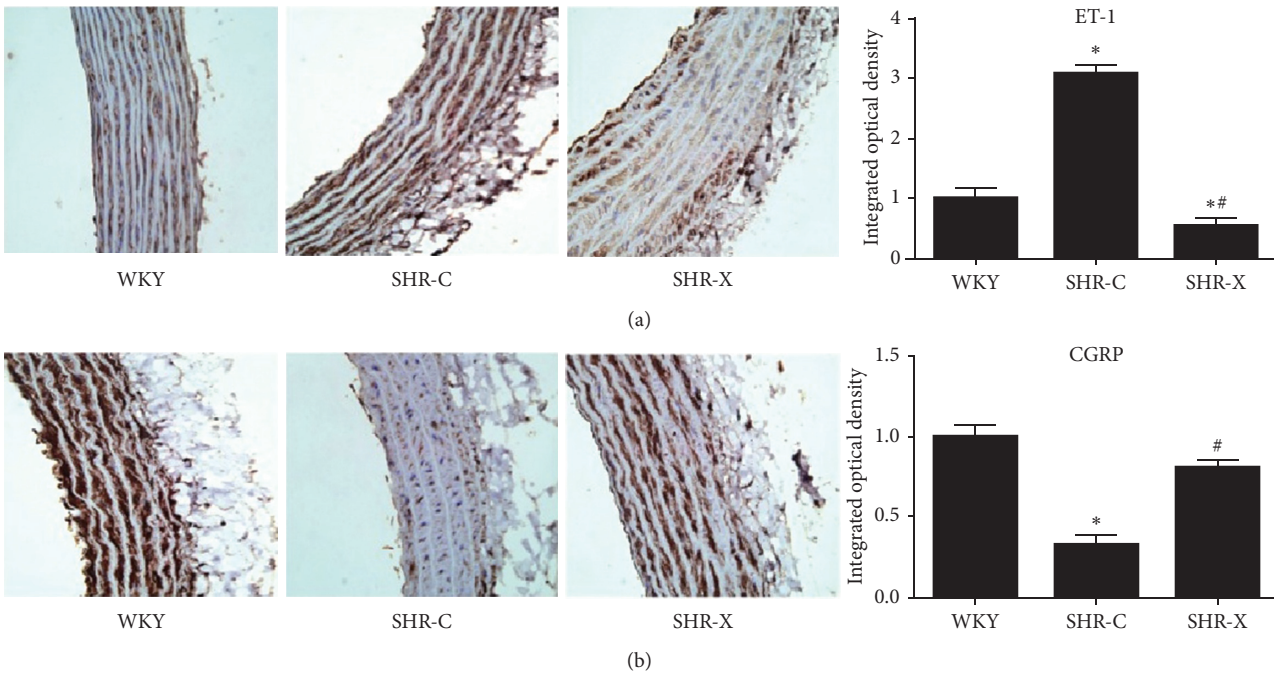

Figure 4: Immunohistochemistry of ET-1 and CGRP. Values are means ± SD. WKY: Wistar-Kyoto rats; SHR: spontaneously hypertensive rats; SHR-C: SHR control; SHR-X: SHR Xinmaitong treatment; ET-1: endothelin-1; CGRP: calcitonin gene-related peptide. $^{*}P < 0.05$ versus WKY. $^{\#}P < 0.05$ versus SHR-C.

In summary, the present study for the first time provide data to confirm the beneficial impact of traditional Chinese herbal medicine Xinmaitong treatment where it reduces the blood pressure in SHRs, and this antihypertensive effect might be partly related to the improvement of arterial tone.

Further investigation is under way in our laboratory in order to unravel the potential mechanism and clinical application of Xinmaitong in hypertension.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Both Bin Zhang and Dong Li contributed equally to this study. Bin Zhang and Dong Li performed the study and statistical analysis and wrote the manuscript. Wenfeng Tan and Gexiu Liu investigated the study subjects and performed laboratory analysis and statistical analysis. Gaoxing Zhang and Jun Guo designed the study and reviewed the manuscript.

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Research Article

Rejuvenated Circulating Endothelial Progenitor Cells and Nitric Oxide in Premenopausal Women with Hyperhomocysteinemia

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Hyperhomocysteinemia (HHcy) induced endothelial dysfunction is associated with disturbance in circulating endothelial progenitor cells (EPCs). Nevertheless, whether this unfavorable effect of HHcy on circulating EPCs also exists in premenopausal women is still unknown. Therefore, this leaves an area for the investigation of the difference on the number and activity of circulating EPCs in premenopausal women with hyperhomocysteinemia and its underlying mechanism. The number of circulating EPCs was measured by fluorescence-activated cell sorter analysis, as well as DiI-acLDL and lectin fluorescent staining. The migration and proliferation of circulating were evaluated by the Transwell chamber assay and MTT. Additionally, the endothelial function and levels of nitric oxide (NO), VEGF, and GM-CSF in plasma and culture medium were determined. The number or activity of circulating EPCs and flow-mediated dilatation (FMD) in premenopausal women with or without HHcy were higher than those in postmenopausal women. However, no significant effect of HHcy on the number or activity of circulating EPCs in premenopausal women was observed. A similar alteration in NO level between the four groups was observed. There was a correlation between FMD and the number or activity of EPCs, as well as NO level in plasma or secretion by EPCs. For the first time, our findings illuminated the quantitive or qualitative alterations of circulating EPCs and endothelial function in premenopausal patients with HHcy are preserved, which was associated with retained NO production. The recuperated endothelial repair capacity is possibly the potential mechanism interpreting cardiovascular protection in premenopausal women with HHcy.

1. Introduction

Hyperhomocysteinemia (HHcy) induced endothelial dysfunction, accelerating vascular injury in part as a result of atherosclerosis, is one of the independent risk factors for coronary heart diseases (CHD) and other cardiovascular diseases (CVD) [1–5]. Numerous clinical and epidemiological have demonstrated HHcy was significantly associated with flow-mediated dilatation (FMD) reduction, indicting potential vascular endothelial injury [6]. This injury-induced endothelial dysfunction plays a crucial role in the initiation of atherosclerosis. The nature of endothelial dysfunction ultimately represents an imbalance between the magnitude of injury and the repair capacity, increased the peripheral resistance, and further aggravating the endothelial injury, indeed constituted a vicious cycle [5]. Therefore, it is critical to accelerate endothelial repair and maintenance vascular endothelial integrity for the prevention and treatment of CVD by HHcy.

Endothelial progenitor cells (EPCs) are a kind of the primitive cells derived from the bone marrow, which could accelerate reendothelialization, repair the endothelial injury, and improve endothelial function [7–9]. Owing to physiological or pathological factors, the EPCs in the bone marrow

will enter the peripheral blood circulation to circulate and proliferate, which can be differentiated into mature endothelial cells, and they participate in vascular repair or formation. The following study shows that the cyclic endothelial cells are involved in repairing damaged endothelial cells, which plays an important role in maintaining the endothelial integrity of the vessel after an arterial injury [10–12]. A growing body of evidence has demonstrated that patients with HHcy have decreased the number of endothelial progenitor cells, increased cell apoptosis rate, and impaired EPCs proliferation and adhesion ability [5, 13]. This phenomenon limited the capacity of EPCs repair and beneficial effect for vascular endothelial.

Clinical and epidemiological have demonstrated that premenopausal women have a decreased prevalence of CVD. However, postmenopausal women have an increased prevalence of cardiovascular diseases, such as congestive heart failure, coronary atherosclerosis, stroke, and arrhythmias [14-15]. These results indicate that estrogen may play a crucial role in preventing cardiovascular disease. A previous study showed that the activity of circulating EPCs in premenopausal women with prehypertension or diabetes mellitus was preserved [7, 11]. However, further study is needed to determine whether the number and activity of circulating EPCs are still retained in HHcy premenopausal women. Therefore, we hypothesize that the number and functional activity of circulating EPCs in HHcy premenopausal women may be different form postmenopausal women with HHcy. In addition, nitric oxide (NO), vascular endothelial growth factor (VEGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) plays an important role in regulating mobilization, as well as migration and proliferation of circulating EPCs [16–19].

In this study, we measured the number and functional activity of circulating EPCs in HHcy patients, investigated the level of NO, VEGF, and GM-CSF in plasma and EPCs culture medium, and elucidated the possible mechanism which is responsible for alteration in endothelial repair capacity in HHcy patients.

2. Methods

2.1. Subject Characteristics. Peripheral blood samples were collected from 80 subjects in our study: twenty healthy premenopausal women, twenty HHcy premenopausal women, twenty healthy postmenopausal women, and twenty HHcy postmenopausal women. The serum homocysteine level was measured by an automatic fluorescence immunoassay method (Abbott, USA). HHcy was defined as a plasma fasting total homocysteine concentration >15 μmol/L [13, 20]. Patients with a history of autoimmune disease, mental disease, diabetes, hypohepatia, renal insufficiency, malignant tumor, gestation period, suckling period, or unwilling to accept the test subjects were excluded. The experimental protocol was approved by the Ethics Committee of our hospitals. Table 1 shows the baseline characteristics of the four subjects. Peripheral blood samples were used for determining EPCs, blood urea nitrogen, triglycerides, high density lipoprotein cholesterol, LDL-cholesterol, serum creatinine, plasma glucose, high density lipoprotein, total cholesterol, total homocysteine, and so on.

2.2. Isolation and Cultivation of EPCs. EPCs were isolated and cultured as previously described [7, 11, 21–24]. In brief, peripheral blood mononuclear cells were obtained from four groups that were isolated by Ficoll density-gradient centrifugation, and it was cultured in endothelial cell growth medium-2 (EGM-2) (500 μmL; Clonetics, San Diego, CA, USA). The cell suspension was incubated at 37°C incubator. After 4 days, we removed nonadherent cells and replaced the medium.

2.3. Flow Cytometry. Circulating EPCs were assessed by the number of $CD34^+KDR^+$, peripheral blood mononuclear cells (PBMNCs) by flow cytometry analysis (Beckman Coulter, Fullerton, CA, USA) as previously described [11, 24]. In brief, peripheral blood (100 μL) was incubated with Phycoerythrin (PE) anti-human kinase-insert domain receptor (KDR; 1 : 20; 4A Biotech, Co., Ltd., Beijing, China), fluorescein isothiocyanate (FITC) anti-human CD45 (1 : 10; cat. 4A Biotech, Co., Ltd), and PE-Cy7 anti-human CD34 (1 : 10; 4A Biotech, Co., Ltd). Analysis was done by ACEA NovoCyteTM (ACEA Biosciences, San Diego, CA, USA). The ratio of $CD45^-CD34^+KDR^+$cells was defined as circulating EPCs.

2.4. Double-Positive Fluorescence Identification. After 7 days culture, the plated EPCs on the cell culture flasks were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-acetylated low density lipoprotein (acLDL) (DiI-acLDL, 10 μL/mL, Molecular probes) at 37°C for 1 h and they were incubated with FITC-labeled Ulex europaeus agglutinin (lectin, 10 μg/mL, Sigma). After staining, the samples were observed under a phase-contrast fluorescence microscope (magnification, ×200). Cell demonstrating double-positive fluorescence were identified as differentiating EPCs.

2.5. Migration and Proliferation of EPCs In Vitro

2.5.1. EPCs Migration. EPC migration was determined using a modified Boyden chamber as described in a previous study [22, 23]. In brief, EPCs migration was evaluated by using a transwell chamber (Costar Transwell ® assay, 8 μm pore size, Corning, NY). A total of 2×104 EPCs were placed in the upper chamber. The chambers were placed in 24-well culture dish containing 500 μL EBM-2 and human recombinant VEGF (50 ng/mL). After 24 h, EPCs were stained by DAPI. Nonmigratory cells were removed from the upper chamber with the use of an absorbent tip. Cells were migrating into the lower counted by a fluorescence microscope.

2.5.2. EPCs Proliferation. EPCs proliferation was evaluated by 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) as described in previous study [22]. In brief, after 7 days of

Table 1: Clinical and biochemical characteristics.

Characteristics	Premenopausal women		Premenopausal women	
	Control ($n = 20$)	HHcy ($n = 20$)	Control ($n = 20$)	HHcy ($n = 20$)
Age (years)	46.1 ± 4.3	44.3 ± 4.8	55.3 ± 4.9$^{\#}$	56.7 ± 4.4$^{\#}$
Height (cm)	161.2 ± 5.5	160.3 ± 5.1	167.9 ± 4.6$^{\#}$	166.6 ± 5.5$^{\#}$
Weight (kg)	62.1 ± 5.4	60.0 ± 5.3	64.7 ± 5.5	65.3 ± 4.5$^{\#}$
BMI (kg/cm^2)	23.9 ± 2.1	23.4 ± 2.1	22.9 ± 1.7	23.6 ± 2.0
Systolic blood pressure (mmHg)	117.9 ± 10.4	119.9 ± 8.1	121.7 ± 10.8	122.3 ± 11.2
Diastolic blood pressure (mmHg)	75.9 ± 10.0	72.9 ± 11.1	76.6 ± 8.4	74.1 ± 8.3
AST (mmol/L)	26.0 ± 5.6	26.1 ± 5.6	23.5 ± 5.6	24.3 ± 6.2
ALT (mmol/L)	23.4 ± 4.8	23.0 ± 6.9	20.5 ± 5.4	22.6 ± 5.5
BUN (mmol/L)	5.4 ± 0.9	5.5 ± 1.1	5.1 ± 1.1	5.5 ± 1.0
Cr (mmol/L)	68.9 ± 12.4	71.3 ± 14.7	67.1 ± 16.5	72.4 ± 16.5
LDL (mmol/L)	2.74 ± 0.24	2.86 ± 0.25	2.68 ± 0.41	2.79 ± 0.35
TC (mmol/L)	4.65 ± 0.35	4.80 ± 0.28	4.57 ± 0.51	4.71 ± 0.37
HDL (mmol/L)	1.41 ± 0.25	1.37 ± 0.20	1.44 ± 0.22	1.40 ± 0.15
TG (mmol/L)	1.38 ± 0.18	1.43 ± 0.17	1.36 ± 0.19	1.41 ± 0.14
FPG (mmol/L)	4.58 ± 0.65	4.77 ± 0.64	4.35 ± 0.52	4.63 ± 0.69
Homocysteine (μmol/L)	10.4 ± 1.6	20.7 ± 3 4*	9.4 ± 1.8	21.8 ± 4.5*
Estradiol (pmol/L)	209.2 ± 20.7	202.4 ± 29.8	99.6 ± 16.9$^{\#}$	107.5 ± 16.7$^{\#}$
FMD (%)	9.46 ± 1.33	8.39 ± 1.22*	8.25 ± 1.07$^{\#}$	5.09 ± 0.92$^{\#\star}$

Abbreviation: BMI: body mass index; AST: aspartate amino transferals; ALT: alanine transaminase; BUN: blood urea nitrogen; Cr: serum creatinine; LDL: low density lipoprotein; TC: total cholesterol; HDL: high density lipoprotein; TG: triglyceride; FPG: fasting plasma glucose; FMD: flow-mediated brachial artery dilatation. Notes: Data are given as mean ± SD. *vs. normal weight; $^{\#}$ vs. premenopausal women.

culture, EPCs were digested by 0.25% trypsin and were cultured in serum-free medium in 96-well culture plates for 24 h. Then, EPCs were cultured with MTT (5 g/L; Fluka; Honeywell International, Inc., Shanghai, China) and incubated for a further 4 h. Measurement of EPCs' proliferation by the optical density value at 490 nm.

2.6. Measurement of NO, GM-CSF, and VEGF Levels in Plasma and Secretion by EPCs. NO, VEGF, and GM-CSF levels in plasma and secretion by EPCs were evaluated as we previously described [7, 11].

2.7. Endothelial Function Evaluation. As described previously, FMD measurement in the brachial artery was performed with subjects in the supine position for the evaluation of endothelial function. Brachial artery diameter was imaged with a 5–12-MHz linear transducer on an HDI 5000 system (Washington, USA). The brachial artery diameters at baseline ($D0$) and after reactive hyperemia ($D1$) was recorded. The FMD $[(D1–D0)/D0 \times 100\%]$ was used as a measure of endothelium-dependent vasodilation. Pressure in an upper-forearm sphygmomanometer cuff was raised to 250 mmHg for 5 min and FMD calculated as the percentage increase in mean diastolic diameter after reactive hyperemia 55 to 65 s after deflation to baseline. After a further 15 min, 400 μg sublingual glyceryl trinitrate (GTN) was given and diastolic diameter was remeasured after 5 min for measurement of endothelial-independent dilatation.

2.8. Statistic Analysis. The statistic software was SPSS V22.0 (SPSS Inc., Chicago, Illinois). All the data were presented as mean values ± SD. Comparisons between the four groups were analyzed by two-factor analysis of variance (premenopausal and postmenopausal women, status of no-HHcy or HHcy). When indicated by a significant F-value, a post hoc test using the Newman-Keuls method identified significant differences among mean values. Univariate correlations were calculated using Pearson's coefficient (r). Statistical significance was assumed if a null hypothesis could be rejected at $P < 0.05$.

3. Results

3.1. Subject Characteristics. As Table 1 shows, all of the subjects had enrolled 80 volunteers. In the baseline values, there was no significant difference in terms of BMI between the four groups. Evidently, the homocysteine level in HHcy premenopausal women and HHcy postmenopausal women was significantly higher than that in the control group ($P < 0.05$). Compared with the postmenopausal women group, the level of estradiol in premenopausal women was higher ($P < 0.05$). In addition, the FMD in postmenopausal women was lower than premenopausal women. The FMD in the HHcy group was lower than the control group, ($P < 0.05$), but there was no significant difference in terms of FMD between HHcy premenopausal women and healthy premenopausal women. There was no significant difference in systolic blood pressure, diastolic blood pressure, HDL, Cr, BUN, LDL, TC, TG, AST, ALT, and FPG for four groups ($P > 0.05$).

3.2. The Number, Migratory Capacity, and Proliferative Activities of Circulating EPCs. The number of circulating EPCs

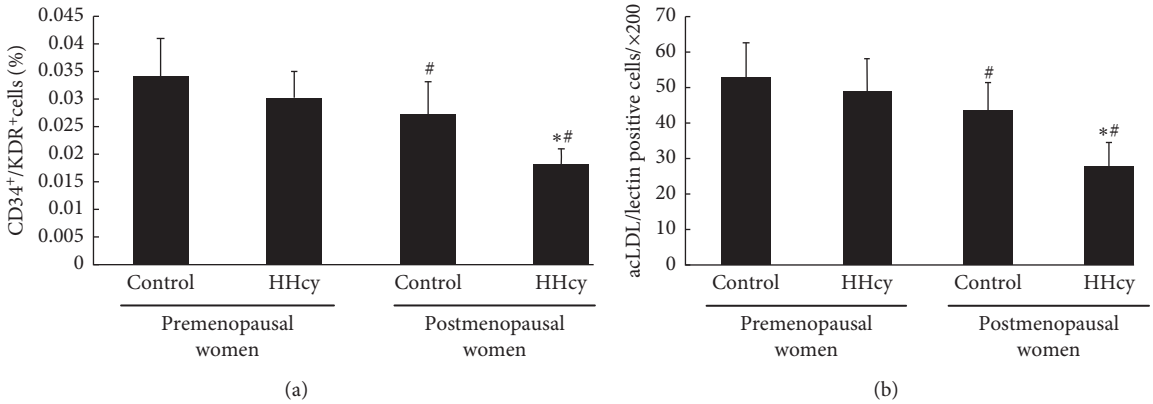

Figure 1: The number of circulating EPCs in the four groups is shown as follows. Evaluated by (a) FACS analysis and (b) phase-contrast fluorescent microscope, the number of circulating EPCs in postmenopausal women was lower than those in premenopausal women. The EPC number in hyperhomocysteinemia in postmenopausal women was lower than that in control postmenopausal women. However, there was no significant difference in the level of the number of circulating EPCs between control and hyperhomocysteinemia premenopausal women. Data are given as mean ± SD. ☆vs. control; # vs. premenopausal women.

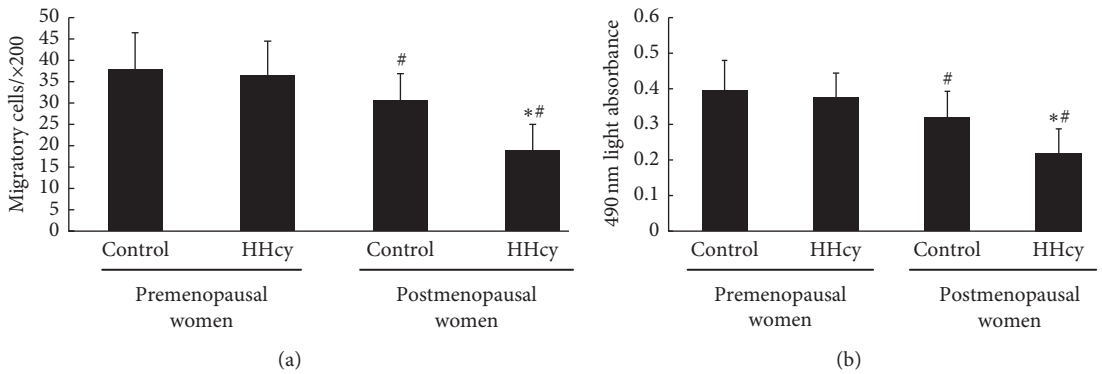

Figure 2: The activity of circulating EPCs in the four groups is shown as follows. The migratory (a) and proliferative (b) activities of circulating EPCs in postmenopausal women were lower than those in premenopausal women. There was no difference in the migratory (a) and proliferative (b) activity between control and hyperhomocysteinemia premenopausal women. Nevertheless, the EPC function in hyperhomocysteinemia postmenopausal women was lower than that in control postmenopausal Women. Data are given as mean ± SD. ☆vs. control; # vs. premenopausal women.

in the four groups is shown in Figure 1. The number of circulating EPCs of circulating EPCs in postmenopausal women was lower than those in premenopausal women. The EPC number in HHcy postmenopausal women was lower than that in control postmenopausal women. However, no significant difference in the level of the number of circulating EPCs between control and HHcy premenopausal women.

As shown in Figure 2, the migratory (a) and proliferative (b) activities of circulating EPCs in postmenopausal women were lower than those in premenopausal women. There was no difference in the migratory (a) and proliferative (b) activity between control and HHcy premenopausal women. Nevertheless, the EPC function in HHcy postmenopausal women was lower than that in healthy postmenopausal women.

3.3. Plasma NO, GM-CSF, and VEGF Levels in Each Group. As Figure 3 shows, the plasma NO, VEGF, and GM-CSF levels in the four groups were as follows. (a) The plasma NO level in postmenopausal women was lower than that in premenopausal women. The plasma NO level in HHcy postmenopausal women was lower than that in control postmenopausal women, but there was a similarity in control and HHcy premenopausal women. (b) There was no significant difference in the plasma VEGF level between the four groups. (c) There was no significant difference in the plasma GM-CSF level between the four groups.

3.4. NO, GM-CSF, and VEGF Secretion by EPCs in Four Groups. As shown in Figure 4. The NO, VEGF, and GM-CSF secretion by EPCs in the four groups was as follows. (a) The NO secretion by EPCs in postmenopausal women was lower than that in HHcy premenopausal women. No difference in NO secretion by EPCs between control and HHcy premenopausal women was found. However, the NO secretion level in HHcy postmenopausal women was lower than that in control premenopausal postmenopausal women. (b) There was no significant difference in VEGF

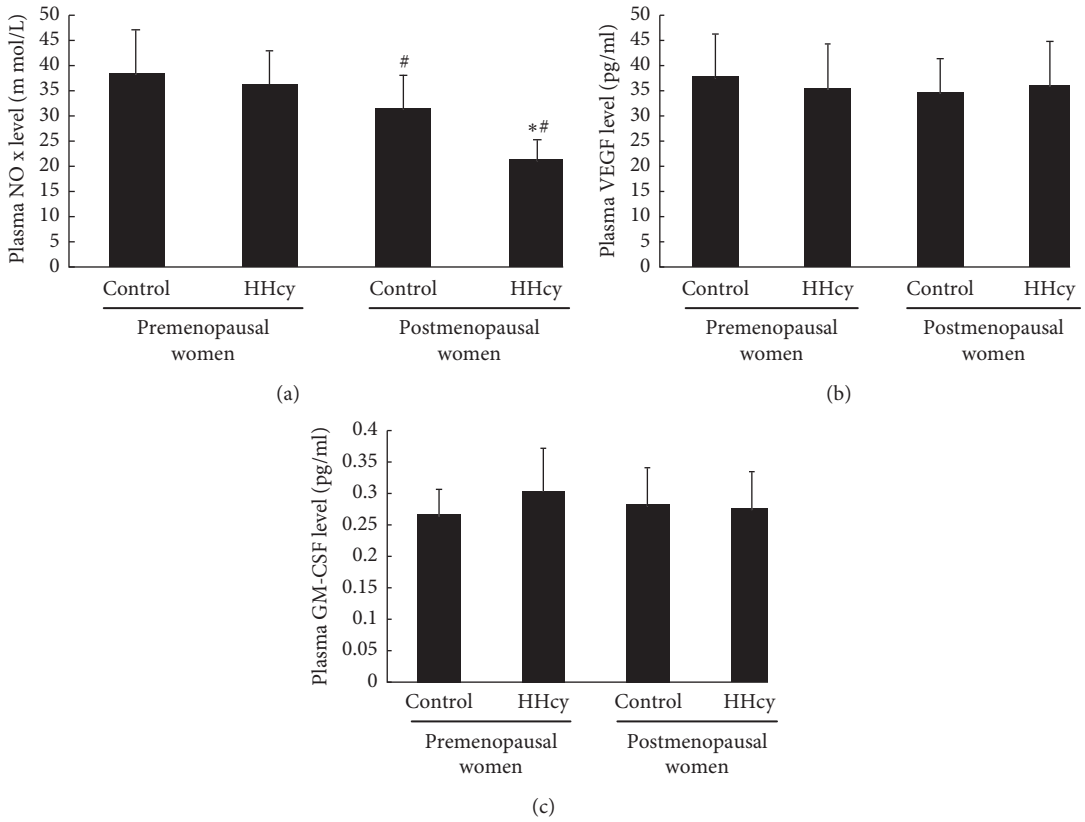

Figure 3: The plasma NO, VEGF, and GM-CSF levels in the four groups were shown as follows. (a) The plasma NO level in postmenopausal women was lower than that in premenopausal women. The plasma NO level in hyperhomocysteinemia postmenopausal Women was lower than that in control, but there was a similarity in control and hyperhomocysteinemia premenopausal women. (b) There was no significant difference in the plasma VEGF level between the four groups. (c) There was no significant difference in the plasma GM-CSF level between the four groups. Data are given as mean ± SD. *vs. control; $^{\#}$ vs. premenopausal women.

secretion by EPCs between the four groups. (c) There was no significant difference in GM-CSF secretion by EPCs between the four groups.

3.5. The Correlation between the Migratory and Proliferative Activities of Circulating EPCs or Plasma NO Level. As Figure 5 shows, the correlation between circulating EPCs or NO level and FMD was as follows. The number of circulating EPCs evaluated by FACS (a) or by cell culture (b) correlated with the FMD. There was a correlation between EPC proliferation (c) or migratory (d) and FMD. In addition, there was a correlation between the plasma NO level (e) or NO secretion by EPCs (f) and FMD.

4. Discussion

In this study, the effect of age difference on the number and activity of circulating EPCs in HHcy women was detected. We found the vascular endothelial function evaluated by FMD in HHcy premenopausal women, as well as the number and activity of circulating EPCs was preserved. Similarly, NO level in plasma or secretion by EPCs in premenopausal women also remained. In addition, we also demonstrated that the number of circulating EPCs, as well as NO in plasma or secretion by EPCs, were significantly reduced, and migratory and proliferative activities of circulating EPCs were also impaired, indicating that the endothelial function-decreased may be closely related to HHcy. There was a significant correction between the number, proliferation, migration of circulating EPCs, and FMD. Similarly, there was a close correction between FMD and NO production in plasma or secretion by EPCS. Therefore, in the present study, which is at least in part associated with the enhanced NO production.

The effects of HHcy on endothelial function have been studied extensively. Accumulating pieces of evidence have shown that HHcy-induced endothelial injury and endothelial dysfunction result in the damage of endothelial integrity, which may accelerate HHcy-related vascular atherosclerosis [13, 25–28]. FMD is a reliable and effective noninvasive new technique [7, 29], and it is widely used to evaluate endothelial dysfunction in CVD. In the current study, we revealed that the FMD in HHcy premenopausal women was preserved. Besides, there is a close correlation between the number and function properties of EPCs and FMD, suggesting increased endothelial repair capacity in premenopausal women. Additionally, we also observed decreased FMD in HHcy men compared with the healthy group, indicating HHcy is a risk factor for vascular endothelial dysfunction.

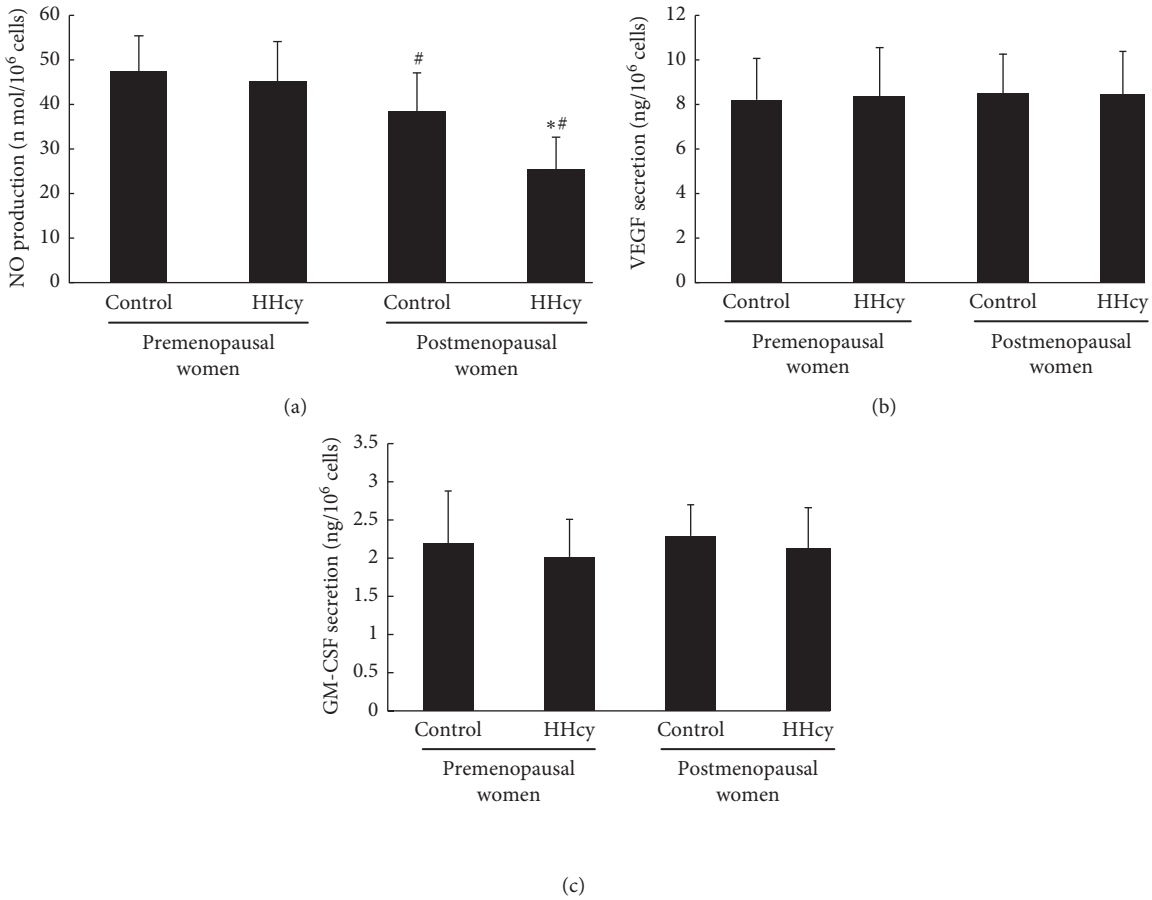

Figure 4: The NO, VEGF, and GM-CSF secretion by EPCs in the four groups was showed as follows. (a) The NO secretion by EPCs in postmenopausal women was lower than that in premenopausal women. No difference in NO secretion by EPCs between control and hyperhomocysteinemia premenopausal women was found. However, the NO secretion level in hyperhomocysteinemia postmenopausal women was lower than that in control. (b) There was no significant difference in VEGF secretion by EPCs between the four groups. (c) There was no significant difference in GM-CSF secretion by EPCs between the four groups. Data are given as mean ± SD. *vs. control; $^{\#}$ vs. premenopausal women.

EPCs can repair injury-induced endothelium [7, 23]. Increasing pieces of evidence have suggested that EPCs contribute up to 25% of endothelial cells in newly formed vessels [13]. The decrease in the number and activity of circulating EPCs may be the related mechanism of endothelial dysfunction and endothelium damage [30]. In our study, we have demonstrated a significant decrease of the number, migratory, and proliferative activities of circulating EPCs in the HHcy postmenopausal women group, but it was preserved in HHcy premenopausal women group, indicating endogenous prevention for endothelial injury in premenopausal women. Therefore, maintaining endothelial integrity is essential for the HHcy postmenopausal women group.

NO not only modulates the mobilization of EPCs from the bone marrow but also improves the function of EPCs. Decreased NO bioavailability by HHcy may reduce nitric oxide-mediated endothelium-dependent vasodilation, which was associated with elevated peroxynitrite in pathological conditions [2]. VEGF and GM-CSF could regulate the number and activity of circulating EPCs [16]. Therefore, we hypothesized that the number and activity of EPCs my be related to NO, GM-CSF, and VEGF. In our study, plasma NO level was restored in HHcy premenopausal women. Besides, plasma NO level in HHcy postmenopausal women was lower than the premenopausal women group, indicating that the decreased number and activity of circulating EPCs may be closely associated with decreased NO production in HHcy postmenopausal women. In addition, a close correction has been observed between NO level in plasma or secretion by EPCs and FMD, indicating NO-mediated prevention of vascular may reverse HHcy-mediated endothelial injury. Furthermore, we discovered the NO secretion by EPCs in HHcy postmenopausal women was lower than healthy postmenopausal women group, indicating inhibited NO production by EPCs may result in a decreased number of circulating EPCs and attenuated activity of EPCs. Decreased NO production secreted by EPCs was a key factor in endothelial dysfunction. The present study indicated that restored exogenous NO production could reverse the number or activity of circulating EPCs in HHcy premenopausal women.

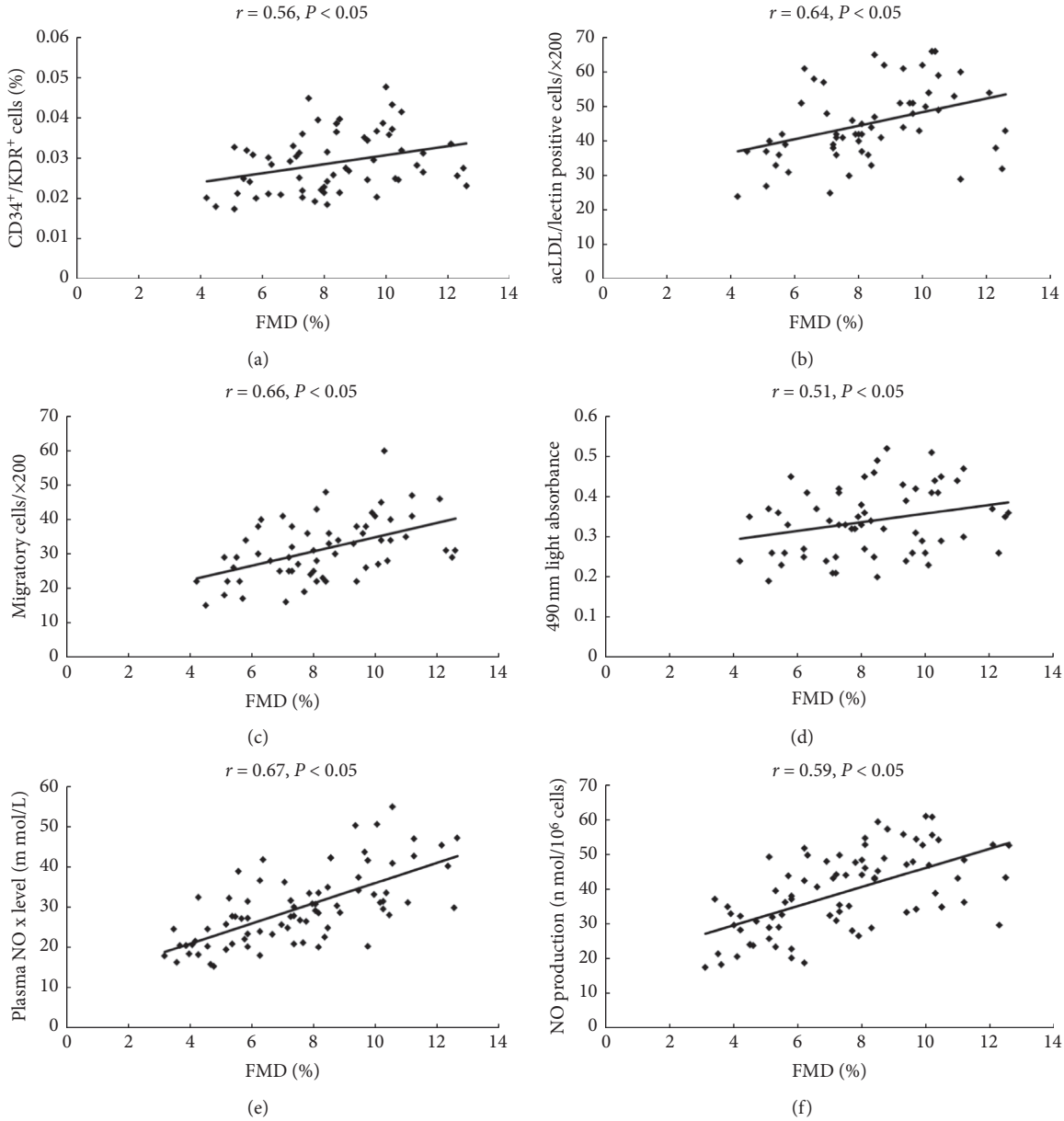

Figure 5: The correlation between circulating EPCs or NO level and FMD was shown as follows. The number of circulating EPCs evaluated by FACS (a) or by cell culture (b) correlated with the FMD. There was a correlation between the EPC proliferation (c) or migratory (d) and FMD. In addition, there was a correlation between the plasma NO level (e) or NO secretion by EPCs (f) and FMD.

5. Limitation

Our research had a few limitations. There were no enough subjects included in this study. In order to reveal whether HHcy can affect the number and function of endothelial progenitor cells in premenopausal women, more research subjects need to be included. Each experimental group should receive more biochemical tests, such as serum insulin and C-peptide, to rule out the influence of confounding factors.

6. Conclusions

In conclusion, compared with previous researches, the present study for the first time demonstrated that there exists the effect of estradiol on circulating EPCs in the HHcy group, and the number, migratory, and proliferative activities of circulating EPCs in HHcy premenopausal women are preserved, which may be related with enhanced NO production. In addition, we observed the number and activity of circulating EPCs in HHcy postmenopausal women were attenuated, indicating the decreased endogenous endothelial repair capacity may be the important underlying mechanism accounting for vascular impair which contribute to augment MACE. Therefore, our study provides new insight that increasing the number of circulating EPCs and improving the function of circulating EPCs; meanwhile, enhancing NO production will be a potential target for reversing HHcy-related vascular injury.

Data Availability

The data used to support the findings of this study are available from the corresponding author.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Long Peng, Qianlin Gu, and Zhenhua Huang contributed equally to this work.

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Research Article

Out-of-Hospital Cardiac Arrest due to Drowning in North America: Comparison of Patient Characteristics between Survival and Mortality Groups

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Out-of-hospital cardiac arrest (OHCA) due to drowning carries high morbidity and mortality. There are a few studies on drowning-related out-of-hospital cardiac arrest (OHCA), in which patients are followed from the scene to hospital discharge. This study aims to compare patient characteristics between the survival group and mortality group of OHCA due to drowning. OHCA due to drowning cases were selected from the North America Termination of Resuscitation Association database between 2011 and 2015. The retrospective analysis of epidemiological characteristics and clinical features of all OHCA patients were performed. Of the 17,094 OHCA cases in the registry, 54 cases of OHCA due to drowning were included in this study. Among the 54 OHCAs due to drowning, 7 (13.0%) survived, while 47 (87.0%) died. Compared to the mortality group, the survival group had a higher bystander witness rate (57.1% versus 17.0%, $p < 0.05$), higher asystole rate (42.9% versus 78.7%, $p < 0.05$), and higher mild therapeutic hypothermia rate (28.6% versus 2.1%, $p < 0.05$). In addition, a large proportion of survivors were children (71.4%) and males (71.4%). Survival among OHCA's due to drowning was found to be improved with a higher bystander rate, higher asystole rate, and higher mild hypothermia rate. In addition, children and males comprised the majority of survivors.

1. Introduction

Drowning is a major global public health concern with high morbidity and mortality, resulting in significant healthcare-related, societal, and financial burdens [1–3]. In 2004, at least 382,000 people documented by the World Health Organization (WHO) died because of drowning. Adult males comprised the largest mortality group due to drowning [4]. Out-of-hospital cardiac arrest (OHCA) due to drowning mostly occurred in prehospital circumstances, and OHCA due to drowning comprised almost 0.5%–1.0% of total OHCA [5].

However, at present, there is a limited research on OHCA due to drowning around the world. Besides some case reports, there is limited systematic analysis of patient characteristics, e.g., between the survival group and mortality group. Therefore, this study aims to compare the patient characteristics between the survival group and mortality group from OHCA due to drowning cases included in the North American Termination of Resuscitation studies between 2011 and 2015.

2. Method

2.1. Study Population. The OHCA due to drowning cases were selected from the North American Termination of Resuscitation Studies data between 2011 and 2015 (total of 17,094 OHCA cases), covering data from the United States and Canada.

2.2. Inclusion and Exclusion Criteria. The inclusion criteria were patients who experienced OHCA due to drowning with clear outcomes from the North American Termination of

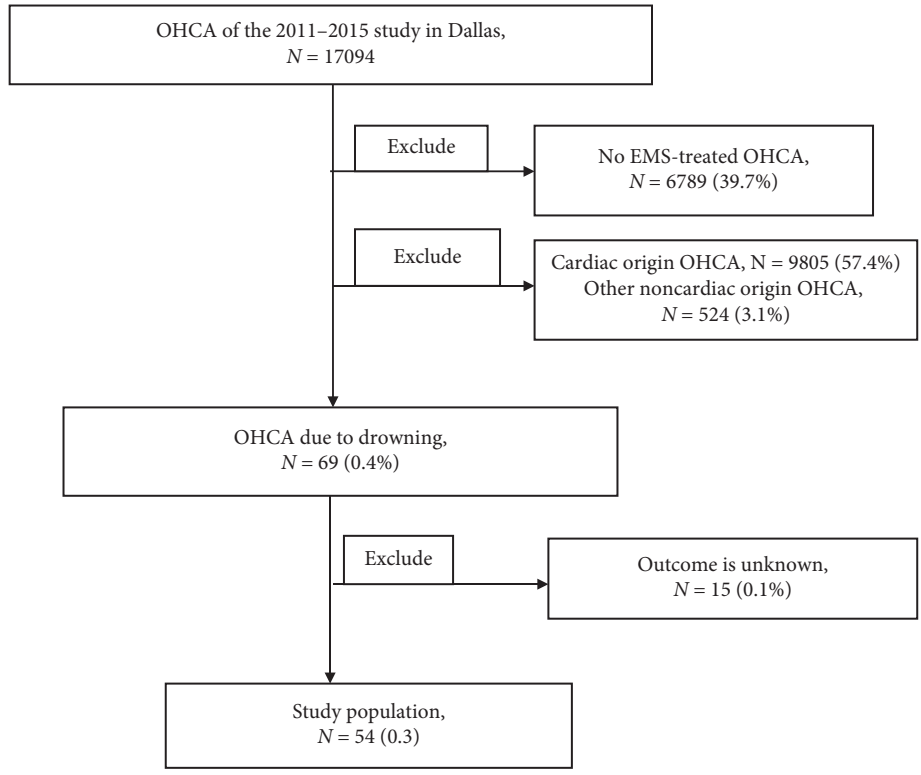

Figure 1: Target cohort and exclusions. OHCA indicates out-of-hospital cardiac arrest; Epistry, Epidemiological Cardiac Arrest Registry; PRIMED, prehospital resuscitation using an impedance valve and early versus delayed analysis; DFW, Dallas–Fort Worth; ROC, Resuscitation Outcomes Consortium; ROSC, return of spontaneous circulation; (*N*), number.

Resuscitation Studies from 2011 to 2015. The exclusion criteria were cases without the emergency medical services (EMS) record, cases with patients who have signed do not resuscitate (DNR) orders, OHCA with nondrowning etiology, and cases without clear outcomes.

2.3. Data Processing. Included cases were divided into the survival group and death group according to hospital discharge outcome. The following patient characteristics were identified: age, gender, witness status (EMS or bystander witness), bystander cardiopulmonary resuscitation (yes or no), location of cardiac arrest (private or public), EMS response time (interval from call for ambulance until ambulance arrival), adrenaline dosage (large dosage was defined as ≥ 3 mg), application of prehospital advanced airway management, return of spontaneous circulation (ROSC) in prehospital environment (yes or no), and survival at discharge (yes or no).

2.4. Statistical Analysis. All statistical calculations were performed using the statistical program SPSS 20.0 (IBM Inc., Armonk, NY, USA). The quantitative variables are expressed as the mean ± standard deviation (SD), while the qualitative variables are expressed as the absolute value and percentage. The independent sample *t*-test was used in terms of the comparison of quantitative variables between groups. The continuous variables were nonnormal distribution, and the four digit (IQR) test was used in the aspects of the quantitative variables, while the chi-square test was used in the comparison of the qualitative variables. All of the above statistical tests were double-sided tests, which were considered statistically significant in $p < 0.05$.

3. Results

3.1. Patient Characteristics. Out of the 17,094 OHCA cases from the database, there were 69 OHCA due to drowning, of which 15 were excluded for unknown outcomes, leaving us 54 cases (Figure 1). Of these, 43 (79.6%) cases were males, while the rest were females, and the median age was 30 (IQR 8.5–51.5). The specific epidemiological characteristics of patients with OHCA due to drowning are shown in Table 1. As shown in Table 1, the number of EMS witnessed was 0, and bystander witnessed was 12 (22.2%). Bystander CPR was performed in 26 cases (48.1%). The median EMS response time was 4.7 (3.4–5.5) minutes, and the duration of CPR was 20.1 (14.8–26.8) minutes. Application of prehospital advanced airway was accomplished in 22 (40.7%) patients. The usage rate of adrenaline was 83.3%. There were 5 patients with prehospital ROSC and 3 patients with mild therapeutic hypothermia (MTH). Among the 54 drowning OHCAs, compared to the death group, within the survival group, there was a higher instance of bystander witnessed rate (57.1% versus 17.0%, $p < 0.05$), lower asystole rate (42.9% versus 78.7%, $p < 0.05$), shorter duration time of cardiopulmonary resuscitation (CPR) (10.5 min versus 22.8 min,

Table 1: Comparison of epidemiological characteristics of out-of-hospital cardiac arrest due to drowning.

Demographics and characteristics	Total, $N = 54$	Discharged alive, $N = 7$ (13.0)	Died, 47 (87.0)	p
Baseline characteristics				
Age (years), median (IQR)	30 (8.5–51.5)	9.5 (2.8–48.8)	30 (10–53)	0.235
Male, *N* (%)	43 (79.6)	5 (71.4)	38 (80.9)	0.621
Public place, *N*(%)	18 (33.3)	2 (28.6)	16 (34.0)	1.000
Event characteristics	—	—	—	—
Witnessed status				
EMS-witnessed, *N* (%)	**0**	**0**	**0**	—
Bystander-witnessed, *N* (%)	12 (22.2)	4 (57.1)	8 (17.0)	0.036
Bystander CPR, *N* (%)	26 (48.1)	3 (42.9)	23 (48.9)	1.000
AED shock delivered, *N* (%)	0	0	0	—
Initial ECG rhythm				
VF/VT, *N* (%)	3 (5.6)	2 (28.6)	1 (2.1)	0.046
PEA, *N* (%)	7 (13.0)	1 (14.3)	6 (12.8)	1.000
Asystole, *N* (%)	40 (74.1)	3 (42.9)	37 (78.7)	0.031
Perfusing, *N* (%)	0	0	0	—
AED—no shock advised *N* (%)	1 (1.9)	1 (14.3)	0	—
EMS interventions				
911 call—EMS arrival (min), median (IQR)	4.7 (3.4–5.5)	3.5 (3.0–4.7)	4.8 (3.5–5.7)	0.067
CPR duration (min), median (IQR)	20.1 (14.8–26.8)	10.5 (8.7–17.5)	22.8 (16.4–30.7)	0.003
Advanced airway attempted, *N* (%)	22 (40.7)	3 (42.9)	19 (40.4)	1.000
Epinephrine administered, *N* (%)	45 (83.3)	4 (57.1)	41 (87.2)	0.081
Dose epinephrine (mg), median	3 (1–4)	1.1 (0.1–3.5)	0.137	0.137
EMS shock delivered, *N* (%)	8 (14.8)	2 (28.2)	6 (12.8)	0.291
Prehospital ROSC	5 (9.3)	4 (57.1)	1 (2.1)	—
Therapeutic hypothermia, *N* (%)	3 (5.6)	2 (28.6)	1 (2.1)	0.041

IQR, interquartile range; EMS, emergency medical services; VF, ventricular fibrillation; VT, ventricular tachycardia; PEA, pulseless electrical activity; AED, automated external defibrillator; CPR, cardiopulmonary resuscitation; ICU, intensive care unit.; DNR, do not resuscitate.

$p < 0.05$), and higher mild therapeutic hypothermia rate (28.6% versus 2.1%, $p < 0.05$).

3.2. Clinical Characteristics of Survivors after Hospital Discharge. As shown in Table 2, among all of the OHCA due to drowning, only 7 (13.0%) survived to hospital discharge, and the large proportion of survivors were children (71.4%) and males (71.4%). Most of the submersion incidents occurred in uptown (71.4%), and average EMS response time was 3.7 minutes. Among the survivors, 4 (57.1%) cases were witnessed by bystanders, and the initial first recognized rhythms were ventricular fibrillation (VF)/ventricular tachycardia (VT), pulseless electrical activity (PEA), and asystole. Furthermore, 4 (57.1%) patients had prehospital ROSC, and CPR was performed by bystanders in the 4 patients with CPR duration time of 11.9 min. Advanced airway management was accomplished in 2 (28.6%) patients. With a long hospitalization time, survivors had a good neurological outcome indicated and were assessed according to the cerebral performance categories. The use of epinephrine did not appear to affect the outcome of OHCA due to drowning (data deficiencies).

4. Discussion

4.1. Characteristics of the Drowned OHCA. These were the few reports available to evaluate the OHCA characteristics of drowning in North America. There were 17,094 OHCAs in North America from OHCA Registry between 2011 and 2015, of which 69 (0.4%) were drowned OHCA. In this investigation, the amount of included patients with OHCA due to drowning were only 54 (0.3%). The mortality was as much as 87%, with the hospital discharge survival rate of 13%.

Of these survivors, we found that patients were younger, and it appeared that children were more prone to survive drowning. Previous studies have also showed that children suffering from OHCAs had better outcomes than adults [6–8]. Previous studies also found higher survival and discharge rates when the downing was bystander witnessed [4,6]. In the survival group, the probability of occurrence in living residence was higher (71.4%), 4 (57.1%) patients were witnessed by bystanders, and bystander CPR was commenced in 4 (57.1%) cases with the average CPR duration time of 11.9 minutes. Drowning occurrences in the homes were more likely to have family members or friends nearby, which can account for its high witness rate. When directly witnessed, calling for medical help and early bystander CPR was much more prompt, which can explain the mean EMS time of 3.7 minutes in the survival group. Furthermore, 4 (57.1%) patients had prehospital return of spontaneous circulation (ROSC), and the advanced air way managements were accomplished in 3 (42.9%) patients after the medical intervention was carried out.

4.2. Characteristics of Survival Outcomes. Our study found that children are more likely to survive from drowning, as found in other previous studies [6,9]. This is possibly

Table 2: The clinical characteristics of patients who survived to hospital discharge.

	Age	Sex	First known rhythm	Place of CA	Bystander witnessed	Bystander CPR	Response time (min)	Advanced airway	Dose of epinephrine (mg)	CPR duration (min)	Days in CCU	MRS
1	11	Male	VF/VT	Place of recreation	Yes	No	4.7	No	Unknown	13.1	Unknown	Unknown
2	2	Male	PEA	Home residence	No	No	3.0	No	0	10.2	2	0
3	51	Male	Asystole	Home residence	No	No	5.4	Yes	4	20	25	0
4	3	Female	AED no shock	Home residence	Yes	Yes	3.1	No	Unknown	3.2	12	4
5	Unknown	Male	VF/VT	Place of recreation	Yes	No	3.5	Yes	Unknown	8.7	20	3
6	8	Female	Asystole	Home residence	No	Yes	3.7	No	0	10.5	31	5
7	48	Male	Asystole	Home residence	Yes	Yes	2.5	No	2	17.5	Unknown	Unknown

EMS, emergency medical services; VF, ventricular fibrillation.; VT, ventricular tachycardia.; PEA, pulseless electrical activity; AED, automated external defibrillator; CPR, cardiopulmonary resuscitation; ICU, intensive care unit; DNR, do not resuscitate; ROSC, return of spontaneous circulation; ROSC, return of spontaneous circulation.

explained by the advantages associated with children, who are generally younger, healthier, with hearts not yet plagued by coronary heart disease compared to the elderly.

4.2.1. Witness Status. The survey of Dyson et al. [4] demonstrates that the survival discharge rate in patients with OHCA caused by drowning in Australia is 7.8%, which is less than the 13% in our study. The witness rate in the survey of the Kylie Dyson study is only 17.9%, while it is 57.1% in our study. This supports our study in which a high witness rate is associated with a significantly increased survival rate.

4.2.2. Advanced Air Way Management. There is little research or information on the effect of the advanced air way management connected to OHCA due to drowning. As known, the immediate cause of cardiac arrest due to drowning is hypoxia caused by dyspnea. The advanced air way managements, such as a trachea cannula and tracheotomy, can directly correct the hypoxia situation of the body. In this study, the advanced air way management was carried out in 22 cases. Of these cases, 3 patients survived to hospital discharge. The advanced air way management rate in both groups did not differ significantly (42.9% versus 40.4, $p > 0.05$), but because of the lack of samples available of patients with advanced air way management performed, it is unclear whether the correction of hypoxia of the body is significantly related to the survival discharge rate among patients with OHCA due to drowning. We need more data illustrating the relationship between prompt advanced air way management and survival rate after hospital discharge to make any further conclusions.

4.2.3. Presence of Shockable Rhythm. The initial ECG rhythm in patients with OHCA due to hypoxia caused by drowning is most often asystole. Compared to the survival group, there are higher asystole rates (78.7% versus 42.9%, $p < 0.05$) in the death group. With the presence of nonshockable rhythm (asystole), it is difficult to reverse the heart rhythm to sustain stable hemodynamics. The research of Zheng et al. [10] in 2016 displayed that conversion to shockable rhythms is associated with better outcomes in out-of-hospital cardiac arrest patients with initial asystole but not in those with pulseless electrical activity. We have a similar finding in this study.

4.2.4. Mild Therapeutic Hypothermia. The previous study of Reinikainen M in 2012 demonstrated that the early use of mild therapeutic hypothermia treatment in OHCA can improve the survival rate [11]. As shown in Table 2, there are higher mild therapeutic hypothermia rates (28.6% versus 2.1%, $p < 0.05$) in the survival group as compared to the death group. To make a conclusion that the early use of mild therapeutic hypothermia treatment in OHCA due to drowning is connected to a significantly increased survival rate, we need to collect more data. As of now, its relationship to better outcomes is still unclear.

5. Limitations

Our study has several limitations.

First, as a retrospective observational study, the present analysis may be the subject to biases caused by unmeasured confounding factors. Therefore, our results need to be interpreted with caution.

Second, the patients with OHCA caused by drowning included in our study were only 54. Due to the limited data, this study can only conduct a descriptive analysis. We cannot study which factors are favorable for survival and which are harmful by using the logistic regression model or other statistical models. But we believe that this study is meaningful because we know little about drowning-related OHCA, and we insist more studies should be conducted in the future.

6. Conclusion

The survival rate of OHCA caused by different etiologies is not obviously different. Important predictors of survival are age and witnessed status. With the presence of advanced air way management and the early use of mild therapeutic hypothermia in OHCA due to drowning, the survival rate will likely be higher.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Zhenhua Huang, Wanwan Zhang, and Jinli Liao contributed equally to this article.

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Research Article

Vitamin D (1,25-$(OH)_2D_3$) Improves Endothelial Progenitor Cells Function via Enhanced NO Secretion in Systemic Lupus Erythematosus

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It has been proven that vitamin D was decreased and function of circulating endothelial progenitor cells (EPCs) was injured in systemic lupus erythematosus (SLE) patients. However, the effect of vitamin D on the function of EPCs *in vitro* and its mechanism need further study. Therefore, we investigated whether vitamin D improved the function of EPCs *in vitro*. The peripheral blood mononuclear cells of the participants were isolated from SLE patients and control subjects and cultured to EPCs. After the EPCs were treated with vitamin D (1,25-$(OH)_2D_3$), we evaluated the number, migratory and proliferative activities, and nitric oxide (NO) production of EPCs *in vitro* and detected vascular endothelial function by flow-mediated dilatation (FMD). We found that vitamin D in a dose-dependent manner improved number and migratory and proliferative activities of EPCs from SLE patients. Additionally, vitamin D upregulated NO production from EPCs *in vitro*. A significant correlation between the FMD and plasma NO level was found. There was also a correlation between number, migration, and proliferation of EPCs and NO production. Thus, the present findings indicated that vitamin D improved the function of EPCs from SLE patients via NO secretion.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototype of autoimmune disease, which mainly affects young women. SLE has a high disability and mortality rate, which caused a heavy burden to families and society [1]. With the progression of diagnosis and treatment, the survival time of SLE patients has been greatly improved in recent years. However, the mortality and disability rates of atherosclerosis are gradually increasing [2]. In SLE patients, atherosclerosis occurs early and severely. Even after removing the impact of traditional risk factors, the relative risk of coronary heart disease in lupus patients is still as high as 8–10 times [3]. So far, the mechanism of atherosclerosis induced by systemic lupus erythematosus remains unclear.

Studies have shown that vascular endothelial structure and function damage was the initiating mechanism of atherosclerosis in SLE [4, 5]. The essence of vascular dysfunction is the imbalance between vascular injury and vascular repair, which is an important factor leading to target organ damage, such as the heart, brain, and kidney damage, and cardiovascular events [6, 7]. Therefore, the repair of vascular endothelial injury is an important measure to effectively prevent and treat SLE atherosclerosis and its complications. In recent years, endothelial progenitor cells (EPCs) have been found to be precursors of vascular

endothelial cells, which can effectively repair vascular endothelial injury [8–10]. When the vascular endothelium is damaged, EPCs are released from the bone marrow to peripheral circulation passively, and by chemotaxis, adhesion, migration, and proliferation, EPCs accelerate vascular reendothelialization, which plays a key role in the process of vascular endothelial injury repair [10, 11]. A large number of studies have confirmed that cardiovascular risk factors can lead to different degrees of vascular endothelial injury, the number and function of EPCs decreased, and endothelial function also decreased accordingly, which indicates that this endothelial dysfunction is closely related to the number and function of EPCs [12].

Brachial artery endothelium-dependent vasodilation, as detected by flow-mediated dilatation (FMD), is a noninvasive and repeatable new technique for detecting vascular function, which can accurately reflect cardiovascular endothelial dysfunction [13]. A large number of studies have pointed out that FMD and EPCs are closely related in number and function, which indicates that EPCs are cell biological indicators that reflect the changes of vascular endothelial function [14]. The main physiological function of vitamin D is to regulate calcium and bone metabolism. Vitamin D deficiency is very common in SLE patients, and it can affect the function of vascular endothelium and lead to the occurrence and development of atherosclerosis [5, 15–17]. Therefore, what is the effect of vitamin D on endothelial progenitor cells in SLE patients? The relevant mechanism needs further study.

Nitric oxide (NO) is an endothelial relaxing factor and a highly active free radical. Previous studies have found that NO plays an important role in regulating endothelial progenitor cell adhesion, migration, and proliferation and mediating vascular repair as well [8, 14, 18]. Therefore, we hypothesized that the decrease of EPCs in SLE patients is related to the low level of active vitamin D, which may be regulated by NO. By observing the effect of vitamin D on EPCs and NO level in SLE patients, this study elucidated the underlying causes of atherosclerosis in SLE patients, thus laying a foundation for the clinical prevention and treatment of cardiovascular diseases in SLE patients and the discovery of new drug targets.

2. Method

2.1. Subject Recruitment. 20 SLE patients and 20 age- and gender-matched control subjects were enrolled from the First Affiliated Hospital of Sun Yat-sen University in Guangzhou, China. The control subjects had no hypertension, heart disease, diabetes, or stroke history. The peripheral blood samples of another 40 SLE patients were collected and cultured to EPCs for 1,25-$(OH)_2D_3$ stimulation experiments. The protocol of this study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. The general clinical characteristics of subjects are listed in Table 1. Serum 25(OH)D was measured by highly sensitive enzyme-linked immunosorbent assay (ELISA, R&D Systems, Germany) according to the manufacturer's instructions.

2.2. Measurement of Flow-Mediated Dilation (FMD). As previously described [19, 20], the brachial artery FMD was measured by a 5–12 MHz linear transducer on an HDI 5000 system (Philips Healthcare, the US).

2.3. Measurement of NO, VEGF, and GM-CSF Level Secreted by EPCs. NO, vascular endothelial growth factor (VEGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels in plasma or secretion by EPCs were evaluated as previously described [14]. We used ELISA kits to determine NO, VEGF, and GM-CSF levels according to the manufacturer's instructions.

2.4. Flow Cytometry and Cell Culture Assay to Assess the Number of Circulating EPCs. As previously described [14], the peripheral blood mononuclear cells of the participants were isolated using Ficoll density gradient centrifugation, following 7 days of culture. EPCs were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL (DiI-acLDL, Thermo Fisher Scientific, the US) and incubated with fluorescein isothiocyanate- (FITC-) labeled lectin (Sigma-Aldrich, Germany). The samples were observed by two independent researchers using a phase-contrast fluorescence microscope (magnification, ×200). Cells demonstrating double-positive fluorescence were identified as differentiating EPCs. The EPCs were counted using a kinase-insert domain receptor (KDR; 4A Biotech, China) and antihuman CD34 (4A Biotech, China). The ratio of CD34 + KDR + cells per 100 peripheral blood mononuclear cells was the number of circulating EPCs.

2.5. Migration and Proliferation Assay of EPCs. EPC migration and proliferation assays were described in a previous study [14]. Migration was analyzed using a modified Boyden chamber. Briefly, 2×10^4 EPCs, resuspended in 250 μl EBM-2, were pipetted in the upper chamber of a modified Boyden chamber. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The proliferation of EPCs was analyzed as follows: EPCs were supplemented with 10 μl MTT (Fluka, the US) and measured by optical density at 490 nm.

2.6. Treatment of Circulating EPCs by 1,25-$(OH)_2D_3$. Forty SLE patients' blood samples were divided into four groups randomly. The peripheral blood mononuclear cells were cultured to EPCs, which were digested by 0.25% trypsin to form a single cell suspension. Then, each group cells were treated with 1,25-$(OH)_2D_3$ (0, 1, 10, 50 nM, Sigma-Aldrich, the US) for 72 h, respectively.

2.7. Statistical Analysis. Normally distributed variables were expressed as mean ± SD and compared using unpaired t tests between two groups. Correlation coefficients were analyzed using Pearson's correlation. Differences were considered significant when $p < 0.05$. All statistical analyses were

Table 1: General clinical characteristics.

Characteristics	SLE ($n = 20$)	Control ($n = 20$)
Age (years)	38.7 ± 12.2	35.1 ± 16.1
BMI (kg/cm^2)	22.3 ± 3.5	23.5 ± 3.8
Systolic blood pressure (mmHg)	132.8 ± 15.9	130.5 ± 18.4
Diastolic blood pressure (mmHg)	78.4 ± 9.6	76.4 ± 7.4
Heart rate (beats/min)	69.4 ± 8.4	68.5 ± 9.7
AST (mmol/L)	31.4 ± 20.8	38.2 ± 20.7
ALT (mmol/L)	28.2 ± 31.9	33.2 ± 18.7
BUN (mmol/L)	9.0 ± 8.5	7.0 ± 4.8
Cr (mmol/L)	213.7 ± 290.7	185.0 ± 205.1
GLU (mmol/L)	5.9 ± 3.5	5.2 ± 0.8
Serum 25(OH)D (ng/mL)	13.2 ± 6.3$^{\#}$	26.6 ± 4.9

Abbreviation: BMI, body mass index; AST, aspartate amino transferals; ALT, alanine transaminase; BUN, blood urea nitrogen; Cr, serum creatinine; GLU: glucose. Notes: Data are given as mean ± SD. $^{\#}P < 0.05$ vs control.

performed with SPSS statistical software 21.0 (SPSS, Inc., the US).

3. Result

3.1. Baseline Characteristics. As listed in Table 1, no significant differences were observed in age, body mass index (BMI), aspartate aminotransferase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), serum creatinine (Cr), and lipids level between SLE patients and, while the level of serum 1,25-$(OH)_2D_3$ in the SLE group was apparently lower than that of the control group ($p < 0.05$).

As shown in Figure 1, compared with the healthy control subjects, the FMD and plasma NO level of SLE patients group are significantly decreased (Figures 1(a) and 1(b)). We also found that the FMD was correlated with the NO level, indicating that endothelium damage may be related NO decrease in SLE patients (Figure 1(c)).

As shown in Figure 2, the number of EPCs was significantly decreased in the peripheral blood mononuclear cells from SLE patients, compared with control subjects (Figure 2(a)). Consistently, the migration and proliferation activities of EPCs from SLE patients were notably decreased compared to those from control subjects (Figures 2(b) and 2(c)).

3.2. The Effect of 1,25-$(OH)_2D_3$ on the Number, Migration, and Proliferation of EPCs. After being cultured *in vitro*, the suspension EPCs from SLE patients were stimulated with different concentrations of 1,25-$(OH)_2D_3$ (0, 1, 10, and 50 nM) for 72 h. The number of EPCs, migration, and proliferation were observed. We found that the number, migration and proliferation of EPCs were all robustly increased after culturing in different concentrations of 1,25-$(OH)_2D_3$ (Figures 3(a)–3(c)).

3.3. The Effect of 1,25-$(OH)_2D_3$ on the NO, VEGF, and GM-CSF Secretion by Cultured EPCs. As shown in Figure 4, different concentrations of 1,25-$(OH)_2D_3$ induced a significant upregulation of NO secretion by cultured EPCs (Figure 4(a)), while no difference was observed in VEGF and GM-CSF levels after culturing with 1,25-$(OH)_2D_3$ (Figures 4(b) and 4(c)).

3.4. Correlation between NO Level In Vitro and the Number and Activity of Circulating EPCs. There was a significant linear regression relationship between the NO secretion level and the number of cultured EPCs (Figure 5(a)). Similarly, there was a significant linear regression relationship between the NO secretion level and the migratory and proliferative activities of EPCs (Figures 5(b) and 5(c)).

4. Discussion

Cardiovascular disease in SLE began with vascular endothelial injury [21]. Recent studies have found that there was a serious deficiency of active vitamin D in SLE, which led to vascular endothelial dysfunction [15, 21]. Our previous clinical observation showed that there was a positive correlation between vitamin D concentration in SLE patients and EPC levels in peripheral blood. However, the effect of 1,25-$(OH)_2D_3$ on the number and function of EPCs in SLE *in vitro* and its mechanism remain unclear.

Several studies have confirmed that vitamin D can improve vascular endothelial function [22, 23]. Pittarella et al. [24] found that 1,25-$(OH)_2D_3$ could directly act on umbilical vein endothelial cells and influence their migration and proliferation through the NO pathway. Martínez-Miguel et al. [25] found that 1,25-$(OH)^{2D}{}_3$ can simultaneously upregulate endothelin-1 and NO synthesis in endothelial cells, which play an important role in maintaining the functional balance of endothelial cells.

EPCs in human peripheral blood are a kind of endothelial precursor cells, called circulating EPCs [26]. They are mobilized into peripheral blood by the bone marrow. The cell phenotype is mainly CD34-positive, which can be homing to the site of vascular injury and can differentiate into mature vascular endothelial cells, which can promote angiogenesis in the injured area and repair the injured intima. It has paracrine function and secretes NO, VEGF, and fibroblast factor-2. It also has important repair and protection effects on the endothelial function. Therefore, it plays an important protective role in the occurrence and

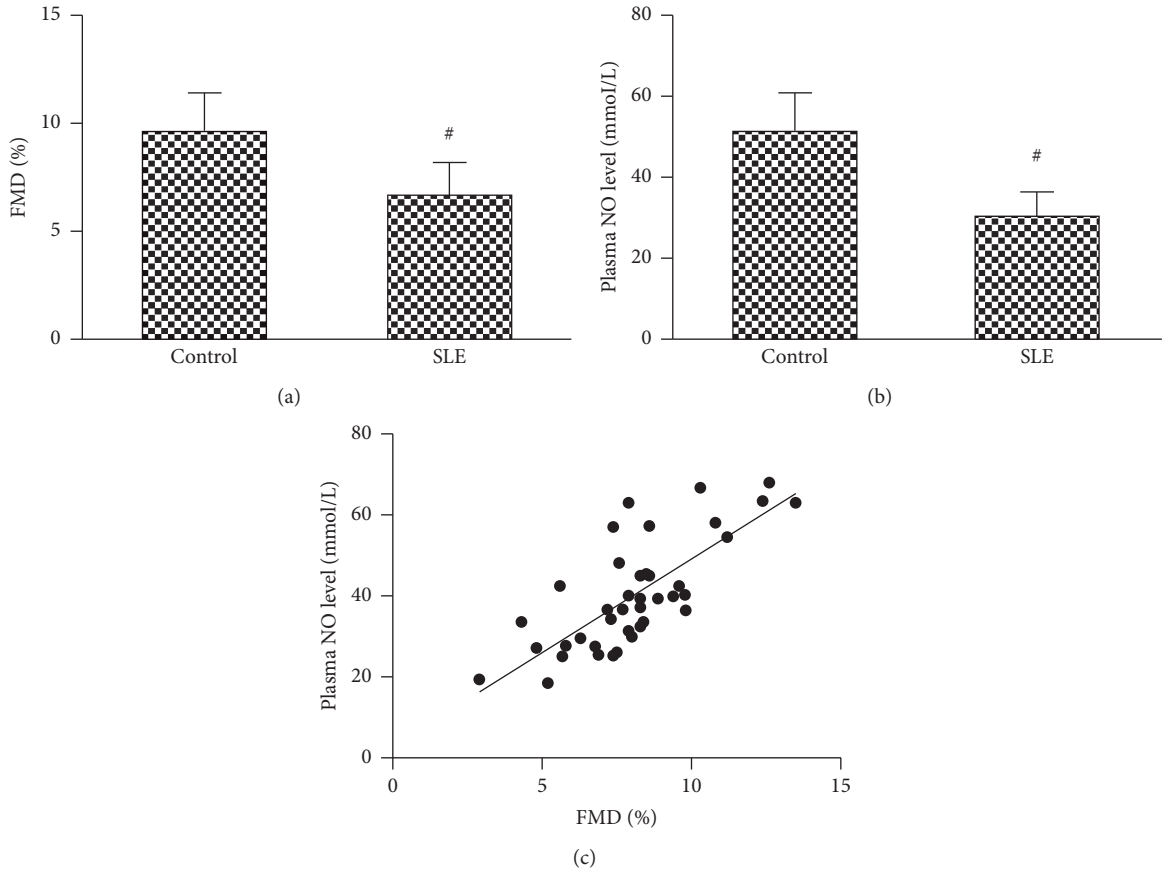


Figure 1: The FMD and plasma NO level in the two groups. The brachial artery FMD (a) and plasma NO level (b) in SLE patients were lower than that in the control subjects. FMD was positively correlated to plasma NO level (c). $^{\#}P<0.05$ vs. control subjects.

development of cardiovascular diseases, such as coronary atherosclerotic heart disease and hypertension [8, 14, 27]. In view of the intersection of 1,25-$(OH)^{2D}{}_3$ and EPCs in the endothelial function, some scholars began to explore the direct correlation between 1,25-$(OH)^{2D}{}_3$ and EPCs.

Cianciolo et al. [28] confirmed that vitamin D receptors also existed in peripheral blood circulation EPCs, and the number of circulating EPCs increased by supplementing active vitamin D to selected dialysis patients. Brodowski et al. [11] found that the endothelial progenitor cell function of preeclampsia pregnant women was improved after vitamin D supplementation. This study further confirmed that there was a positive correlation between vitamin D concentration in SLE patients and the EPCs level in peripheral blood, and 1,25-$(OH)^{2D}{}_3$ could directly improve the number, migration, and proliferation of EPCs from human peripheral blood *in vitro*. Therefore, how does 1,25-$(OH)^{2D}{}_3$ improve the activity of EPCs?

Vitamin D receptors are divided into nuclear receptors and membrane receptors. They are the main receptors for 1,25-$(OH)^{2D}{}_3$ to play biological roles. The vitamin D receptor exists in all kinds of human tissue cells and endothelial cells [30]. Based on the study by Cianciolo et al. [28], we speculated that 1,25-$(OH)^{2D}{}_3$ can activate some cell signal transduction pathways by acting on the vitamin D receptors of EPCs, affecting the proliferation, differentiation, and paracrine function of EPCs. Our experiment further examined the function of EPCs to secrete NO. It was found that 1,25-$(OH)_2D_3$ promoted the secretion of NO by EPCs in SLE in a concentration-dependent manner. It was also found that the NO level was closely related to the number, migration, and proliferation of EPCs in SLE *in vitro*. It indicated that the endothelial function of 1,25-$(OH)_2D_3$ in SLE may be achieved by the NO level.

Since the concentration of active vitamin D in the human body is far less than 100 nmol/L [31], we speculated that 1,25-$(OH)^{2D}{}_3$ has a limited effect on EPCs *in vivo*. It may only promote the homing of EPCs in peripheral circulation but has little effect on the function of EPCs themselves. More experimental data are needed to further confirm this.

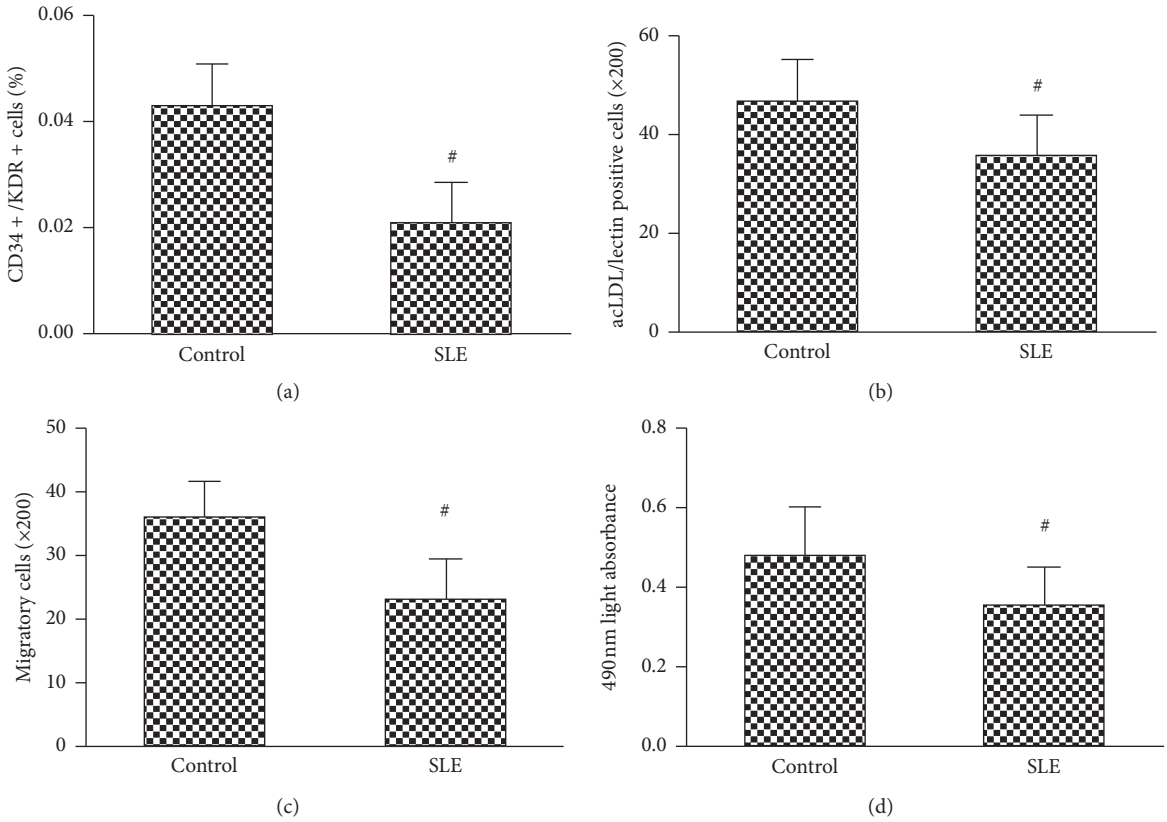


Figure 2: The number of circulating EPCs in the SLE patients and healthy controls. Evaluated by (a) FACS analysis and (b) phase-contrast fluorescent microscope, the number of circulating EPCs in SLE patients was lower than those in the control subjects. The migratory (c) and proliferative (d) activities of circulating EPCs in SLE patients were lower than those in the healthy control. Data are given as mean ± SD. $^{\#}P<0.05$ vs. control subjects.

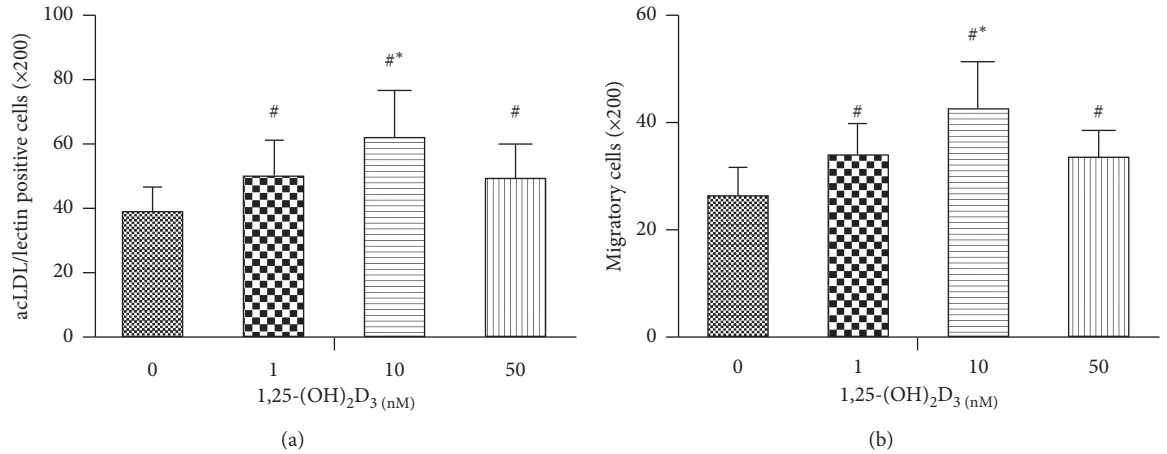
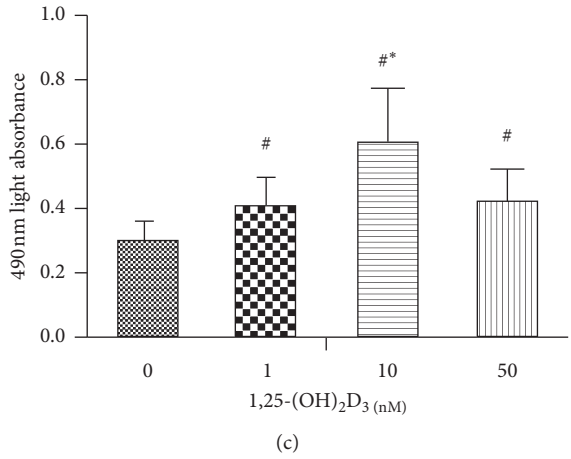


Figure 3: Continued.



(c)

Figure 3: The effect of 1,25-$(OH)_2D_3$ on the number, migration, and proliferation of EPCs *in vitro*. The number, migration, and proliferation of EPCs were significantly increased after culturing in different concentrations of 1,25-$(OH)_2D_3$ (a)–(c). $^{\#}P < 0.05$ vs blank control (0 nM 1,25-$(OH)_2D_3$), $^{*}P < 0.05$ vs 1 nM 1,25-$(OH)_2D_3$.

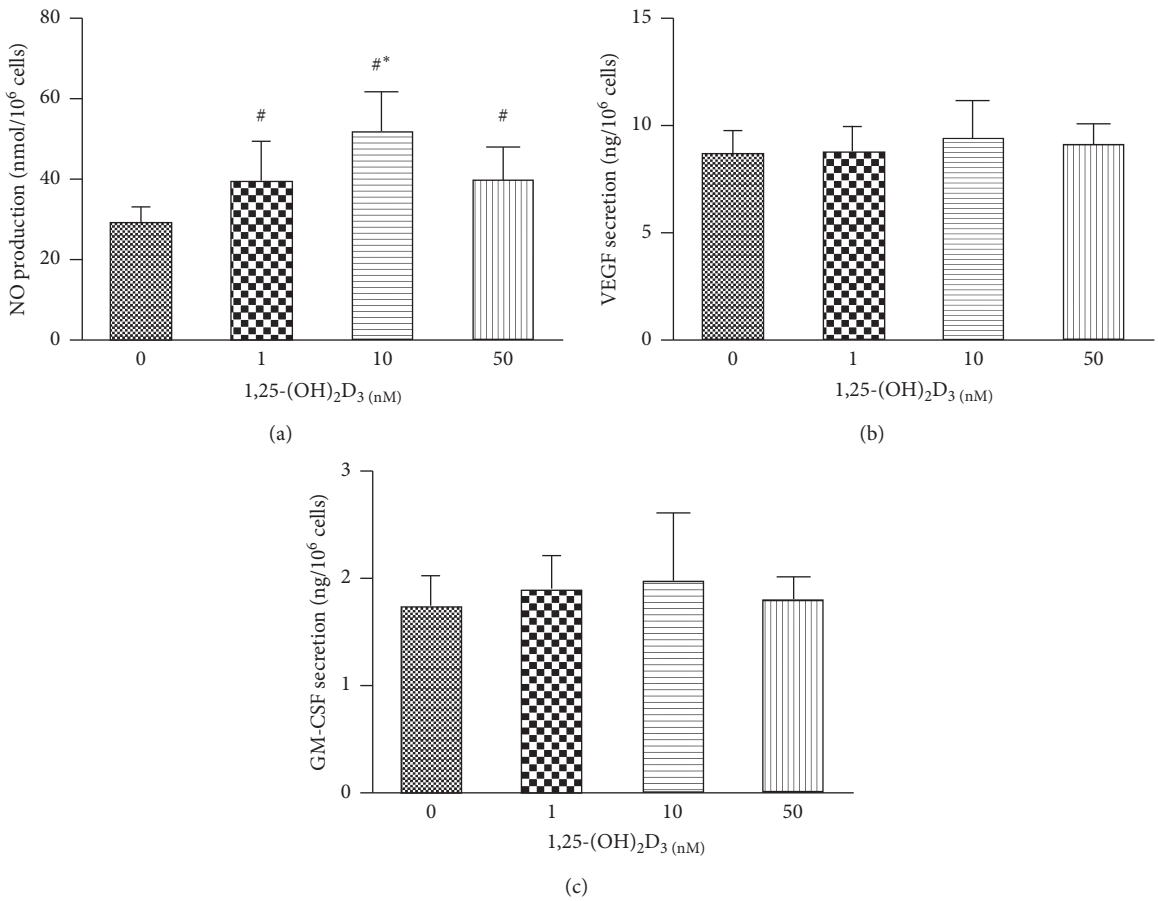


Figure 4: The NO, VEGF, and GM-CSF secretion by EPCs. Different concentrations of 1,25-$(OH)_2D_3$ induced a significant upregulation of NO secretion by cultured EPCs (a), while no difference was observed in VEGF and GM-CSF levels after culturing with 1,25-$(OH)_2D_3$ (b), (c). $^{\#}P < 0.05$ vs blank control (0 nM 1,25-(OH)2D3), $^{*}P < 0.05$ vs 1 nM 1,25-$(OH)_2D_3$.

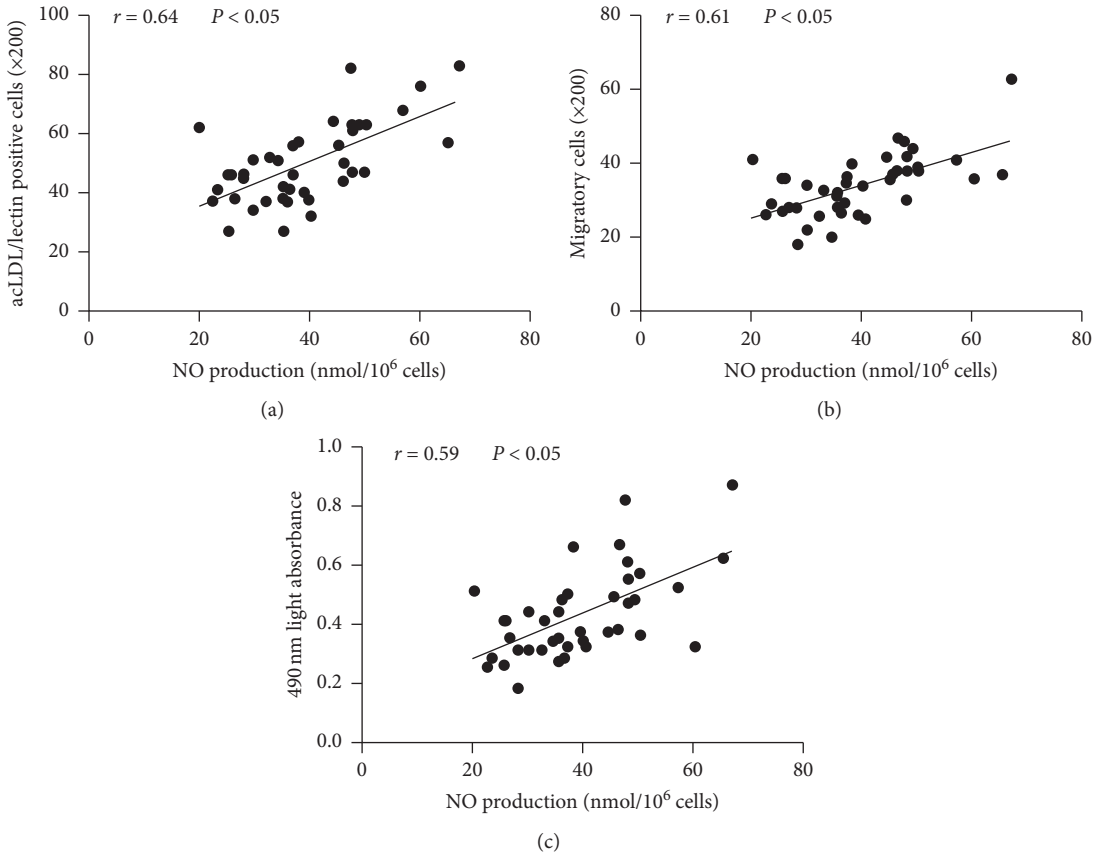


Figure 5: Correlation between the NO level *in vitro* and the number and activity of circulating EPCs. The linear regression relationship between the NO level *in vitro* and promoted circulating EPCs in response to 1,25-$(OH)_2D_3$. There was a significant linear regression relationship between the NO secretion level and the number of cultured EPCs (a). There was a significant linear regression relationship between the NO secretion level and the migratory and proliferative activities of EPCs (b), (c).

5. Conclusion

The present findings indicate that 1,25$(OH)^{2D}{}_3$ improves the function of EPCs from SLE patients via NO secretion.

Data Availability

The data used to support the findings of this study are available from the corresponding author or the first author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Zhenhua Huang, Lixiang Liu, and Shufen Huang contributed equally to this work.

Acknowledgments

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Research Article

The Effect of Sex Differences on Endothelial Function and Circulating Endothelial Progenitor Cells in Hypertriglyceridemia

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Background. Men have a higher risk and earlier onset of cardiovascular diseases compared with premenopausal women. Hypertriglyceridemia is an independent risk factor for the occurrence of ischemic heart disease. Endothelial dysfunction is related to the development of ischemic heart disease. Whether sex differences will affect the circulating endothelial progenitor cells (EPCs) and endothelial function in hypertriglyceridemia patients or not is not clear. *Methods*. Forty premenopausal women and forty age- and body mass index (BMI)-matched men without cardiovascular and metabolic disease were recruited and then divided into four groups: normotriglyceridemic women (women with serum triglycerides level <150 mg/dl), hypertriglyceridemic women (women with serum triglycerides level ≥150 mg/dl), normotriglyceridemic men (men with serum triglycerides level <150 mg/dl), and hypertriglyceridemic men (men with serum triglycerides level ≥150 mg/dl). Peripheral blood was obtained and evaluated. Flow-mediated dilatation (FMD), the number and activity of circulating EPCs, and the levels of nitric oxide (NO), vascular endothelial growth factor (VEGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) in plasma and culture medium were measured. *Results*. The number and activity of circulating EPCs, as well as the level of NO in plasma or culture medium, were remarkably increased in premenopausal females compared with those in males both in the hypertriglyceridemic group and the normotriglyceridemic group. The EPC counts and activity, as well as the production of NO, were restored in hypertriglyceridemic premenopausal women compared with those in normal women. However, in hypertriglyceridemic men, the EPC counts and activity, as well as levels of NO, were significantly reduced. The values of VEGF and GM-CSF were without statistical change. *Conclusions*. The present study firstly demonstrated that there were sex differences in the number and activity of circulating EPCs in hyperglyceridemia patients. Hypertriglyceridemic premenopausal women displayed restored endothelial functions, with elevated NO production, probably mediated by estradiol. We provided a new insight to explore the clinical biomarkers and therapeutic strategies for hypertriglyceridemia-related vascular damage.

1. Introduction

It is well known that atherosclerosis is a progressive inflammatory disease characterized by plaque consisting of cholesterol, fat, calcium, and other substances deposition in the arterial wall. Epidemiological data have suggested that there are remarkably sex differences in the onset of cardiovascular disease (CVD), with atherosclerosis occurring approximately ten years later and myocardial infarction about 20 years later in premenopausal women than in man [1]. However, the risk of CVD rises about ten times in postmenopause women, while only increases 4.6 times in age-matched men [2]. The mechanisms of sex differences in the occurrence of CVD are not fully elucidated. Researches have indicated that estrogen [3] and androgen [4] may play a crucial role in the development of CVD.

Hypertriglyceridemia is commonly defined as fasting triglyceride serum level >150 mg/dl (>1.7 mmol/l) [5]. There is growing evidence suggesting that

hypertriglyceridemia is an independent risk factor for ischemic cardiovascular disease [6–11], even slightly elevation of triglycerides was associated with a higher cardiovascular risk [8] and mortality rate [9]. In the general population, compared with the individuals with nonfasting triglycerides of 70 mg/dL (0.8 mmol/L), individuals with levels of 580 mg/dL (6.6 mmol/L) have sharply increased risks of myocardial infarction for 5.1-fold, ischemic heart disease, and ischemic stroke for 3.2-fold, respectively, and all-cause mortality for 2.2-fold [11]. Lipid-lowering treatment with statins was proved to exert a positive effect on patients with atherosclerosis through restoring the endothelial function and increased circulating endothelial progenitor cells (EPCs) [12, 13]. However, some researches have indicated patients with atherosclerosis may not get enough protection by statins alone [14], especially in patients with systemic inflammation [15]. Besides, statins may have some side effects such as statin-associated symptoms (SAS), including muscle or central nervous system symptoms and diabetes [16].

Vascular endothelium cell, nature's blood container, is the dynamic regulator of hemostasis and thrombosis with the function of recruitment and activation of platelet and coagulation cascade [17]. Therefore, vascular endothelium dysfunction was proven to be a significant risk factor for vascular diseases, such as atherosclerosis [18] and insulin resistance [19, 20]. Asahara et al. have firstly described that EPCs isolated from peripheral blood play a crucial role in angiogenesis [21]. Early EPCs, derivatives of CD14+ monocytic cell lineage [22], promote angiogenesis by secreting inflammatory cytokines and paracrine angiogenic factors [23]. While late EPCs, originating from CD34+ hematopoietic stem cells [24, 25], are associated with endothelial tubulogenesis and neovascularization via enhanced expression of proliferation and angiogenesis genes [23]. EPCs promote angiogenesis or vascular repair through activation of resident endothelial cells and recruitment of endogenous monocytes/macrophages to sites of ischemia, which were mediated by paracrine factors [26] such as vascular endothelial growth factor (VEGF) [27–29], granulocyte-macrophage colony-stimulating factor (GM-CSF) [29–32], and nitric oxide (NO) [32–34]. Dyslipidemia, hyperglycemia, insulin resistance, and induced EPC dysfunction via disrupting the SDF-1/CXCR-4 and NO pathways and the p53/SIRT1/p66Shc axis are critical for mobilization, migration, homing, and vasculogenic properties [35]. Researches also have indicated that EPC therapy is a safe and efficient way to delay the progression of atherosclerosis [36] and improve the heart function [37] for patients with coronary heart disease.

Our previous study has demonstrated that premenopausal women in prehypertension status present an increased circulating EPC number and elevated NO level, which may associate with the vascular protection effect of premenopausal women [38]. In this study, we will further investigate the sex differences of the endothelial function and circulating EPCs in patients of hypertriglyceridemia, and the probable underlying mechanisms.

2. Materials and Methods

The materials and methods section should contain sufficient detail so that all procedures can be repeated. It may be divided into headed subsections if several methods are described.

2.1. Characteristics of Subjects. Forty premenopausal women and forty age- and BMI-matched men without cardiovascular and metabolic disease were recruited. Based on the Adult Treatment Panel III (ATP-III) guidelines [5], according to the triglyceride level and sex, we divided the subjects into four groups: normotriglyceridemic women (women with serum triglycerides level <150 mg/dl), high triglyceridemic (HTG) women (women with serum triglycerides level ≥150 mg/dl), normotriglyceridemic men (men with serum triglycerides level <150 mg/dl), HTG men (men with serum triglycerides level ≥150 mg/dl). Patients with diabetes, tumor or cancer, and infection or inflammatory disease were excluded. Besides, we also excluded the smokers, alcohol abusers, pregnant, women undergoing breastfeeding, or patients with a history of hysterectomy, oophorectomy, or irregular menstrual cycles. All the subjects were given informed consent, and the experimental protocols were approved by the ethics committee of our hospital. The characteristics of the subjects are listed in Table 1.

2.2. Blood Samples. The peripheral blood was obtained from the patients in the early morning after overnight fasting. Caffeinated beverage or alcohol was forbidden for at least 12 hours before blood draw. None of the patients were taking any medicines such as statins, antiplatelet, or anti-inflammatory that may have an impact on the parameters of EPCs. The following items were detected and evaluated: AST (aspartate aminotransferase), ALT (alanine aminotransferase), BUN (blood urea nitrogen), Cr (creatinine), LDL (low-density lipoprotein), TC (total cholesterol), HDL (high-density lipoprotein), TG (triglyceride), FPG (fasting plasma glucose), estradiol, and so on, which are presented in Table 1.

2.3. Evaluation of the EPC Number and Activity. The number of circulating EPCs was evaluated by flow cytometry analysis and cell culture assay, and EPC activity was measured by EPC migration and proliferation assay, which were demonstrated in our previous studies [38, 39].

2.4. Measurement of the Plasma Levels of NO, VEGF, and GM-CSF and Secretion by EPCs. The plasma levels of NO, VEGF, and GM-CSF and secretion by cultured EPCs were evaluated by methods as we described previously [38, 39].

2.5. Measurement of FMD. Flow-mediated dilation (FMD) of the brachial artery was used to evaluate the endothelial function. The detailed method was demonstrated in our previous studies [40, 41].

Table 1: Clinical and biochemical characteristics.

Characteristics	Normotriglyceridemic women ($n = 20$)	HTG women ($n = 20$)	Normotriglyceridemic men ($n = 20$)	HTG men ($n = 20$)
Age (years)	44.8 ± 3.4	45.3 ± 3.28	46.6 ± 4.4	46.2 ± 3.6
Height (cm)	162.7 ± 5.4	160.6 ± 6.7	168.1 ± 6.0#	169.2 ± 6.2#
Weight (kg)	68.6 ± 6.5	69.1 ± 6.0	75.0 ± 5.3#	75.3 ± 4.3#
BMI (kg/cm^2)	25.9 ± 2.3	26.8 ± 2.2	26.2 ± 2.2	26.7 ± 1.6
Systolic blood pressure (mmHg)	123.2 ± 9.0	125.2 ± 3.4	124.3 ± 8.7	127.0 ± 4.4☆
Diastolic blood pressure (mmHg)	75.3 ± 7.2	78.0 ± 5.9	76.1 ± 7.5	78.3 ± 5.1☆
Heart rate (beats/min)	78.8 ± 9.3	81.3 ± 8.3	77.5 ± 7.7	78.3 ± 8.8
AST (mmol/L)	28.1 ± 6.2	26.7 ± 5.1	25.8 ± 6.3	28.8 ± 3.1
ALT (mmol/L)	23.3 ± 6.9	22.4 ± 5.5	21.4 ± 5.1	24.3 ± 5.7
BUN (mmol/L)	4.6 ± 0.8	5.0 ± 1.1	4.9 ± 0.7	5.1 ± 0.8
Cr (mmol/L)	60.4 ± 11.7	64.6 ± 13.6	61.3 ± 12.0	64.7 ± 12.1
LDL (mmol/L)	2.81 ± 0.45	2.69 ± 0.45	2.73 ± 0.42	2.57 ± 0.40
TC (mmol/L)	4.70 ± 0.50	4.50 ± 0.56	4.51 ± 0.62	4.34 ± 0.63
HDL (mmol/L)	1.31 ± 0.23	0.97 ± 0.15☆	1.34 ± 0.15	0.93 ± 0.14☆
TG (mmol/L)	1.53 ± 0.17	3.42 ± 0.73☆	1.48 ± 0.16	3.66 ± 0.75☆
FPG (mmol/L)	4.66 ± 0.84	4.47 ± 0.50	4.44 ± 0.45	4.83 ± 0.45
Estradiol (pmol/L)	224.48 ± 33.4	212.3 ± 35.7	104.2 ± 19.9#	110.92 ± 16.6#
FMD (%)	8.97 ± 1.99	8.22 ± 1.56	7.60 ± 1.66#	5.85 ± 1.77#☆

BMI, body mass index; LDL, low-density lipoprotein; TC, total cholesterol; HDL, high-density lipoprotein; TG, triglyceride; FPG, fasting plasma glucose; hrCRP, hypersensitive C-reactive protein; FMD, flow-mediated brachial artery dilatation; HTG, hypertriglyceridemic. Notes: data are given as mean ± SD. ☆vs normotriglyceridemic subjects; # vs. premenopausal women.

2.6. Statistical Analysis. SPSS Version 26.0 statistical software (SPSS Inc., Chicago, Illinois) was used for data analysis. Results were expressed as mean value ± standard deviation. Two-factor ANOVA was used for comparisons between the four groups (sex and the status of normal triglyceridemia or hypertriglyceridemia). When there was a significant F value, a post hoc test was then performed with the Newman–Keuls method to identify significant differences among mean values. Univariate correlations were analyzed by Pearson's coefficient (r). $P < 0.05$ was considered as statistically significant.

3. Results and Discussion

3.1. Baseline Clinical Characteristics. As we have shown in Table 1, there were no significant differences in age, BMI, and serum TG, TC, LDL, and HDL among the four groups ($P > 0.05$). The serum TG levels of both premenopausal HTG women ($P < 0.05$) and HTG men ($P < 0.05$) were remarkably increased than those in the normotriglyceridemic groups. The serum level of estradiol and the value of FMD were higher in the female groups than in the male groups (both normotriglyceridemic and HCG groups, $P < 0.05$ and $P < 0.05$, respectively). Moreover, FMD was obviously lower in the HMG men compared with that in the normotriglyceridemic men ($P < 0.05$). However, the value of FMD in normotriglyceridemic women and HMG women did not have remarkable differences ($P > 0.05$).

3.2. EPC Number and Activity in the Four Groups. The number of EPCs evaluated by FACS analysis and cell culture assay of four groups is shown in Figure 1. The number of EPCs in the normotriglyceridemic groups was close to that in the hypertriglyceridemic groups of the same sex, both men and women ($P > 0.05$ and $P > 0.05$, respectively) (Figures 1(a) and 1(b)). However, the number of EPCs in men, both normotriglyceridemic and hypertriglyceridemic groups, was drastically lower than that in women ($P < 0.05$) (Figures 1(a) and 1(b)).

The migratory and the proliferative activities of EPCs were significantly higher in both normotriglyceridemic and hypertriglyceridemic premenopausal female groups than those in the male groups ($P < 0.05$ and $P < 0.05$, respectively) (Figures 2(a) and 2(b)). Besides, in the male groups, the migratory and the proliferative activities of EPCs were statistically decreased in the hypertriglyceridemic men compared with those in the normotriglyceridemic men ($P < 0.05$) (Figures 2(a) and 2(b)). However, the activity of EPCs in normotriglyceridemic premenopausal women was not remarkably different from that in hypertriglyceridemic premenopausal women ($P > 0.05$) (Figures 2(a) and 2(b)).

3.3. Levels of NO, VEGF, and GM-CSF in Plasma or Culture Media in the Four Groups. The level of NO from plasma or culture media of the normotriglyceridemic and hypertriglyceridemic men groups was remarkably lower than that of the premenopausal women groups ($P < 0.05$ and $P < 0.05$, respectively) (Figures 3(a) and 4(a)). However, the differences in plasma level of NO or the level of NO secreted by cultured EPCs between the normotriglyceridemic and hypertriglyceridemic premenopausal female groups did not reach statistical significance ($P > 0.05$ and $P > 0.05$, respectively) (Figures 3(a) and 4(a)). In addition, the level of NO in plasma or cultured media of the hypertriglyceridemic men

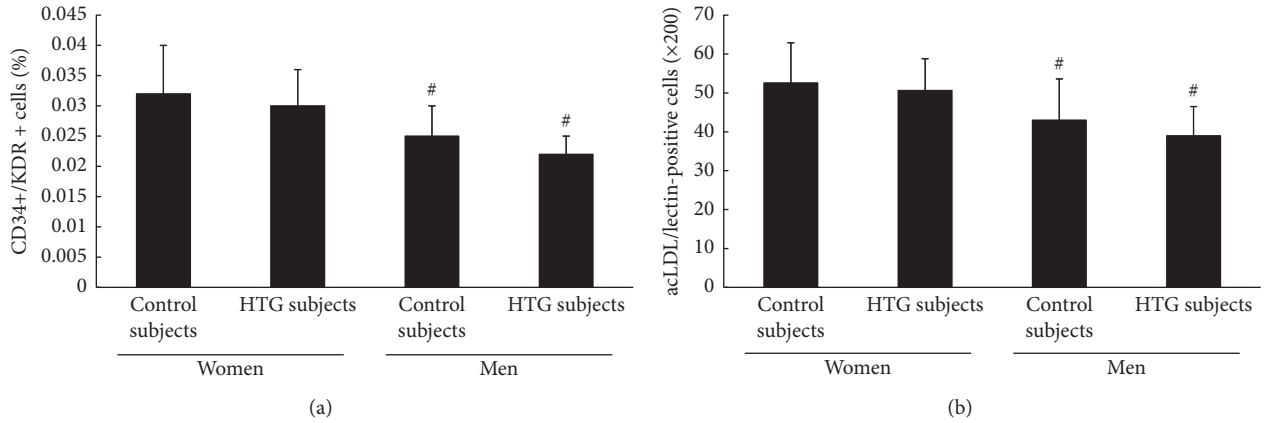

Figure 1: (a) fluorescence-activated cell sorting analysis. (b) Phase-contrast fluorescent microscope. There was no significant difference in the level of circulating EPCs between the normotriglyceridemic and the hypertriglyceridemic women. In both the normotriglyceridemic and hypertriglyceridemic patients, the number of EPCs in women groups was statically higher than that in the men groups. Data were presented as mean ans standard deviation. #vs premenopausal women.

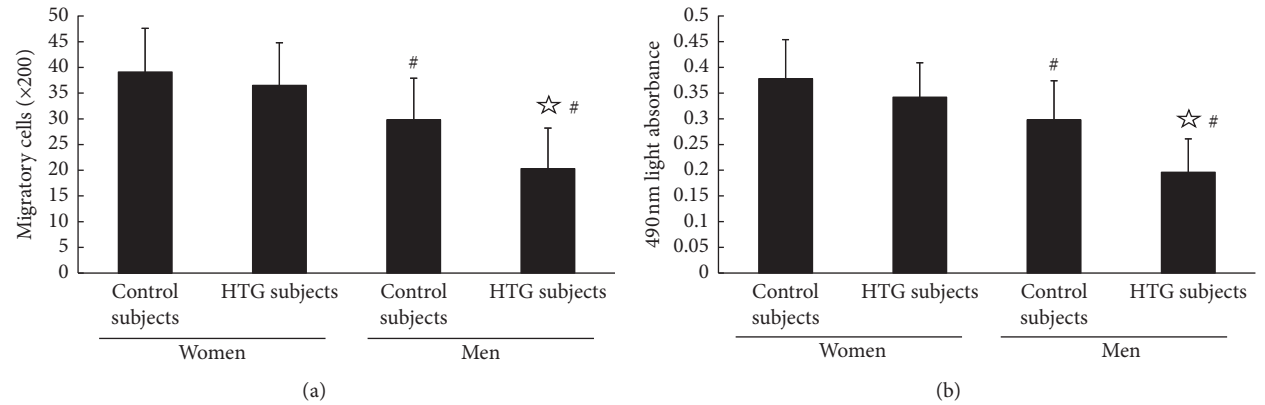

Figure 2: (a) The migratory and (b) the proliferative activities of EPCs were statically elevated in the female groups (both normotriglyceridemic and hypertriglyceridemic) than those in the male groups. The migratory and the proliferative activities of EPCs were lower in the hypertriglyceridemic men group than those in the normotriglyceridemic men group. However, the migratory and the proliferative activities of EPCs in normotriglyceridemic premenopausal women were similar to those in hypertriglyceridemic premenopausal women. Data were presented as mean and standard deviation. ☆vs. normotriglyceridemic. #vs premenopausal women.

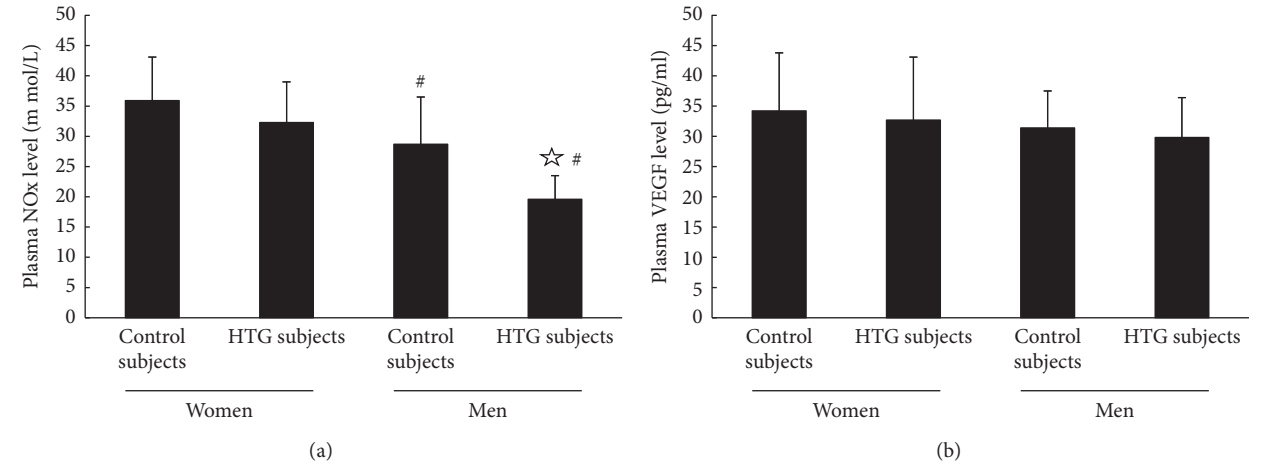

Figure 3: Continued.

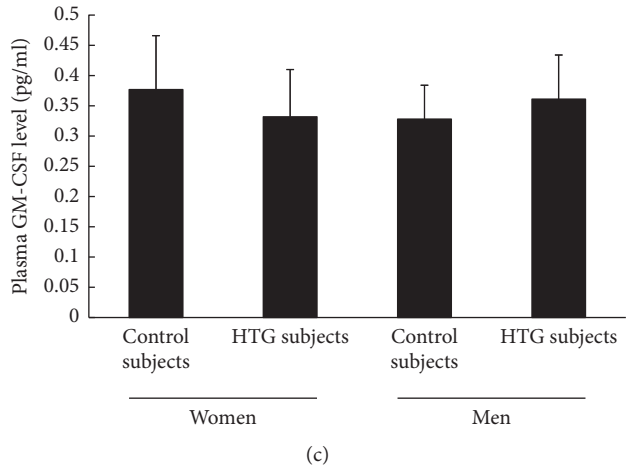

Figure 3: The plasma levels of NO, VEGF, and GM-CSF. (a) The plasma levels of NO in normotriglyceridemic and hypertriglyceridemic men were statically lower than those in the female groups ($P < 0.05$). In the male groups, the level of NO was decreased in hypertriglyceridemic subjects than that in normotriglyceridemic subjects ($P < 0.05$). The plasma levels of NO between normotriglyceridemic and hypertriglyceridemic premenopausal female groups displayed no significant difference ($P > 0.05$). (b) and (c) The plasma level of VEGF and GM-CSF had no statistical difference between the four groups.

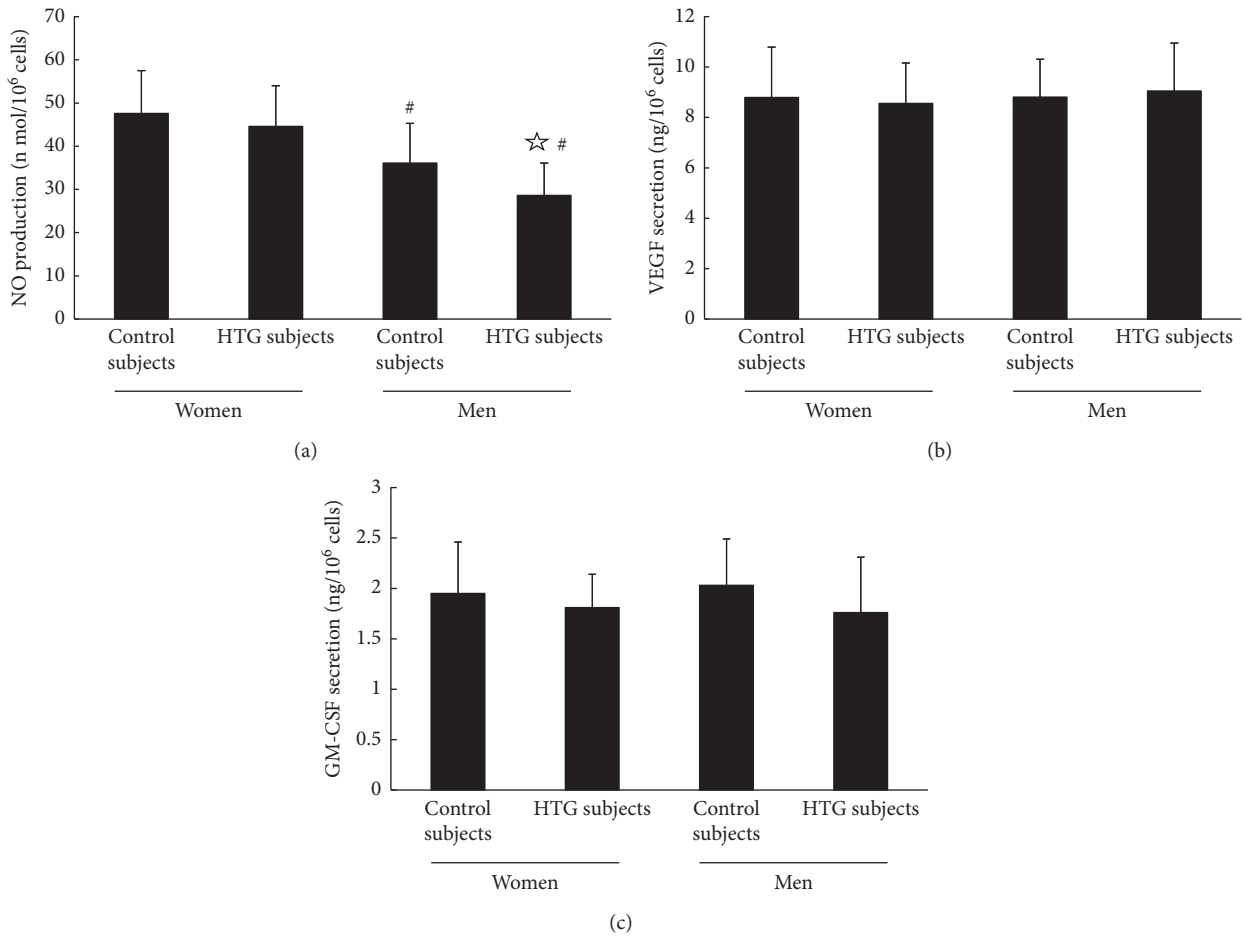

Figure 4: The levels of NO, VEGF, and GM-CSF secreted by cultured EPCs. (a) The level of NO produced by cultured EPCs of the normotriglyceridemic and hypertriglyceridemic men groups was remarkably lower than that of the premenopausal women groups. Besides, the secretion of NO from hypertriglyceridemic men was statically elevated compared with that from normotriglyceridemic men. Nevertheless, the level of NO in the EPCs cultural media of the hypertriglyceridemic premenopausal women was not significantly different from that of the normotriglyceridemic premenopausal women). (b) and (c) There was no obvious difference in VEGF or GM-CSF secretion between four groups.

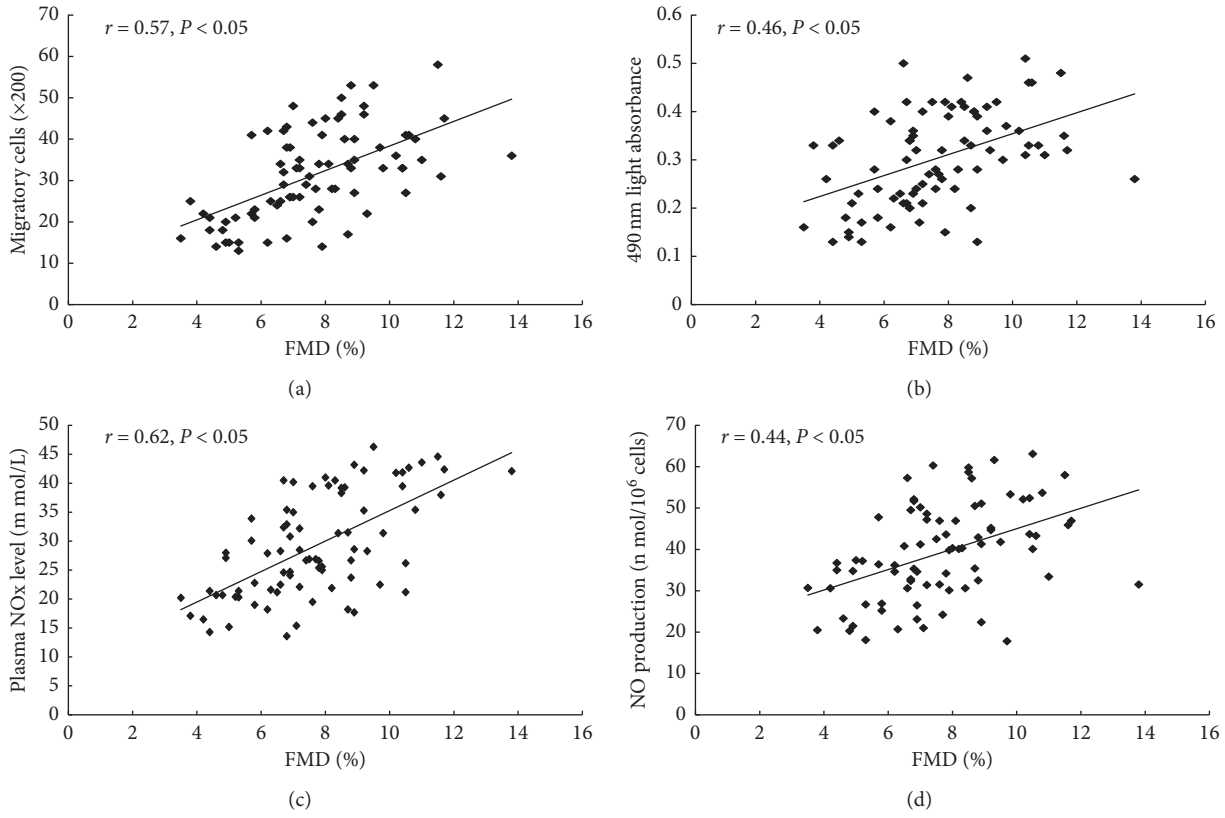

Figure 5: The correlation between FMD and EPCs or NO level. (a) and (b) There was a positive relevance between FMD and the proliferative or migratory activity of circulating ECPs. Besides, (c) and (d) FMD was positively correlated with the plasma NO level or NO level detected from cultured media.

was statistically elevated compared with that from the normotriglyceridemic men ($P < 0.05$ and $P < 0.05$, respectively) (Figures 3(a) and 4(a)). Nevertheless, there were no drastic differences in VEGF or GM-CSF plasma level or secretion between four groups (Figures 3(b), 3(c), 4(b), and 4(c)).

3.4. Correlation between FMD and EPCs or NO Level. FMD, which reflected the endothelial function, was positively relevant with the migratory ($r = 0.57$, $P < 0.05$) and the proliferative ($r = 0.46$, $P < 0.05$) activity of circulating ECPs (Figures 5(a) and 5(b)). Moreover, there was a positive correlation between FMD and the plasma NO level ($r = 0.62$, $P < 0.05$) or level of NO secreted by cultured EPCs ($r = 0.44$, $P < 0.05$) (Figures 5(c) and 5(d)).

4. Discussion

Our research firstly demonstrated sex effects on the endothelial function and circulating endothelial progenitor cells in hypertriglyceridemic patients, which may be probably attributed to the disturbance of NO production. We have evaluated that the number and activity of EPCs were preserved in premenopausal women compared with those in the age-matched men in hypertriglyceridemic status. Besides, the plasma level of NO or NO level secreted by EPCs in the culture media was higher in the premenopausal hypertriglyceridemic women than in men. Moreover, there was a positive correlation between EPCs activity or the NO level and endothelial functions evaluated by FMD.

It is well documented that there are sex differences in the occurrence of ischemic heart disease. Generally, males tend to have earlier onset [1] of occlusive coronary artery disease (CAD) [42] with more severe infarction foci and poor recovery [43–45] than females. EPCs are essential for the intrinsic repair and regeneration process of the injured myocardium [46]. Ischemic heart disease was proved to be associated with depletion of circulating EPCs [47, 48] and higher local density of EPCs [48]. Coincidently, in comparison with men or postmenopausal women, premenopausal women exhibit a higher level of circulating EPCs with better colony-forming capacity and migratory activity [49–52]. This phenomenon was consistent with that of the previous studies of the sex differences in the occurrence of ischemic heart disease, which implied that the better cardiovascular repaired and protective capacity in premenopausal women might partly attribute to the enhanced number and activity of EPCs. Hypertriglyceridemia is proved to be an independent risk factor of cardiovascular disease [6–11]. However, there were no scientific data revealing the relationship between sex differences and hypertriglyceridemia and EPCs. Hence, we postulated that the sex differences in circulating EPCs might also exist in hypertriglyceridemia.

We have studied the number and activity differences in circulating EPCs among four groups. Generally, the number of circulating EPCs in male groups (both the hypertriglyceridemia and the control) was higher than that in the age-matched premenopausal female groups, while the activity of EPCs was depleted compared with that of female groups, which were consistent with the previous studies [49–52]. Furthermore, we noticed that, in the male groups, the number and activity of EPCs are drastically reduced in patients with hypertriglyceridemia, which suggested the depletion of the number and activity of EPCs in hypertriglyceridemia may at least partly explain the higher risk of ischemic heart disease in hypertriglyceridemic male patients. Interestingly, we found that the number and activity of circulating EPCs in hypertriglyceridemic premenopausal women was nearly identical to that in normotriglyceridemic premenopausal women. The present results suggested the restoration of endogenous vascular endothelial repair capacity in premenopausal women, even in hypertriglyceridemia status. The reduction of number and activity of EPCs only existed in hypertriglyceridemic males rather than in the hypertriglyceridemic premenopausal female, indicating that a higher tendency of ischemic heart disease in hypertriglyceridemic men than in hypertriglyceridemic premenopausal women may be due to the sex differences in endogenous endothelial repair capacity.

It is well known that EPCs promoting angiogenesis or vascular repair is mediated by paracrine molecules such as VEGF [27–29], GM-CSF [29–32], and NO [32–34]. Previous studies elucidated that elevation of triglycerides was inversely correlated with the level of NO [53–55]. Therefore, we hypothesized that the sex differences in EPCs in patients with hypertriglyceridemia might be related to the alteration of the production of VEGF, GM-CSF, or NO. We detected the plasma level of NO, VEGF, and GM-CSF in the four groups. Similar to the sex differences in the changes of circulating EPCs in hypertriglyceridemia status, we found that the NO plasma level was lower in male groups than in premenopausal female groups, which was consistent with those of the previous studies in healthy subjects [56]. Besides, the plasma NO level was restored in premenopausal women in hypertriglyceridemia status compared with that in women in the control group. In our previous research, we have elucidated the positive correlation between plasma NO level and the number or activity of circulating EPCs [38]. Therefore, it suggested that the restoration of number and activity of circulating EPCs in hypertriglyceridemic premenopausal women may be attributed to the stability of exogenous NO production. Nevertheless, we did not observe a significant change in the plasma level of VEGF or GM-CSF among four groups, implicating that VEGF and GM-CSF may not be the key factors contributing to the sex differences in hypertriglyceridemia.

NO was generated by endothelial nitric oxide synthase (eNOS), which acts as a key regulator for the homeostasis of endothelial function [57]. Therefore, we further investigated the sex differences in NO production by EPCs. And, we found that the level of NO secreted by EPCs was similar to the change of the NO plasma level. As we have mentioned before, NO secretion by EPCs is significantly correlated with the number and activity of circulating EPCs [38]. Our result suggested that enhanced endogenous NO production would probably help to preserve the number and activity of circulating EPCs in hypertriglyceridemic women. In addition, there was no remarkable difference in the production of VEGF and GM-CSF, which further suggested that VEGF and GM-CSF did not participate in the sex differences in the EPCs of hypertriglyceridemia.

FMD is an indicator of endothelial function. We further investigated the relationship between FMD and EPCs or NO. The result revealed that the activity of EPCs and NO exogenous or endogenous production was positively correlated with the endothelial function.

In general, we discovered the conservation of endothelial function with EPC counts and activity in hypertriglyceridemic premenopausal women, which may be attributed to the enhanced production of NO. It is well documented that NO plays an essential role in the maintenance of vascular homeostasis, including regulation of vascular dilator tone, modulation of local cell growth, and protection of vessels [58]. Endothelial cells, as well as vascular smooth muscle cells, are crucial targets of estradiol [59]. Furthermore, estradiol activates early and late endothelial eNOS via binding the estrogen receptors through nongenomic and genomic pathways [60]. Undoubtedly, the remarkable difference between premenopausal women and age-matched men is the estradiol level. Hence, we inferred that estradiol augmented NO production and thereby increased the number and activity of circulating EPCs in hypertriglyceridemia.

The present study demonstrated a new insight into the evaluation and therapeutic targets of hypertriglyceridemia-related endothelium injury. Firstly, we showed the sex differences in circulating EPC counts and activity, indicating that EPCs and FMD could act as an essential clinical biomarker to detect hypertriglyceridemia-related vascular injury. Secondly, the correlation of higher cardiovascular risk with the reduction of EPC counts and activity in men compared with that in premenopausal women reminded us that enhancement of circulating EPC counts and activity is a potential therapeutic target to reverse the hypertriglyceridemia-related endothelial damage. Furthermore, our study also revealed that enhanced NO production was essential for the improvement of EPC number and activity. Hence, we advocated that patients with hypertriglyceridemia could adopt some strategies, such as exercises [39, 61], to stimulate NO production and thereby improve the endothelial repair capacity.

5. Conclusions

The present study raised a new view that there were sex differences in the number and activity of circulating EPCs in hyperglyceridemia patients. Hypertriglyceridemic premenopausal women displayed restored endothelial functions, which was associated with the enhanced NO production, probably mediated by estradiol. We provided a new insight to explore the clinical biomarkers and therapeutic strategies for hypertriglyceridemia-related vascular damage.

Data Availability

The datasets used in the current study can be made available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Zi Ren, Jiayi Guo, and Xingxing Xiao these authors contributed equally to this work.

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Research Article

Upregulation of MicroRNA-125b Leads to the Resistance to Inflammatory Injury in Endothelial Progenitor Cells

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Objectives. MicroRNA-125b (miR-125b) has been recognized as one of the key regulators of the inflammatory responses in cardiovascular diseases recently. This study sought to dissect the role of miR-125b in modulating the function of endothelial progenitor cells (EPCs) in the inflammatory environment of ischemic hearts. *Methods*. EPCs were cultured and transfected with miR-125b mimic and negative control mimic. Cell migration and adhesion assays were performed after tumor necrosis factor-α (TNF-α) treatment to determine EPC function. Cell apoptosis was analyzed by flow cytometry. The activation of the NF-κB pathway was measured by western blotting. EPC-mediated neovascularization in vivo was studied by using a myocardial infarction model. *Results*. miR-125b-overexpressed EPCs displayed improved cell migration, adhesion abilities, and reduced cell apoptosis compared with those of the NC group after TNF-α treatment. miR-125b overexpression in EPCs ameliorated TNF-α-induced activation of the NF-κB pathway. Mice transplanted with miR-125b-overexpressed EPCs showed improved cardiac function recovery and capillary vessel density than the ones transplanted with NC EPCs. *Conclusions*. miR-125b protects EPCs against TNF-α-induced inflammation and cell apoptosis by attenuating the activation of NF-κB pathway and consequently improves the cardiac function recovery and EPC-mediated neovascularization in the ischemic hearts.

1. Introduction

The role of EPCs in vascular and tissue repair in ischemic conditions, such as coronary or peripheral vascular diseases, has been well recognized [1]. Circulating EPCs are recruited into the ischemic sites, and they enhance repair through paracrine effects or by incorporating into newly formed vessels after ischemic injury [2–4]. Of note, the functional activities of EPCs are impaired in patients with coronary artery disease (CAD). Compelling evidence suggests that the number and function of EPCs inversely correlate with risk factors for CAD, such as hypertension, diabetes, dyslipidemia, smoking, and age [5–7]. Furthermore, the hostile inflammatory environment in the ischemic sites can induce the apoptosis of EPCs and, consequently, impede the EPC-mediated repair [8, 9]. Hence, improving the function and survival of EPCs in the ischemic sites is critical for EPC-mediated repair.

MicroRNAs (miRNAs) are known as a class of non-coding RNAs that modulate the gene expression at a posttranscriptional level. miR-125, known as one of the major regulators in the development of hematological malignancies, is a family of highly conserved miRNAs throughout diverse species [10]. Emerging evidence suggests that miR-125 is involved in the regulation of the innate immune and inflammatory responses [11, 12]. More importantly, the role of miR-125b in cardiovascular diseases has been drawing increasing attention recently. Dr. Wang et al. reported that the target of miR-125b in the mouse heart is TNF receptor-associated factor 6 (TRAF6), an adaptor molecule in the NF-kB pathway. Overexpression of miR-125b in the mouse heart protects the myocardium from ischemia/reperfusion injury by suppressing the TRAF6-mediated NF-kB activation [13]. However, the role of miR-125b in the regulation of EPCs is still unclear, and further studies are required to study its role.

In the present study, we focused on the role of miRNA-125b in regulating the inflammatory response and function of EPCs in the ischemic hearts. Our results confirmed that upregulation of miRNA-125b ameliorated TNF-α-induced functional defects in EPCs in vitro and enhanced EPC-mediated neovascularization in the ischemic hearts. miR-125b-mediated inhibition of TNF-α/NF-κB pathway activation is involved in the beneficial effects we observed.

2. Methods and Materials

2.1. Cell Culture and miRNA Transfection. Bone marrow mononuclear cells were isolated by density gradient centrifugation from the mouse bone marrow and cultured in endothelial cell basal medium-2 (EBM-2) supplemented with endothelial growth medium SingleQuots as indicated by the manufacturer (Clonetics, San Diego). After 4 days of culture, nonadherent cells were removed by washing the plates with phosphate-buffered solution (PBS), and a new medium was applied. EPCs were transfected for 24 h on day 6 with 50 nmol/L microRNA mimics for miR-125b (MC10148, Ambion) or miR-negative control (AM17010, Ambion), using the Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol [13]. Cells were treated with or without 10 ng/mL tumor necrosis factor-α (TNF-α, Peprotech) for 1 h and then used for the following experiments at day 7. Cultured EPCs were identified by the flow cytometry analysis. Based on the isolation and cultivation protocol, the adherent mononuclear cells were identified as EPCs similar to the previous studies.

2.2. EPC Adhesion to Endothelial Cells In Vitro. 2×10^5 human umbilical vein endothelial cells (HUVECs) were seeded in each well of a four-well plate 48 h before the assay to prepare a monolayer of ECs. Then, 1×10^5 EPCs labelled with CM-DiI (CellTrackerTM CM-DiI, Invitrogen) were added to each well and incubated for 3 h at 37°C. Nonattached cells were gently washed and removed with PBS, and adherent EPCs were fixed with 4% paraformaldehyde and counted by independent investigators blinded to treatment randomly.

2.3. EPC Migration In Vitro. A total of 2×10^4 EPCs were harvested and resuspended in 250 μL EBM-2 after TNF-α treatment and pipetted into the upper chamber of a modified Boyden chamber (Costar Transwell assay, 8 μm pore size, Corning, NY), which was placed in a 24-well culture plate containing 500 μL EBM-2 medium supplemented with 100 ng/mL SDF-1. Transmigrated cells were counted after 24 h incubation at 37°C by independent investigators blinded to the treatment randomly.

2.4. EPC Apoptosis Assay. EPCs were treated with 10 ng/mL tumor necrosis factor-α (TNF-α, Peprotech) for 1 h, and cell apoptosis was detected by AnnexinV-staining (Roche, Penzberg, Germany). Briefly, EPCs were cultured with TNF-α (10 ng/mL) for 1 h. Then, EPCs were collected and washed for three times. Annexin V-FITC and propidium iodide (PI) were added to the washed cells (1×10^6 cells/mL in FACS buffer) for 15 min at room temperature in the dark. FACS buffer was added, and cells were analyzed immediately by flow cytometry analysis.

2.5. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction. miR-125b-5p were quantified using specific Taqman assays for miR (Applied Biosystems, USA). Specific primers for miR-125b-5p were obtained from Applied Biosystems. miR-125b levels were normalized to the U6 small nucleolar RNA. Primer sequences for gene encoding for TNF-α, IL-1β, IL-6, and β-actin were reported in Table 1. The results were normalized to the mRNA levels of β-actin.

2.6. Western Blotting. Proteins were extracted with cell lysis buffer (Cell Signaling Technology) and analyzed with by western blotting by using p-NFκB p65 antibody (Ser 276) (1 : 1000, Santa Cruz, sc-101749), NF-κB p65 (1 : 1000, Cell Signaling, 8242T) and rabbit anti-β-actin antibodies (1 : 3000; Cell Signaling Technology). The intensities of protein bands were quantified densitometrically by using the NIH IMAGE J software.

2.7. Surgical Induction of Myocardial Infarction (MI) and EPC Transplantation. The mice were anesthetized by 5.0% isoflurane, and anaesthesia was maintained by inhalation of 1.5% to 2% isoflurane driven by oxygen flow using a rodent ventilator. The hearts were exposed, and the left anterior descending (LAD) coronary artery was ligated with an 8-0 silk ligature. 2×10^5 EPCs suspended in 20 μl PBS were injected at 5 different sites at the infarct border zone using an 20 μl Hamilton syringe with a 30-gauge needle. 6–8 mice were used for LAD ligation in each group. The cardiac function was evaluated by echocardiography. The study protocol was approved by the Ethics Committee of Sun Yat-sen University.

2.8. Histological Assessments. Cardiac tissues were fixed in 4% paraformaldehyde for 4 hours and then snapfrozen. Serial cryosectioning was performed starting at 1 mm below the LAD ligation moving toward the apex. To evaluate PC endothelial differentiation and capillary density, immunohistochemical staining was performed using fluorescent anti-CD31 (Santa Cruz) antibodies. All surgical procedures and pathohistological analyses were performed by investigators blinded to treatment assignments.

2.9. Statistical Analyses. All values are reported as mean ± SEM. Two-tailed Student's t test was used to compare 2 means. One-way or 2-way ANOVA with a Bonferroni correction was used to compare multiple (>2) means with 1 or 2 independent variables, respectively. $p < 0.05$ was considered significant.

Table 1: Primer sequences.

Gene	Forward primer	Reverse primer
TNF-alpha	AGGGATGAGAAGTTCCCAAATG	AGGGATGAGAAGTTCCCAAATG
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	TCGGAGGCTTAATTACACATGTTC	TGCCATTGCACAACTCTTTTCT

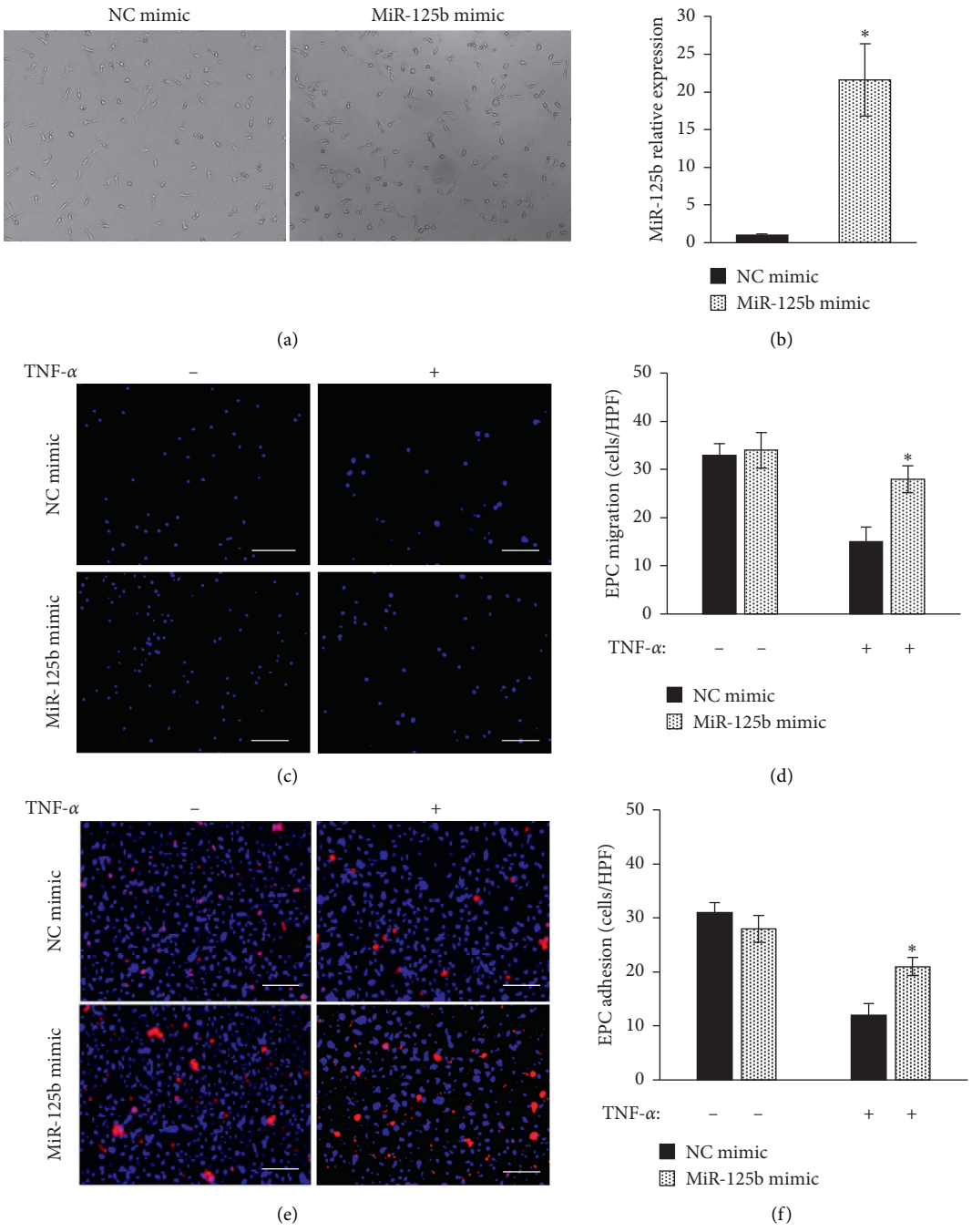

Figure 1: miR-125b overexpression in EPCs preserved its migration and adhesion function after TNF-α treatment. EPCs were transfected with miR-125b mimic and negative control mimic for 24 h. (a) The level of miR125b measured by qRT-PCR (b). EPC migration and adhesion measured after transfection. Representative (c) and quantification (d) of the migratory activity of EPCs. Representative (e) and quantification (f) of DiI-labeled EPC adhesion to HUVECs with TNF-α activation (scale bar = 100 μm, $^*p < 0.05$ vs. NC mimic with TNF-α treatment, $n = 5$).

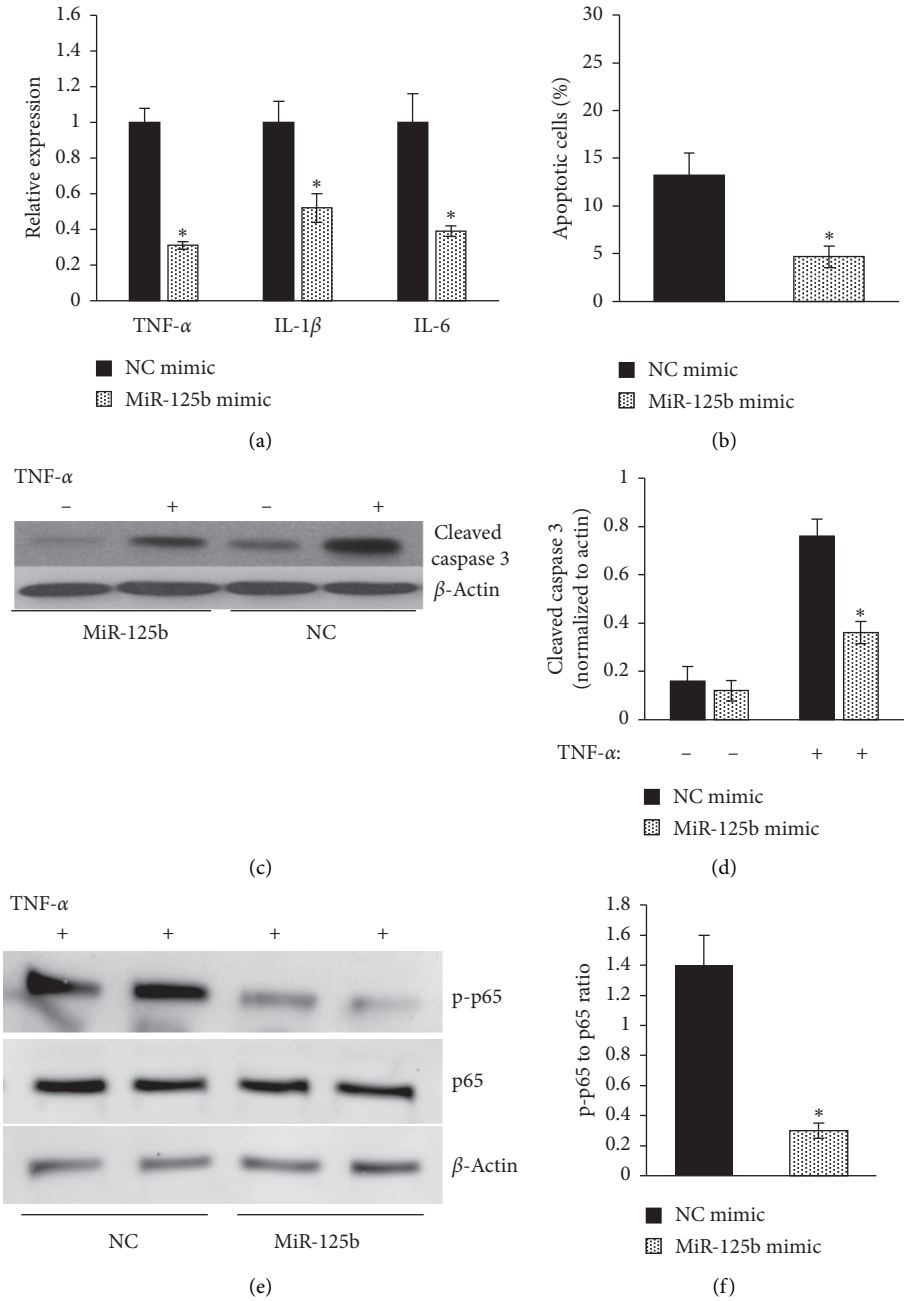

Figure 2: Upregulation of miR-125b in EPCs attenuated the expression of proinflammatory factors and ameliorated the TNF-α-induced cell apoptosis. EPCs were treated with TNF-α (10 ng/mL) for 1 h after transfection. The mRNA levels of proinflammatory factors (TNF-α, IL-1β, and IL-6) were measured by qRT-PCR (a). Cell apoptosis was determined by flow cytometry using annexin V staining (b). The activation of caspase3 was analyzed by western blotting. Representative (c) and quantification (d) of caspase3 level (normalized to β-actin). The activation of NF-κB was determined by the level of p-p65 in EPCs using western blotting. Representative (e) and quantification (f) of p-p65 level (normalized to p65) ($^*p < 0.05$ vs. NC mimic with TNF-α treatment, $n = 5$).

3. Results

3.1. Overexpression of miRNA-125b in EPCs Ameliorates TNF-α Induced Functional Defects in EPCs. The level of miR-125b in the EPC transfected with miR125b mimic is about 21-folds increase compared with the negative control (NC) group (Figures 1(a) and 1(b)). Transfected EPCs were treated with or without 10 ng/mL TNF-α for 1 h and then tested for cell migration and adhesion in vitro. The NC group showed significant reduced cell migration (Figures 1(c) and 1(d)) and adhesion (Figures 1(e) and 1(f)) capacity after TNF-α treatment compared with the one without TNF-α treatment, while TNF-α treatment slightly reduced the cell migration and adhesion function in the miR-125b-overexpressed group in comparison with cells without TNF-α treatment. After TNF-α treatment, miR-125b-overexpressed EPCs

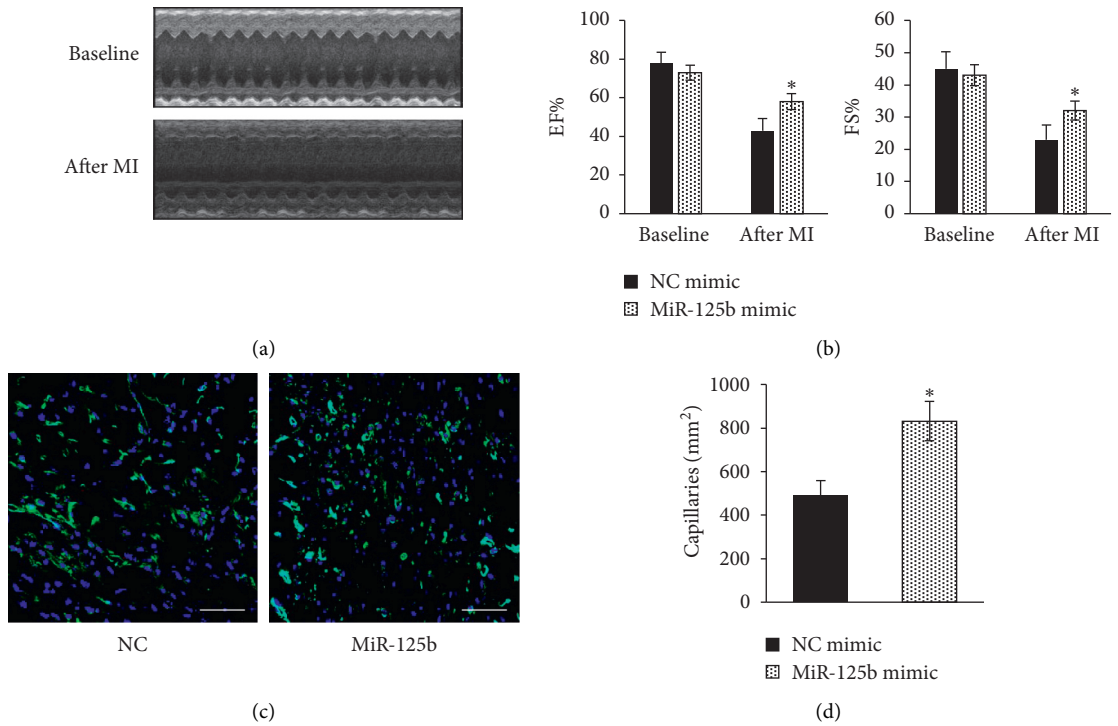

Figure 3: miR-125b overexpression enhanced the EPC-mediated neovascularization and cardiac function recovery in the ischemic hearts. The cardiac function was assessed by echocardiography at baseline and after MI (28 days). Representative (a) and quantification (b) of echocardiography analyses. EPC-mediated neovascularization in the ischemic hearts analyzed by CD31 staining (GFP) (c) and capillary density quantified (d) (scale bar = 100 μm, $^*p < 0.05$ vs. NC mimic, $n = 5$).

showed significantly better preserved migration and adhesion function than those of the NC group.

3.2. Overexpression of miRNA-125b in EPCs Attenuates TNF-α Induced Expression of Proinflammatory Factors and Cell Apoptosis. The mRNA level of the proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in EPCs was measured by qRT-PCR after 1 h TNF-α treatment. We found that miR-125b-overexpressed EPCs showed significantly lower TNF-α, IL-1β, and IL-6 mRNA expression levels than those of the NC group (Figure 2(a)). Moreover, the percentage of apoptotic cells (Annexin V positive cells) is significantly lower in the miR-125b-overexpressed group compared with that of the NC group after TNF-α treatment (Figure 2(b)). In parallel with these flow cytometry results, our western blotting results showed markedly lower cleaved caspase 3 level in the overexpression group than in the NC group after TNF-α treatment (Figures 2(c) and 2(d)) which indicated reduced cell apoptosis.

3.3. Effects of miRNA-125b on EPC-Mediated Neovascularization in the Ischemic Hearts. To investigate the effect of miR-125b on the regulation of TNF-α induced NF-κB pathway activation, western blotting was performed to analyze the level of p65 phosphorylation (p–p65). As shown in Figures 2(e) and 2(f), the level of p–p65 in miR-125b-overexpressed EPCs was markedly lower than of the NC group after TNF-α treatment. To further investigate the role of miRNA-125b in EPC-mediated neovascularization, miR-125b-overexpressed and NC control EPCs were transplanted by intramyocardial injections into the mice immediately after surgical-induction of MI, the cardiac function was evaluated by echocardiography, and the capillary density in the infarct border zone was assessed by immunohistology staining of CD31. The cardiac function of the mice transplanted with miR-125b-overexpressed EPCs was improved compared with that of the ones transplanted with NC control EPCs 28 days after MI (Figures 3(a) and 2(b)). The capillary density in the infarct border zone of the mice transplanted with miR-125b-overexpressed EPCs was about 2-folds higher than the ones transplanted with NC control EPCs (Figures 3(c) and 2(d)). These data suggest miR-125b overexpression in EPC-enhanced EPC-mediated neovascularization and cardiac function recovery in the ischemic hearts.

4. Discussion

In this study, we have identified a novel role of miR-125b in the regulation of EPC functions. Upregulation of miR-125b in EPCs ameliorates the inflammatory and apoptotic responses of EPCs in the ischemic heart by inhibiting the activation of the TNF-α/NF-κB pathway. Using the myocardial infarction model, we demonstrated that miR-125b overexpression enhanced the EPC-mediated neovascularization and cardiac function recovery in the ischemic hearts. To the best of our knowledge, this is the first

study to address the role of miR-125b in modulating EPC-mediated neovascularization in the ischemic hearts.

Although EPC-related cell therapy has been studied extensively, the majority of the cell-therapy trials achieve only modest efficacy [14–16]. The low survival rate of the transplanted EPCs in the ischemic hearts is one of the major obstacles to the success of this therapy [17, 18]. After the myocardial infarction, a large number of inflammatory cells are recruited to the ischemic heart where a hostile, pro-inflammatory environment is created. Compelling evidence showed that the EPC level can be significantly affected by systemic inflammation [19]. It has been reported that lipopolysaccharide-induced systemic inflammatory reaction led to a decrease in the number of circulating EPCs [20]. Of note, patients with long-term inflammatory disease, like active ulcerative colitis, had significantly lower levels of circulating EPCs [21]. In this study, our data indicate that TNF-α treatment in EPCs markedly impaired its migration and adhesion function. More importantly, EPCs treated with TNF-α showed increased cell apoptosis, which might partly explain the low survival rate of transplanted EPCs in the ischemic hearts. Collectively, others and our study suggest that enhancing the survival of EPCs in the proinflammatory environment in the ischemic hearts is crucial for achieving satisfactory outcome of cell therapy.

miR-125 family is well known as one of the major regulators in the development of hematological malignancies and autoimmune diseases [22–24]. Recently, accumulating studies demonstrate that miR-125b negatively regulates the activation of the NF-κB pathway by targeting TRAF6 [25]. More important, evidence showed that miR-125b-mediated repression of the NF-κB pathway exerts a protective effect on ischemic hearts [13]. However, the role of miR125b in the regulation of EPC function is still unclear. Our study for the first time demonstrated that the overexpression of miR-125b led to resistance to TNF-α-induced functional defects and cell apoptosis in EPCs and consequently enhanced the EPC-mediated neovascularization in the ischemic hearts. It is well recognized that the TNF-α/NF-κB pathway is central to most of the inflammatory processes and exerts negative regulatory effect on vascular repair [26]. In consistency with the reports from other cell types [27], our data showed that the overexpression of miR-125b blunted the TNF-α induced proinflammatory responses in EPCs and restored the functions of EPCs and, more importantly, protected against TNF-α induced apoptosis. Furthermore, mice transplanted with miR-125b-overexpressed EPCs showed enhanced neovascularization compared with that of the one with NC control EPCs. Taking together, our study for the first time unveils the protective effect of miR-125b on EPCs.

Although our data have demonstrated that upregulation of miR-125b blunted the TNF-α-induced NF-κB pathway activation in EPCs, the detailed mechanisms underlying miR-125b-mediated negative regulation of NF-κB pathway in EPCs has not been revealed in this study. Reports from others have characterized TRAF6 as the target of miR-125b in the NF-κB pathway activation [13, 25, 27]. However, whether miR-125b exerts its effect on TNF-α/NF-κB pathway by targeting TRAF6 or other molecules in EPCs still requires further investigations to confirm.

In conclusion, our study suggests that miR-125b-mediated inhibition of the TNF-α/NF-κB pathway is crucial for the protection of EPCs in the inflammatory environment and may be a novel therapeutic target for enhancing the effectiveness of cell therapy for ischemic heart disease.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ke Yang and Xing Liu contributed equally to this article.

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Research Article

Aliskiren Improved the Endothelial Repair Capacity of Endothelial Progenitor Cells from Patients with Hypertension via the Tie2/PI3k/Akt/eNOS Signalling Pathway

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Background. Studies show that aliskiren exerts favourable effects not only on endothelial progenitor cells (EPCs) but also on endothelial function. However, the mechanism of the favourable effect of aliskiren on EPCs from patients with hypertension is unclear and remains to be further studied. *Methods*. The object of this study was to investigate and assess the in vitro function of EPCs pretreated with aliskiren. After treated with aliskiren, the human EPCs were transplanted into a nude mouse model of carotid artery injury, and the in vivo reendothelialization of injured artery was estimated by staining denuded areas with Evans blue dye via tail vein injection. *Results*. We found that aliskiren increased the in vitro migration, proliferation, and adhesion of EPCs from patients with hypertension in a dose-dependent manner and improved the reendothelialization capability of these EPCs. Furthermore, aliskiren increased the phosphorylation of Tie2, Akt, and eNOS. After the blockade of the Tie2 signalling pathway, the favourable effects of aliskiren on the in vitro function and in vivo reendothelialization capability of EPCs were suppressed. *Conclusions*. This study demonstrates that aliskiren can improve the in vitro function and in vivo reendothelialization capability of EPCs from patients with hypertension via the activation of the Tie2/PI3k/Akt/eNOS signalling pathway. These findings further indicate that aliskiren is an effective pharmacological treatment for cell-based repair in hypertension-related vascular injury.

1. Introduction

As a major cardiovascular disease, hypertension usually impairs target organs and increases the risk of cardiovascular events. Endothelial dysfunction and vascular endothelial abnormalities are the known molecular mechanisms of endothelial injury in hypertension [1–3]. Increasing evidence suggests that circulating endothelial progenitor cells (EPCs) derived from bone marrow participate in the endothelial repair process in endothelial injury [4–8]. EPCs are able to proliferate and differentiate into endothelial cells and are therefore ideal candidates for application in vascular regeneration [9]. Relevant studies demonstrate that the reendothelialization capability of EPCs is beneficial to maintaining the integrity of the vascular endothelium after arterial injury [8, 9], which is crucial for the prophylaxis and

treatment of cardiovascular disease [8, 10–15]. Recent clinical trials proved that the state of hypertension and prehypertension leads to the declined number and dysfunction of circulating EPCs, implying that this impaired endogenous endothelial repair capacity is involved in mediating hypertension-related endothelial dysfunction and vascular injury [4, 16].

Tie2 tyrosine kinase receptor (Tie2) is a significant endothelial-specific receptor tyrosine kinase [9]. Accumulating experimental and clinical evidence supports the hypothesis that Tie2 and its ligands, e.g., angiopoietin-2 (Ang2), contribute to vasculogenesis and angiogenesis [17]. Ang2/Tie2 signalling plays a pivotal role in the biological processes of EPCs, such as chemotactic migration and cell survival [17, 18]. In addition, phosphoinositide 3-kinase (Pl3k), protein kinase B (Akt), and endothelial nitric oxide synthase (eNOS), which are downstream molecules regulating the Ang1-Tie2 signalling pathway, are related to the Ang2-mediated cellular responses of EPCs [9, 10, 17]. Our previous study further demonstrated that the Tie2/PI3k/Akt/eNOS signalling pathway is a target for the shear stress-mediated augmentation of the in vivo reendothelialization capability of transplanted EPCs in endothelial repair [9]. Therefore, the Tie2-dependent pathway plays a crucial role in regulating the endothelial repair capacity of EPCs.

Aliskiren, an active direct renin inhibitor, exhibits beneficial effects on endothelial function, ischaemia-induced neovascularization, and reduced arterial stiffness [19–21]. Aliskiren not only increases the number of EPCs but also improves the function of EPCs in processes such as adhesion and cellular migration [22–24]. However, the mechanism of the beneficial effect of aliskiren upon EPCs is unclear. Therefore, based on previous studies, we hypothesized that aliskiren might improve the endothelial repair capability of human EPCs via the Tie2/PI3k/Akt/eNOS signalling pathway. To test this hypothesis, we focused on aliskiren affecting the in vitro function and in vivo reendothelialization capability of early EPCs from patients with hypertension, evaluated the regulatory effects of aliskiren on the Tie2/Pl3k/Akt/eNOS signalling pathway in EPCs, and studied the role of this signalling pathway in the aliskiren-mediated regulation of EPC function in vitro and reendothelialization capability in vivo in mice. Our present study may thus provide valuable information to the further understanding of cell-based therapy as a novel pharmacological strategy for treating hypertension-related vascular injury.

2. Materials and Methods

2.1. Characteristics of the Subjects. Eighteen normotensive subjects and eighteen patients with essential hypertension, which had no family history of hypertension, were enrolled. The subjects must have been diagnosed as without cardiovascular diseases or had no ongoing drug and other treatments. The hypertensive patients were diagnosed by sitting blood pressure (after 10 min of rest) measurements obtained three times at 1-week intervals; a systolic blood pressure (SBP) of ≥140 mmHg and (or) a diastolic blood pressure (DBP) of ≥90 mmHg were diagnosed as hypertension. The normotensive subjects had no cardiovascular risk factors, an SBP of <120 mmHg, and a DBP of <80 mmHg, according to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) [25]. The age, sex, and body mass index (BMI) were matched between normotensive subjects and the patients. Complete clinical examination, laboratory tests, and instrumental examination were performed in order to exclude patients with secondary hypertension. Subjects with infection, peripheral artery disease, diabetes, malignant disease, or active inflammatory disorders, as well as those who smoked, were excluded; these conditions above may influence the number or function of EPCs [16]. The consent procedure and experimental protocol were approved by the Ethical Committee of the First Affiliated Hospital of Sun Yat-Sen University (approval no. [2017] 078), and written informed consent was collected.

Peripheral venous blood samples from subjects and patients used for EPC isolation and culture as well as for biochemical tests and routine blood, including the measurement of serum total triglyceride, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, cholesterol, high-sensitivity C-reactive protein (CRP), and levels fasting plasma glucose (FPG), were obtained from the two groups after overnight fasting [16, 26].

2.2. EPC Culture and Identification. EPC isolation and culture were performed as described previously [9, 16, 27–32]. In addition, the procedure for the identification of early EPCs was conducted as described in our previous study [9].

2.3. Migration, Proliferation, and Adhesion Assays with Circulating EPCs. The human EPCs cultured for 7 days were pretreated with 1 μmol/L, 10 μmol/L, or 50 μmol/L aliskiren (SIGMA-ALDRICH) for 12 h. EPC migration, proliferation, and adhesion assays were performed as described in previous studies [16, 32–34].

2.4. Tie2 Knockdown and Pharmacological Inhibition. The experimental protocols for the knockdown of Tie2 and the inhibition of PI3k and eNOS were performed via the protocol described in our previous study [9]. Tie2 expression was knocked down by shRNA lentiviral transduction particles. The viral transduction (Santa Cruz Biotechnology) was operated according to the manufacturer's manual. The human EPCs were preincubated with the inhibitor for 1 d prior to treatment with different concentrations of aliskiren, namely, 1 μmol/L, 10 μmol/L, and 50 μmol/L. Ten micromolar LY294002 (Calbiochem) was used to inhibit PI3k. eNOS was inhibited by 100 μmol/L L-NAME (Calbiochem).

2.5. Western Blot Analysis. As previously described in our studies [9, 26], proteins were extracted from EPCs in a cell lysis solution (Thermo Fisher Scientific). After SDS-PAGE treatment, the protein extract was transferred into PVDF membrane (Cell Signaling Technology). The assay-related

Table 1: Clinical and biochemical characteristics.

Characteristics	Normotensive subjects ($n = 18$)	Hypertensive patients ($n = 18$)
Age (years)	54.9 ± 7.9	55.3 ± 8.1
Height (cm)	166.8 ± 6.4	163.8 ± 6.0
Weight (kg)	63.1 ± 6.6	63.2 ± 5.5
BMI (kg/cm^2)	22.7 ± 2.4	23.6 ± 2.3
Systolic blood pressure (mmHg)	120.4 ± 11.1	148.6 ± 6.3*
Diastolic blood pressure (mmHg)	77.3 ± 6.8	90.4 ± 4.9*
Heart rate (beats/min)	71.3 ± 9.0	72.9 ± 7.8
AST (mmol/L)	25.3 ± 5.8	25.8 ± 5.8
ALT (mmol/L)	22.4 ± 4.1	23.5 ± 4.8
BUN (mmol/L)	5.34 ± 1.14	5.66 ± 1.16
Cr (mmol/L)	59.4 ± 12.8	62.4 ± 12.0
LDL (mmol/L)	2.89 ± 0.46	3.05 ± 0.40
TC (mmol/L)	4.84 ± 0.59	5.00 ± 0.48
HDL (mmol/L)	1.46 ± 0.22	1.42 ± 0.21
TG (mmol/L)	1.40 ± 0.18	1.44 ± 0.17
FPG (mmol/L)	4.95 ± 0.67	4.73 ± 0.73

Abbreviation: BMI, body mass index; LDL, low-density lipoprotein; TC, total cholesterol; HDL, high-density lipoprotein; TG, triglyceride; FPG, fasting plasma glucose. Notes: data are given as mean ± SD. *vs normotensive subjects.

antibodies include Tie2 rabbit mAb, phospho-Akt antibody, anti-Akt, phospho-eNOS antibody, and anti-eNOS (1 : 1000) and mouse anti-Tie2 (1 : 1000) (Cell Signaling Technology). HRP-conjugated anti-rabbit IgG (1 : 2000) (Cell Signaling Technology) was incubated to visualize the protein, and the protein was observed by ECL chemiluminescence system (Thermo Fisher Scientific). The intensity of the immunoreactive bands was analysed, and the results for the phospho-Tie2, phospho-Akt, and phospho-eNOS in human EPCs are expressed as the ratio of each phosphorylated protein to that of the corresponding unphosphorylated protein. The statistical comparisons for the Western blot analysis were made relative to non-aliskiren-treated EPCs from patients with hypertension.

2.6. Animal Model and Reendothelialization Assay. The nude mouse model of carotid artery injury was generated and the reendothelialization experiment was performed as described in our previous study [9].

All experimental programs were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Sun Yat-Sen University and conformed to the Guide for the Care (approval no. [2017]118) and Use of Laboratory Animals published by the US National Institutes of Health (NIH).

2.7. Statistical Analysis. SPSS V20.0 software was used for the statistical analyses. All the values are presented as the means value ± SDs. Student's *t*-test was used to compare the two groups. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Clinical Characteristics. The baseline characteristics of the two groups indicated that no significant differences were found except for the mean systolic and diastolic pressure. The SBP and DBP measurements were lower in the normotensive group than in the hypertensive group (Table 1).

The repair capacity of EPCs and the phospho-Tie2, phospho-Akt and phospho-eNOS in EPCs were lower in hypertensive patients.

The levels of migration and proliferation of EPCs were lower in the hypertensive than in normotensive (Figures 1(a) and 1(b)). The adhesion activity of EPCs was lower in the hypertensive than in the normotensive. Moreover, after the HUVECs (human umbilical vein endothelial cells) were pretreated with TNF-α, the adhesion of EPCs was increased in both the normotensive and hypertensive groups (Figures 1(c) and 1(d)). Compared with EPCs transplanted from patients with hypertension, EPCs transplanted from normotensive subjects had a greater reendothelialization capability, suggesting that hypertension attenuated the reendothelialization capability in vivo of EPCs (Figure 1(e)).

Similarly, the phospho-Tie2, phospho-Akt, and phospho-eNOS in EPCs were lower in the hypertensive than in the normotensive, respectively (Figures 2(a)–2(c)).

3.2. Aliskiren Upregulated the Migration, Proliferation, and Adhesion of EPCs In Vitro. The human EPCs cultured for 7 days were pretreated with 1 μmol/L, 10 μmol/L, or 50 μmol/L aliskiren for 12 h. Treatment with aliskiren resulted in a dose-dependent increase in the migratory (Figure 3(a)) and proliferative (Figure 3(b)) activity of EPCs, respectively. Furthermore, aliskiren markedly enhanced the adhesion of EPCs (Figure 3(c)).

3.3. Aliskiren Increased the Phospho-Tie2, Phospho-PI3k, and Phospho-Akt in EPCs. Aliskiren has a favourable effect on the phospho-Tie2, phospho-Akt, and phospho-eNOS in EPCs from patients with hypertension. The phospho-Tie2, phospho-Akt, and phospho-eNOS in EPCs were markedly lower in the hypertensive than in the normotensive (Figures 4(a)–4(c)). When EPCs from patients with

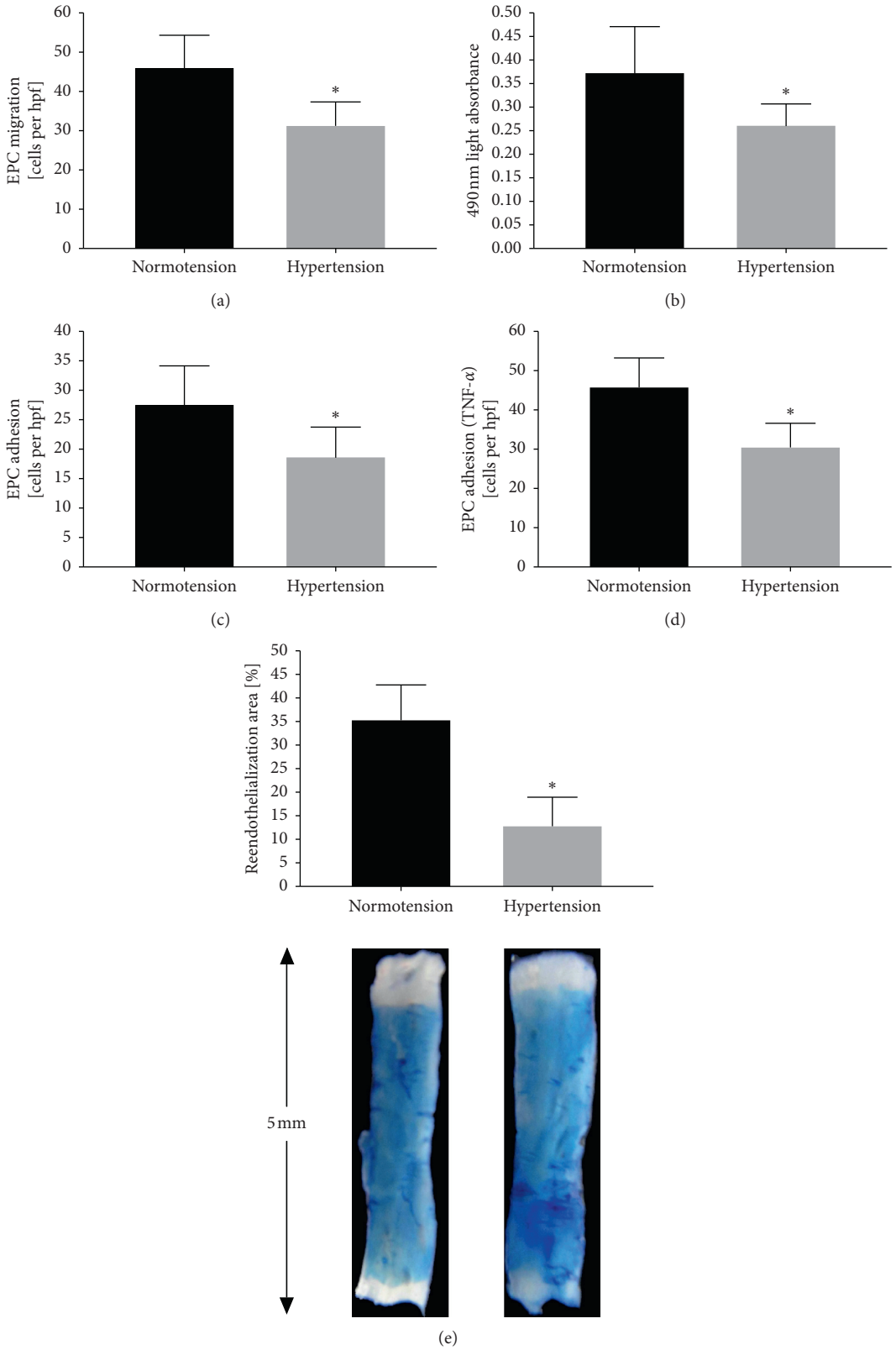

Figure 1: The quantification analysis of the in vitro function and in vivo reendothelialization capacity of human EPCs in the two groups. (a) Quantification analysis of human EPC migration ($^*P < 0.05$ vs. EPCs from normotensive subjects, $n = 18$ per group). (b) Quantification analysis of the proliferative activity of human EPCs ($^*P < 0.05$ vs. EPCs from normotensive subjects, $n = 18$ per group). (c)–(d) Quantification analysis of human EPC adhesion to HUVECs with or without TNF-α activation ($^*P < 0.05$ vs. EPCs from normotensive subjects, $n = 18$ per group). (e) Quantification analysis of the area of carotid artery reendothelialization by transplanted EPCs on day 3 after injury ($^*P < 0.05$ vs. EPCs from normotensive subjects, $n = 18$ per group). hpf = high-power field.

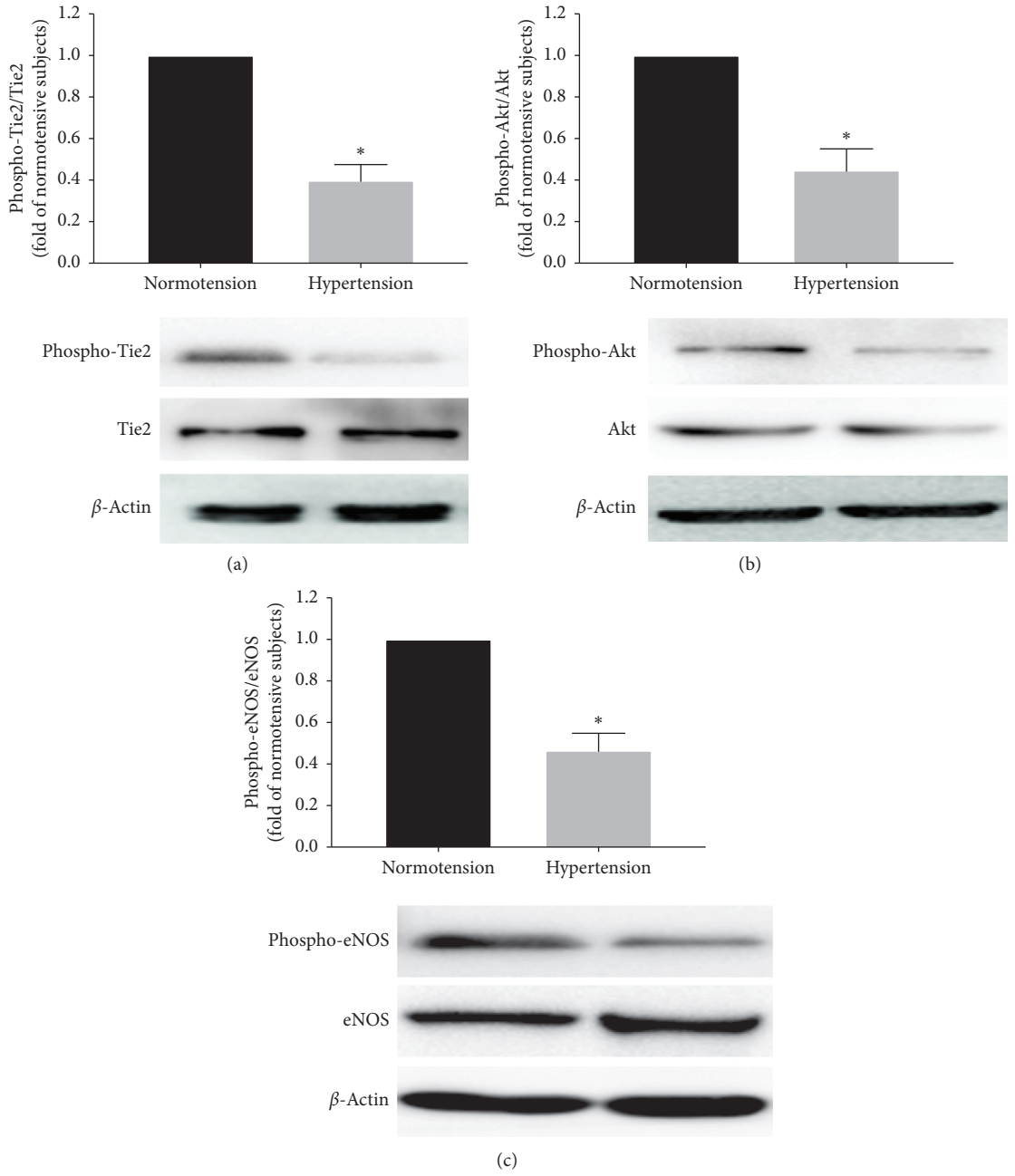

Figure 2: The quantification analysis of Tie2, Akt, and eNOS phosphorylation in human EPCs in the two groups. (a–c) Quantification analysis showing that the phosphorylation of Tie2, Akt, and eNOS in EPCs from patients with hypertension was significantly lower than that in EPCs from normotensive subjects ($^*P < 0.05$ vs. EPCs from normotensive subjects, $n = 18$ per group).

hypertension were treated in vitro with 1 μmol/L, 10 μmol/L, or 50 μmol/L aliskiren for 12 hours, the phospho-Tie2, phospho-Akt, and phospho-eNOS were enhanced in a dose-dependent manner. For concentrations of 10 μmol/L or 50 μmol/L aliskiren, the phospho-Tie2, phospho-Akt, and phospho-eNOS in EPCs from hypertensive patients were markedly higher than those in EPCs from normotensive subjects, and the level of phosphorylation was relatively high for aliskiren concentrations of both 10 μmol/L and 50 μmol/L (Figures 4(a)–4(c)).

3.4. Aliskiren Modulated the PI3k/Akt/eNOS Signalling Pathway in EPCs. We further investigated whether the aliskiren-induced phosphorylation of eNOS was regulated by the Tie2/PI3k/Akt pathway via Tie2 knockdown and PI3k and eNOS inhibition experiments. Treatment with aliskiren for 12 h increased the phospho-Akt and phospho-eNOS from patients with hypertension (Figures 5(a) and 5(b)). No significant differences were found in the phospho-Akt and phospho-eNOS treated with or without scrambled siRNA (Figures 5(a) and 5(b)). The increased phospho-Akt and

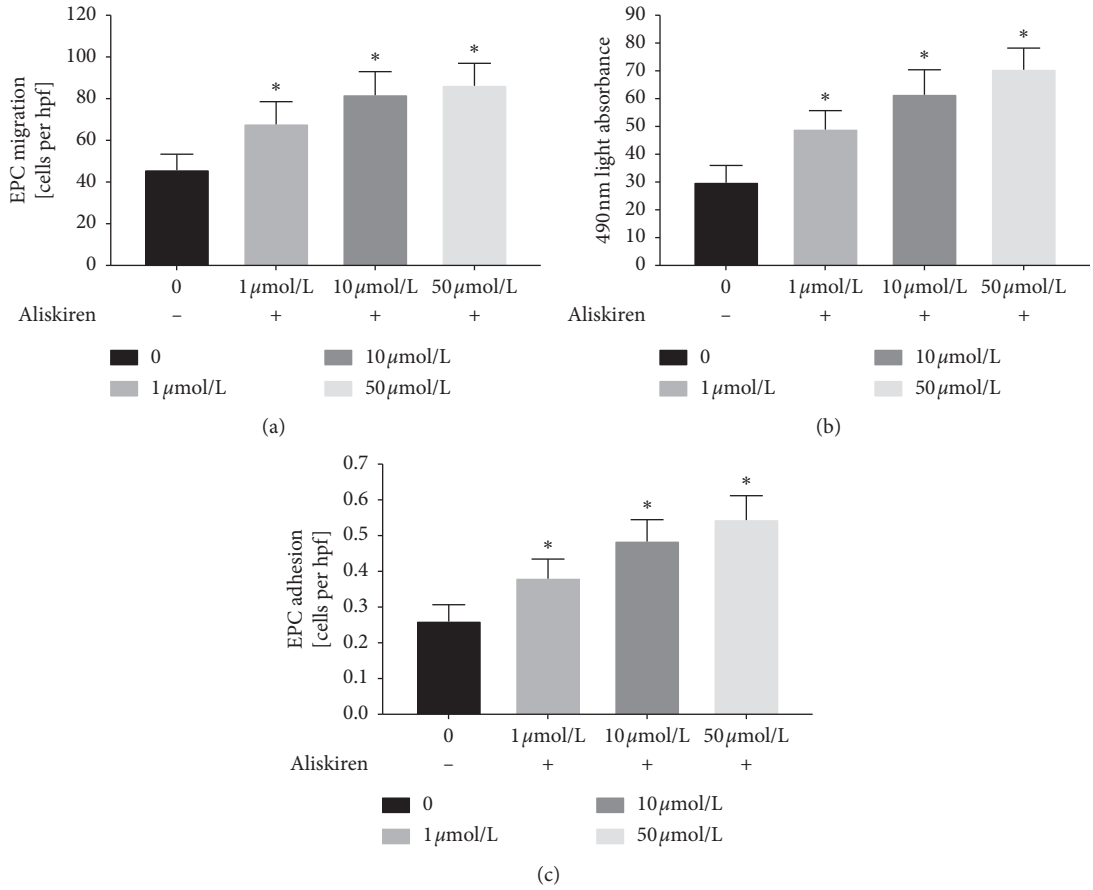

Figure 3: The effect of different concentrations of aliskiren on the in vitro function of patients' EPCs. (a) Quantification analysis of the migration of patients' EPCs treated with 1 μmol/L, 10 μmol/L, or 50 μmol/L aliskiren for 12 h ($^*P < 0.05$ vs. subjects' EPCs without aliskiren treatment, $n = 18$ per group). Quantification analysis of the proliferation (b) and adhesion (c) of patients' EPCs treated as described above ($^*P < 0.05$ vs. subjects' EPCs without aliskiren treatment, $n = 18$ per group). hpf = high-power field.

phospho-eNOS induced by aliskiren were reduced by 63% and 64%, respectively, after Tie2 knockdown (Figures 5(a) and 5(b)), suggesting that Tie2 knockdown abolished the aforementioned upregulation of Akt and eNOS phosphorylation. Furthermore, PI3k inhibition suppressed the favourable effect of aliskiren on the phospho-Akt and phospho-eNOS (Figures 5(a) and 5(b)). The increased phospho-Akt and phospho-eNOS treated with aliskiren were reduced by 64% and 67%, respectively, after treatment with LY294002. Likewise, the increase in phospho-eNOS treated with aliskiren was suppressed by L-NAME (Figure 5(b)). The above results indicated that aliskiren upregulates the phospho-Akt and phospho-eNOS in EPCs from patients with hypertension via the Tie2/PI3k/Akt/eNOS signalling pathway.

3.5. The Tie2/PI3K/Akt/eNOS Signalling Pathway Contributed to the Aliskiren-Induced Upregulation of the In Vitro Function and In Vivo Reendothelialization Capability of Human EPCs. We hypothesized that aliskiren upregulated this signalling pathway to enhance the in vitro function and in vivo reendothelialization capability of human EPCs. The results indicated that aliskiren exerted significant beneficial effects on the in vitro migratory, adhesive, and proliferative activity as well as on the in vivo reendothelialization capability of EPCs (Figures 6(a)–6(d)). However, Tie2 knockdown and PI3k inhibition (LY294002) or eNOS inhibition (L-NAME) suppressed the increase in all the in vitro functions of aliskiren-treated EPCs (Figures 6(a)–6(c)). Similarly, they eliminated the increase in the in vivo reendothelialization capability of transplanted EPCs treated with aliskiren (Figure 6(d)). The transduction of the scrambled siRNA control for the Tie2-siRNA lentiviral particles did not have a statistically significant effect (Figures 6(a)–6(d)).

4. Discussion

Our findings indicated that aliskiren could enhance the in vitro migratory, proliferative, and adhesive activity of EPCs from patients with hypertension and increase the in vivo reendothelialization area of human EPCs in a nude mouse model. Similarly, the phospho-Tie2, phospho-AKt, and phospho-eNOS in EPCs were increased as the result of aliskiren. After blockade of the Tie2/PI3k/Akt/eNOS signalling pathway, the favourable effect of aliskiren on the in vitro function and in vivo reendothelialization capability of EPCs was consequently inhibited. Thus, aliskiren can

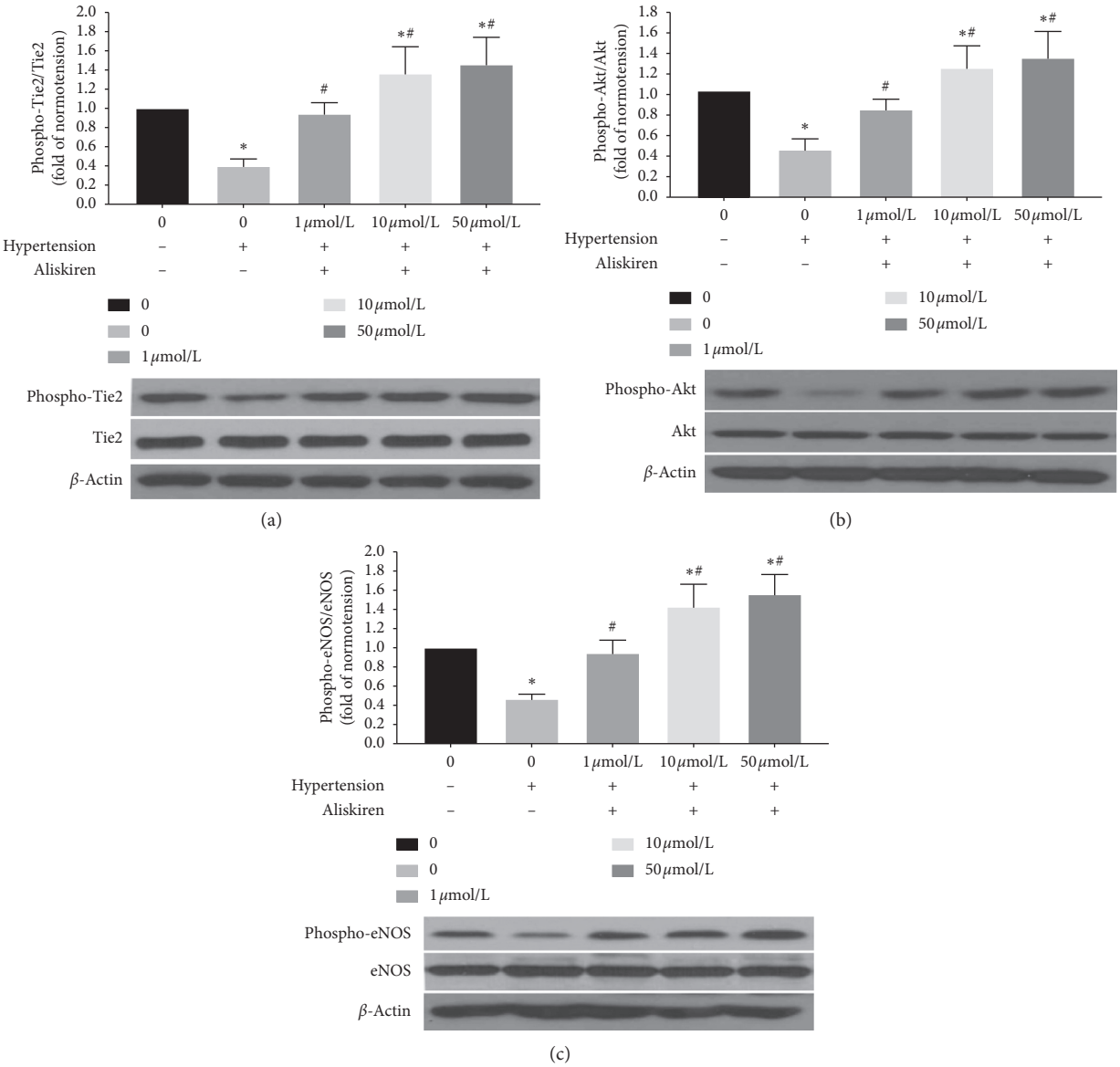

Figure 4: The quantification analysis of Tie2, Akt, and eNOS phosphorylation in patients' EPCs treated with different concentrations of aliskiren. Quantification analysis of Tie2 (a), Akt (b), and eNOS (c) phosphorylation in patients' EPCs treated with 1 μmol/L, 10 μmol/L, or 50 μmol/L aliskiren for 12 h ($^{*}P < 0.05$ vs. non-aliskiren-treated EPCs from normotensive subjects, $n = 18$; $^{\#}P < 0.05$ vs. non-aliskiren-treated EPCs from hypertensive patients, $n = 18$).

improve the in vitro function and in vivo reendothelialization capability of EPCs from patients with hypertension at least partly through the Tie2/PI3k/Akt/eNOS signalling pathway.

This study indicated that the number and function of EPCs was reduced in patients with essential hypertension [4], which further confirmed the diminished endogenous endothelial repair capacity in hypertension [16]. In addition, our results revealed that the phospho-Tie2, phospho-Akt, and phospho-eNOS in EPCs were reduced in patients with hypertension; this reduction might be related to the impaired function and endothelial repair capacity of EPCs. Therefore, the downregulation of the Tie2/PI3K/AKt/eNOS signalling pathway in EPCs is the crucial mechanism underlying the reduced endogenous vascular repair capacity in hypertension.

Previous studies demonstrated that aliskiren has a favourable effect on endothelial function in patients with essential hypertension by improving the carotid-femoral pulse wave velocity (cfPWV), the reactive hyperaemia peripheral arterial tonometry (RH PAT) index, and flow-mediated dilatation (FMD). Aliskiren has been proven to enhance the number of EPCs [19–21], which may be responsible for its favourable effect on endothelial function. However, the underlying mechanism of the beneficial effect of aliskiren is still unclear. Tie2-dependent signalling has been generally accepted to contribute to the increase in EPC function and subsequent in vivo angiogenesis [9, 17, 1

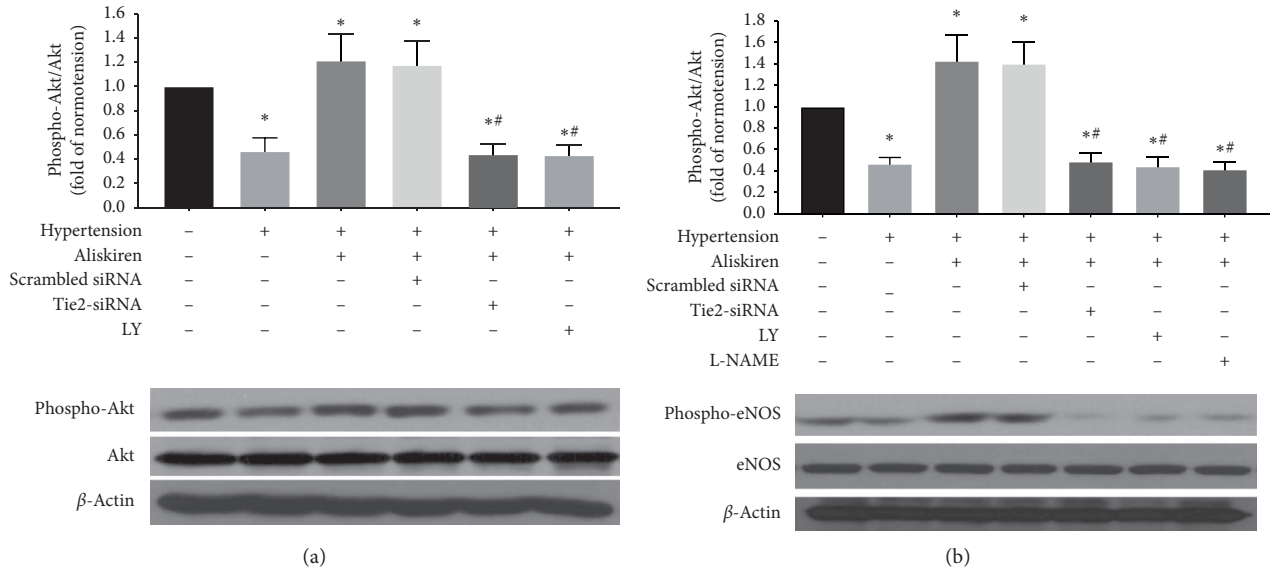

FIGURE 5: The role of the Tie2/PI3k/AKT pathway in the aliskiren-induced phosphorylation of eNOS. (a) Quantification analysis of aliskiren-induced Akt phosphorylation in EPCs with Tie2 knockdown or after PI3k inhibition (LY294002) ($^{*}P < 0.05$ vs. non-aliskiren-treated EPCs from normotensive subjects, $n = 18$ per group;$^{\#}P < 0.05$ vs. aliskiren-treated EPCs from hypertensive patients, $n = 18$ per group). (b) Quantification analysis of aliskiren-induced eNOS phosphorylation in EPCs with Tie2 knockdown or after PI3k or eNOS inhibition (L-NAME) ($^{*}P < 0.05$ vs. non-aliskiren-treated EPCs from normotensive subjects, $n = 18$ per group; $^{\#}P < 0.05$ vs. aliskiren-treated EPCs from hypertensive patients, $n = 18$ per group).

8, 35–37]. Moreover, recent studies indicated that aliskiren could activate the PI3K/Akt/eNOS signalling pathway in spontaneously hypertensive rats (SHRs) [38]. Accordingly, we hypothesized that Tie2, one of the upstream signalling mediators of the PI3K/Akt/eNOS pathway, might be activated by aliskiren and might subsequently regulate the PI3K/Akt/eNOS pathway, thus leading to an increase in the in vitro function and in vivo reendothelialization capability of EPCs in patients with hypertension. To address this hypothesis, the effect of aliskiren on the Tie2/PI3K/Akt/eNOS pathway in EPCs from patients with hypertension was investigated. Similar to the results of previous studies, we found that aliskiren can enhance the in vitro migratory activity, proliferative activity, and adhesion of EPCs in a dose-dependent manner in patients with hypertension. Furthermore, aliskiren can accelerate the reendothelialization mediated by EPCs in patients with hypertension, indicating that the aliskiren-induced increased endothelial repair capacity was associated with its beneficial effect on the in vitro function of EPCs. Additionally, aliskiren can increase the phospho-Tie2, phospho-Akt, and phospho-eNOS in EPCs in a dose-dependent manner, which was in parallel with its effect on the in vitro function. Accordingly, the aliskiren-mediated increase in EPC function was related to alterations in the Tie2/PI3K/Akt/eNOS signalling pathway. In order to further verify this hypothesis, we first investigated the modulatory effect of aliskiren on Tie2-dependent signalling. When blocked by Tie2-siRNA or inhibited by LY, the aliskiren-mediated increase in phospho-Akt and phospho-eNOS was abolished, suggesting that aliskiren activates the phospho-Akt and phospho-eNOS via Tie2-dependent signalling. These results confirmed the regulatory effect of aliskiren on the Tie2/PI3K/Akt/eNOS pathway in EPCs from patients with hypertension.

Furthermore, when this signalling pathway was blocked, the aliskiren-induced enhancement in the in vitro migratory, proliferative, and adhesive activity of EPCs from patients with hypertension was inhibited, indicating the role of Tie2-dependent signalling in aliskiren-regulated EPC function in the setting of hypertension. Similarly, the increased EPC-mediated reendothelialization in patients with hypertension was attenuated after the blockade of this signalling pathway, further supporting the relationship between Tie2-dependent signalling and the endothelial repair capacity. The present results suggested that the beneficial effect of aliskiren on increasing both the in vitro function and the in vivo reendothelialization capability of EPCs are at least partly regulated by the Tie2/PI3K/Akt/eNOS signalling pathway.

The findings obtained in this study provide some valuable information, as follows. First, our study revealed that hypertension leads the diminished phosphorylation level of Tie2, Akt, and eNOS in EPCs and then leads to a reduction in the in vitro function and in vivo reendothelialization capability of EPCs; this process is the crucial mechanism underlying the reduced vascular repair capacity in the hypertensive. Second, aliskiren can activate the Tie2 signalling pathway and subsequently increase the in vitro function as well as the in vivo reendothelialization capability of EPCs from patients with hypertension, which indicates the favourable effect of aliskiren on the endothelial repair capacity in the setting of hypertension, as well as its possible mechanism. The present findings suggest an important pharmacological therapeutic target for the EPC-based repair for hypertension-related vascular damage.

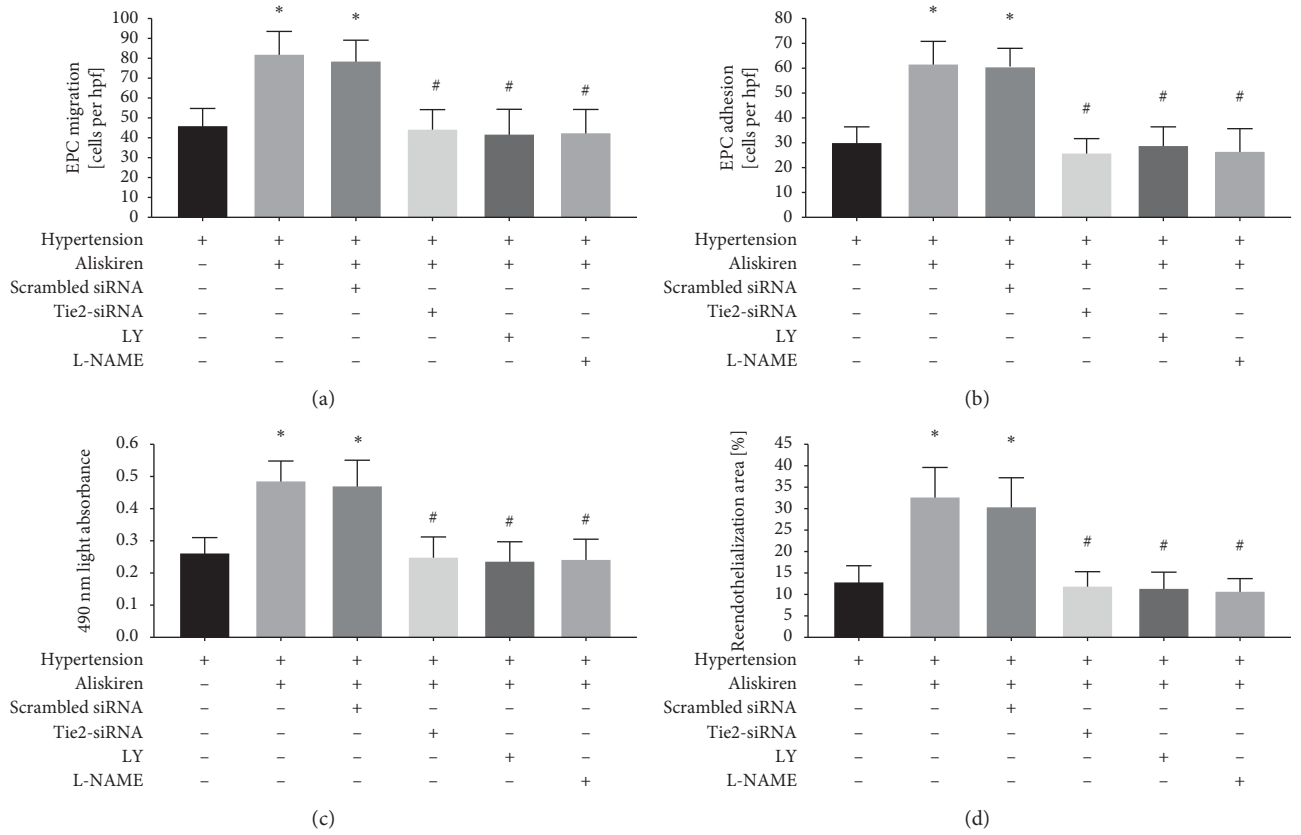

Figure 6: Tie2/PI3k/AKT signalling pathway blockade inhibits the in vitro function and in vivo reendothelialization capacity of EPCs treated with aliskiren. (a–c) Quantification analysis of aliskiren-mediated migration ($^{*}P < 0.05$ vs. non-aliskiren-treated EPCs from normotensive subjects, $n = 18$ per group; $^{\#}P < 0.05$ vs. aliskiren-treated EPCs from hypertensive patients, $n = 18$ per group) (a), adhesion ($^{*}P < 0.05$ vs. non-aliskiren-treated EPCs from normotensive subjects, $n = 18$ per group; $^{\#}P < 0.05$ vs. aliskiren-treated EPCs from hypertensive patients, $n = 18$ per group) (b), and proliferation ($^{*}P < 0.05$ vs. non-aliskiren-treated EPCs from normotensive subjects, $n = 18$ per group; $^{\#}P < 0.05$ vs. aliskiren-treated EPCs from hypertensive patients, $n = 18$ per group) (c) of EPCs with Tie2 knockdown or after PI3k or eNOS inhibition. hpf = high-power field. (d) Quantification analysis of the aliskiren-mediated reendothelialization capacity of EPCs with Tie2 knockdown or after PI3k or eNOS inhibition ($^{*}P < 0.05$ vs. non-aliskiren-treated EPCs from normotensive subjects, $n = 18$ per group; $^{\#}P < 0.05$ vs. aliskiren-treated EPCs from hypertensive patients, $n = 18$ per group).

However, this study has some limitations. First, because of the restriction on the use of aliskiren in clinical trials in China, our study did not test the effect of the oral administration of aliskiren on the Tie2/PI3K/Akt/eNOS pathway in circulating EPCs in patients with hypertension. However, the accelerated reendothelialization mediated by circulating EPCs, along with the relationship between this increase in reendothelialization capability and the Tie2/PI3K/Akt/eNOS signalling pathway in response to in vitro treatment with aliskiren, can partly elucidate both the favourable effect of aliskiren on the endothelial repair capability and its underlying mechanism. Second, early EPCs play important roles in endothelial repair processes [39], and late-outgrowth EPCs are involved in angiogenesis in response to the stimulus of ischaemia. In this investigation, we did not study the effect of aliskiren on the number and function of late-outgrowth EPCs. A recent study demonstrated that aliskiren can not only increase the number of EPCs but can also improve ischaemia-induced neovascularization in mice with diabetes via an SDF-1-related mechanism [22]; however, the populations of EPCs used in this study were not clearly stated. In addition, the Tie2 signalling pathway has been reported to contribute to vasculogenesis and angiogenesis [17]. Accordingly, aliskiren can be inferred to increase the number and function of late-outgrowth EPCs and subsequently promote ischaemia-induced neovascularization via the Tie2 signalling pathway. The effect of aliskiren on late-outgrowth EPCs, as well as the underlying mechanism, remains to be further investigated.

5. Conclusion

This study, for the first time, indicates that aliskiren can improve the in vitro function and in vivo reendothelialization capability of circulating EPCs in patients with hypertension via the Tie2/PI3k/Akt/eNOS signalling pathway. All our findings indicate that Tie2-dependent signalling is a crucial target for the EPC-based repair of hypertension-related endothelial injury, as well as provide new insights into a pharmacological therapeutic approach for treating vascular injury in hypertension.

Abbreviations

Ang2: Angiopoietin-2
Akt/PKB: Protein kinase B
EPCs: Endothelial progenitor cells
eNOS: endothelial nitric oxide synthase
HUVECs: Human umbilical vein endothelial cells
PI3K: Phosphatidylinositol 3′-kinase
Tie2: Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical Approval

This study was approved by the ICE for Clinical Research and Animal Trials of the First Affiliated Hospital of Sun Yat-sen University.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Da-Jun Hu and Xing Liu conceptualized and designed the study. Shun Yao and Chen Su conducted the in vitro and in vivo experiments. Shao-Hong Wu analysed the data. Shun Yao wrote the manuscript. Shun Yao and Chen Su contributed equally to this study.

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Research Article

Sex Differences in the Outcomes of Elderly Patients with Acute Coronary Syndrome

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Background. The impact of sex on the outcome of patients with acute coronary syndrome (ACS) has been suggested, but little is known about its impact on elderly patients with ACS. *Methods*. This study analyzed the impact of sex on in-hospital and 1-year outcomes of elderly (≥75 years of age) patients with ACS hospitalized in our department between January 2013 and December 2017. *Results*. A total of 711 patients were included: 273 (38.4%) women and 438 (61.6%) men. Their age ranged from 75 to 94 years, similar between women and men. Women had more comorbidities (hypertension (79.5% vs. 72.8%, $p = 0.050$), diabetes mellitus (35.2% vs. 26.5%, $p = 0.014$), and hyperuricemia (39.9% vs. 32.4%, $p = 0.042$)) and had a higher prevalence of non-ST-segment elevation ACS (NSTE-ACS) (79.5% vs. 71.2%, $p = 0.014$) than men. The prevalence of current smoking (56.5% vs. 5.4%, $p < 0.001$), creatinine levels (124.4 ± 98.6 vs. 89.9 ± 54.1, $p < 0.001$), and revascularization rate (39.7% vs. 30.0%, $p = 0.022$) were higher, and troponin TnT and NT-proBNP tended to be higher in men than in women. The in-hospital mortality rate was similar (3.5% vs. 4.4%, $p = 0.693$), but the 1-year mortality rate was lower in women than in men (14.7% vs. 21.7%, $p = 0.020$). The multivariable analysis showed that female sex was a protective factor for 1-year mortality in all patients (OR = 0.565, 95% CI 0.351–0.908, $p = 0.018$) and in patients with STEMI (OR = 0.416, 95% CI 0.184–0.940, $p = 0.035$) after adjustment. *Conclusions*. Among the elderly patients with ACS, the 1-year mortality rate was lower in women than in men, which could be associated with comorbidities and ACS type.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death for both men and women worldwide [1]. Acute coronary syndrome (ACS), a major clinical manifestation of atherothrombosis, refers to a wide spectrum of clinical presentations, such as ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation ACS (NSTE-ACS), and increases with age, and the outcomes of ACS in elderly patients are generally worse than those in young patients [2]. Almost one out of every two patients hospitalized for ACS is over 75 years of age [3, 4], and the in-hospital mortality due to ACS ranges from 4% to10% [4, 5].

Women account for approximately 30% of patients presenting with ACS [4, 6] and have long been described as being "older and sicker" than their male counterparts [7]. In particular, significant sex-related differences exist in ACS presentation, management, and outcomes [7]. A large contemporary registry study in China demonstrated that women hospitalized for ACS received acute treatments and secondary prevention less frequently and had a higher in-hospital mortality than men due to poor clinical profiles and little evidence for acute treatments [4]. To date, little is known about the sex-related differences in elderly patients with ACS.

The Italian Elderly ACS study included patients with NSTE-ACS and ≥75 years of age and showed that women had poor 1-year primary outcomes, including death, non-fatal myocardial infarction, disabling stroke, cardiac

rehospitalization, and severe bleeding [8]. According to a nationwide registry study in the Netherlands, the relation between sex and mortality appeared to be age-dependent, with increased mortality in women at a young age and decreased mortality in women at an advanced age [9]. It is still unclear whether these differences can be solely explained by sex or by other factors such as age, extent or impact of risk factors, clinical presentation, and treatment strategies. Therefore, this study focused on patients with ACS and ≥75 years and aimed to investigate the sex differences in the clinical characteristics, in-hospital management, adverse events, and 1-year mortality among those patients.

2. Methods

2.1. Study Design and Patients. This retrospective single-center study included 711 consecutive patients with ACS and ≥75 years of age who were hospitalized in our department between January 2013 and December 2017. Only patients who were initially admitted to our center were included; those who were transferred from other centers were excluded. The diagnostic criteria for ACS were based on the presence of chest pain or discomfort, electrocardiogram (ECG) findings, and myocardial injury biomarker measurements. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Human Research Committee of the Second Xiangya Hospital of Central South University. The need for individual consent was waived by the committee.

2.2. Definitions and Endpoints. ACS was defined in accordance with the guidelines published by the American College of Cardiology for the diagnosis and management of patients with ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation ACS (NSTE-ACS) [10, 11]. Renal insufficiency (CKD ≥3) was defined as an estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m^2. In our study, severe heart failure indicated class III-IV heart failure, according to the Killip or New York Heart Association classification system. Readmission was defined as any readmission after discharge. Stroke was defined as the sudden onset of focal neurological deficit resulting from either cerebral infarction or hemorrhage. According to the Bleeding Academic Research Consortium (BARC) criteria, BARC types 2 and 3 were included as in-hospital bleeding events [12]. A BARC type 2 event was defined as clinically overt hemorrhage requiring medical attention, whereas a BARC type 3 event was defined as bleeding, including gastrointestinal bleeding, respiratory bleeding, and genitourinary bleeding, with a hemoglobin decrease of at least 3 g/dl, requiring transfusion or surgical intervention.

The primary outcome was 1-year all-cause mortality. The secondary outcomes included the rates of revascularization, readmission, and stroke over 1 year of follow-up.

2.3. Data Collection. The following data were collected: body weight, height, and body mass index (BMI) during hospitalization, diabetes, atrial fibrillation, chronic kidney disease, history of chest pain, history of CVD treatment, smoking, laboratory parameters, length of hospital stay, demographic characteristics, medication, in-hospital management, and adverse events. The following biochemical parameters were also extracted from the medical charts: hemoglobin, total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglycerides, and glycated hemoglobin (HbA1c).

2.4. Follow-Up. In-hospital outcomes were ascertained by a hospital chart review. After discharge, participant follow-up was carried out by means of outpatient visits and telephone calls for up to one year.

2.5. Statistical Analysis. Categorical variables were presented as numbers (percentages) and compared using the Pearson chi-squared tests or Fisher's exact test. Continuous variables were presented as mean ± SD and compared using Student's *t*-test if the data were normally distributed. Nonnormally distributed continuous variables were presented as medians (interquartile ranges) and compared using nonparametric tests. Odds ratios (ORs) were presented with 95% confidence intervals (CIs). Univariable factor logistic regression was used to analyze the risk factors associated with 1-year all-cause mortality. A multivariable logistic regression analysis was used to define the independent determinants of 1-year all-cause mortality after adjusting for comorbidities, presentation, and clinical profiles. Meaningful factors, defined by the univariable $p < 0.05$, including age (Model 1); hypertension; current smoking; severe heart failure (Model 2); hemoglobin, platelet, total cholesterol, HDL-C, LDL-C, creatinine, and serum uric acid levels (Model 3), were included in the multivariable logistic regression analysis. A two-tailed p-value <0.05 indicated statistical significance. All statistical analyses were conducted using SPSS 22.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Characteristics of the Patients. There were 273 (38.4%) women and 438 (61.6%) men. They were 75–94 years of age, and the age distribution was similar between women and men (78 [76–81] vs. 78 [76–80], $p = 0.381$).

3.2. Characteristics of the Patients according to Sex. The baseline characteristics of each group are listed in Table 1. Women had a higher prevalence of traditional risk factors for CVD, including hypertension (79.5% vs. 72.8%, $p = 0.050$), diabetes mellitus (35.2% vs. 26.5%, $p = 0.014$), and hyperuricemia (39.9% vs. 32.4%, $p = 0.042$), but a lower prevalence of current smoking (5.4% vs. 56.5%, $p < 0.001$) than men. The prevalence of severe heart failure on admission (56.8% vs. 46.0%, $p = 0.005$) and NSTE-ACS (79.5% vs. 71.2%, $p = 0.014$) was significantly higher, while STEMI (20.5% vs. 28.8%, $p = 0.014$) was less frequent in women than in men. Platelet counts (202.9 ± 64.9 vs. 174.3 ± 61.4, $p < 0.001$), total cholesterol (4.1 ± 1.0 vs. 3.8 ± 0.9, $p < 0.001$),

Table 1: Differences between women and men.

	Women ($n = 273$)	Men ($n = 438$)	p
Demographics and medical history			
Age, yrs (median, IQR)	78 (76–81)	78 (76–80)	0.381
Body mass index (median, IQR)	22.7 (20.6–25.4)	23.1 (20.9–25.4)	0.427
Diabetes mellitus, no. (%)	96 (35.2)	116 (26.5)	0.014
Hypertension, no. (%)	217 (79.5)	319 (72.8)	0.050
Atrial fibrillation, no. (%)	30 (11.2)	53 (12.3)	0.656
CKD ≥ 3	13 (4.8)	26 (5.9)	0.506
Stroke, no. (%)	32 (11.9)	71 (16.5)	0.098
Previous chest pain, no. (%)	181 (67.5)	286 (66.2)	0.716
Previous PCI, no. (%)	36 (13.5)	78 (18.1)	0.112
Previous CABG, no. (%)	3 (1.1)	10 (2.3)	0.255
Current smoking, no. (%)	14 (5.4)	239 (56.5)	<0.001
Clinical presentation			
Hyperuricemia, no. (%)	109 (39.9)	142 (32.4)	0.042
Severe heart failure, no. (%)	155 (56.8)	201 (46.0)	0.005
STEMI, no. (%)	56 (20.5)	126 (28.8)	0.014
NSTE-ACS, no. (%)	217 (79.5)	312 (71.2)	0.014
Laboratory data			
WBC, 10^9/L (mean ± SD)	7.4 ± 3.3	7.2 ± 3.2	0.568
Hemoglobin, g/L (mean ± SD)	110.4 ± 18.3	121.8 ± 20.2	<0.001
Platelets, 10^9/L (mean ± SD)	202.9 ± 64.9	174.3 ± 61.4	<0.001
Fasting glucose, mmol/l (median, IQR)	6.0 (4.9–7.6)	5.7 (4.8–7.1)	0.181
HbA1C, % (median, IQR)	6.7 (6.0–7.4)	6.1 (5.6–6.5)	0.005
ALT, u/l (median, IQR)	17.5 (11.7–28.3)	19.9 (13.6–33.5)	0.012
AST, u/l (median, IQR)	22.6 (17.1–39.5)	22.6 (17.8–49.9)	0.579
Albumin, g/L (mean ± SD)	35.1 ± 4.5	35.1 ± 4.1	0.902
Triglycerides, mmol/l (mean ± SD)	1.6 ± 1.0	1.6 ± 4.2	0.960
Total cholesterol, mmol/l (mean ± SD)	4.1 ± 1.0	3.8 ± 0.9	<0.001
HDL-C, mmol/l (mean ± SD)	1.1 ± 0.3	1.0 ± 0.3	0.002
LDL-C, mmol/l (mean ± SD)	2.4 ± 0.9	2.2 ± 0.8	0.002
Creatinine, μmmol/l (mean ± SD)	89.9 ± 54.1	124.4 ± 98.6	<0.001
Serum uric acid, μmmol/l (mean ± SD)	349.2 ± 119.1	381.4 ± 110.1	<0.001
PT, sec (mean ± SD)	13.7 ± 4.3	13.5 ± 2.8	0.674
APTT, sec (mean ± SD)	39.5 ± 14.4	41.4 ± 22.2	0.562
CK-Mb, u/l (mean ± SD)	35.0 ± 71.4	44.3 ± 119.0	0.266
TnT, pg/ml (median, IQR)	19.3 (9.7–603.3)	33.2 (12.3–1461.0)	0.141
hs-CRP, mg/l (median, IQR)	4.3 (1.3–23.2)	5.1 (1.4–20.2)	0.849
NT-proBNP, pg/ml (median, IQR)	935.9 (290.4–3082.5)	1337.0 (394.9–3737.7)	0.067
EF, % (mean ± SD)	53 ± 10.4	53 ± 10.9	0.460
In-hospital management			
Aspirin, no. (%)	227 (83.2)	376 (86.2)	0.262
Clopidogrel, no. (%)	234 (86.0)	391 (89.5)	0.168
ACEI/ARB, no. (%)	168 (61.5)	268 (61.2)	0.925
Beta blocker, no. (%)	206 (75.5)	306 (69.9)	0.106
Statin, no. (%)	265 (97.8)	422 (96.3)	0.283
Diuretic, no. (%)	128 (47.6)	210 (48.6)	0.791
PPI, no. (%)	227 (84.4)	340 (78.7)	0.063
IABP, no. (%)	7 (2.6)	18 (4.2)	0.255
Revascularization	82 (30.0)	174 (39.7)	0.022
In-hospital events			
Heart failure, no. (%)	42 (15.7)	77 (17.9)	0.458
Bleeding, no. (%)	22 (8.2)	39 (9.1)	0.706
Ventricular tachycardia, no. (%)	23 (8.6)	36 (8.3)	0.920
Stroke, no. (%)	5 (1.9)	2 (0.5)	0.071
Death, no. (%)	4 (3.5)	8 (4.4)	0.693
One-year follow-up			
Revascularization	4 (1.6)	7 (1.9)	0.801
Readmission, no. (%)	96 (36.9)	159 (39.9)	0.436
Stroke, no. (%)	13 (5.2)	18 (4.8)	0.827
Death, no. (%)	40 (14.7)	95 (21.7)	0.020

STEMI: ST-segment elevation myocardial infarction; NSTE-ACS: non-ST-elevation acute coronary syndrome; WBC: white blood cell; ALT: glutamic-pyruvic transaminase; AST: glutamic-oxaloacetic transaminase; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; PT: prothrombin time; APTT: activated partial thromboplastin time; ACEI/ARB: angiotensin-converting enzyme inhibitors/angiotensin receptor blockers; PPI: proton pump inhibitor; IABP: intra-aortic balloon pump; CKD: chronic kidney disease; and CKD ≥ 3: estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m^2.

Table 2: Differences between women and men according to ACS type.

	NSTE-ACS			STEMI		
	Women ($n = 217$)	Men ($n = 312$)	p	Women ($n = 56$)	Men ($n = 126$)	p
Demographics and medical history						
Age, yrs (median, IQR)	78 (76–81)	78 (76–80)	0.085	77 (76–80)	78 (76–81)	0.218
Body mass index, (median, IQR)	22.7 (20.5–25.4)	23.2 (20.9–25.5)	0.275	22.3 (20.7–25.3)	22.3 (19.8–24.6)	0.703
Diabetes mellitus, no. (%)	69 (31.8)	91 (29.2)	0.517	27 (48.2)	25 (19.8)	<0.001
Hypertension, no. (%)	182 (83.7)	248 (79.5)	0.204	35 (62.5)	71 (56.3)	0.437
Atrial fibrillation, no. (%)	19 (8.9)	35 (11.4)	0.362	11 (19.6)	18 (14.4)	0.374
CKD ≥ 3	9 (4.2)	14 (4.5)	0.853	4 (7.1)	12 (9.5)	0.601
Stroke, no. (%)	25 (11.8)	54 (17.6)	0.068	7 (12.5)	17 (13.7)	0.825
Previous chest pain, no. (%)	148 (69.8)	219 (71.3)	0.708	33 (58.9)	67 (53.6)	0.505
Previous PCI, no. (%)	32 (15.1)	69 (22.5)	0.037	4 (7.3)	9 (7.2)	0.986
Previous CABG, no. (%)	3 (1.4)	10 (3.3)	0.187	0 (0)	0 (0)	NA
Current smoking, no. (%)	7 (3.4)	166 (55.5)	<0.001	7 (12.5)	73 (58.9)	<0.001
Clinical presentation						
Hyperuricemia, no. (%)	91 (41.9)	98 (31.4)	0.013	18 (32.1)	44 (34.9)	0.715
Severe heart failure, no. (%)	132 (60.8)	149 (47.9)	0.003	23 (41.1)	52 (41.3)	0.980
Laboratory data						
WBC, 10^9/L (mean ± SD)	6.8 ± 2.6	6.5 ± 2.4	0.076	9.4 ± 4.6	9.1 ± 4.0	0.752
Hemoglobin, g/L (mean ± SD)	110.3 ± 19.0	122.4 ± 20.2	<0.001	110.9 ± 15.4	120.3 ± 20.1	0.001
Platelets, 10^9/L (mean ± SD)	200.3 ± 62.3	171.0 ± 60.0	<0.001	212.7 ± 73.7	182.5 ± 64.2	0.009
Fasting glucose, mmol/l (median, IQR)	5.9 (4.9–6.9)	5.6 (4.8–6.8)	0.083	6.8 (4.9–8.2)	6.3 (5.3–7.7)	0.873
HbA1C, % (median, IQR)	6.5 (5.9–7.0)	6.2 (5.7–6.8)	0.115	7.0 (6.1–7.7)	5.8 (5.5–6.1)	0.035
ALT, u/l (median, IQR)	16.0 (10.9–22.9)	18.0 (12.4–27.8)	0.006	33.0 (20.7–48.5)	31.7 (16.7–58.8)	0.533
AST, u/l (median, IQR)	21.0 (16.2–26.9)	19.8 (16.9–27.2)	0.813	76.0 (25.7–286.4)	67.3 (28.7–187.7)	0.759
Albumin, g/L (mean ± SD)	35.7 ± 4.5	35.8 ± 3.8	0.785	32.9 ± 3.5	33.4 ± 4.1	0.494
Triglycerides, mmol/l (mean ± SD)	1.6 ± 1.1	1.7 ± 5.0	0.725	1.6 ± 0.8	1.3 ± 0.9	0.009
Total cholesterol, mmol/l (mean ± SD)	4.0 ± 1.0	3.7 ± 0.9	<0.001	4.4 ± 1.1	3.9 ± 0.9	0.002
HDL-cholesterol, mmol/l (mean ± SD)	1.1 ± 0.3	1.0 ± 0.3	0.002	1.1 ± 0.3	1.0 ± 0.3	0.373
LDL-cholesterol, mmol/l (mean ± SD)	2.3 ± 0.9	2.2 ± 0.8	0.018	2.7 ± 1.0	2.3 ± 0.8	0.005
Creatinine, μmmol/l (mean ± SD)	91.3 ± 53.7	124.7 ± 109.1	<0.001	84.3 ± 56.0	123.4 ± 66.6	<0.001
Serum uric acid, μmmol/l (mean ± SD)	352.6 ± 121.5	380.8 ± 106.9	0.005	336.6 ± 109.1	383.0 ± 118.1	0.013
PT, sec (mean ± SD)	13.7 ± 4.6	13.2 ± 3.0	0.367	13.6 ± 1.8	14.4 ± 1.7	0.164
APTT, sec (mean ± SD)	38.3 ± 10.4	39.4 ± 21.3	0.741	45.8 ± 27.5	47.9 ± 24.9	0.830
CK-Mb, u/l (mean ± SD)	19.6 ± 34.8	27.1 ± 91.3	0.280	91.0 ± 124.5	82.4 ± 158.2	0.725
TnT, pg/ml (median, IQR)	15.8 (8.9–49.1)	16.4 (10.8–66.4)	0.380	3092.5 (2437.8–4858.0)	2117.0 (1057.9–4761.0)	0.288
hs-CRP, mg/l (median, IQR)	2.7 (1.1–15.1)	3.4 (1.1–12.9)	0.813	24.5 (12.1–44.8)	14.9 (4.4–55.7)	0.328
NT-proBNP, pg/ml (median, IQR)	644.5 (225.5–2074.6)	755.3 (256.7–2273.2)	0.458	2751.7 (1418.2–6079.7)	3387.9 (1561.7–7932.8)	0.268
EF, % (mean ± SD)	55.3 ± 10.1	55.1 ± 10.1	0.862	47.9 ± 9.6	48.0 ± 11.5	0.961
In-hospital management						
Aspirin, no. (%)	174 (80.2)	261 (83.9)	0.267	53 (94.6)	115 (92.0)	0.524
Clopidogrel, no. (%)	183 (84.7)	273 (87.8)	0.312	51 (91.1)	118 (93.7)	0.533
ACEI/ARB, no. (%)	129 (59.4)	190 (60.9)	0.737	39 (69.6)	78 (61.9)	0.315
Beta blocker, no. (%)	167 (77.0)	218 (69.9)	0.072	39 (69.6)	88 (69.8)	0.979
Statin, no. (%)	210 (97.7)	298 (95.5)	0.191	55 (98.2)	124 (98.4)	0.923
Diuretic, no. (%)	93 (43.7)	124 (40.4)	0.457	35 (62.5)	86 (68.8)	0.405
PPI, no. (%)	173 (81.2)	223 (72.6)	0.024	54 (96.4)	117 (93.6)	0.441
IABP, no. (%)	1 (0.5)	8 (2.6)	0.089	6 (10.7)	10 (8.3)	0.609
Revascularization	49 (22.6)	114 (36.5)	0.002	33 (41.1)	60 (47.6)	0.159
In-hospital events						

Table 2: Continued.

	NSTE-ACS			STEMI		
	Women ($n = 217$)	Men ($n = 312$)	p	Women ($n = 56$)	Men ($n = 126$)	p
Heart failure, no. (%)	25 (11.8)	29 (9.5)	0.404	17 (30.9)	48 (38.4)	0.335
Bleeding, no. (%)	14 (6.6)	17 (5.6)	0.618	8 (14.3)	22 (17.6)	0.579
Ventricular tachycardia, no. (%)	16 (7.5)	19 (6.2)	0.554	7 (12.5)	17 (13.6)	0.840
Stroke, no. (%)	3 (1.4)	1 (0.3)	0.310	2 (3.6)	1 (0.8)	0.177
Death, no. (%)	2 (2.2)	4 (3.0)	0.719	2 (8.7)	4 (8.7)	1.000
One-year follow-up						
Revascularization	4 (2.0)	6 (2.2)	0.891	0 (0.0)	1 (1.1)	0.480
Readmission, no. (%)	78 (37.3)	119 (40.5)	0.475	18 (35.3)	40 (38.5)	0.702
Stroke, no. (%)	10 (4.9)	16 (5.7)	0.698	3 (6.4)	2 (2.1)	0.193
Death, no. (%)	29 (13.4)	47 (15.1)	0.583	11 (19.6)	48 (38.1)	0.014

LDL-C (2.4 ± 0.9 vs. 2.2 ± 0.8, $p = 0.002$), and HDL-C (1.1 ± 0.3 vs. 1.0 ± 0.3, $p = 0.002$) were significantly higher, whereas hemoglobin levels (110.4 ± 18.3 vs. 121.8 ± 20.2, $p < 0.001$), creatinine levels (89.9 ± 54.1 vs. 124.4 ± 98.6, $p < 0.001$), and serum uric acid (349.2 ± 119.1 vs. 381.4 ± 110.1, $p < 0.001$) were significantly lower in women than in men. Regarding in-hospital management, the coronary revascularization rate was significantly lower in women than in men (30.0% vs. 39.7%, $p = 0.022$), while the medication rate and rate of intra-aortic balloon pump (IABP) use were similar between the two groups. There were no differences in in-hospital adverse events. A total of 135 patients died (19.0%), and the 1-year mortality rate was significantly lower in women than in men (14.7% vs. 21.7%, $p = 0.020$). The percentages of rehospitalization, revascularization, stroke, and bleeding after discharge were similar between the two groups.

3.3. Differences between Women and Men according to ACS Type. The results of the subgroup (NSTE-ACS and STEMI) comparisons are shown in Table 2. There were 182 (25.8%) patients with STEMI and 529 (74.2%) with NSTE-ACS. Among the 256 (36%) patients who underwent coronary revascularization, 93 (51.1%) were STEMI patients, and 163 (30.8%) were NSTE-ACS patients. Sex-related differences, including the prevalence of current smoking, and hemoglobin, platelet, total cholesterol, LDL-C, creatinine, and serum uric acid levels were still observed in both STEMI and NSTE-ACS subgroups. In the NSTE-ACS subgroup, the rates of hyperuricemia (41.9% vs. 31.4%, $p = 0.013$), severe heart dysfunction on admission (60.8% vs. 47.9%, $p = 0.003$), high HDL-C (1.1 ± 0.3 vs. 1.0 ± 0.3, $p = 0.002$), and the use of proton pump inhibitors (PPIs) (81.2% vs. 72.6%, $p = 0.024$) were significantly higher, while the coronary revascularization rate (22.6% vs. 36.5%, $p = 0.002$) was lower in women than in men, but these differences were not observed in the STEMI subgroup. In patients with STEMI, the rate of diabetes mellitus (48.2% vs. 19.8%, $p < 0.001$) was higher in women than in men and 1-year mortality (19.6% vs. 38.1%, $p = 0.014$) was lower.

3.4. Multivariable Analyses between Women and Men. To evaluate whether the residual sex difference in mortality could be explained by disparities in the risk factors, we examined the independent determinants of 1-year all-cause mortality. As shown in Table 3, female patients had a significantly lower unadjusted risk of death (unadjusted OR = 0.620, 95% CI 0.413–0.929, $p = 0.021$) than male patients. The multivariable logistic regression analysis showed similar results (OR = 0.597, 95% CI 0.397–0.900, $p = 0.014$) after adjusting for age (Model 1). Additional variables included diabetes mellitus, hypertension, current smoking, and severe heart failure (Model 2) and hemoglobin, platelet, total cholesterol, HDL-C, LDL-C, creatinine, and serum uric acid levels (Model 3). The same associations were observed in Model 2 (OR = 0.531, 95% CI 0.347–0.811, $p = 0.003$) and Model 3 (OR = 0.565, 95% CI 0.351–0.908, $p = 0.018$). Similar results were observed in elderly patients with STEMI but not in elderly patients with NSTE-ACS.

Regarding the adjustment variables in Model 3, in all patients, age (OR = 1.08, 95% CI: 1.02–1.15, $p = 0.010$), severe heart failure (OR = 1.77, 95% CI: 1.14–2.75, $p = 0.011$), PLT levels (OR = 1.004, 95% CI: 1.001–1.007, $p = 0.018$), and creatinine levels (OR = 1.005, 95% CI: 1.003–1.007, $p < 0.001$) were associated with mortality, along with sex (OR = 0.57, 95% CI: 0.35–0.91, $p = 0.018$). In patients with STEMI, severe heart failure (OR = 3.84, 95% CI: 1.89–7.78, $p < 0.001$) was associated with mortality, along with sex (OR = 0.42, 95% CI: 0.18–0.94, $p = 0.035$). In patients with NSTE-ACS, age (OR = 1.09, 95% CI: 1.01–1.18, $p = 0.033$), hemoglobin (OR = 0.98, 95% CI: 0.97–1.00, $p = 0.027$), LDL (OR = 1.49, 95% CI: 1.07–2.08, $p = 0.018$), and creatinine (OR = 1.004, 95% CI: 1.002–1.007, $p = 0.001$) were associated with mortality.

4. Discussion

The main findings of the present study are as follows: (1) elderly women had more comorbidities and were more likely to present with NSTE-ACS than men in the total ACS cohort; (2) in women with NSTE-ACS, the prevalence of severe heart failure on admission was higher, and they underwent coronary revascularization less often than men,

Table 3: Multivariable logistic regression analysis of one-year all-cause mortality.

	Unadjusted			Model 1			Model 2			Model 3		
	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p	OR	95% CI	p
Total												
Women vs. men	0.62	0.41–0.93	0.021	0.60	0.40–0.90	0.014	0.53	0.35–0.81	0.003	0.57	0.35–0.91	0.018
Age	—	—	—	1.10	1.04–1.16	0.001	1.09	1.03–1.15	0.003	1.08	1.02–1.15	0.010
Severe heart failure	—	—	—	—	—	—	—	—	—	1.77	1.14–2.75	0.011
PLT	—	—	—	—	—	—	—	—	—	1.004	1.001–1.007	0.018
Creatinine	—	—	—	—	—	—	—	—	—	1.005	1.003–1.007	<0.001
STEMI												
Women vs. men	0.40	0.19–0.84	0.016	0.40	0.19–0.84	0.016	0.36	0.17–0.79	0.011	0.42	0.18–0.94	0.035
Severe heart failure	—	—	—	—	—	—	3.21	1.66–6.22	0.001	3.84	1.89–7.78	<0.001
NSTE-ACS												
Women vs. men	0.87	0.53–1.43	0.584	0.81	0.49–1.34	0.405	0.72	0.43–1.23	0.230	0.71	0.38–1.33	0.283
Age	—	—	—	1.13	1.05–1.20	<0.001	1.10	1.02–1.18	0.009	1.09	1.01–1.18	0.033
Severe heart failure	—	—	—	—	—	—	1.79	1.05–3.07	0.033	—	—	—
Hemoglobin	—	—	—	—	—	—	—	—	—	0.98	0.97–1.00	0.027
LDL	—	—	—	—	—	—	—	—	—	1.49	1.07–2.08	0.018
Creatinine	—	—	—	—	—	—	—	—	—	1.004	1.002–1.007	0.001
Serum uric acid	—	—	—	—	—	—	—	—	—	1.002	1.000–1.004	0.090

Data are expressed as OR ± 95% CIs (reported in parentheses) as assessed by univariate (unadjusted) or multivariate logistic regression analyses. Other covariates included in multivariate logistic regression models were as follows: Model 1: age; Model 2: age, diabetes mellitus, hypertension, current smoking, and severe heart failure; Model 3: adjustment for variables included age, diabetes mellitus, hypertension, current smoking, severe heart failure, hemoglobin, PLT, total cholesterol, HDL-C, LDL-C, creatinine level, and serum uric acid. The adjustment parameters which were statistically significant were shown.

but in-hospital adverse events and 1-year mortality were similar between women and men; (3) in STEMI patients, there were no differences in the in-hospital treatments and in-hospital adverse events between women and men, but women had lower 1-year mortality than men; and (4) female sex was a protective factor for 1-year mortality in all populations, especially in patients with STEMI.

Although traditional atherosclerotic disease risk factors are important for both men and women with ACS, some factors accumulated more often in female patients. In the present study, women had higher rates of traditional risk factors, including hypertension, diabetes, hyperuricemia, elevated LDL-C, and severe heart failure, than men. In contrast, men were more likely to be smokers and have elevated creatinine levels. Notably, these differences were similar across both types of ACS (STEMI and NSTE-ACS). These risk factor distribution patterns have been confirmed by other studies in the whole populations [7] and in elderly patients [4, 9]. In addition, women have sex-specific risk factors, such as pregnancy and menopause [7, 13]. It is accepted that the cardiovascular risk profile of women worsens during postmenopause, and the prevalence of coronary artery disease (CAD) steeply increases with age thereafter. We also observed that among the elderly patients with ACS, women were more likely to present with NSTE-ACS than men, whereas men presented with STEMI more often than women. This finding is in agreement with data derived from the Improving Care for Cardiovascular Disease in China (CCC) Project, in that the prevalence of STEMI was significantly lower in women than in men, and women were more likely to present with NSTE-ACS compared to their male counterparts [4]. An increased burden of plaque erosion, coronary vasospasm, spontaneous coronary artery dissection, and stress-related cardiomyopathy in women might partly be related to the observed phenomenon [7].

In terms of in-hospital management among patients with NSTE-ACS, there were no differences between women and men, except for the use of PPIs. We believe that the use of PPIs occurred more often in women than in men, which could be explained by the increased bleeding risk in women with ACS [8, 14, 15]. Nevertheless, no difference was observed regarding in-hospital bleeding in the present study; the use of PPIs might partly contribute to this finding. In the present study, we observed a lower coronary revascularization rate in women hospitalized for NSTE-ACS than in men. This result is in line with data from the CCC project, which showed that eligible women with NSTE-ACS were less likely to undergo timely percutaneous coronary intervention (PCI) than men with NSTE-ACS (30.5% vs. 34.2%, $p < 0.001$) [4]. We believe that the fear of complications associated with invasive treatments might, in part, explain this finding because women, especially older women, might be considered too fragile to undergo aggressive treatments. A surprising finding in our study was the similar in-hospital clinical outcomes between women and men, despite fewer PCI carried out in women. This finding was in contrast with a previous report that showed that women with NSTE-ACS had higher crude in-hospital mortality rates than men with NSTE-ACS [4]. Because our cohort included only patients of ≥75 years of age, the above difference might originate from the different age groups. In addition, a recent report confirmed that women with NSTE-ACS ≥70 years of age had better outcomes than those <70 years [8]. Another study using data from the National Inpatient Sample (NIS) database in the United States indicated that women had lower risk-adjusted in-hospital mortality than men after accounting for differences in age and comorbidities [16]. These findings suggested that the relation between sex and mortality was age-dependent, with increased mortality in women at a young age and decreased mortality in women at an advanced age.

Among the patients with STEMI, there were no differences regarding in-hospital treatments and in-hospital adverse events between women and men, but women had better 1-year outcomes than men in the present study. Some studies demonstrated increased rates of mortality among women, some studies indicated no difference, and other studies showed lower rates of mortality in women than in men [17]. These controversial results may be explained by potential interactions between age and sex; significant differences in in-hospital mortality rates between women and men with STEMI were demonstrated when the cohort was stratified by age groups (<55 years, 55–64 years, and >75 years) [4]. Younger age was associated with higher 30-day mortality rates in women with STEMI, but this difference decreased after age 60 and was no longer observed in elderly women [18]. In fact, mortality in elderly women was lower than that in age-matched men, as shown by previous studies [19, 20]; this result was further confirmed by the Netherlands National Trial Register, which showed that excess mortality in women mostly occurred in young patients with STEMI, while older women had a better outcome than men of the same age [9]. These findings suggest that there is an age-dependent relationship among the outcomes between male and female patients with STEMI. Our results show that elderly female patients with STEMI have lower 1-year mortality than elderly male patients with STEMI.

Previous studies have shown that sex differences in early mortality after ACS could be largely explained by the clinical differences at presentation [21, 22]. To evaluate whether the sex differences associated with 1-year mortality could be explained by disparities in clinical characteristics, we adjusted for comorbidities, presentation, and clinical profiles. After adjusting for age (Model 1); age, diabetes mellitus, hypertension, current smoking, and severe heart failure (Model 2); and hemoglobin, platelet counts, total cholesterol, HDL-C, LDL-C, creatinine, and serum uric acid (Model 3), the female sex was consistently shown as an independent protective factor for 1-year mortality in the whole cohort, especially among patients with STEMI. Nevertheless, a delay in presentation [1, 4, 23] and angiographic severity of coronary lesions [17], which may also contribute to the sex difference in mortality after ACS, were not adjusted in the present study. In addition, a previous study indicated that the more favorable mortality rate in older women could be attributed to the shorter exposure to obstructive coronary disease and longer life expectancy in women than in men [18]. In all patients, sex, age, severe heart failure, PLT, and creatinine were all independently associated with mortality. In patients with STEMI, only sex and severe heart failure were independently associated with mortality. Since all patients were menopausal, the differences cannot be attributed to estrogens, and other factors are also involved in the mortality risk. In patients with NSTE-ACS, age, hemoglobin, LDL, and creatinine were independently associated with mortality, but not sex. Therefore, in NSTE-ACS, other traditional risk factors for mortality play more important roles in the risk of mortality. Nevertheless, the mechanism for the sex disparity in 1-year mortality, especially in elderly patients with STEMI, still needs to be further investigated in future studies.

5. Limitations

This study has some limitations. First, this was a single-center experience and included a small number of patients. Second, residual measured and unmeasured confounding factors, including changes in ECG parameters, might have contributed to some of these findings but were not included in the regression model. Third, the data were based on the routine clinical parameters measured in the management of ACS, which do not include sexual hormone levels. Finally, the details of the procedural characteristics, especially the time intervals for STEMI and angiographic severity of coronary lesions, are important in view of the previously described sex differences in the literature, but these factors were not analyzed in this study.

6. Conclusion

Our study showed that elderly women with ACS had more comorbidities and were more likely to present with NSTE-ACS than men in the total cohort, similar to other studies. A surprising finding was the better 1-year outcome in elderly women with STEMI than in elderly men with STEMI, while in-hospital and 1-year outcomes were similar between elderly women and men with NSTE-ACS. It is worth noting that the female sex was an independent protective factor for 1-year mortality in the whole ACS cohort, especially in patients with STEMI.

Data Availability

The data used to support the findings of this study are included within the Supplementary Materials.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

A supplementary Excel datasheet file was used for statistical analysis. (*Supplementary Materials*)

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Research Article

Smoking-Induced Inhibition of Number and Activity of Endothelial Progenitor Cells and Nitric Oxide in Males Were Reversed by Estradiol in Premenopausal Females

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Objectives. The number and activity of circulating EPCs were enhanced in premenopausal women contrast to postmenopausal females and age-matched males. Here, we investigated whether this favorable effect exists in premenopausal women and age-matched men with cigarette smoking. *Methods*. In a cross-sectional study, the number and activity of circulating EPCs and nitric oxide production (NO) as well as flow-mediated vasodilation (FMD) in both premenopausal women and age-matched men with or without cigarette smoking were studied. *Results*. Compared with age-matched men with or without smoking, the number and function of circulating EPCs as well as NO level in premenopausal women were obviously higher than that in the former and not affected by smoking. The number and function of circulating EPCs as well as NO level in male smokers were shown to be the most strongly inhibited. Furthermore, there was significant correlation between EPC number and activity, plasma NO level, and NO secretion by EPCs and FMD. *Conclusions*. Estradiol was deemed to play an important role in enhancing the number and activity of EPCs and NO production in premenopausal women even when affected by smoking, which may be the important mechanisms underlying vascular protection of estradiol in premenopausal women, but not in age-matched men.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of deaths among both men and women globally [1]. However, sexual dimorphism exists in the incidence of CVD with a phenomenon that premenopausal women tend to have a lower prevalence, but menopausal females tend to have a higher prevalence compared to age-matched males [2]. It is widely believed that the differences in sex hormones, especially female estrogens, may partly account for this favorable phenomenon [3], but the possible mechanisms of the cardiovascular protections associating with premenopausal women are yet to be explored.

Endothelial progenitor cells (EPCs) are originated from bone marrow under various physiological or pathological conditions and then circulating in the peripheral blood, involving in the process of endothelial repairing by adhering to the inner wall of injured blood vessels and differentiating into mature endothelial cells [4, 5]. Furthermore, it has been discovered that levels of EPCs may be an important

predictor of vascular function and cardiovascular incidence and cardiac deaths [6, 7]. Our previous studies have demonstrated that the number and activity of circulating EPCs were reduced in elderly men, coronary artery disease, and essential hypertension [8–10]. Investigations revealed that the number and activity of circulating EPCs were enhanced in premenopausal women contrast to postmenopausal females and age-matched men [11, 12]. Our results concur with these previous studies in which the number and activity of circulating EPCs were preserved in prehypertensive premenopausal women due to the restoration of nitric oxide (NO) production [13]. The situation changes; however, when prehypertensive premenopausal women combined with diabetes mellitus, the number and activity of circulating EPCs were predominantly hampered [14].

Cigarette smoking is one of most important risk factors for cardiovascular disease, and after 5 years of smoking cessation, CVD risk obviously declined but still remained higher than that in never smokers [15]. Unfortunately, the number of female smokers is increasing significantly [16]. It has reported that the number of circulating EPCs was reduced in chronic smokers and this makes smokers more vulnerable to CVD [17]. However, whether the detrimental effect still exists in young female smokers is not clear. Accordingly, we evaluated the numbers and activity of circulating EPCs as well as flow-mediated vasodilation (FMD) in both premenopausal women and age-matched men with or without cigarette smoking. The present study will extend our knowledge of the effects of cigarette smoking on EPCs and FMD in premenopausal women, which may shed some light on the mechanisms behind the cardioprotective effects particularly possessed in young women, especially those with CAD risk factors.

2. Materials and Methods

2.1. Study Details and Inclusion and Exclusion Criteria. Eighty healthy volunteers (female : male = 1 : 1) aged between 18 and 50 years old from the community were enrolled in the study and divided into four groups: female smoker, female nonsmoker, male smoker, and male nonsmoker. All the women in the study were in normal menstrual state. Detailed medical history and both physical and laboratory examination were taken in all volunteers and subjects with CVD, diabetes, hyperlipidemia, infectious disease, and severe trauma and receiving operation in early last month were excluded. This study was approved by the Sixth Affiliated Hospital of Sun Yat-sen University Ethics Review Board. Informed consent was obtained from all subjects enrolled in this study. The clinical characteristics of the population studied are summarized in Table 1.

2.2. The Count of Circulating EPCs by Flow Cytometry Analysis and Cell Culture Assay. Detection of EPCs was performed as in our previous studies [10, 13, 14]. Flow cytometry analysis was performed according to the protocol, and the count of circulating EPCs was determined by the ratio of CD34 + KDR + cells per 100 peripheral blood mononuclear cells (PBMNCs).

The circulating EPCs were isolated and cultured in vitro and then quantified by determining the uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate-labeled acetylated LDL (DiI-acLDL) and the staining of FITC-labeled Ulex europeus agglutin (lectin).

2.3. The Migration and Proliferation of EPCs. Migration and proliferation assays were performed in our previous studies [10, 13, 14]. EPC migration was determined using a modified Boyden chamber. Briefly, 2×10^4 EPCs were placed in the upper chamber and the whole chamber was incubated in EBM-containing human recombinant VEGF (50 ng/mL) at 37°C for 24 h. Afterwards, the lower side of the filter was fixed with 2% paraformaldehyde and stained with DAPI for cell nuclei and then counted manually in 3 random microscopic fields.

EPC proliferation was determined by the MTT method in accordance with the protocol. After 24 h of serum-free pretreatment, EPCs were supplemented with 10 μl MTT (Fluka Co. Product) and incubated for another 4 h, and then the EPC preparation was shaken with DMSO and the OD value was measured at 490 nm.

2.4. Measurement of NO, VEGF, and GM-CSF Levels from Plasma and EPCs Secretion. Nitrite, the stable metabolite of NO, was measured using the Greiss method as described previously [18], in which the total NO was determined based on the enzymatic conversion of NO3− to NO2− by nitrate reductase and detection of nitrite as an azo dye product of the Greiss reaction. Levels of VEGF and GM-CSF were measured by highly sensitive ELISA assays (R&D Systems, Wiesbaden, Germany) in accordance with our previous studies.

2.5. Measurement of Flow-Mediated Vasodilation in the Brachial Artery. For evaluation of endothelial function in subjects, flow-mediated vasodilation measurement in the brachial artery was performed as we described previously [14]. The brachial artery diameter was imaged with a 5–12 MHz linear array transducer ultrasound system at a location 3 to 7 cm above the right elbow, and the diameters at baseline (D0) and after reactive hyperemia (D1) and sublingual nitroglycerine (D2) were recorded. The flow-mediated vasodilation [(D1−D0)/D0×100%] was regarded as endothelium-dependent vasodilation. The nitroglycerine-mediated vasodilatation [$(D2 - D0)/D0 \times 100\%$] was regarded as endothelium-independent vasodilatation. The repeatability coefficients of flow-mediated vasodilation and nitroglycerine-mediated vasodilation on the same person in a 2-day interval were 0.93 and 0.91, respectively.

2.6. Statistical Analysis. Data were presented as mean ± SD. Statistical analysis was performed with SPSS 23.0 software for Windows (SPSS Software, Chicago, IL). Comparisons

Table 1: Clinical and biochemical characteristics of four groups.

Characteristics	Female nonsmokers ($n = 20$)	Female smokers ($n = 20$)	Male nonsmokers ($n = 20$)	Male smokers ($n = 20$)
Age (years)	42.8 ± 3.6	43.4 ± 3.9	44.6 ± 3.7	43.6 ± 3.8
Smoking (pack-years)	NA	14.8 ± 4.5	NA	16.6 ± 5.5
Height (cm)	162.3 ± 5.7	161.9 ± 5.3	168.4 ± 4.9$^{\#}$	169.6 ± 6.6$^{\#}$
Weight (kg)	60.2 ± 5.2	58.7 ± 5.1	64.6 ± 6.2$^{\#}$	66.4 ± 5.0$^{\#}$
BMI (kg/cm^2)	22.9 ± 2.3	22.4 ± 1.8	22.8 ± 2.2	23.1 ± 1.5
Systolic blood pressure (mmHg)	118.7 ± 10.0	121.5 ± 8.9	117.3 ± 9.6	120.6 ± 9.3
Diastolic blood pressure (mmHg)	73.3 ± 6.5	75.5 ± 7.4	72.8 ± 6.1	74.6 ± 7.0
Heart rate (beats/min)	73.2 ± 6.3	72.9 ± 7.8	74.4 ± 8.0	71.1 ± 7.7
AST (mmol/L)	23.6 ± 5.0	25.7 ± 6.0	26.5 ± 5.2	24.4 ± 5.7
ALT (mmol/L)	20.6 ± 4.8	23.6 ± 7.1	24.7 ± 5.6	22.2 ± 5.5
BUN (mmol/L)	4.6 ± 0.8	4.8 ± 0.9	4.9 ± 0.8	5.0 ± 0.7
Cr (mmol/L)	63.2 ± 13.2	65.3 ± 15.0	69.1 ± 14.7	68.3 ± 15.7
LDL (mmol/L)	2.68 ± 0.38	2.78 ± 0.41	2.58 ± 0.37	2.66 ± 0.30
TC (mmol/L)	4.65 ± 0.49	4.77 ± 0.52	4.48 ± 0.61	4.61 ± 0.47
HDL (mmol/L)	1.39 ± 0.24	1.35 ± 0.22	1.41 ± 0.18	1.43 ± 0.17
TG (mmol/L)	1.41 ± 0.20	1.45 ± 0.20	1.38 ± 0.18	1.36 ± 0.16
FPG (mmol/L)	4.81 ± 0.54	4.51 ± 0.50	4.40 ± 0.41	4.61 ± 0.53
Estradiol (pmol/L)	203.6 ± 20.6	198.3 ± 22.9	103.5 ± 15.0$^{\#}$	95.7 ± 11.0$^{\#}$
FMD (%)	9.72 ± 1.73	8.87 ± 1.67	8.34 ± 1.59$^{\#}$	6.89 ± 1.72$^{\#*}$

BMI, body mass index; LDL, low-density lipoprotein; TC, total cholesterol; HDL, high density lipoprotein; TG, triglyceride; FPG, fasting plasma glucose; FMD, flow-mediated brachial artery dilatation. Smokers are defined as individuals with smoking ≥10 pack-year. Nonsmokers are defined as individuals who never smoked. Data are given as mean ± SD. *vs nonsmokers; $^{\#}$vs premenopausal women.

among the four groups were analyzed by two-factor analysis of variance. Univariate correlations were calculated using Pearson's coefficient (r). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baseline Characteristics. All healthy volunteers who participated in the study were included and excluded according to the methodology of the abovementioned trial design. As shown in Table 1, the four groups were similar in terms of age, body mass index, systolic blood pressure, diastolic blood pressure, and heart rate. There were no differences between the levels of AST, ALT, BUN, creatinine, LDL, HDL, total cholesterol, triglyceride, and fasting plasma glucose. Compared with men, the height and weight were lower in premenopausal women, in the condition of smoking or not ($P < 0.05$). Estradiol was higher and flow-mediated vasodilation was better in premenopausal women than that in men with or without smoking ($P < 0.05$).

3.2. Effect of Smoking on the Gender-Related Decline in the Number of Circulating EPCs. As shown in Figure 1, the number of circulating EPCs characterized by FACS analysis or fluorescence staining in male with or without smoking was lower than that in premenopausal female ($P < 0.05$). Compared with male nonsmokers, the number of circulating EPCs was further lower in male smokers ($P < 0.05$). However, the variance of EPC level related to smoking was disappeared in premenopausal women.

3.3. Effect of Smoking on the Gender-Related Decline in the Activity of Circulating EPCs. As shown in Figure 2, the migration and proliferation of circulating EPCs in male with or without smoking was lower than that in premenopausal female ($P < 0.05$). Compared with male nonsmokers, the migration and proliferation of circulating EPCs were further lower in male smokers ($P < 0.05$). However, the variance of EPC function related to smoking was disappeared in premenopausal women.

3.4. Effect of Smoking on the Gender-Related Decline in the Plasma NO Level. As shown in Figure 3, the plasma NO level in male with or without smoking was lower than that in premenopausal female ($P < 0.05$). Compared with male nonsmokers, the plasma NO level was further lower in male smokers ($P < 0.05$). However, the VEGF and GM-CSF levels were related to neither gender nor smoking.

3.5. Effect of Smoking on the Gender-Related Decline in the NO Secretion by EPCs. As shown in Figure 4, the NO secreted by EPCs in male with or without smoking was less than that in premenopausal female ($P < 0.05$). Compared with male nonsmokers, the NO secreted by EPCs was further less in male smokers ($P < 0.05$). However, the VEGF and GM-CSF secreted by EPCs were related to neither gender nor smoking.

3.6. Correlation between Circulating EPCs or NO Level and FMD. As shown in Figure 5, there was a significant correlation between the FMD and the number of circulating

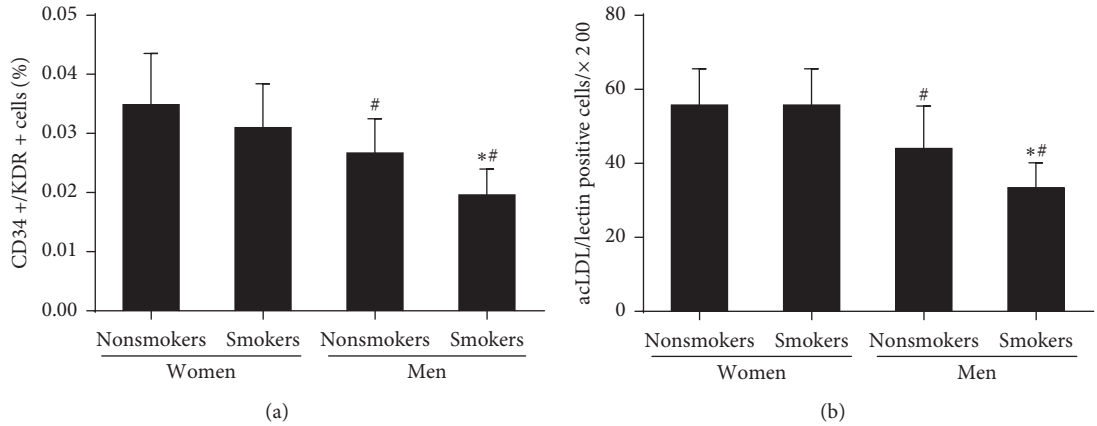

Figure 1: The number of circulating EPCs in the four groups, evaluated by (a) FACS analysis and using (b) phase-contrast fluorescent microscope; the number of circulating EPCs in male nonsmokers and smokers was lower than those in female premenopausal nonsmokers and smokers. The EPC number in male smokers was lower than that in female premenopausal smokers. However, no significant difference in the level of number of circulating EPCs between female premenopausal nonsmokers and smokers was found. Data are given as mean ± SD. *Vs nonsmokers; #vs premenopausal women.

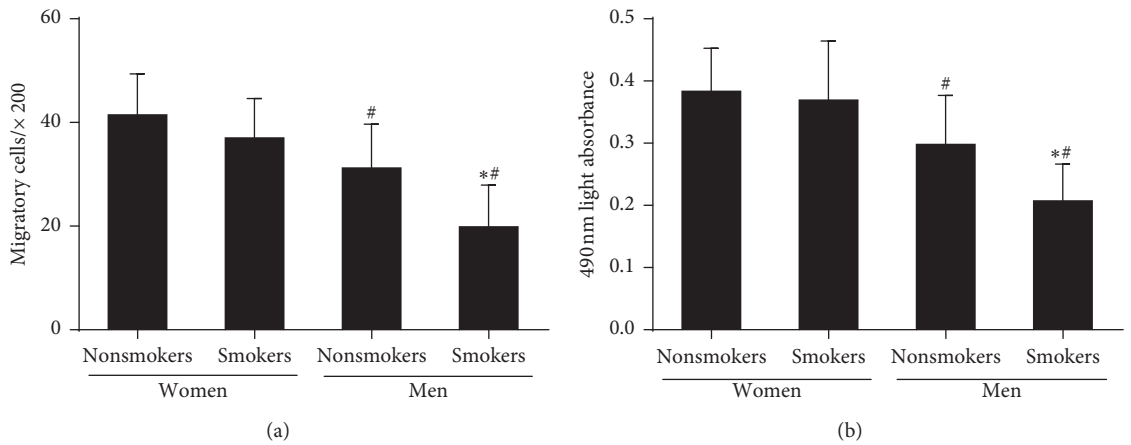

Figure 2: The activity of circulating EPCs in the four groups. The migratory (a) and proliferative (b) activities of circulating EPCs in male nonsmokers and smokers were lower than those in female premenopausal nonsmokers and smokers. There was no difference in the migratory (a) and proliferative (b) activity between female premenopausal nonsmokers and smokers. Nevertheless, the EPC function in male smokers was lower than that in male nonsmokers. Data are given as mean ± SD. *Vs nonsmokers; #vs premenopausal women.

EPCs evaluated by FACS ($r = 0.44$, $P < 0.05$) or by cell culture ($r = 0.52$, $P < 0.05$). Similarly, there was a significant correlation between the FMD and the migration ($r = 0.58$, $P < 0.05$) or proliferation ($r = 0.49$, $P < 0.05$) of circulating EPCs. We also found that the plasma NO level ($r = 0.63$, $P < 0.05$) or NO secretion ($r = 0.45$, $P < 0.05$) by EPCs significantly correlated with the FMD.

4. Discussion

Our present study indicated that the number and function of circulating EPCs were enhanced in premenopausal women contrast to age-matched men, which was consistent with other previous research [11, 12]. As an aside, it is interesting to note that the number and function of circulating EPCs were further attenuated in male smokers; however, the impairments of EPC number and function caused by smoking were disappeared in premenopausal women. Cigarette smoking is one of most important risk factors for cardiovascular CVD and which could be attributed to the inhibition of circulating EPCs in chronic smokers [15, 17]. However, the detrimental effect of smoking on EPCs did not exist in premenopausal female. As mentioned above, the level of estradiol was significantly higher in premenopausal women than that in men with or without smoking; in view of the comparable

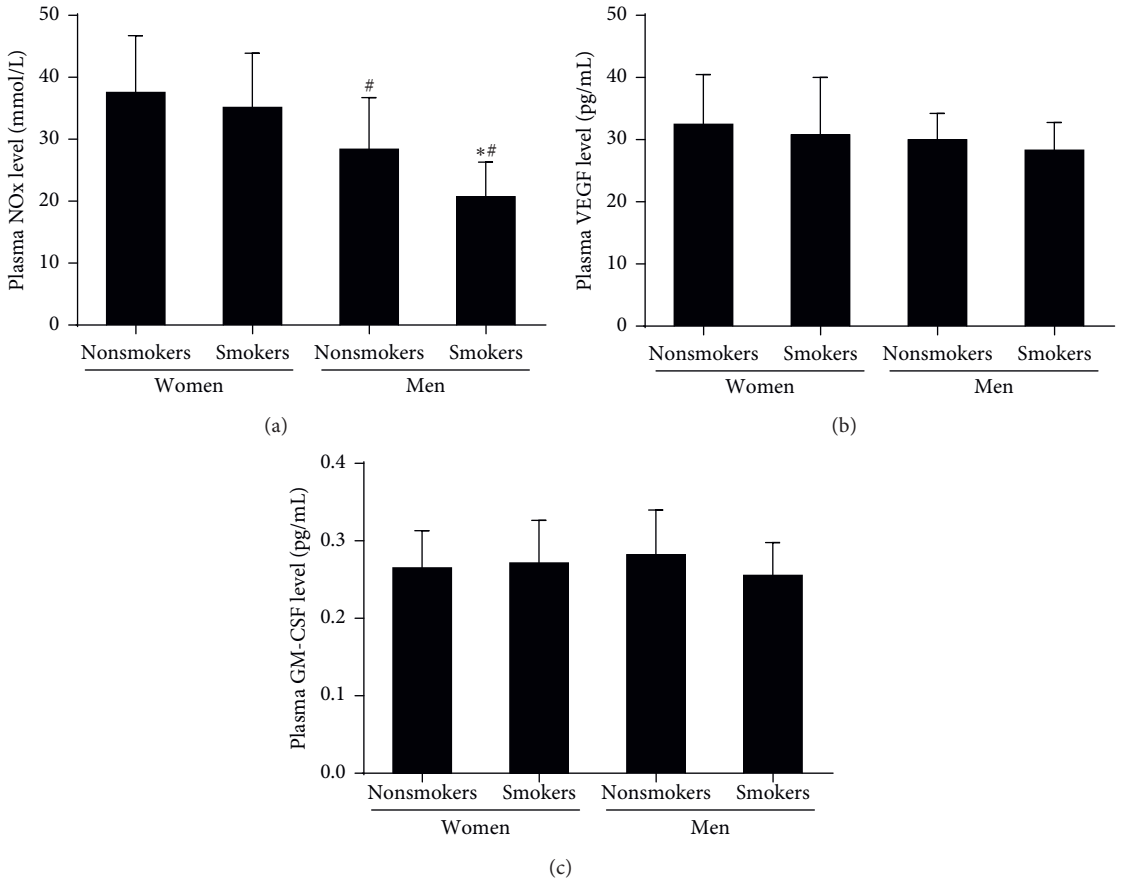

Figure 3: The plasma NO, VEGF, and GM-CSF levels in the four groups. (a) The plasma NO level in male nonsmokers and smokers was lower than that in female premenopausal nonsmokers and smokers. No difference in plasma NO level between female nonsmokers and smokers was found. The plasma NO level in male smokers was lower than that in male smokers. (b) There was no significant difference in the plasma VEGF level between the four groups. (c) There was no significant difference in the plasma GM-CSF level between the four groups. Data are given as mean ± SD. *vs nonsmokers; #vs premenopausal women.

clinical characteristics among the four groups, it is reasonable to infer that estradiol may account for the higher degree of EPC number and function in premenopausal women and the protection against unfavorable effect of smoking on EPCs. It is widely recognized that female estrogens exhibit protective effects on the cardiovascular system in different ways [3], and our findings demonstrated that estradiol-induced enhancements of EPC number and function could be one of the pathways, whether there was smoking or not.

NO, VEGF, and GM-CSF play important roles in the regulation of the number and function of circulating EPCs [18–21]. Furthermore, many studies indicated that smoking was associated with the generation and activity of NO, VEGF, and GM-CSF [22–24]. Results obtained in our research demonstrated that NO level in male with or without smoking was lower than that in premenopausal female, whatever in plasma or cell supernatant secreted by cultured EPC, and compared with male nonsmokers, NO level was further lower in male smokers. However, the VEGF and GM-CSF levels were related to neither gender nor smoking. The changes of NO were consistent with the variances of EPC number and function, which provided a clue that decline in the count and activity of circulating EPCs may attribute to the inhibition of NO production. NO has been shown to be an important signaling molecule to accommodate functional homeostasis of EPCs. Insufficient NOS/NO/MMP9 pathway resulted in impaired mobilization of EPCs in hypertension [25]. In patients with diabetes mellitus, altered eNOS activity led to the suppression of EPC mobilization and function, which seems to contribute to the pathogenesis of vascular disease in diabetes [26]. Even in healthy subjects, acute exercise-induced NO production appeared to be strictly related to the upregulation of circulating EPCs [18].

A lot of attention has been paid to the intriguing phenomenon that premenopausal women have a lower prevalence of CVD until into menopause, and after that,

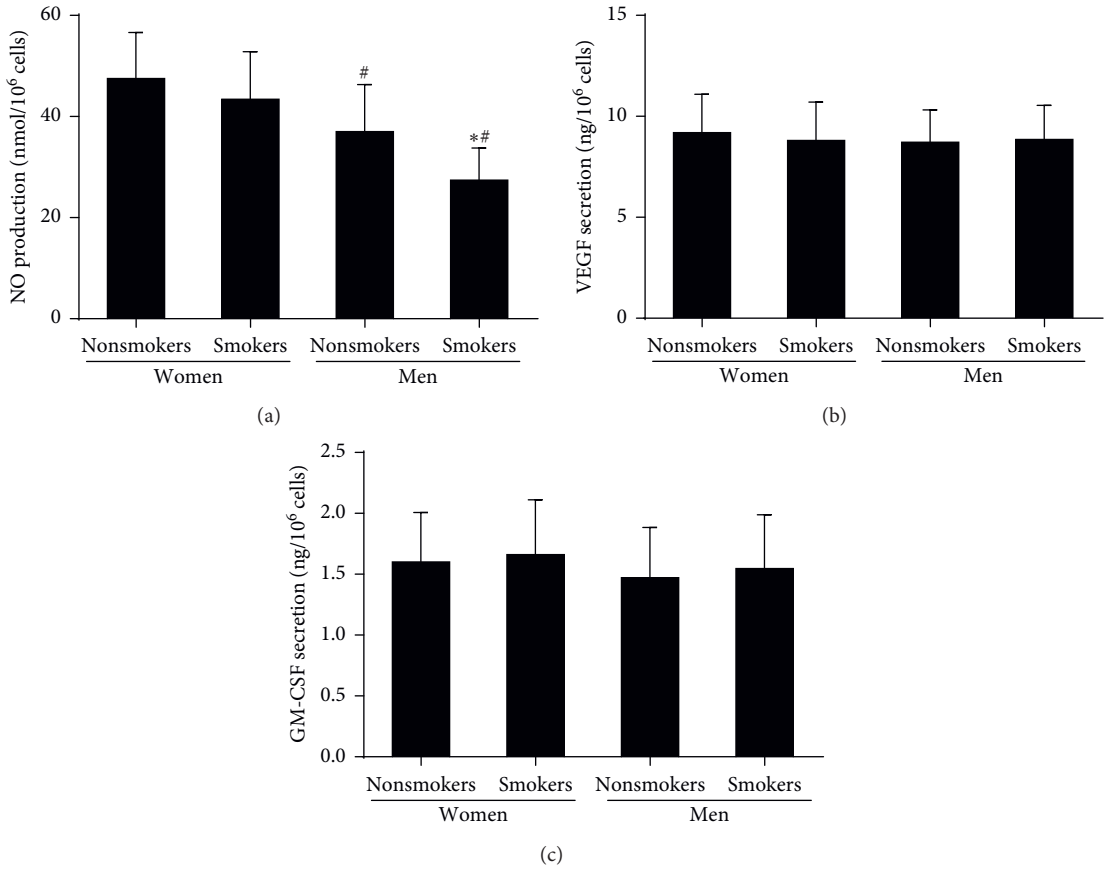

Figure 4: The NO, VEGF, and GM-CSF secretion by EPCs in the four groups. (a) The NO secretion by EPCs in male nonsmokers and smokers was lower than that in female premenopausal nonsmokers and smokers. No difference in NO secretion by EPCs between female premenopausal nonsmokers and smokers was found. However, the plasma NO level in male smokers was lower than that in male smokers. (b) There was no significant difference in VEGF secretion by EPCs between the four groups. (c) There was no significant difference in GM-CSF secretion by EPCs between the four groups. Data are given as mean ± SD. *Vs nonsmokers; #vs premenopausal women.

the incidence surges and may eventually surpass that of men [27]. It was generally accepted that the estrogens play a key role in maintaining of the lower rates of CVD in premenopausal women [3]. The production of NO has been recognized as the most important process which mediates the vascular protection of estradiol [28]. It can thus be inferred that the higher level of estradiol in premenopausal women in the presence of smoking or not prompted the production of NO and thus enhanced the number and activity of circulating EPCs. However, in age-matched males who have a low-level of estradiol, the production of NO and the EPC number and activity were suppressed, especially when affected by tobacco. This result was supported by a previous study in which smoking was associated with reduced NO generation and eNOS activity [24].

As a manifestation of endothelial function, impaired FMD was supposed to be one of the earliest adverse effects of cigarette smoking on vascular function, and smoking-induced reduced NO production may probably be responsible for the downregulation of FMD [29, 30]. Here, we found that not only EPC number and activity correlated with FMD but also the plasma NO level and NO secretion by EPCs correlated with FMD. The study backed up this idea that reduced NO production may suppress the number and activity of EPCs and ultimately lead to endothelial dysfunction in men, especially when affected with tobacco. This suggested that estrogen may play a role in reversing the impairments of smoking on EPCs and vascular homeostasis. These results have intensified our understandings of the noxious effects of smoking and the favorable effects of estradiol on CVD and the balance between these two conflicting factors would determine the degree of the alteration of EPCs and endothelial function.

However, it should be noted that the present study has the limitation of lacking detailed molecular pathways to elaborate the mechanisms underlying the alteration of number and activity of EPCs and NO production among the four groups.

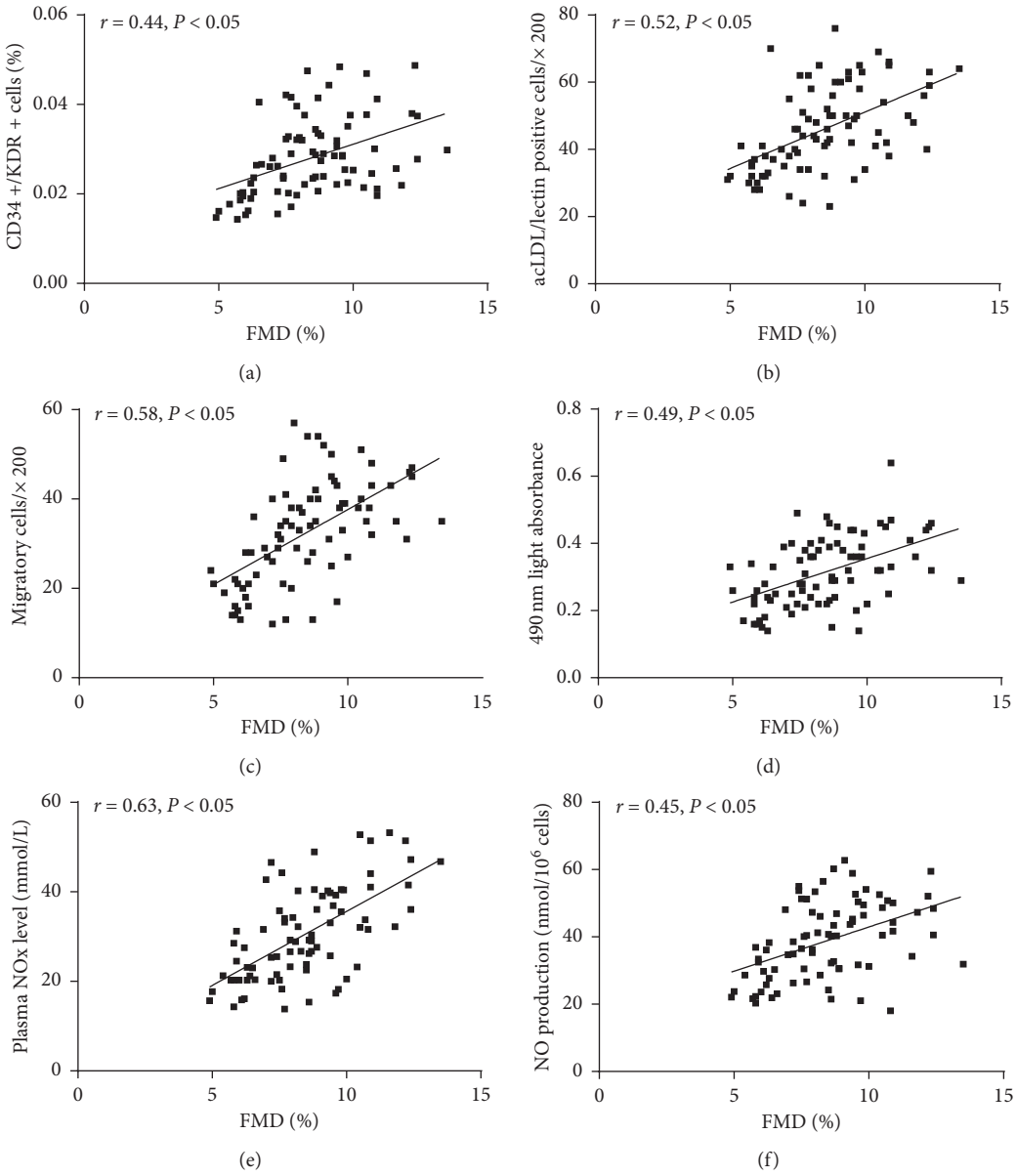

Figure 5: The correlation between circulating EPCs or NO level and FMD. The number of circulating EPCs evaluated by FACS (a) or by cell culture (b) correlated with the FMD. There was a correlation between the proliferatory (c) or migratory EPCs (d) and FMD. In addition, there was a correlation between the plasma NO level (e) or NO secretion by EPCs (f) and FMD.

5. Conclusions

To our knowledge, this was the first time to demonstrate that estradiol may exert vital roles in enhancing the number and activity of EPCs and NO production in premenopausal women even in the presence of smoking, which may be the important mechanism underlying vascular protection of estradiol. Our findings provided a new insight into the vascular protection of high-level estradiol in premenopausal women even in the presence of smoking, and the number and activity of EPCs and NO production may be responsible for the alteration of vascular function.

Data Availability

The data used to support the findings of this study are included within the tables of the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Yijia Shao and Liang Luo contributed equally to this work.

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Research Article

Conversion from Nonshockable to Shockable Rhythms and Out-of-Hospital Cardiac Arrest Outcomes by Initial Heart Rhythm and Rhythm Conversion Time

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Background. The conversion from a nonshockable rhythm (asystole or pulseless electrical activity (PEA)) to a shockable rhythm (pulseless ventricular tachycardia or ventricular fibrillation) may be associated with better out-of-hospital cardiac arrest (OHCA) outcomes. There are insufficient data on the prognostic significance of such conversions by initial heart rhythm and different rhythm conversion time. *Methods*. Among 24,849 adult OHCA patients of presumed cardiac etiology with initial asystole or PEA in the Resuscitation Outcomes Consortium Cardiac Epidemiologic Registry (version 3, 2011–2015), we examined the association of shockable rhythm conversion with prehospital return of spontaneous circulation (ROSC), survival, and favorable functional outcome (modified Rankin Scale score ≤3) at hospital discharge by initial rhythm and rhythm conversion time (time from cardiopulmonary resuscitation (CPR) initiation by emergency medical providers to first shock delivery), using logistic regression adjusting for key clinical characteristics. *Results*. Of 16,516 patients with initial asystole and 8,333 patients with initial PEA, 16% and 20% underwent shockable rhythm conversions; the median rhythm conversion time was 12.0 (IQR: 6.7–18.7) and 13.2 (IQR: 7.0–20.5) min, respectively. No difference was found in odds of prehospital ROSC across rhythm conversion time, regardless of initial heart rhythm. Shockable rhythm conversion was associated with survival and favorable functional outcome at hospital discharge only when occurred during the first 15 min of CPR, for those with initial asystole, or the first 10 min of CPR, for those with initial PEA. The associations between shockable rhythm conversion and outcomes were stronger among those with initial asystole compared with those with initial PEA. *Conclusions*. The conversion from a nonshockable rhythm to a shockable rhythm was associated with better outcomes only when occurred early in initial nonshockable rhythm OHCA, and it has greater prognostic significance when the initial rhythm was asystole.

1. Introduction

The prognosis of out-of-hospital cardiac arrest (OHCA) remains poor [1–3]. OHCA patients with nonshockable rhythms (i.e., asystole and pulseless electrical activity (PEA)) are unlikely to benefit from an electrical defibrillation and suffer the worst outcomes [4]. Given that patients with nonshockable rhythms include the majority of presentations with the worst outcomes and represent the greatest opportunity to improve survival, the identification of prognostic factors in these patients is of clinical importance.

The conversion from a nonshockable rhythm to a shockable rhythm (i.e., pulseless ventricular tachycardia or ventricular fibrillation) has been shown to be associated with

better short- or long-term outcomes in some, but not all OHCA populations [5–10]. In a previous meta-analysis involving 1,108,281 OHCA patients across 12 studies, we showed that the conversion from a nonshockable rhythm to a shockable rhythm and the subsequent electrical defibrillation attempt were associated with better outcomes only when the initial rhythm was asystole [5]. In a subgroup analysis, we found that the association between shockable rhythm conversion and 1-month favorable functional outcomes in patients with initial nonshockable rhythms tended to be weaker when rhythm conversion occurred late compared to early. In that analysis, however, only data from 2 studies, both conducted in Japan, were included [11, 12]. Findings were not stratified by initial heart rhythm, and the full spectrum of OHCA outcomes was not examined. Further investigation is thus needed to thoroughly assess the prognostic significance of shockable rhythm conversion by initial rhythm and rhythm conversion time and across different outcomes in initial nonshockable rhythm OHCA.

Using data from the Resuscitation Outcomes Consortium (ROC) Cardiac Epidemiologic Registry (version 3, 2011– 2015), a North American population-based registry that included more than 60,000 EMS-treated OHCA events from 264 Emergency Medical Service (EMS) agencies and per-protocol ascertainments of multiple outcomes, we sought to thoroughly investigate the associations of conversion from a nonshockable rhythm to a shockable rhythm and prehospital return of spontaneous circulation (ROSC), survival, and favorable functional outcome at hospital discharge, stratified by initial heart rhythm and across the spectrum of rhythm conversion time in OHCA patients with initial nonshockable rhythms.

2. Materials and Methods

2.1. Study Design and Population. This study is a secondary analysis using data of the Resuscitation Outcomes Consortium (ROC) Cardiac Epidemiologic Registry from April 2011 to June 2015 (version 3). ROC is a network of clinical research of out-of-hospital cardiac arrest consisting of ten North Regional Centers (Ottawa, Toronto, Vancouver, Birmingham, Dallas, Pittsburgh, Milwaukee, Portland, Seattle/King County, and San Diego) across the United States and Canada and their respective EMS systems [13–16]. The present study population was derived from 67,204 patients who were treated by EMS providers in the ROC Cardiac Epidemiologic Registry from April 2011 to June 2015 (version 3). Patients with the following characteristics were included in the present study: age between 18 and 89 years, no existing do-not-resuscitate order, cardiac arrest of no obvious causes (presumed cardiac etiology), known initial rhythm, shock delivery status, and documented OHCA outcomes (Figure 1).

Study data were obtained from the National Institutes of Health (NIH) Biological Specimen and Data Repository Information Coordinating Center (https://biolincc.nhlbi.nih.gov/studies/roc_cardiac_epistry_3/?q=roc). The present study is a retrospective, observational analysis of this dataset approved by the Institutional Review Boards (IRBs) of ROC and NIH and then downloaded from the NIH website. The requirement of written informed consent was waived because of the nature of an anonymous dataset.

3. Methods of Measurements

The first recorded electrical defibrillation delivery during cardiopulmonary resuscitation (CPR) was used as the surrogate for the conversion from a nonshockable rhythm to a shockable rhythm. The time of shockable rhythm conversion was defined as the interval from the first chest compression by an EMS provider to the time of the first electrical shock delivery. Time-stamped data (hours: minutes: seconds) on chest compression initiation and shock deliveries were automatically recorded by monitor-defibrillators. All other covariables were ascertained based on standard ROC Cardiac Epidemiologic Registry protocols.

3.1. Outcomes. Three outcomes were assessed in this study: prehospital ROSC, survival to hospital discharge, and favorable functional outcome at hospital discharge, which was defined as a modified Rankin Scale score of ≤3. Outcomes were ascertained by research personnel at each participating center through review of prehospital data streams, audio recordings, and hospital records. Modified Rankin Scale scores at hospital discharge were assigned using a standardized chart review instrument.

3.2. Data Analysis. Patient characteristics overall and stratified by initial rhythm (asystole or PEA) and categories of rhythm conversion time were summarized using descriptive statistics. The associations between shockable rhythm conversion (compared to no rhythm conversion) and outcomes were assessed using logistic regression with adjustment for age, sex, witness status (not witnessed vs. bystander witnessed vs. witnessed by EMS personnel), bystander CPR, location of OHCA (public vs. nonpublic), use of advanced airway, EMS response time, and use and dosage of epinephrine. Shockable rhythm conversion time was first modelled as a continuous variable, and cubic splines with knots at the 5^{th}, 35^{th}, 65^{th}, and 95^{th} percentiles and the referent point at the 35^{th} percentile (conversion time = 10 min), were used to visualize associations across conversion time. Rhythm conversion time was then categorized (<10 min, 10–15 min, and ≥15 min), and logistic regressions were repeated, comparing shockable rhythm conversion with nonshockable rhythm conversion, by conversion time categories. All analyses by continuous or categorical rhythm conversion time were stratified by initial rhythm (asystole or PEA). A two-sided α-value of 0.05 was chosen as the cutoff for statistical significance. Statistical analyses were conducted using SPSS 20.0 (IBM Inc., Armonk, New York) and Stata 15.1 (StataCorp, College Station, Texas).

4. Results

4.1. Patient Characteristics. Of the 24,849 patients with initial nonshockable rhythm OHCA, 16,516 (66%) had

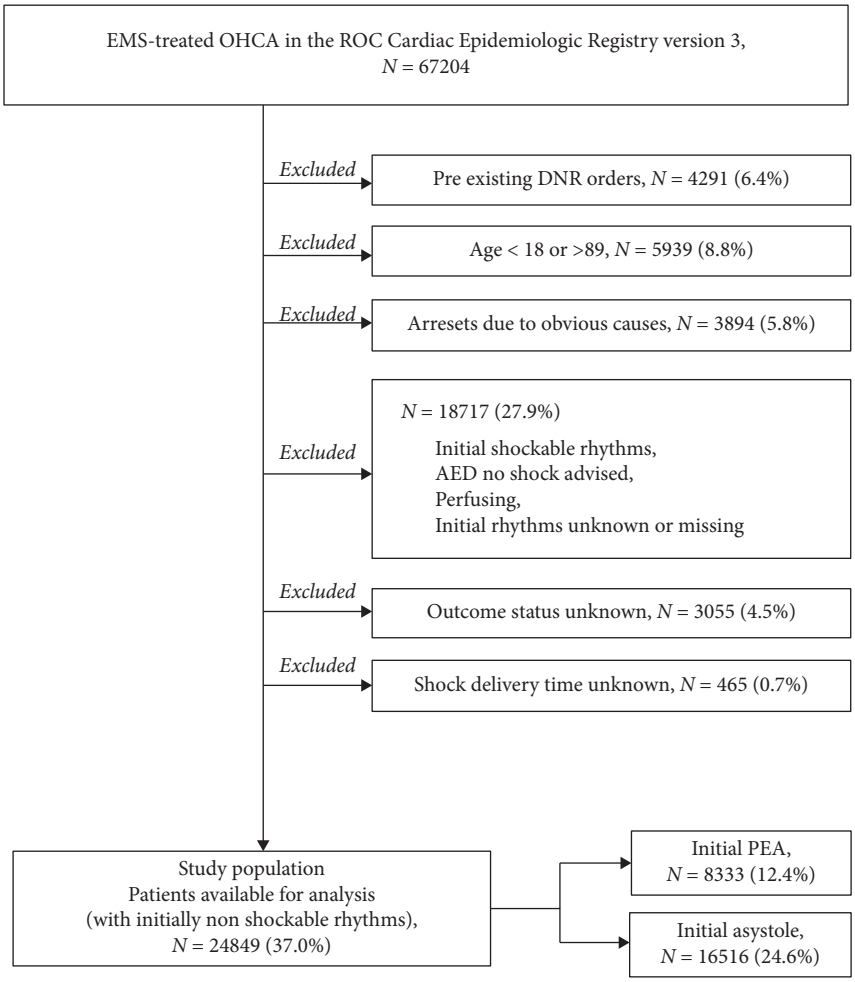

Figure 1: Study population selection process. EMS, Emergency Medical Services; OHCA, out-of-hospital cardiac arrest; ROC, Resuscitation Outcomes Consortium; DNR, do-not-resuscitate; AED, automatic external defibrillator; PEA, pulseless electrical activity; *N*, number.

initial asystole and 8,333 (34%) had initial PEA (Table 1). Among patients with initial asystole and those with initial PEA, respectively, the median age was 66 (IQR: 54–77) and 70 (IQR: 59–79), 10,224 (62%) and 5,120 (61%) were men, 2,581 (16%) and 1,655 (20%) underwent shockable rhythm conversions, and the median rhythm conversion time was 12.0 (IQR: 6.7–18.7) and 13.2 (IQR: 7.0–20.5) min. Patient characteristics were comparable between those with initial asystole and those with initial PEA, except that the proportion of patients with OHCA witnessed by EMS personnel or bystander was higher among those with initial PEA. There was no statistical difference in patient characteristics across rhythm conversion time among those who underwent shockable rhythm conversions.

4.2. Shockable Rhythm Conversion and Prehospital ROSC. Among patients with initial asystole ($N = 16{,}516$) and those with initial PEA ($N = 8{,}333$), respectively, 3,361 (20%) and 3,106 (37%) had prehospital ROSC. Of the 1061, 567, and 953 patients with shockable rhythm conversions from initial asystole at <10 min, 10–15 min, and ≥15 min of CPR, 323 (30%), 164 (29%), and 322 (34%), respectively, underwent prehospital ROSC. Of the 616, 330, and 705 patients with shockable rhythm conversions from initial PEA at <10 min, 10–15 min, and ≥15 min of CPR, 238 (39%), 123 (37%), and 278 (39%), respectively, underwent prehospital ROSC (Table 2). Using the 35th percentile of rhythm conversion times (10 min) as the referent point, there was a trend towards increasing odds of prehospital ROSC with rhythm conversion time, when the initial rhythm was asystole and shockable rhythm conversion occurred within 10 min (Figure 2(a)), after adjustment for age, sex, witness status, bystander CPR, OHCA location, use of advanced airway, EMS response time, and use of epinephrine. After categorizing rhythm conversion time into <10 min, 10–15 min, and ≥15 min of CPR and using nonshockable rhythm conversion as the reference, however, there was no observable difference in association between shockable rhythm conversion and prehospital ROSC by rhythm conversion time among those with asystole (Table 2). Among those with initial PEA, the association between shockable rhythm conversion and

Table 1: OHCA patient characteristics overall, by initial rhythm, and by time of spontaneous shockable rhythm conversion.

Variable	Overall	Initial asystole					Initial pulseless electrical activity				
		No rhythm conversion	Spontaneous rhythm conversion				No rhythm conversion	Spontaneous rhythm conversion			
			Total	Conversion in <10 min	Conversion in 10–15 min	Conversion in ≥15 min		Total	Conversion in <10 min	Conversion in 10–15 min	Conversion in ≥15 min
N	24849	13935	2581	1061	567	953	6678	1655	616	330	705
Median age, year (IQR)	67 (55, 78)	66 (54, 77)	65 (54, 76)	65 (55, 77)	64 (55, 76)	65 (53, 76)	70 (59, 79)	70 (58, 79)	69 (56, 79)	69 (59, 79)	71 (60, 79)
Men, *n* (%)	15344 (61.7)	8498 (61.0)	1726 (66.9)	736 (69.4)	383 (67.5)	607 (63.7)	4026 (60.3)	1094 (66.1)	435 (70.6)	222 (67.3)	437 (61.6)
Witnessed OHCA											
By EMS, *n* (%)	2492 (10.0)	601 (4.3)	114 (4.4)	46 (4.3)	24 (4.2)	44 (4.6)	1479 (22.1)	298 (18.0)	112 (18.2)	61 (18.5)	125 (17.6)
By bystander, *n* (%)	7747 (31.2)	3260 (23.4)	889 (34.4)	374 (35.2)	200 (35.3)	315 (33.1)	2833 (42.4)	765 (46.2)	275 (44.6)	158 (47.9)	332 (46.8)
Bystander resuscitation, *n* (%)	10738 (43.2)	6280 (45.1)	1147 (44.4)	481 (45.3)	254 (44.8)	412 (43.2)	2612 (39.1)	699 (42.2)	286 (46.4)	139 (42.1)	274 (38.6)
Public location, *n* (%)	2426(9.8)	1060 (7.6)	301 (11.7)	129 (12.2)	73 (12.9)	99 (10.4)	802 (12.0)	263 (15.9)	125 (20.3)	48 (14.5)	90 (12.7)
Median EMS response time, min (IQR)	5.4 (4.2, 6.9)	5.3 (4.1, 6.8)	5.5 (4.2, 7.0)	5.5(4.3, 7.0)	5.4 (4.1, 7.0)	5.4 (4.1, 7.0)	5.5 (4.2, 7.0)	5.6 (4.3,7.0)	5.6 (4.3, 7.0)	5.5 (4.2, 7.0)	5.7 (4.3, 7.0)
Median epinephrine use, mg (IQR)	3 (3, 5)	3(3, 4)	4(3, 6)	4(3, 5)	4 (3, 5)	4 (3, 6)	3 (2, 4)	4(3, 6)	4 (3, 6)	4 (3, 5)	5(3, 6)
Advanced airway applied, *n* (%)	21592 (86.9)	11964 (85.9)	2341 (90.7)	933 (87.9)	512 (90.3)	896 (94.0)	5807 (87.0)	1480 (89.4)	530 (86.0)	289 (87.6)	661 (93.2)
Median time of rhythm conversion, min (IQR)	—	—	12.0 (6.7, 18.7)	6.0 (3.9, 7.9)	12.4 (11.2, 13.7)	21.4 (17.9, 26.5)	—	13.2 (7.0, 20.5)	5.5 (3.1, 7.7)	12.6 (11.1, 14.0)	21.9 (18.4, 26.8)
Median time of rhythm conversion, min (IQR)	—	—	12.0 (6.7, 18.7)	6.0 (3.9, 7.9)	12.4 (11.2, 13.7)	21.4 (17.9, 26.5)	—	13.2 (7.0, 20.5)	5.5 (3.1, 7.7)	12.6 (11.1, 14.0)	21.9 (18.4, 26.8)

OHCA, out-of-hospital cardiac arrest; EMS, Emergency Medical Services; *n*, number; N, total number; IQR, interquartile range; min, minute; mg, milligram.

Table 2: Results from multivariable logistic regression analysis, assessing the associations of spontaneous rhythm conversion with prehospital ROSC, survival to hospital discharge, and favorable functional outcome in initial nonshockable rhythm OHCA stratifying by time of spontaneous shockable rhythm conversion.

	N total	Prehospital ROSC		Survival to hospital discharge		Favorable functional outcome at hospital discharge	
		N of events (proportion, %)	OR (95% CI)	*N* of events (proportion, %)	OR (95% CI)	*N* of events (proportion, %)	OR (95% CI)
Initial asystole							
No spontaneous rhythm conversion	13935	2552 (18.2)	Reference	118 (0.8)	Reference	47 (0.3)	Reference
Spontaneous conversion in <10 min	1061	323 (30.4)	1.93 (1.67, 2.23)	34 (3.2)	4.39 (2.95, 6.53)	14 (1.3)	4.28 (2.32, 7.89)
Spontaneous conversion in 10–15 min	567	164 (28.9)	1.76 (1.45, 2.13)	12 (2.1)	3.05 (1.65, 5.62)	7 (1.2)	4.38 (1.94, 9.90)
Spontaneous conversion in ≥15 min	953	322 (33.8)	2.23 (1.92, 2.59)	9 (0.9)	1.60 (0.80, 3.20)	2 (0.2)	0.90 (0.22, 3.74)
Initial pulseless electrical activity							
No spontaneous rhythm conversion	6678	2467 (36.9)	Reference	238 (3.6)	Reference	126 (1.9)	Reference
Spontaneous conversion in <10 min	616	238 (38.6)	1.26 (1.06, 1.50)	34 (5.5)	2.09 (1.42, 3.08)	20 (3.2)	2.26 (1.37, 3.75)
Spontaneous conversion in 10–15 min	330	123 (37.3)	1.15 (0.91, 1.45)	12 (3.6)	1.50 (0.82, 2.77)	3 (0.9)	0.72 (0.23, 2.33)
Spontaneous conversion in ≥15 min	705	278 (39.2)	1.32 (1.12, 1.56)	11 (1.6)	0.88 (0.47, 1.65)	4 (0.6)	0.67 (0.24, 1.85)

Covariables in regression models include age, sex, witnessed OHCA (by EMS vs. bystander vs. not), bystander CPR, location of OHCA (public vs. not), use of advanced airway, Emergency Medical Services response time, and dose of epinephrine administered. Favorable functional outcome at hospital discharge is defined as a Modified Rankin Scale score of ≤3. OHCA, out-of-hospital cardiac arrest; EMS, Emergency Medical Services; CPR, cardiopulmonary resuscitation; N, number; min, minute; OR, odds ratio.

prehospital ROSC was weaker compared to those with initial asystole and did not differ by rhythm conversion time.

4.3. Shockable Rhythm Conversion and Survival to Hospital Discharge. Among patients with initial asystole ($N = 16{,}516$) and those with initial PEA ($N = 8{,}333$), respectively, 173 (1%) and 295 (4%) survived to hospital discharge. Of the 1061, 567, and 953 patients with shockable rhythm conversions from initial asystole at <10 min, 10–15 min, and ≥15 min of CPR, 34 (3%), 12 (2%), and 9 (1%), respectively, survived to hospital discharge. Of the 616, 330, and 705 patients with shockable rhythm conversions from initial PEA at <10 min, 10–15 min, and ≥15 min of CPR, 34 (6%), 12 (4%), and 11 (2%), respectively, survived to hospital discharge. Using the 35th percentile of rhythm conversion times (10 min) as the referent point, there was a linear trend towards decreasing odds of survival to hospital discharge with rhythm conversion time for both patients with initial asystole and those with initial PEA, adjusting for covariates (Figure 2(b)). After categorizing rhythm conversion time into <10 min, 10–15 min, and ≥15 min of CPR, higher odds of survival to hospital discharge was observed with shockable rhythm conversion, when the initial rhythm was asystole and shockable rhythm conversion occurred within the first 10 min (odds ratio (OR) 4.39; 95% confidence interval (CI): 2.95, 6.53) or 10–15 min of CPR (OR 3.05; 95% CI: 1.65, 5.62), or when the initial rhythm was PEA and shockable rhythm conversion occurred within the first 10 min of CPR (OR 2.09; 95% CI: 1.42, 3.08).

4.4. Shockable Rhythm Conversion and Favorable Functional Outcome. Among patients with initial asystole ($N = 16{,}516$) and those with initial PEA ($N = 8{,}333$), respectively, 70 (0.4%) and 153 (2%) had a favorable functional outcome at hospital discharge. Of the 1061, 567, and 953 patients with shockable conversions from initial asystole at <10 min, 10–15 min, and ≥15 min of CPR, 14 (1%), 7 (1%), and 2 (0.2%), respectively, had a favorable functional outcome at discharge. Of the 616, 330, and 705 patients with shockable conversions from initial PEA at <10 min, 10–15 min, and ≥15 min of CPR, 20 (3%), 3 (1%), and 4 (1%), respectively, had a favorable functional outcome at discharge. Using the 35th percentile of rhythm conversion times (10 min) as the referent point, there was a trend towards decreasing odds of favorable functional outcome at discharge with shockable rhythm conversion time, most prominently when occurred

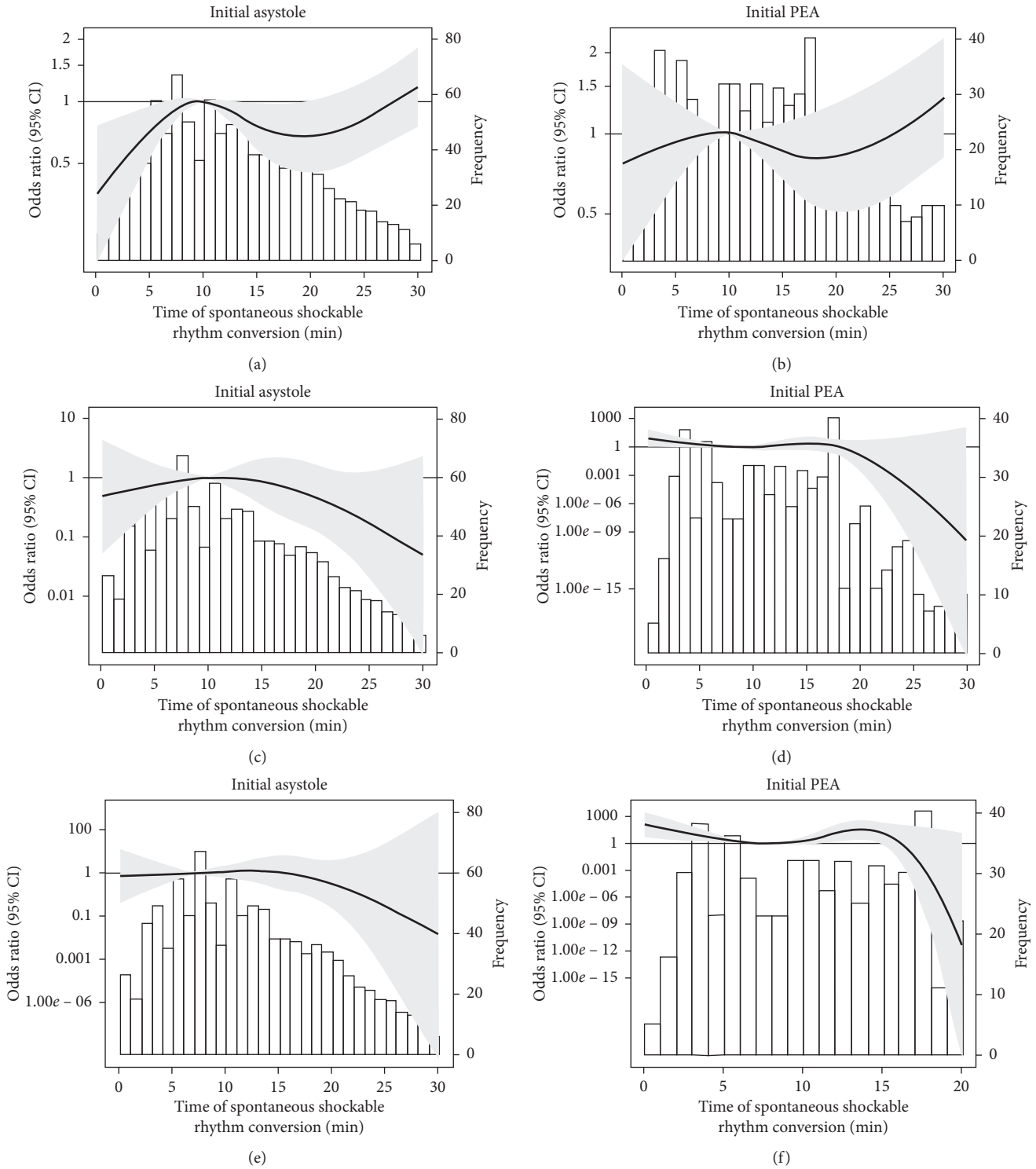

Figure 2: Adjusted odds ratios and 95% confidence intervals comparing shockable rhythm conversion and nonshockable rhythm conversion for prehospital return of spontaneous circulation, survival to hospital discharge, and favorable functional outcome at hospital discharge by time of rhythm conversion in initial heart rhythm in the ROC Cardiac Epidemiologic Registry (version 3). (a) Adjusted odds ratios and 95% confidence intervals for prehospital return of spontaneous circulation in OHCA patients with initial asystole or PEA. (b) Adjusted odds ratios and 95% confidence intervals for survival to hospital discharge in OHCA patients with initial asystole or PEA. (c) Adjusted odds ratios and 95% confidence intervals for favorable functional outcome at hospital discharge in OHCA patients with initial asystole or PEA. OHCA, out-of-hospital cardiac arrest; PEA, pulseless electrical activity; CI, confidence interval.

beyond the first 15 min of CPR among those with initial asystole, adjusting for all covariates (Figure 2(c)). After categorizing rhythm conversion time into <10 min, 10–15 min, and ≥15 min of CPR, higher odds of favorable functional outcome at discharge was observed with shockable rhythm conversion, when the initial rhythm was asystole and conversion occurred within the first 10 min (OR 4.28; 95% CI: 2.32, 7.89) or 10–15 min of CPR (OR 4.38; 95% CI: 1.94, 9.90), or when the initial rhythm was PEA and conversion occurred within the first 10 min (OR 2.26; 95% CI: 1.37, 3.75).

5. Discussion

In this retrospective analysis of 24,849 OHCA patients with initial nonshockable rhythms in a North American population-based registry, we found that shockable rhythm conversion was associated with survival and better functional outcomes at hospital discharge in patients with initial asystole, only when rhythm conversion occurred within the first 15 min of CPR. In patients with initial PEA, shockable rhythm conversion was associated with survival and better functional outcomes at hospital discharge, only when occurred within the first 10 min of CPR, and the associations were weaker compared to among those with initial asystole.

The conversion from a nonshockable rhythm to a shockable rhythm in OHCA remains a subject of clinical importance. Some studies have demonstrated strong associations between shockable rhythm conversion and better outcomes in OHCA patients with initial nonshockable rhythms, whereas others did not [6–10]. Factors underlying the differing prognostic significance of shockable rhythm conversion across populations have been relatively understudied, and there has been little published data on the interactions across initial heart rhythm, rhythm conversion time, and shockable rhythm conversion in initial nonshockable rhythm OHCA. To our knowledge, only two studies have thus far analyzed data on shockable rhythm conversion and outcomes stratified by rhythm conversion time. Goto et al. studied 569,937 OHCA patients enrolled in a Japanese national registry between 2005 and 2010 [12], and Funada et al. studied 430,443 OHCA patients enrolled in the same registry between 2011 and 2014 [11]. Both studies involved only Japanese patients, categorized rhythm conversion times into 10-min intervals, assessed outcomes at one-month post-OHCA and did not stratify analyses by initial arrest rhythm (which has previously been shown to interact with shockable rhythm conversion for its associations with OHCA outcomes) [5, 17]. These researchers concluded that the first 20 min of CPR could be a threshold beyond which shockable rhythm conversion may no longer be associated with better outcomes in OHCA patients with initial nonshockable rhythms [11, 12]. In contrast to these studies, the present study provides a more thorough delineation of the prognostic significance of shockable rhythm conversion stratified by initial heart rhythm, across the continuous spectrum of rhythm conversion time, and multiple OHCA outcomes that were assessed from at the field till hospital discharge.

Our findings may have clinical implications and provide a basis for the development of better CPR strategies. The current *American Heart Association Guidelines for Cardiopulmonary Resuscitation and Emergency Cardiovascular Care* recommend "appropriate rhythm-based strategies" for patients whose heart rhythms have evolved during CPR [18], which would indicate attempts to electrical defibrillation in patients who had undergone shockable rhythm conversions from nonshockable rhythms. However, as demonstrated in the present study, when such rhythm conversions occurred beyond certain time thresholds (i.e., 15 min for initial asystole and 10 min for initial PEA), electrical shocks may no longer confer survival or functional outcome benefits, possibly because the arresting heart had entered a "metabolic phase" where there was irreversible ischemic damage, and the heart muscles had become more susceptible to reperfusion injury [19]. Continued chest compressions to maximize circulation, in these scenarios, may therefore be preferable to electrical defibrillation attempts.

Strengths of this study include its large sample size and per-protocol ascertainment of shock delivery time and multiple OHCA outcomes. However, our study has several limitations. First, because our data originated from a North American registry, the generalizability of our findings to other populations may be limited. Second, like all observational studies, our findings may be affected by uncontrolled confounding. Nonetheless, because of the rigorous design of the ROC Cardiac Registry Epistry and its focus on per-protocol ascertainment of pertinent OHCA covariables and outcomes, we believe the influence of confounding and measurement errors was reduced to the greatest extent possible. Third, only data collected after the initiation of CPR by EMS personnel were available. We are thus unable to account for the duration of cardiac arrest or CPR performed before EMS arrival. Fourth, we did not have access to continuous heart rhythm readings and the use of first electrical shock delivery as the surrogate for shockable rhythm conversion may result in misclassifications. Further, without heart rhythm readings, we were unable to ascertain whether shockable rhythm conversions resulted in fine ventricular fibrillations, as opposed to coarse ventricular fibrillations, which are more likely to respond to electrical shocks. Nonetheless, all EMS providers participating in the ROC were instructed to adhere to clinical practice guidelines, minimizing the chances of inappropriate delivery of shocks in the absence of shockable heart rhythms.

In conclusion, the conversion from a nonshockable rhythm to a shockable rhythm was associated with better outcomes only when occurred early in initial nonshockable rhythm OHCA. These findings may facilitate the advancement of OHCA resuscitation strategies.

Data Availability

The data that support the findings of this study are openly available in the National Institutes of Health (NIH) Biological Specimen and Data Repository Information Coordinating Center (https://biolincc.nhlbi.nih.gov/studies/roc_cardiac_epistry_3/). Requests for the date can be

submitted through the website of the National Institutes of Health (NIH): https://biolincc.nhlbi.nih.gov/studies/roc_cardiac_epistry_3/ (https://biolincc.nhlbi.nih.gov/studies/rocprimed/?q=primed).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Wanwan Zhang, Shengyuan Luo, and Daya Yang contributed equally to this work.

Acknowledgments

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Research Article

Omentin-1 Ameliorated Free Fatty Acid-Induced Impairment in Proliferation, Migration, and Inflammatory States of HUVECs

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Objectives. Endothelial cell injury is a critical pathological change during the development of atherosclerosis. Here, we explored the effect of omentin-1 on free fatty acid- (FFA-) induced endothelial cell injury. *Methods*. An FFA-induced endothelial cell injury model was established to investigate the role of omentin-1 in this process. Cell proliferation was analyzed with the Cell Counting Kit assay and flow cytometry. Scratch and transwell assays were used to evaluate cell migration. Factors secreted by endothelial cells after injury were detected by western blotting, reverse-transcription quantitative polymerase chain reaction, and cellular fluorescence assay. *Results*. Omentin-1 rescued the FFA-induced impaired proliferation and migration capabilities of human umbilical vein endothelial cells (HUVECs). It decreased the number of THP-1 cells attached to HUVECs in response to injury and inhibited the FFA-induced proinflammatory state of HUVECs. *Conclusion*. Omentin-1 could partly ameliorate FFA-induced endothelial cell injury.

1. Introduction

The vascular endothelium is a continuous single-cell lining of the circulatory system that forms an interface between the blood and the vascular wall. This layer possesses multiple functions, including maintenance of thrombohemorrhagic balance and regulation of inflammatory response [1]. Some pathological conditions such as dyslipidemia and hyperglycemia may induce endothelial cell injury, which is characterized with increased cell apoptosis, decreased cell proliferation, and activation of proinflammatory signaling pathways [2, 3]. Such injuries interrupt the homeostasis of the vascular endothelium and cause inflammatory responses, thereby contributing to the early stage of coronary heart disease (CHD) [4].

The coronary artery is surrounded by the epicardial adipose tissue (EAT), a special visceral adipose tissue without fascia separation. The volume of the EAT is positively associated with CHD, independent of blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and some other traditional risk factors [5]. The adipose tissues, including EAT, secrete high levels of nonesterified fatty acids during lipolysis [6, 7]. These free fatty acids (FFAs) activate Toll-like receptor (TLR)-4, eventually leading to an increase in nuclear factor kappa B (NF-κB) activity. This changes the profile of endothelial cells toward an inflammatory state [8]. FFAs could also downregulate the synthesis of vascular endothelial growth factor (VEGF) and inhibit the repair of endothelial wounds [9]. Furthermore, excessive secretion of FFAs impairs the cardiac structure [10]. FFAs may also contribute to the onset and development of CHD.

Omentin-1 is a novel adipocytokine expressed in the EAT, omental adipose tissue, and other visceral adipose tissue depots [11]. It inhibits the tumor necrosis factor-α (TNF-α)-induced endothelial cell inflammatory state [12], downregulates the extracellular matrix synthesis of vascular smooth muscle cells [13], and attenuates arterial calcification in osteoprotegerin-deficient mice [14]. Despite these vascular protective effects, omentin-1 is known to predict coronary collateral circulation [15]. Further, the plasma level of omentin-1 is associated with CHD and heart failure in

senile patients [16]. These findings suggest that omentin-1 may exert antiatherosclerosis effects. However, the role of omentin-1 in FFA-induced endothelial cell injury is still unknown. Here, a FFA-induced endothelial cell injury model was established to explore the effect of omentin-1 on this process.

2. Materials and Methods

2.1. Cell Culture and Treatment. Human umbilical vein endothelial cells (HUVECs) were obtained from Procell (CL-0122) and cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S). Cells from passages 3 to 8 were used for experiments. Palmitic acid (PA; P5585, Sigma), a representative FFA in vivo, was prepared in dimethyl sulfoxide (DMSO). Confluent HUVECs were serum-starved for overnight in non-FBS DMEM, and then the medium was replaced with FBS-containing DMEM. HUVECs were incubated with PA and/or recombinant human omentin-1 (9137-IN-050, R&D) as follows: control (0 mM PA in phosphate-buffered saline [PBS]), DMSO (0 mM PA in DMSO), 1 mM PA, 1 mM PA + 100 ng/mL omentin-1, 1 mM PA + 150 ng/mL omentin-1, or 1 mM PA + 200 ng/mL omentin-1 for 24 h.

THP-1 cells were purchased from Procell (CL-0233) and cultured in Roswell Park Memorial Institute (RPMI)-1640 (HyClone) medium supplemented with 15% FBS and 1% P/S. Cells from passages 3 to 5 were used for experiments.

2.2. Cell Proliferation Assay. Serum-starved cells (1×10^3) were seeded into 96-well plates and treated with PA and/or recombinant human omentin-1 (concentration as mentioned above) for 24 h. Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) (40203ES76, Yeasen) assay according to the manufacturer's recommendation.

2.3. Cell Cycle Analysis. Cell Cycle Analysis Kit (FXP021, 4A BIOTECH) was used to analyze changes in cell cycle phases following treatment of HUVECs with PA and/or recombinant human omentin-1 for 24 h. The cells were collected after treatment, washed thrice with PBS, and fixed in 95% ethanol at 4°C overnight. Cells (1×10^6) were stained with propidium iodide (PI) and examined using flow cytometry. Data were analyzed using FlowJo 7.6.1 software.

2.4. Scratch Assay. HUVECs were seeded into six-well plates at 2×10^5 cells/well and serum-starved for 24 h. The cell layer on the surface of each well was gently scratched using 10-μL pipette tips. Wells were rinsed thrice with PBS to remove any cell debris and filled with the medium containing 2% FBS with PA and/or recombinant human omentin-1 (concentration as mentioned above). Images were obtained at 0, 12, and 24 h using a microscope. Wound closure rate ((initial wound area - wound area at 24 h)/initial wound area) was analyzed and calculated using Image Pro Plus 6.0 software.

2.5. Migration Assay. The migration of HUVECs was investigated using a 24-well modified Boyden chamber (8 μm, Corning). After treatment with PA and/or recombinant human omentin-1 (concentration as mentioned above) for 24 h, HUVECs were collected and resuspended in serum-free DMEM. Approximately 5×10^4 cells in 200 μL of serum-free DMEM were seeded in the upper chamber, while the lower chamber was filled with 600 μL DMEM containing 10% FBS. After 24 h incubation, the cells on the upper face of the membrane were wiped, while those that migrated to the lower face of the membrane were fixed with 4% paraformaldehyde for 20 min and stained with crystal violet solution (C0121, Beyotime) for 5 min. Images were obtained using a microscope (Leica DM5000 B). The number of migrated HUVECs was counted in three random fields (200× magnification).

2.6. Western Blotting Analysis. After treatment for 24 h, HUVECs were lysed using radioimmunoprecipitation assay (RIPA) buffer (WB3100, NCM Biotech) containing 1% phenylmethylsulphonyl fluoride (PMSF G2008, Servicebio). The cell lysis solution was further dissociated with ultrasound treatment and centrifuged at 12000 × g at 4°C for 30 min to obtain supernatant. Protein concentration in the supernatant was determined with the bicinchoninic acid (BCA) assay (23227, ThermoFisher Scientific). Proteins (20 μg) were separated on 10% Bis-Tris gel, and the separated bands were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore IPVH00010). The membrane was blocked in 5% defatted milk at 25°C for 2 h and incubated overnight at 4°C with primary antibodies against the following proteins: tubulin (1 : 4000 dilution; ab6046, Abcam); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1 : 4000 dilution; T0004, Affinity); intercellular adhesion molecule-1 (ICAM-1) (1 : 800 dilution; 4915S, CST); monocyte chemoattractant protein-1 (MCP-1) (1 : 1000 dilution; DF7577, Affinity); NF-κB (1 : 1000 dilution; #4764, CST); and NF-kappa-B inhibitor alpha (IκBα) (1 : 1000 dilution; #4812, CST). The PVDF membrane was then probed with corresponding secondary antibodies conjugated to horseradish peroxidase (HRP; 1 : 5000 dilution; #S0001, #S0002, Affinity) at 25°C for 70 min and observed using enhanced chemiluminescence (p10100, NCM Biotech) with ChemiDoc XRS Plus (Bio-Rad). The relative protein expression level was analyzed using Image Lab 3.0 software.

2.7. Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA from HUVECs was extracted using TRIzol reagent (15596018, Invitrogen) and reverse-transcribed with HiScript II QRT SuperMix (R223-01, Vazyme). RT-qPCR was performed on a ViiA 7 system (Applied Biosystems) using the All-in-One qPCR Mix (GeneCopoeia) for 40 cycles to detect the mRNA expression levels of *ICAM-1*, *MCP-1*, *interlukin-1* (*IL-1*), *interlukin-6* (*IL-6*), and *TNF-α*. GAPDH was used for normalization. The primers used in this research are shown in Supplementary Table 1.

2.8. THP-1 Cell Adhesion Analysis. Starved HUVECs (1×10^5) were seeded into 12-well plates and treated with PA and/or recombinant human omentin-1 (concentration as mentioned above) for 24 h. The medium was replaced. THP-1 cells were incubated with 10 μM of 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) fluorescent probe for 30 min in the dark. Cells were collected, washed thrice with PBS, and resuspended in PBS. 100 μL of THP-1 cell suspension was added to the wells of a 12-well plate containing HUVECs, and the cells were cocultured for 1 h. The medium was discarded, and each well was gently washed thrice with PBS. Images were acquired using a fluorescence microscope (Leica DM5000B). The number of THP-1 cells attached to HUVECs was analyzed using Image Pro Plus 6.0 software.

2.9. Immunofluorescence of HUVECs. Starved HUVECs (5×10^4) were seeded into 24-well plates and treated with PA and/or recombinant human omentin-1 (concentration as mentioned above) for 24 h. The medium was discarded, and the cells were rinsed thrice with PBS. The cells were fixed with 4% paraformaldehyde for 20 min, permeated with 0.5% Triton-X100 for 15 min, blocked with normal goat serum for 60 min, and incubated overnight at 4°C with primary antibodies against the following proteins: ICAM-1 (1 : 200 dilution; 4915S, CST); MCP-1 (1 : 200 dilution; GB11199, Servicebio); and NF-κB (1 : 200 dilution; #4764, CST). The cells were then incubated with the corresponding secondary antibody Alexa Fluor 488 goat anti rabbit IgG H&L (1 : 500 dilution; ab150077, Abcam) for 40 min at 37°C. The cells were stained for 5 min with 4′,6-diamidino-2-phenylindole (DAPI; 1 : 1000 dilution; 564907, BD Pharmingen), and images were acquired using a fluorescence microscope (Leica DM5000B). Fluorescence intensity was analyzed using Image Pro Plus 6.0 software.

2.10. Statistical Analysis. Data are presented as the means ± SEM. Data were compared using the unpaired Student's *t*-test and one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test. Statistical analyses were performed using SPSS 19 software. A value of P of <0.05 was considered statistically significant.

3. Results

3.1. Omentin-1 Reversed the PA-Induced Impairment in the Proliferation and Migration of HUVECs. The treatment of HUVECs with PA alone resulted in the inhibition of their proliferation ability as compared with the control and DMSO treatment (Figure 1(a)). The simultaneous treatment with PA and omentin-1 partly ameliorated the impaired proliferation ability of these cells (Figure 1(a)). Flow cytometry results showed that the proportion of cells in S and G2 phases after treatment with PA alone was lower than that observed after control and DMSO treatment (Figure 1(b)). Thus, HUVEC division was inhibited by PA. The treatment with omentin-1 resulted in the upregulation in S and G2 phases cells as compared with PA treatment, indicating that omentin-1 reversed the PA-induced inhibition of cell division (Figure 1(b)). The scratch and transwell assays demonstrated the impairment in the migration ability of HUVECs by PA treatment and that the cotreatment with omentin-1 could alleviate this effect (Figures 1(c) and 1(d)). These results suggest that PA induces endothelial injury and omentin-1 ameliorates the damage.

3.2. Omentin-1 Reduced the PA-Induced Attachment of THP-1 Cells to HUVECs. Western blotting analysis showed that the protein expression levels of ICAM-1, an atherosclerosis-associated endothelial-leukocyte adhesion molecule, and MCP-1, an important chemotactic factor, were significantly higher in PA-treated HUVECs than in the control and DMSO treated HUVECs (Figure 2(a)). Omentin-1 inhibited the PA-induced upregulation of ICAM-1 and MCP-1 expression (Figure 2(a)). The PA-induced upregulation in the mRNA expression levels of *ICAM-1* and *MCP-1* was also inhibited following the simultaneous treatment with omentin-1 (Figure 2(b)). Cellular immunofluorescence results further confirmed these findings (Figures 2(c) and 2(d)). The number of THP-1 cells attached to HUVECs significantly increased after treatment with PA alone as compared with that observed in the control and DMSO treatment (Figure 2(e)). However, the cotreatment with omentin-1 reduced the PA-induced increase in the attachment of THP-1 cells to HUVECs (Figure 2(e)). These findings indicate that omentin-1 could prevent the PA-induced adhesion of THP-1 cells to HUVECs and that the role of omentin-1 in the inhibition of the upregulation of PA-induced ICAM-1, and MCP-1 expression, at least in part, contributed to this preventive effect.

3.3. Omentin-1 Inhibited the PA-Induced Inflammatory State of HUVECs. Both ICAM-1 and MCP-1 are target genes of NF-κB; hence, we detected the protein expression level of NF-κB by western blotting. As a result, we found that the PA-induced upregulation in the expression of NF-κB was ameliorated by omentin-1 cotreatment (Figure 3(a)), and the PA-induced degradation of IκBα was prevented by omentin-1 (Figure 3(a)). Thus, omentin-1 inhibited the activation of NF-κB. To further investigate the effect of omentin-1 on the expression of NF-κB, a cellular immunofluorescence assay was performed to detect the expression of NF-κB. The results showed that the fluorescence intensity for NF-κB expression in the cells from PA group was significantly higher than that reported for the control and DMSO treatment groups. On the contrary, omentin-1 reduced the PA-induced change in the fluorescence intensity for NF-κB (Figure 3(b)). The PA-induced upregulation in the mRNA expression levels of *IL-1*, *IL-6*, and *TNF-α*, the target genes of NF-κB, was also ameliorated by omentin-1 (Figure 3(c)). Together these results demonstrate that omentin-1 could partly relieve the PA-induced proinflammatory state of HUVECs by preventing the activation of NF-κB.

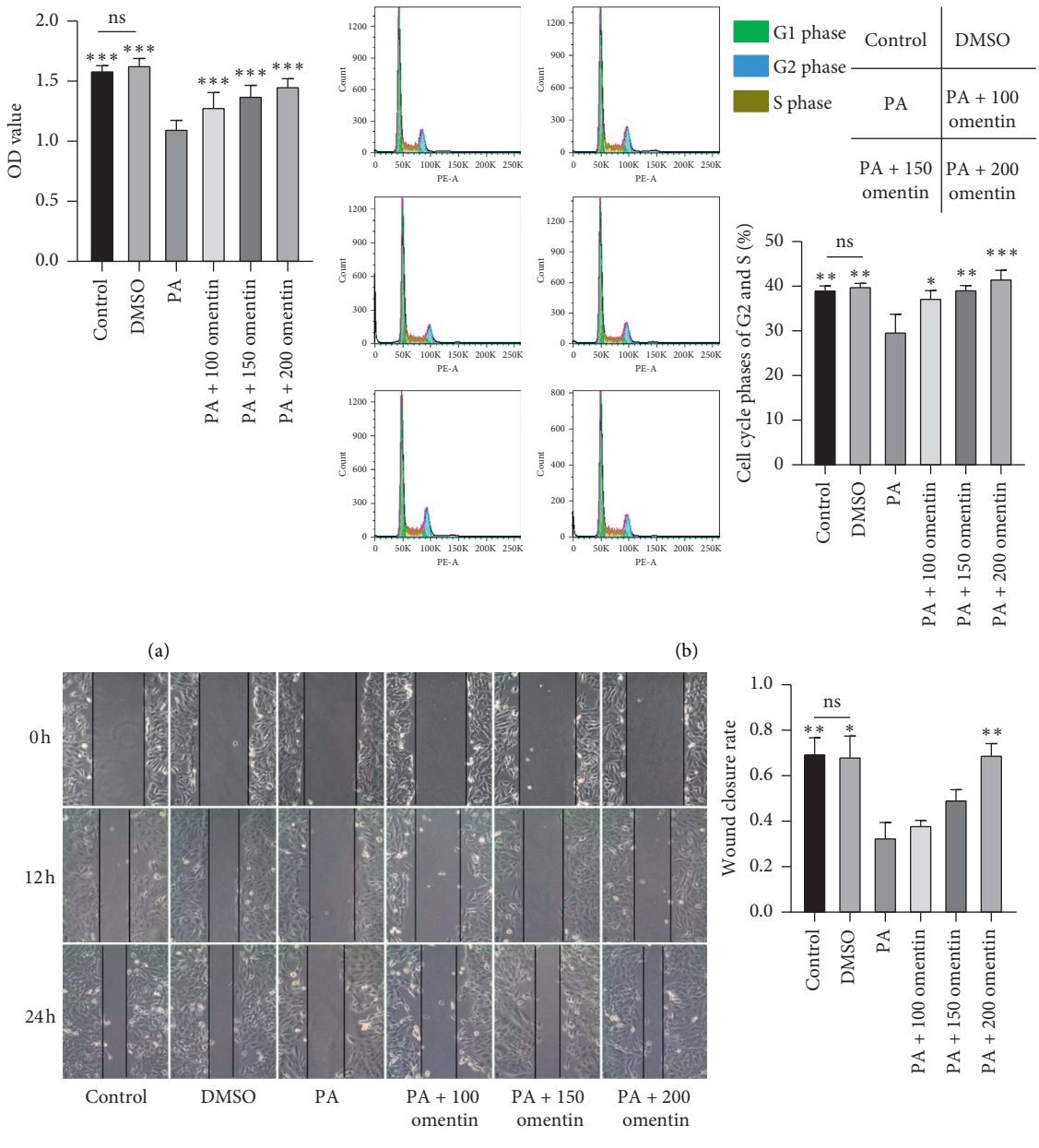

Figure 1: Continued.

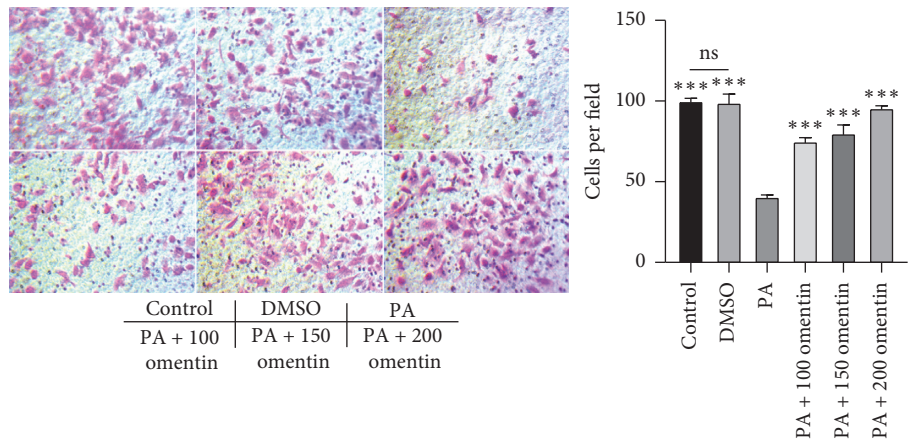

Figure 1: Omentin-1 reversed the PA-induced impairment in the proliferation and migration of HUVECs. (a) Proliferation ability of HUVECs was determined using CCK-8 assay following different treatments. (b) Representative flow cytometry results of cell cycle analysis and quantitative analysis. (c) Representative images of scratch assay (100x magnification) and quantitative analysis of wound closure rate demonstrated the migration capability of HUVECs under different treatments. (d) Representative images of transwell assay (200x magnification) and the number of migrated HUVECs. The values are mean ± SEM of three independent experiments. $^{*}P < 0.05$ vs. PA group, $^{**}P < 0.001$ vs. PA group, and $^{***}P < 0.0001$ vs. PA group.

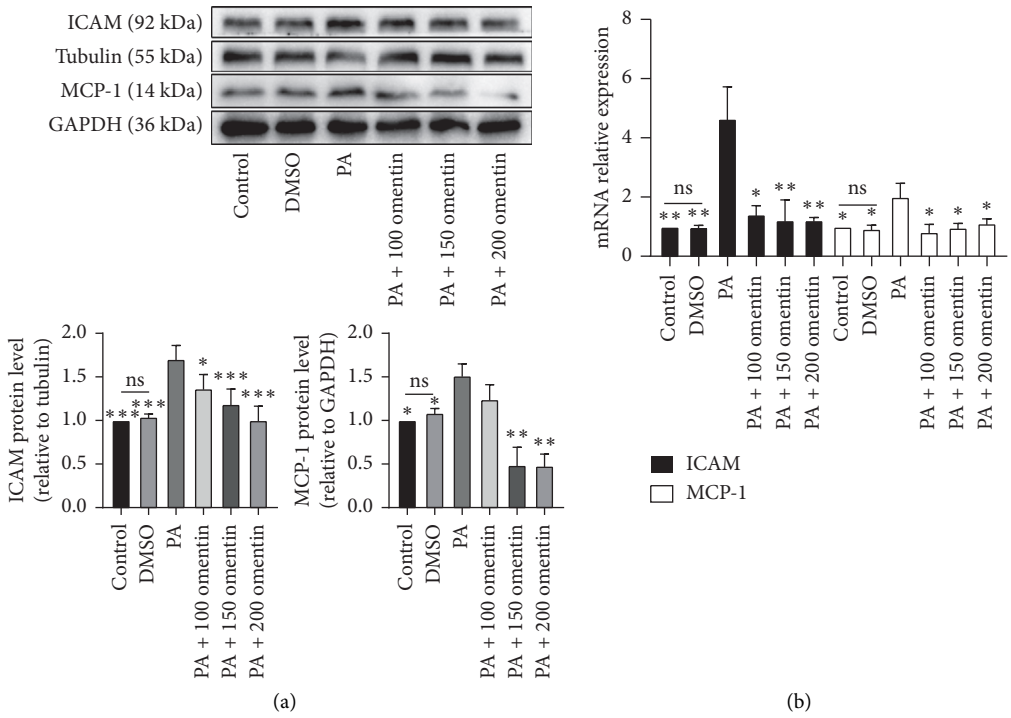

Figure 2: Continued.

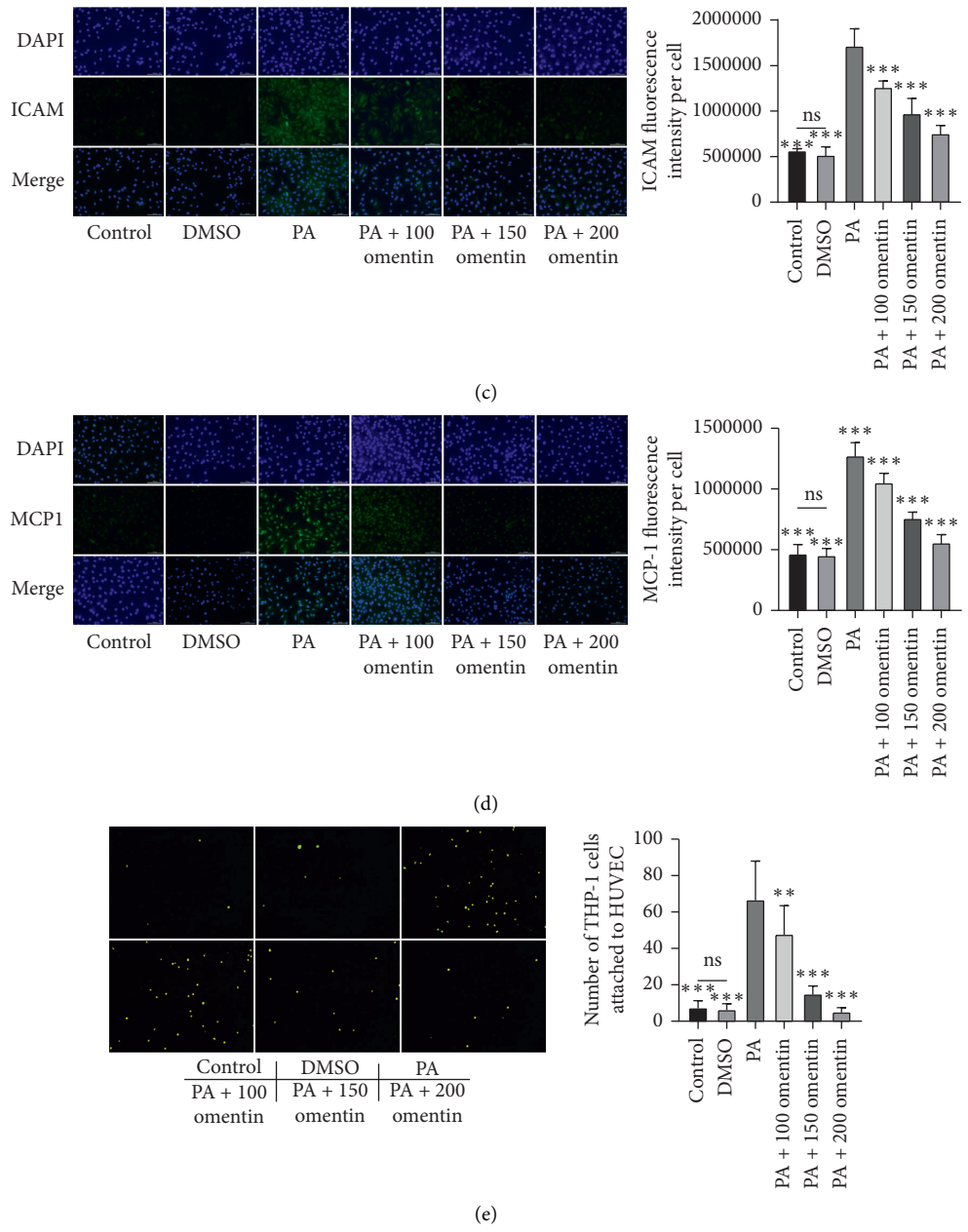

Figure 2: Omentin-1 reduced the PA-induced attachment of THP-1 cells to HUVECs. ICAM-1 and MCP-1 protein expression levels in HUVECs from different groups were detected with western blotting (a) using tubulin or GAPDH as loading control. Quantitative analysis of protein expression is shown as bar graphs. The mRNA expression levels of *ICAM-1* and *MCP-1* were determined with RT-qPCR (b). (c, d) Representative images of cellular fluorescence assay for ICAM-1 and MCP-1 (400x magnification) and the quantitative analysis of fluorescence intensity per cell. (e) The representative pictures of BCECF-AM-labeled THP-1 cells attached to HUVECs in different treatments groups are shown (400x magnification); the number of fluorescent cells was counted. The values are mean ± SEM of three independent experiments. $^{*}P < 0.05$ vs. PA group, $^{**}P < 0.001$ vs. PA group, and $^{***}P < 0.0001$ vs. PA group.

4. Discussion

CHD, one of the most serious clinical manifestations of atherosclerosis, has become a burden on the global health industry [17]. As a chronic arterial disease, atherosclerosis is characterized with the appearance of fatty steak, development of atheroma, and formation of plaque. The plaque itself or upon its rupture may result in thrombosis and occlusion of the arteries, resulting in the induction of hypoperfusion and damage of the related organ [18]. Endothelial cells play important roles in the cardiovascular system in the maintenance of the vascular tone and regulation of inflammation and thrombosis [19]. Pathological stimuli such as diabetes mellitus, hypertension, and dyslipidemia may provoke endothelial cell injury, characterized with an alteration in the normal functions [20]. To date, endothelial cell injury is one of the earliest pathological changes that can be detected during the development of atherosclerosis [4]. A recent

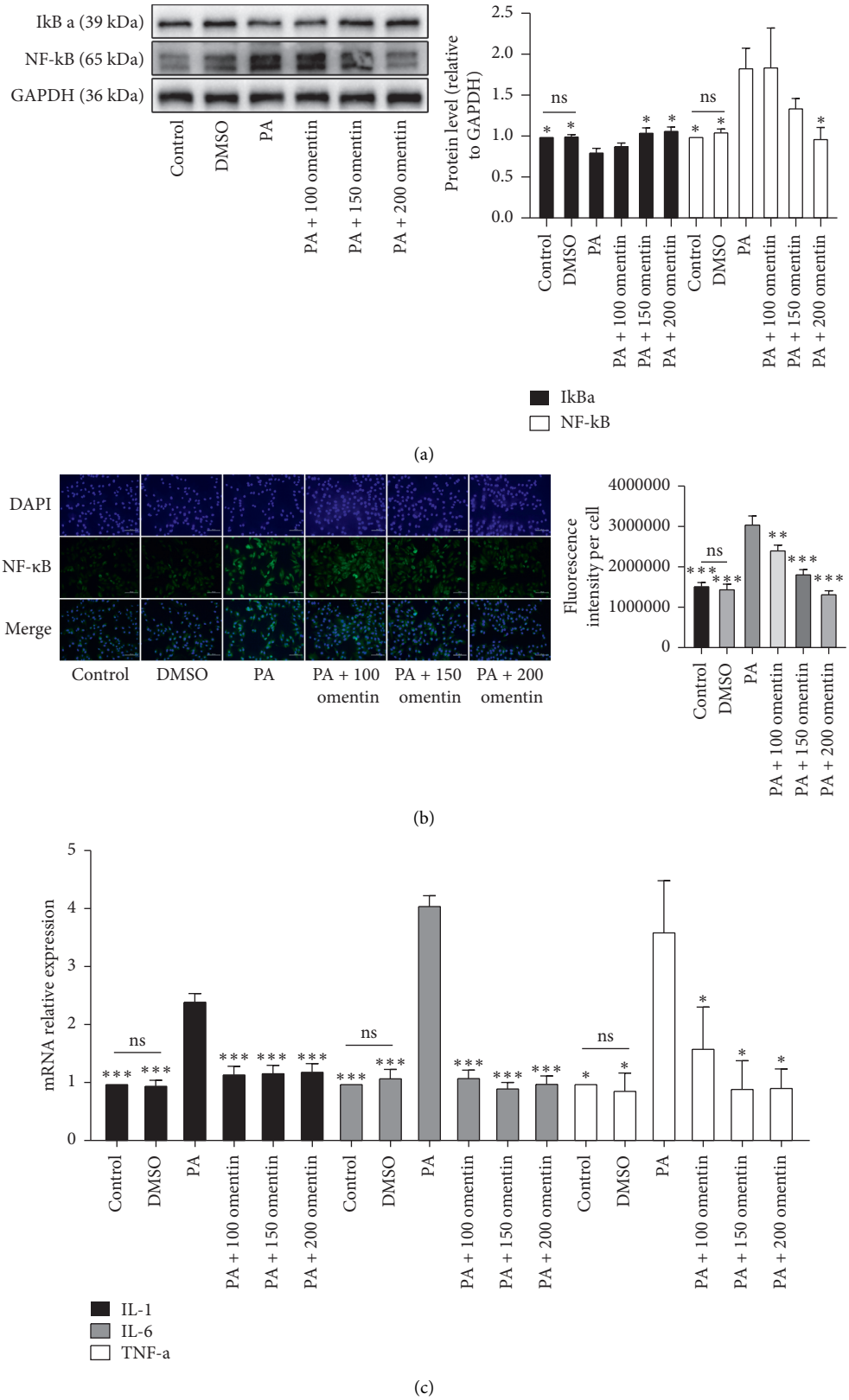

Figure 3: Omentin-1 inhibited the PA-induced inflammatory state of HUVEC. I$\kappa B\alpha$ and NF-κB protein expression levels in HUVECs from different groups were detected with western blotting (a) using tubulin as loading control. Quantitative analysis of proteins expression is shown in the right bar graph. (b) Representative images of cellular fluorescence assay for NF-κB (400x magnification) and the quantitative analysis of fluorescence intensity per cell. (c) The mRNA expression levels of *IL-1*, *IL-6*, and *TNF-α* in HUVECs from different treatment groups using RT-qPCR. The values are mean ± SEM of three independent experiments. $^{*}P < 0.05$ vs. PA group, $^{**}P < 0.001$ vs. PA group, and $^{***}P < 0.0001$ vs. PA group.

study reported the ability of FFAs to induce endothelial cell injury *in vitro* [21]. In the present study, a model of PA-induced endothelial cell injury was established to determine the role of omentin-1 in this process.

Omentin-1 is a novel adipocytokine abundantly expressed in the EAT, omental adipose tissue, and some other organs [11]. Although the specific receptor of omentin-1 has not been identified, the role of omentin-1 has been extensively explored. Omentin-1 mediated cardiovascular protective effects [22], modulated the functions of insulin [23], and regulated bone metabolism [14]. It was recently reported that the circulating level and mRNA expression level of *omentin-1* in the EAT were significantly downregulated in patients with coronary artery disease (CAD) as compared with those in patients without CAD. In addition, the protein and mRNA expression levels of omentin-1 were lower in the EAT surrounding coronary stenotic segments compared with those in the EAT adjacent to nonstenotic segments, indicating that omentin-1 may be an antiatherosclerosis adipocytokine [24]. However, its role in PA-induced endothelial cell injury is unclear.

The vascular endothelium comprises a continuous monolayer of endothelial cells and regulates coagulation and inflammatory reactions [1]. The integrity of the monolayer of the endothelial cells is important for maintaining these functions [24]. In the present study, the proliferation and migration capabilities of HUVECs were inhibited by PA treatment. These capabilities, which could boost the repair of the single-cell lining, are crucial for the integrity of the vascular endothelium. In comparison with PA treatment, the cotreatment with omentin-1 increased the proportion of S and G2 phase cells, thereby stimulating HUVEC division. Omentin-1 also rescued the PA-induced impaired migration ability of HUVECs. These findings suggest that omentin-1 may contribute to the maintenance of the integrity of the vascular endothelium. The expression levels of ICAM-1 and MCP-1 were also upregulated following exposure of HUVECs to PA alone. As an endothelial-leukocyte adhesion molecule, ICAM-1 expression level increases in atherosclerosis lesions [25]. MCP-1 could recruit and accumulate monocytes in the lesion and accelerate the development of atherosclerosis [26]. The upregulation in the expression of adhesion molecule and chemokine resulted in an increase in the number of THP-1 cells attached to HUVECs as compared with the control and DMSO groups. During the early stages of atherosclerosis development, monocytes are recruited to the region of injured endothelial cells owing to the chemoattractant gradient. These cells attach to the endothelial cells via adhesion molecules such as ICAM-1 and then migrate into the vascular intima, wherein they differentiate into macrophages, internalize lipoprotein particles, and eventually become foam cells [27]. The PA-induced elevation in the expression levels of ICAM-1 and MCP-1 was prevented by the cotreatment with omentin-1; the number of THP-1 cells attached to HUVECs reduced following cotreatment with omentin-1 as compared with that observed after treatment with PA alone. These results demonstrate that omentin-1 may prevent the adhesion of monocytes to endothelial cells. The PA-induced upregulated expression of ICAM-1 and MCP-1 suggests the change in the profile of HUVECs to a proinflammatory state. Treatment with PA increased the protein expression level of NF-κB and provoked the degradation of IκBα. It is known that IκBα binds to NF-κB and covers the nuclear localization sequence of NF-κB, which acts as an inhibitor of NF-κB. Diverse proinflammatory stimuli could activate IκB kinase complex which specifically phosphorylates IκBα, leading to polyubiquitinated and degraded of IκBα. Under such circumstance, NF-κB could translocate into nucleus and bind target genes including *IL-1*, *IL-6*, and *TNF-α* and stimulate their transcription eventually resulting in a proinflammatory state of HUVECs [27, 28]. In the present study, the mRNA expression levels of *IL-1*, *IL-6*, and *TNF-α* were also upregulated after the exposure of HUVECs to PA alone as compared with those observed after control and DMSO groups. The upregulation in these proinflammatory factors further validated the PA-induced activation of NF-κB. These inflammatory factors induce differentiation of monocytes into macrophages and accelerate the formation of foam cells [29]. The activation of NF-κB and the proinflammatory signaling cascade was inhibited by the cotreatment with omentin-1. However, the exact mechanism underlying these protective effects is unknown. Previous studies have shown that omentin-1 stimulated the AMP-activated protein kinase (AMPK) signaling pathway to suppress the TNF-α-induced endothelial cell inflammatory state, inhibit myocardium hypertrophy, and reverse myocardial ischemic injury [12, 30, 31]. Further studies are warranted to evaluate the exact mechanism underlying the effect of omentin-1 on PA-induced endothelial cell injury. The expression level of omentin-1 is decreased in patients with CAD [32]. Increasing omentin-1 expression may prevent the onset and development of atherosclerosis and could be a potential therapeutic strategy for CAD treatment.

In conclusion, omentin-1 could rescue the PA-induced impaired proliferation and migration capabilities of HUVECs, reduce the increased number of THP-1 cells attached to PA-induced HUVECs, and inhibit the PA-induced proinflammatory state of HUVECs. These findings demonstrate the ability of omentin-1 to ameliorate PA-induced endothelial cell injury and its potential role as an antiatherosclerosis adipocytokine.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

The primers used in this research. (*Supplementary Materials*)

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Review Article

The Roles of AMPK in Revascularization

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Coronary heart disease (CHD) is the most common and serious illness in the world and has been researched for many years. However, there are still no real effective ways to prevent and save patients with this disease. When patients present with myocardial infarction, the most important step is to recover ischemic prefusion, which usually is accomplished by coronary artery bypass surgery, coronary artery intervention (PCI), or coronary artery bypass grafting (CABG). These are invasive procedures, and patients with extensive lesions cannot tolerate surgery. It is, therefore, extremely urgent to search for a noninvasive way to save ischemic myocardium. After suffering from ischemia, cardiac or skeletal muscle can partly recover blood flow through angiogenesis (de novo capillary) induced by hypoxia, arteriogenesis, or collateral growth (opening and remodeling of arterioles) triggered by dramatical increase of fluid shear stress (FSS). Evidence has shown that both of them are regulated by various crossed pathways, such as hypoxia-related pathways, cellular metabolism remodeling, inflammatory cells invasion and infiltration, or hemodynamical changes within the vascular wall, but still they do not find effective target for regulating revascularization at present. 5′-Adenosine monophosphate-activated protein kinase (AMPK), as a kinase, is not only an energy modulator but also a sensor of cellular oxygen-reduction substances, and many researches have suggested that AMPK plays an essential role in revascularization but the mechanism is not completely understood. Usually, AMPK can be activated by ADP or AMP, upstream kinases or other cytokines, and pharmacological agents, and then it phosphorylates key molecules that are involved in energy metabolism, autophagy, anti-inflammation, oxidative stress, and aging process to keep cellular homeostasis and finally keeps cell normal activity and function. This review makes a summary on the subunits, activation and downstream targets of AMPK, the mechanism of revascularization, the effects of AMPK in endothelial cells, angiogenesis, and arteriogenesis along with some prospects.

1. Introduction

Coronary heart disease (CHD) is the main cause of death globally; it is estimated that 17.9 million people died of cardiovascular diseases (CVDs) in 2016, representing 31% of all global deaths. The basic pathophysiology process is atherosclerosis, which tends to create plaque and block vascular cavity, resulting in myocardial ischemia, hypoxia or necrosis. Presently, the therapies for CHD mainly include coronary artery intervention (PCI) or coronary artery bypass grafting (CABG) [1]. However, postsurgical restenosis and low operative tolerance of aging and patients with extensive lesions limit its efficacy in CHD. Therefore, it is important to search for other alternative methods. Ischemic zones can actually recover blood perfusion by recruiting new vessels or expanding and remodeling produce arterioles; this process is also called revascularization and includes angiogenesis and arteriogenesis [2]. The mechanism of these processes has been widely studied. Angiogenesis is induced by hypoxia and involves three cells: tip cells, stalk cells, and phalanx cells [3–5], while the main stimulus of arteriogenesis is fluid shear stress (FSS), which is sensed by endothelial cells and consequently attracts leukocytes and promotes the phenotype transformation of vascular smooth muscle cells (VSMCs) [6–9]. Signal pathways of these two ways both include vascular endothelial growth factor (VEGF) pathway and nitric oxide- (NO-) dependent pathway [10–14] and both of them can be regulated by a highly conserved eukaryotic

kinase, 5′adenosine monophosphate-activated protein kinase (AMPK) [15–17]; SNF1 and SnRK1 are its orthologues in yeast and several plants [18].

AMPK, a heterotrimeric complex combined by α, β, γ subunits, is activated by upstream kinases and regulated by the ratio of ADP/ATP or AMP/ATP or posttranslational modifications including phosphorylation and ubiquitylation, which exerts vital roles in maintaining energy homeostasis, protecting endothelial cellular function, regulating cellular autophagy, oxidative stress, and aging [19]. AMPK is ubiquitously expressed in a lot of tissues and cells, such as the endothelial cells (ECs), skeletal muscle, liver, and brain [20]. The roles of AMPK in revascularization have been widely researched, and it seems that the findings are varying in different conditions. In ischemia or hypoxia, AMPK activation facilitates angiogenesis but in tumor microenvironment inhibits it. Similarly, some findings show that AMPK promotes arteriogenesis by regulating inflammation but others suggest AMPK play a negative role in collateral circulation [15, 17].

2. AMPK

2.1. Subunits of AMPK. AMPK, a heterotrimeric protein complex, includes α subunit (encoded by protein kinase AMP-activated-α (PRKAA)) [21], β (PRKAB) [22], and γ (PRKAG) [23]. These isoforms play distinct roles in the AMPK stability and activity, but all three are essential for full activity. α (two isoforms) are catalytic subunits; β subunit (two isoforms) and γ subunit (three isoforms) contain the regulatory site, which could be combined by 12 various ways [24].

Both α subunits are similar in that their N termini have traditional serine/threonine kinase domains (α-KD) as well as the conserved threonine residue (α1 Thr183 and α2 Thr172), which are key phosphorylated sites [25]. The following are the inhibitory domains (α-AID), which negatively regulate AMPK. The C termini of AMPK is C-terminal domain (α-CTD) with nuclear export sequence (NES), whose crystal structure has not been resolved. Between α-AID and α-CTD is "α linker," which is locked around the γ subunit (Figure 1). These two isoforms have various subcellular locational pattern; α1 isoform majorly appears to distribute in the cytoplasm or to associate with the plasma membrane of carotid body type 1 cells. However, α2 prefers locating in the nuclei of some cell types, such as skeletal muscle [26]. They have specificity of tissue distribution; for instance, AMPKα1 isoform is in the adipose tissue [27] while skeletal muscle expresses much higher AMPKα2 [28]. Interestingly, ECs have both of these isoforms, although AMPKα1 predominates at a much higher level than AMPKα2 [29].

Most of the parts of β subunits are highly conserved except the first 65 residues of NH2-terminus. AMPKβ1 is nearly expressed in all cell types while β2 is mainly distributed in muscle. From N-terminus to C-terminus, β subunits have myristoylated N-terminal regions, carbohydrate-binding modules (β-CBM), β-linker regions, and the C-terminal domains (β-CTD) (Figure 1) [22, 30]. The crystal

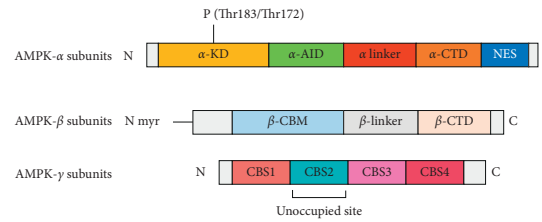

Figure 1: The structure of AMPK subunits: AMPK have three subunits, including α, β, γ. α is catalytic while β and γ are regulatory. Both α1 and α2 subunits have a crucial site in Thr183 and Thr172, whose phosphorylation is necessary for AMPK maximal activation. The β subunits could act as a scaffold, which makes the AMPK complex located on lysosomes, an exception from having phosphorylation, myristoylation, and carbohydrate-binding sites. The γ subunits bind the nucleotides by three sites, which are the structural basis for this energy sensor.

structures of β-CBM and β-CTD are completely resolved but the structures of N-terminal regions and β-linker are still unclear. Significantly, there is compelling evidence that N-terminal myristoylation of β subunits plays an indispensable role in AMPK lysosomal localization and activation in an AMP/ADP/ATP-independent manner in the process of glucose depletion [31, 32]. And N-myristoylation of AMPK β subunits also controls T cell inflammatory function [33, 34]. Hardie et al. have demonstrated that glycogen inhibits AMPK activation by binding the β-CBM of AMPK, which suggest that AMPK equilibrates cellular energy by sensing not only the change of AMP/ATP or ADP/ATP but also glycogen [35]. β-CTD interacts with γ N-terminal regions, which let AMPK become an intact complex to exert its normal function [36].

Although γ subunits have different lengths (γ1 331 < γ3 489 < γ2 569 residues), each one shares the same COOH-terminal having about 300 residues, a variable N-terminal domain that interacts with β-CTD and four tandem repeats of a motif termed CBS repeat (Figure 1) [18]. Excepting CBS2 which is an unoccupied site, CBS1, CBS3, and CBS4 could be bound by AMP or ATP by different affinities, CBS1 site binds ATP with higher affinity, but CBS3 site has higher affinity for AMP, and CBS4 is believed to be a non-changeable site; that is, it binds AMP irreversibly [37, 38]. Furthermore, different isoforms of γ subunits also have distinct affinity with AMP, such as γ3 which is the least sensitive [39]. Like α and β subunits, γ subunits also have tissue distribution specificity; γ1 subunit is widely expressed in all tissues, whereas γ2 and γ3 isoforms are mainly abundant in skeletal muscle [40].

In conclusion, both α1 and α2 subunits have a crucial site in Thr183 and Thr172, whose phosphorylation is necessary for AMPK maximal activation. The β subunits could act as a scaffold, which makes AMPK complex locate on lysosomes, except for having phosphorylation, myristoylation, and carbohydrate-binding sites [18]. The γ subunits bind the nucleotides by three sites, which are structural basis for this energy sensor. Most importantly, the catalytic features of α subunit and regulatory activity of β and γ subunits are all integrant for AMPK correct and normal activation.

2.2. Activation of AMPK. AMPK is activated mainly by three complementary mechanisms: (1) allosteric activation [41–43]; (2) phosphorylation of α1 Thr183 or α2 Thr172 [25]; and (3) inhibiting dephosphorylation of Thr183 or Thr172 [44].

Mammalian AMPK is sensitive to the changes of AMP/ATP or ADP/ATP. Therefore, any cellular metabolic process that reduced ATP levels or increased AMP/ADP can activate AMPK, such as hypoxia, glucose decrease, mitochondrial oxidative stress, or metabolic inhibition of ATP synthesis [20, 45]. However, Lin and Hardie et al. found that AMPK can be activated through an additional AMP-/ADP-independent mechanism in response to glucose reduction both in vivo and in vitro [31]. They demonstrated that different compartmentalized pools of AMPK are activated through distinct ways, which depends on the extent of elevation of cellular AMP [46]. Low increases in AMP activate AMPK only via the AMP-independent, AXIN-based manner in lysosomes, which is regulated by fructose-1,6-bisphosphate (FBP) levels. When FBP decreases, adolase is released and then interacts with vacuolar-type H + -ATPase (V-ATPase), Ragulator, and AMPK-AXIN-LKB1 and finally becomes a complex and activates AMPK. Mild concentrations of AMP also enlarge this to activate cytosolic AMPK by an AXIN-dependent pathway. By comparison, severe glucose starvation activates all pools of AMPK in the AMP-/ADP-dependent manner rather than AXIN. Researches demonstrated a space-time basis for hierarchical activation of AMPK in various compartments in the process of differing the extents of energy stress [47]. But the question of how the FBP-free status of adolase binds vacuolar-type H + -ATPase (V-ATPase) has not been illuminated. Excitedly, Lin and Hardie et al. recently suggested that transient receptor potential cation channels (TRPVs), in low glucose, relay the adolase to the reconfiguration of v-ATPase, activating AMPK [48]. Although α subunit is catalytic, more and more evidence finds that regulatory β and γ subunits also are essential for AMPK optimum function. For example, N-myristoylation of β subunits is necessary for lysosome location of AMPK complex [31].

Besides allosteric activation, upstream two major AMPK kinases, which are liver kinase B1 (LKB1) [48], also known as serine/threonine kinase 11 (STK11) or renal carcinoma antigen NY-REN-19, and the Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) [49] can regulate AMPKα activity through a phosphorylated manner. Researches reveal phosphorylation of the α subunit can depend on, or independently of, its LKB1 activity. CaMKKβ is activated by intracellular concentration of Ca^{2+} [50, 51]. Thus, stimuli that magnify this, such as bradykinin [52] and thrombin [53], also phosphorylate AMPKα subunit in an AMP-/ADP-independent way owing to increased CaMKKβ activity. It is worth mentioning that ubiquitination modification also regulates AMPKα activation. Zhenkun Lou et al. have found that AMPKα1 or AMPKα2 ubiquitination blocks its phosphorylation by LKB1, which could be rescued by the deubiquitinase ubiquitin specific peptidase 10 (USP10) [54]. Other researchers also have shown that AMPKα2 is ubiquitinated by ubiquitin-conjugating enzyme E2O (UBE2O) in a mouse model of breast cancer, which activates the mammalian target of rapamycin-hypoxia inducible factor 1-α (mTOR-HIF1-α) pathway and triggers cancer growth [55]. Similarly, AMPKα1 is also ubiquitinated and degraded by MAGE-A3/6-TRIM28 E3 ubiquitin ligase complex [56].

Briefly, in the case of replete energy, that is, low AMP/ATP or ADP/ATP, phosphatases can keep AMPKα1 Thr183 or α2 Thr172 in an unphosphorylated state by accessing to it. However, when energy decreases, CBS of the AMPK γ subunit is occupied by AMP or ADP, which prohibits the phosphatases from dephosphorylating Thr183 or Thr172, therefore increasing AMPK activity. It is worth mentioning that unlike AMP, ADP has no conspicuous allosteric effect on AMPK [44, 57].

2.3. Downstream Targets of AMPK. Downstream targets of AMPK mainly include molecules involving glucose, lipid, protein metabolism or inflammation, oxidative stress, and aging process.

During lipid metabolism, once being activated, AMPK as a serine/threonine kinase phosphorylates some crucial molecules that regulate lipid metabolism, such as acetyl-CoA carboxylase (ACC) [58], 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) [42], and sterol regulatory element-binding protein 1c (SREBP1c) [59]. Except for the above-mentioned molecules, evidence has shown that AMPK reduces hepatic steatosis in high-fat, high-sucrose (HFHS) diet-fed mice by interacting with and mediates phosphorylation of insulin-induced gene (Insig), a novel effector of AMPK, which plays a critical role in regulating intracellular cholesterol equilibrium [60]. Furthermore, activated AMPK also stimulates skeletal muscle to uptake glucose by phosphorylating Rab-GTPase-activating protein TBC1 domain family member 4 (TBC1D4), which ultimately induces fusion of glucose transporter type 4 (GLUT-4) vesicles with the plasma membrane [61], and phosphorylates 6-phosphofructo-2-kinase (PFK-2) [62], glycogen, and glycogen synthase to promote glycolysis and inhibit glycogen synthesis. In addition, AMPK suppresses the energy-intensive protein biosynthesis process by phosphorylating tuberous sclerosis complex 2 (TSC2) which regulates activity of mammalian target of rapamycin complex 1(mTORC1) promoting protein synthesis [20, 63]. AMPK regulates autophagy by directly and indirectly activating Unc-51 like autophagy activating kinase (ULK1) [64, 65] and mitochondrial biogenesis by regulating peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) which in turn promotes gene transcription in the mitochondria [66, 67]. AMPK participates in the cellular redox regulation and anti-inflammation response. Hong Li et al. have depicted that the Cys130 and Cys174 of AMPKα is oxidized during energy stress, which could be inhibited by Thioredoxin1 (Trx1) and protects AMPK activation in ischemia [68, 69]. In some inflammatory disease, AMPK also impacts a positive role, such as allergic diseases [68], monosodium urate (MSU) crystal-induced inflammation [70], and synovitis [33]. The process of aging,

involving inflammation, oxidative stress, metabolic disorder, and decrease of autophagic clearance, is of course using AMPK as a supervisor that orchestras all the pathways in order to resist bad effects of senescence [71]. For instance, skeletal muscle AMPK knockdown-aged mice show hypoglycemia and hyperketosis during fasting [72].

3. The Mechanism of Revascularization

After the initiation of ischemia, cardiac or skeletal muscle undergoes a series of molecules and hemodynamical changes triggered by hypoxia-related pathways [10], invasion and infiltration inflammatory cells [73, 74], and cellular metabolism remodeling [75, 76], to promote capillary neogenesis (angiogenesis), or arterioles remodeling (arteriogenesis or collateral circulation), and then eventually to restore blood perfusion of ischemic zones.

Angiogenesis is induced by hypoxia via HIF1-α, which depicts the formation of new capillaries by sprouting or splitting from preexistent vessels, which is different from vasculogenesis [3, 5]. The latter is a process of endothelial cells from mesoderm cell precursors which form primitive tubules during the embryonic phase [10, 77, 78]. The process of angiogenesis is completed mainly by three EC subtypes. (1) Firstly, "tip cells" featured migratory capability sense proangiogenic stimuli, such as VEGF, fibroblast growth factor (FGF), and led the newly forming vessel to sprout towards the source of the proangiogenic stimuli. (2) During the migration of the tip cells, proliferative "stalk cells" lengthen neovessels. When neighbouring vessels' sprouts meet and their tip cells fuse, an interconnected, closed, and functional lumen allowing blood flow is formed. (3) Next, the quiescent "phalanx cells" mature neovessels featured by a typical cobblestone shape. (4) Finally, in order to form a tighter vessel for proper stability and barrier function, pericytes secrete platelet-derived growth factor-B (PDGF-B) and subsequently recruit VSMC expressing PDGF receptor β [79, 80]. Recently, the roles of metabolism remodeling of endothelial cells in angiogenesis are attached by many researchers. For example, Katrien and Yiming Xu et al. have found that endothelial 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3, (PFKFB3) plays a critical role in vessel sprouting and angiogenesis [81, 82].

Arteriogenesis or collateral growth, being different from angiogenesis, is a process that the existing interconnected vascular branches between adjacent blood vessels expand and remodel triggered by FSS, which is induced by increased flow across the collateral bed; when the main coronary artery is occluded, the downstream pressure decreases, resulting in an increased pressure drop and flow velocity across collaterals [83–85]. The basic pathophysiological courses of arteriogenesis contain the following. (1) Endothelial cells sense elevated FSS, which is the initiated step of arteriogenesis formation, by some molecules including Trpv4 [86], actin-binding rho activating protein (Abra) [87], and then change morphology and express multiple genes mainly participating in attracting circulatory blood cells and promoting cells adhesion, such as selectins, chemokine (C-C motif) ligand 2 (CCL2), intercellular adhesion molecules (ICAM), vascular cell adhesion molecules (VCAM-1), and VEGF. (2) The second one is inflammatory cell invasion and infiltration; for example, Florian P. Limbourg et al. suggest that endothelium matures macrophage and controls macrophage differentiation via Notch signaling, which in turn promotes arteriole growth [88], and neutrophils signal is enhanced at early ischemic phase [89]. (3) The third is VSMC proliferation, migration, and phenotypic transformation [6, 7]. Although a considerable number of researches using multifarious animal models have uncovered the signaling pathways of arteriogenesis involving the VEGF, PDGF, NO, and rho-pathway [87, 90], clinical trials are somehow disappointing [91].

4. AMPK in Endothelial Cells

ECs, mostly remaining quiescent throughout adult life, retain the capacity to rapidly form new blood vessels in response to injury or in pathological conditions such as hypoxia, ischemic, and hemodynamic changes. They then can respond with suitable regulatory and control processes to maintain cellular or systematic homeostasis. Such responses contain secretion of angiogenic factors promoting proliferation, migration of ECs, differentiation of endothelial progenitor cells (EPCs), or remodeling of endothelial metabolism.

It is widely believed that ECs prefer generating ATP through oxidative phosphorylation to produce more energy (the ratio of ATP yielded by oxidative phosphorylation and glycolysis is 30 : 2 or 32 : 2). In fact, ECs have a lower mitochondrial content and depend primarily on glycolysis [92]. Although the level of ATP per glucose generated is relatively low, high glycolytic flux can generate more ATP at a faster rate than oxidative phosphorylation when glucose is sufficient and is positioned to shunt glucose into glycolysis side branches to synthesize macromolecule such as the hexosamine and pentose phosphate. More advantages of aerobic glycolysis in ECs may include (1) generating less reactive oxygen species (ROS) by decreasing aerobic oxidation, (2) preserving maximal amounts of oxygen to supply perivascular cells, (3) making ECs adapt hypoxic environment they will grow into, and (4) producing lactate which is a proangiogenic signaling molecule [80, 93–95]. Except for glucose, another fuel source for ECs is fatty acids. Given the fact that it modestly contributes total ATPs in ECs, the exact role of fatty acids in ECs is elusive at present and needs more attention in the future. For example, Ulrike et al. show that fatty acid synthase knockdown ($FASN^{KD}$) in ECs impedes vessel sprouting by reducing proliferation [76]. AMPK, as an energy and embolism gauge, can also phosphorylate key rate-limiting enzymes of the above-mentioned anabolism pathways in ECs, and as such the relationship between the AMPK and the ECs metabolism in angiogenesis still needs to be lucubrated.

For amino acid metabolism, arginine is most broadly studied for its conversion to citrulline and NO. The latter is the essential signaling molecule for endothelial function, which is synthesized by endothelial NO synthase (eNOS). eNOS expression and activity are carefully regulated by

multiple interconnected mechanisms at the transcriptional (binding of transcription factors, DNA methylation), posttranscriptional (primary transcript modifications, mRNA stability, and nucleocytoplasmatic transport), and posttranslational levels (phosphorylation, fatty acid acylation, and protein-protein interactions) [96]. Modification of phosphorylation is vital for eNOS activity. In this moment, AMPK is the only kinase identified that can probably phosphorylate eNOS on more than one site, that is, Ser1177 and Ser633 in the reductase domain and inhibitory Thr495 site in the CaM-binding domain of the enzyme. A body of researches have reported AMPK dependent eNOS phosphorylation (on Ser1177) can proceed the following diverse endothelial cell stimulation, such as peroxisome proliferator-activated receptors (PPAR) agonists, AICAR, metformin, VEGF, and adiponectin. It is worth noting that the effects are usually weaker and much less arresting than other stimulation, like thrombin, hypoxia, and shear stress, which also lead to AMPK activation [97, 98].

5. AMPK in Angiogenesis

The roles of AMPK in angiogenesis have not been clarified and somehow are contradictory. A considerable amount of evidence has shown that AMPK exerts its positive impact on angiogenesis mainly in the metabolic syndrome, ischemia diseases, and hypoxia. That mainly includes four parts. (1) It guarantees energy supply of endothelial cells. (2) AMPK regulates EPCs differentiation, ECs proliferation, and migration [99, 100]. (3) AMPK, acting as an upstream kinase, phosphorylates eNOS to produce NO, facilitating vascular vasodilation and angiogenesis [101]. (4) Activation of AMPK under hypoxic conditions promotes autophagy, which somehow enhances VEGF expression [102]. Some earlier studies report that AMPKα1 impedes anoxia-induced apoptosis [103, 104] and protects against diabetes mellitus-induced vascular injury by improving EPCs function and promoting reendothelialization through upregulation of heme oxygenase-1 and stromal cell-derived factor 1 (SDF1) [105, 106], and dominant negative AMPK mutants inhibit both ECs migration and differentiation in vitro under hypoxia and in vivo angiogenesis [103]. In addition, evidence has demonstrated that LKB1/AMPK improve blood perfusion by inducing angiogenesis in hind limbs ischemic model of mice [102, 107] (Figure 2). At present, protective roles of AMPK in angiogenesis or on ECs or EPCs under some adverse condition, such as anoxia, stroke, senescence, and oxidative stress, have been validated [20, 108], and it also can be stimulated by cytokines or pharmacological agents such as VEGF [109], AICAR [109], metformin [100], berberine [110, 111], and adiponectin [112].

However, other researches have also revealed the passive effects of AMPK on angiogenesis. Evidence has demonstrated that AMPK exerts protective roles on retinopathy. Activated AMPK protects retinal vasculature from edema, hemorrhage, and final retinal detachment by decreasing oxidative stress and inflammation, improving circulation in narrow arterioles, inhibiting angiogenesis [113–116]. Studies have shown that metformin inhibits laser-induced choroidal neovascularization by activating AMPK [117]. Similarly, AMPK, being activated by berberine, can inhibit modified LDL-induced injury of Müller cell [118], which is the major glia of the retina; they are maintaining the blood-retinal barriers (BRBs). In addition, a variety of researches have shown that AMPK activation by many pharmacological activators, such as compound C, metformin, AICAR, curcumin, and simvastatin, inhibits tumor invasion and metastasis via the blockage of angiogenesis [119–122]. Furthermore, antifungal drug itraconazole targets mitochondrial protein voltage-dependent anion channel 1 (VDAC1) to suppress angiogenesis by modulating the AMPK/mTOR signaling axis in endothelial cells [123]. Interestingly, there are some studies which have shown that AMPK activation by some agents may play a positive role in tumor growth, even including metformin [124, 125].

Whether AMPK activation promotes angiogenesis or inhibits it depends on different cellular microenvironment. Generally, activation of AMPK in ischemic or hypoxic conditions facilitates angiogenesis but in tumor microenvironment inhibits it, which is attributed to different pathway activation. For example, under ischemic or hypoxic condition, AMPK activation has a positive effect on autophagy by inhibiting mTOR and phosphorylating autophagy modulators [126]. Autophagy somehow stabilizes HIF-1α, which regulates VEGF and other angiogenic molecules, and promotes angiogenesis [127]. The signal pathway of mTOR-HIF-1α-VEGF is activated in cancer cells; metformin or other AMPK activators can impede them, inhibiting angiogenesis [128].

6. AMPK in Arteriogenesis

So far, there is not much evidence on the role of AMPK in arteriogenesis and the ones that exist are inconsistent. One line of evidence shows that AMPKα1(–/–) can impair adult arteriogenesis in that it reduces accumulation of macrophages in ischemic hindlimb and inhibits the expression of growth factors in macrophages [15]. However, another has shown that mitochondrial oxidative stress impedes coronary collateral growth in lean rats in response to repetitive ischemia through activating AMPK and consequently inhibiting mTOR signaling, which is necessary for new protein synthesis and phenotypic switching of endothelial cells [17]. These two cases hint that the effects of AMPK in arteriogenesis under different physiological or pathological circumstances need to be developed further. Researches have shown that FSS, as a key factor which promotes opening and remodeling of collateral circulation, could influence activity of AMPK. For example, Wei Yi et al. have found that FSS can impede the survival and increase the apoptosis of bone marrow mesenchymal stem cells (BMSCs), which partly is attributed to the decrease of AMPK phosphorylation [129, 130]. What is more, exercise, also as an important element for arteriogenesis [131], has been found to play a positive role in AMPK activation. Young has verified that, in physiological condition, rat cardiac AMPK activity increases progressively with exercise intensity [132]. More importantly, Ferguson has also found that interval and continuous

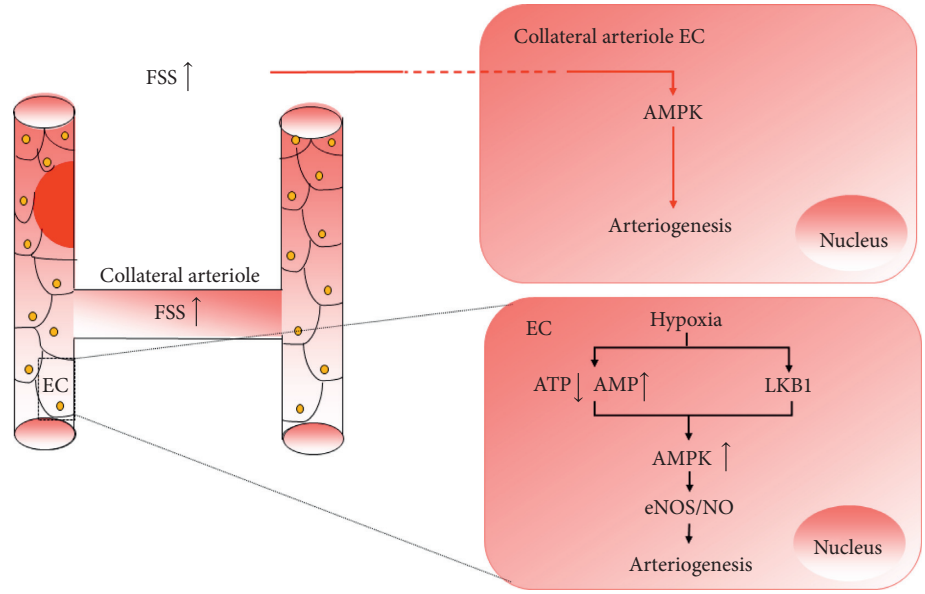

Figure 2: The roles of AMPK in revascularization. After vessels are occluded, remote tissues suffer ischemia and hypoxia, the blood perfusion of collateral arterioles increases, and the FSS is elevated. FSS and hypoxia activate AMPK by different or the same ways.

sprint cycling promotes phosphorylation of human skeletal muscle AMPK αThr172 [133] (Figure 2).

7. Prospect

AMPK, as a key modulator of cellular energy, metabolism, and oxidative-redox homeostasis, plays a complicated regulatory role in the ECs. When AMPK is activated by elevated ratio of AMP/ATP or ADP/ATP, ROS, cytokines, or agents, the kinase will promote catalysis pathways, such as glycolysis, inhibit analysis pathways, such as glycogen or protein synthesis, and regulate inflammatory process and oxidative stress, through phosphorylation of some crucial enzymes such as eNOS, FASN, ACC, PFK-2, mTORC1, and ULK1. Although AMPK also participates in regulating revascularization, the effect of AMPK is contradictory. Generally, activated AMPK promotes angiogenesis in ischemia whereas inhibiting angiogenesis under retinopathy or tumor microenvironment. The role of AMPK during arteriogenesis also is double-faced, which is attributed to different intracellular or extracellular circumstances. Global knockout of AMPKα1 and macrophage-specific knockout mice, which are subjected to hindlimb ischemia brought about by femoral artery ligation, impairs adult arteriogenesis so that it reduces perfusion to the lower limb. However, if cells suffer mitochondrial oxidative stress, activated AMPK does not promote collateral growth; on the contrary, it suppresses arteriole opening or remodeling. As mentioned previously, although up until this moment there is no enough evidence that has shown the definite role of AMPK in arteriogenesis; given that both FSS and exercise also regulate AMPK phosphorylation, it is still worthy of exploring AMPK function in collateral circulation. What is more, AMPK, as a heterotrimeric protein complex, so far, has had many studies focus on the function of AMPK phosphorylation, while the role of other posttranslational modifications in revascularization need to be illuminated, such as ubiquitination, acetylization, and glycosylation. Different isoforms of AMPK may influence this process.

Abbreviations

Abra:	Actin-binding rho activating protein
ACC:	Phosphorylates acetyl-CoA carboxylase
AMPK:	5′-Adenosine monophosphate-activated protein kinase
BMSCs:	Bone marrow mesenchymal stem cells
BRBs:	Blood-retinal barriers
CABG:	Coronary artery bypass grafting
CaMKKβ:	Ca^{2+}/calmodulin-dependent protein kinase kinase β
CCL2:	Chemokine (C-C motif) ligand 2
CHD:	Coronary heart disease
CVDs:	Cardiovascular diseases
eNOS:	Endothelial NO synthase
ECs:	Endothelial cells
EPCs:	Endothelial progenitor cells
FASN:	Fatty acid synthase
FBP:	Fructose 1,6-bisphosphate (FBP)
FBPase-2:	6-Phosphofructo-2-kinase
FGF:	Fibroblast growth factor
FSS:	Fluid shear stress
GLUT-4:	Glucose transporter type 4
HIF1-α:	Hypoxia inducible factor 1-α
HMG-CoA reductase:	3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase
HNF4:	Hepatocyte nuclear factor 4
ICAM:	Intercellular adhesion molecules
Insig:	Insulin-induced gene
LKB1:	Liver kinase B1
MSU:	Monosodium urate
mTORC1:	Rapamycin complex 1

NO: Nitric oxide
PCI: Coronary artery intervention
PDGF: Platelet-derived growth factor
PFKFB3: Fructose-2,6-bisphosphatase, isoform 3
PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPAR: Peroxisome proliferator-activated receptors
ROS: Reactive oxygen species
SDF1: Stromal cell-derived factor 1
SREBP1c: Sterol regulatory element-binding protein 1c
STK11: Serine/threonine kinase 11
TBC1D4: TBC1 domain family member 4
TRPVs: Transient receptor potential cation channels
Trx1: Thioredoxin1
TSC2: Tuberous sclerosis complex 2
UBE2O: Ubiquitin-conjugating enzyme E2O
ULK1: Unc-51 like autophagy activating kinase
USP10: deubiquitinase ubiquitin specific peptidase 10
V-ATPase: Vacuolar-type H + -ATPase
VCAM-1: Vascular cell adhesion molecules
VDAC1: Voltage-dependent anion channel 1
VEGF: Vascular endothelial growth factor
VSMCs: Vascular smooth muscle cells.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Research Article

Reconstruction of a lncRNA-Associated ceRNA Network in Endothelial Cells under Circumferential Stress

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Background. Numerous studies have highlighted that long noncoding RNA (lncRNA) can indirectly regulate the expression of mRNAs by binding to microRNA (miRNA). LncRNA-associated ceRNA networks play a vital role in the initiation and progression of several pathological mechanisms. However, the lncRNA-miRNA-mRNA ceRNA network in endothelial cells under cyclic stretch is seldom studied. *Methods*. The miRNA, mRNA, and lncRNA expression profiles of 6 human umbilical vein endothelial cells (HUVECs) under circumferential stress were obtained by next-generation sequencing (NGS). We identified the differential expression of miRNAs, mRNAs, and lncRNAs using the R software package GDCRNATools. Cytoscape was adopted to construct a lncRNA-miRNA-mRNA ceRNA network. In addition, through GO and KEGG pathway annotations, we analyzed gene functions and their related pathways. We also adopted ELISA and TUNEL to investigate the effect of si-NEAT1 on endothelial inflammation and apoptosis. *Results*. We recognized a total of 32978 lncRNAs, 1046 miRNAs, and 31958 mRNAs in 6 samples; among them, 155 different expressed lncRNAs, 74 different expressed miRNAs, and 960 different mRNAs were adopted. Based on the established theory, the ceRNA network was composed of 13 lncRNAs, 44 miRNAs, and 115 mRNAs. We constructed and visualized a lncRNA-miRNA-mRNA network, and the top 20 nodes are identified after calculating their degrees. The nodes with most degrees in three kinds of RNAs are hsa-miR-4739, NEAT1, and MAP3K2. Functional analysis showed that different biological processes enriched in biological regulation, response to stimulus and cell communication. Pathway analysis was mainly enriched in longevity regulating, cell cycle, mTOR, and FoxO signaling pathway. Circumferential stress can significantly downregulate NEAT1, and after transducing si-NEAT1 for 24 h, inflammatory cytokine IL-6 and MCP-1 were significantly increased; furthermore, fewer TUNEL-positive cells were found in the si-NEAT1 treated group. *Conclusions*. The establishing of a ceRNA network can help further understand the mechanism of vein graft failure. Our data demonstrated that NEAT1 may be a core factor among the mechanical stress factors and that cyclic stress can significantly reduce expression of NEAT1, give rise to inflammation in the early stage of endothelial dysfunction, and promote EC apoptosis, which may play an essential role in vein graft failure.

1. Introduction

Coronary Artery Bypass Grafting (CABG) is an economical and effective treatment for most cases of multiple or left main coronary arteries [1]. During the surgery, the patient's autogenous vein is one of the most commonly used conduits for bypass grafting. However, given that vascular smooth muscle cell (VSMC) could give rise to abnormal neointimal hyperplasia of the veins, as many as 50% of CABG operations end up in failure [2]. Jeremy et al. reported that the

reasons for vein graft failure involve physical, chemical, and biological factors, including the adhesion of platelets and white blood cells, the change of hemodynamics, activated matrix metalloproteinases and excessive release of platelet-derived growth factor-BB and thrombin, and the superposition effect of atherosclerosis [3–6]. After the vein was transplanted from the venous system to the arterial system, the inflammation and apoptosis of ECs could be induced by changing the mechanical environment, which plays an important role in several pathological processes, including intimal hyperplasia, atherosclerosis, and occlusion [7]. So far, no ideal method has been found to prevent and deal with such adverse events. Wadey et al. observed the different responses between arterial and vein endothelial cells to blood flow and attributed to the different responses to the epigenetic memory of vastly different hemodynamic environments [8].

A plethora of evidence has shown that miRNAs are involved in the pathogenesis of vein graft failure. Previous studies have shown that the expression of miR-21 was significantly increased in various vascular injury models, and the downregulated miR-21 expression serves to improve the intimal thickening of arterial vessels after grafting [9, 10]. MiR-145 exhibits an inhibitory effect on the proliferation, migration, and phenotype transformation of smooth muscle [11]. Ohnaka et al. conducted a nonvirus transfection of miR-145 and observed that the transplanted vein neointimal thickening was suppressed in rabbits [12]. Jan Fiedler et al. observed that higher expression of miR-24 in the myocardial infarction (MI) model promoted endothelial cell apoptosis and that suppressed miR-24 expression using local transfection adenovirus promoted the formation of new veins, thereby improving blood perfusion [13]. MiR-92a was reported to play the part of inhibiting the proliferation and migration of EC cells; the downregulated miR-92a expression using the antagomir method led to improved vascular endothelialization after balloon injury and reduced intimal thickening [14].

LncRNAs could competitively combine with the miRNA response element (MRE) to repress miRNA's regulation of target mRNAs. Such a lncRNA-miRNA-mRNA competing endogenous RNA (ceRNA) network has been demonstrated in several diseases, yet its application in vein graft failure is to be clarified. Therefore, it is essential to detect the ceRNA coregulatory of ECs suffering from cyclic stress by conducting bioinformatic analysis, which is of great help to construct the systematic regulatory network and to explore the correlation between the main biomarkers.

2. Materials and Methods

2.1. Cyclic Stretch Stimulation. Human umbilical vein endothelial cells (HUVECs, ScienCell Research Laboratories, San Diego, CA, USA) were maintained in endothelial cell medium (ECM, ScienCell Research Laboratories, San Diego, CA, USA) with 10% fetal bovine serum (FBS, ThermoFisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2. The ECs were then plated on the collagen-coated plates (Flexcell International Corporation, McKeesport, PA, USA). We adopted a computer-controlled circumferential stress unit (Flexcell 5100, Flexcell International Corporation, McKeesport, PA) to compose cyclic stretch to HUVEC for 24 hours including a condition of cyclic deformation at 60 cycles/min and elongation at 18%. The control group was maintained in the 6-well plate under the same condition but without mechanical stretch.

2.2. RNA Isolation. The HUVECs in two groups were harvested after 24 hours. Total RNA was obtained from ECs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. In brief, cells were lysed in the Eppendorf tubes using TRIZOL reagent, and then RNA was separated and precipitated, finally, the total RNA will be dissolved in DEPC-treated water for further experiments. The RNA concentration and purity were checked using NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) with criteria of OD A260/A280 (>1.8) and A260/A230 (>1.6), Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was adopted to access the yield and quality by RIN >7.0, and gDNA contamination was evaluated by gel electrophoresis.

2.3. RNA Library Preparation and Sequencing. Ribo-Zero Magnetic Gold Kit (Illumina, San Diego, CA, USA) and NEBNext RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA) were adopted to prepare the whole transcriptome libraries. Quality control and quantification was done by using the BioAnalyzer 2100 system (Kapa Biosystems, Woburn, MA, USA). The resulting libraries were sequenced on a HiSeq2000 instrument (Illumina, San Diego, CA, USA), and we used approximately 1 μg total RNA to prepare an RNA library according to the protocol. Then, we performed the single-end sequencing (50 bp) on Hiseq2500 (Illumina, San Diego, CA, USA) following the vendor's recommended protocol. We used FastQC software to check for potential sequencing issues and contaminants in the raw sequencing reads. Reads with quality scores below 30, adapter sequences, and primers were trimmed. Reads with a length of <60 bp were discarded sequently. We used TopHat 2.0 to align sequence reads to the human genome (GRCh38), and the results were reconstructed with Cufflinks. All transcriptomes were pooled and merged to generate a final transcriptome using Cuffmerge. After the final transcriptome was produced, Cuffdiff was used to estimate the abundance of all transcripts based on the final transcriptome. For mRNA and lncRNA analyses, the RefSeq and Ensembl transcript databases were chosen as the annotation references. We used the Coding Potential Calculator (CPC) [15] to predict transcripts with coding potential. The transcripts that remained were considered reliably expressed lncRNAs. As for miRNAs, reads with a length <10 nt and >34 nt were discarded. The clean reads were aligned against the miRNA precursor of *Homo sapiens* and other species in miRBase 22.1 [16] to identify known miRNAs. The unannotated sequences were mapped to the human genome to analyze their expression and distribution

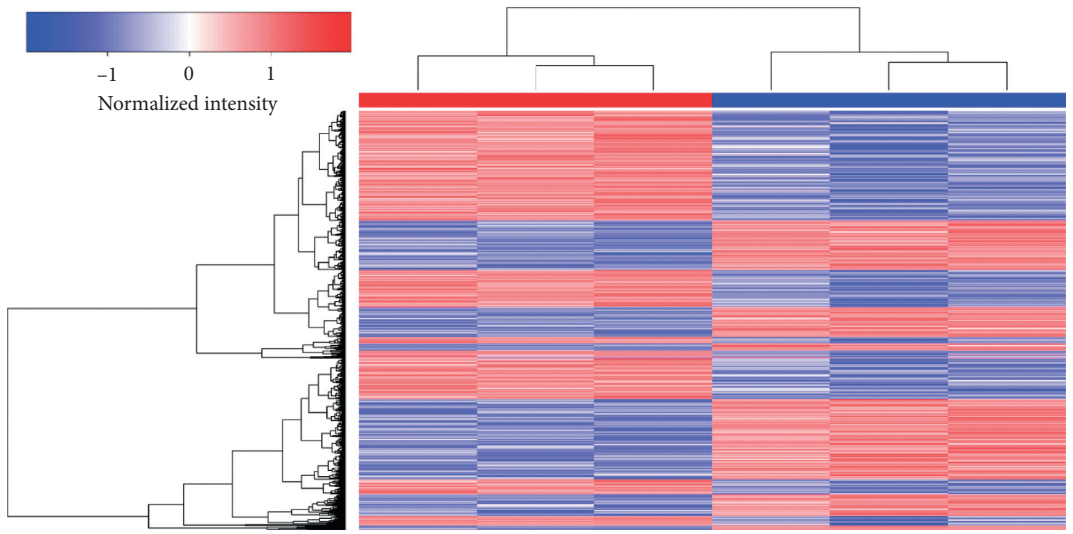

Figure 1: RNA profiling in HUVECs with and without mechanical stretch stimulation. Red boxes represent upregulated genes and blue boxes represent downregulated genes. Each group has 3 replicates.

in the genome and then used to predict potential novel miRNA candidates by the Mireap program.

2.4. Construction of lncRNA-miRNA-mRNA ceRNA Network. The clean reads of 6 EC samples were imported and analyzed by R 3.6 software after transformation. We adopted a R package of GDCRNATools to analyze the sequencing data. It is a novel R package for integrative analysis of RNA-seq data, and it allows users to conduct ceRNA networks and other routine analyses based on online databases [17]. In short, lncRNA, miRNA, and mRNA expression profiles of fold change (FC) ≥1.5 and P value ≤0.05 were retained. To construct the ceRNA network, we predicted miRNA-mRNA and lncRNA-miRNA interactions based on starbase V3.0 [18], miRcode [19], and miRTarBase [20] based on our sequencing data; then, according to ceRNA hypothesis, the miRNAs negatively regulated by lncRNAs and its downregulated target mRNAs were selected, and the common miRNAs interacting with both lncRNAs and mRNAs were seen as an inclusion criteria. After that, the lncRNA-associated ceRNA network was reconstructed and visualized using Cytoscape software V3.5 (San Diego, CA, USA) based on the R output. We used different colors and shapes to represent the three types of RNA, respectively, and all node degrees were calculated simultaneously using the software plugin CytoHubba.

2.5. Functional Enrichment Analysis. To understand the potential regulative role of the lncRNA-miRNA-mRNA network, we used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resources [21] and WEB-based gene set analysis toolkit (Webgestalt) [22]; to enrich the downstream mRNA to molecular functions and pathways, we enriched KEGG pathways using Wedgestalt, and each pathway was listed and ranked by their enrichment ratio. Furthermore, GO analysis was carried out using the DAVID database, molecular functions, and biological processes, and cellular component of the differentially expressed genes were elucidated.

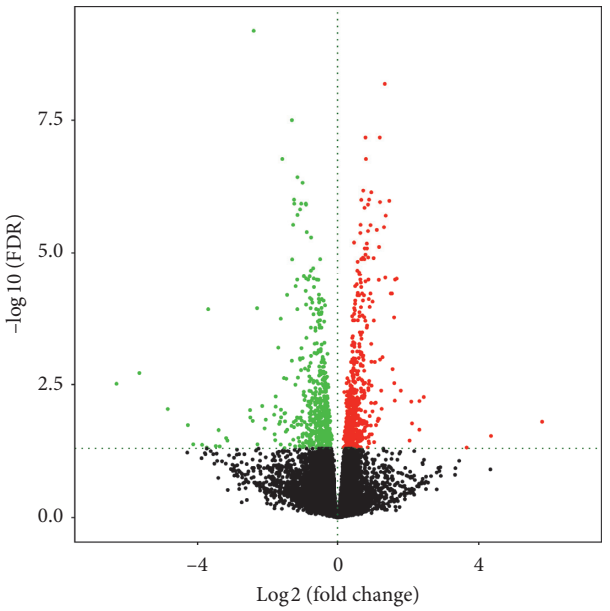

Figure 2: Volcano plot of the differently expressed RNAs. Upregulated genes are marked in light red; downregulated genes are marked in light green. FC ≥ 1.5 and P value ≤0.05 were chosen as selective criteria, 155 different expressed lncRNAs, 74 different expressed miRNAs, and 960 different mRNAs were identified.

2.6. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Validation. To validate the hub gene that we identified in the bioinformatics analysis, qRT-PCR was performed using the riboSCRIPT mRNA/lncRNA qRT-PCR starter kit (RIBO bio, Guangzhou, China) under instructions. Briefly, total RNA was isolated using TRIzol as previously described, then RNA was reverse transcripted to cDNA using random primer and Oligo (dT), and then qPCR was performed using the three-step method. Relative quantification of lncRNA was calculated using the 2-ΔΔCt method and was normalized to GAPDH as a reference. The template sequence for qRT-PCR

Table 1: Top 40 differently expressed lncRNAs in sequencing analysis.

Gene ID	Gene symbol	P	FC	FDR	Regulation
ENSG00000268575	AL031282.2	0.00062	5.79268	0.01580	Up
ENSG00000237813	AC002066.1	0.00341	3.65619	0.04825	Up
ENSG00000234378	AC098828.3	0.00016	2.08850	0.00658	Up
ENSG00000247095	MIR210HG	$8.07E-05$	1.79213	0.00410	Up
ENSG00000231412	AC005392.2	$5.17E-05$	1.60687	0.00297	Up
ENSG00000275216	AL161431.1	$3.17E-09$	1.36434	$1.98E-06$	Up
ENSG00000268621	IGFL2-AS1	0.00059	1.13821	0.01523	Up
ENSG00000261040	WFDC21P	0.00145	1.96944	0.02708	Up
ENSG00000235852	AC005540.1	$7.91E-10$	1.89379	$9.91E-07$	Up
ENSG00000225855	RUSC1-AS1	$5.50E-05$	1.88656	0.00308	Up
ENSG00000258667	HIF1A-AS2	0.00081	1.84409	0.01852	Up
ENSG00000261780	AC105243.1	$1.99E-08$	1.83428	$8.21E-06$	Up
ENSG00000241316	SUCLG2-AS1	$1.58E-07$	1.79216	$3.62E-05$	Up
ENSG00000269926	DDIT4-AS1	$3.87E-08$	1.76033	$1.31E-05$	Up
ENSG00000228437	LINC02474	$1.87E-05$	1.71344	0.00141	Up
ENSG00000231721	LINC-PINT	$3.13E-07$	1.69794	$6.14E-05$	Up
ENSG00000223901	AP001469.1	0.00123	1.69227	0.02438	Up
ENSG00000167046	AL357033.1	$2.17E-07$	1.67088	$4.62E-05$	Up
ENSG00000214049	UCA1	0.00172	1.66915	0.03007	Up
ENSG00000235904	RBMS3-AS3	$1.30E-07$	1.64632	$3.35E-05$	Up
ENSG00000230552	AC092162.2	$2.81E-05$	−5.64846	0.00191	Down
ENSG00000248802	AC078850.2	0.00027	−4.84467	0.00909	Down
ENSG00000234871	LINC01032	0.00285	−3.87282	0.04276	Down
ENSG00000272482	AC254633.1	$8.34E-07$	−3.69988	0.00012	Down
ENSG00000259437	AC093334.1	0.00308	−3.48527	0.04517	Down
ENSG00000227848	SUCLA2-AS1	0.00314	−3.37469	0.04590	Down
ENSG00000258561	AL359232.1	0.00047	−2.50748	0.01311	Down
ENSG00000225339	AL354740.1	$7.71E-07$	−2.31612	0.00012	Down
ENSG00000233818	AP000695.2	0.00022	−2.17093	0.00797	Down
ENSG00000255864	AC069208.1	$6.51E-06$	−1.71555	0.00064	Down
ENSG00000258969	LINC02307	0.00028	−1.64003	0.00936	Down
ENSG00000257114	LINC02450	0.00036	−1.54184	0.01101	Down
ENSG00000227925	LINC01655	$3.40E-07$	−1.46588	$6.51E-05$	Down
ENSG00000275119	AC244131.2	0.00281	−1.38628	0.04225	Down
ENSG00000259635	AC100830.1	0.00085	−1.33988	0.01888	Down
ENSG00000225791	TRAM2-AS1	0.00091	−1.27027	0.01999	Down
ENSG00000238042	LINC02257	$7.40E-10$	−1.26935	$9.91E-07$	Down
ENSG00000261094	AC007066.2	0.00312	−1.23834	0.04567	Down
ENSG00000257176	AC009318.1	0.00047	−1.18344	0.01311	Down
ENSG00000226370	LINC00375	0.00076	−1.17222	0.01790	Down

is NEAT1 5′-GGCAGGTCTAGTTTGGGCAT-3′; 5′-CCTCATCCCTCCCAGTACCA-3′; GAPDH 5′-CATGGCCTTCCGTGTTCCTA-3′; 5′-CGCCTCCTTTTCCTCTCAT-3′.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). The levels of inflammatory cytokines secreted by endothelial cells transduced with si-NEAT1 (RIBO bio, Guangzhou, China) and control reagents were measured by ELISA. We transduced the si-NEAT1 into endothelial cells after 3-4 passages using Lipofectamine 3000 Reagent (ThermoFisher Scientific, Waltham, MA, USA) according to the established protocol. After that, ELISA was performed on a 96-well plate using ELISA kits (BD Biosciences, San Diego, CA) according to the manufacturer's protocol. We used a spectrophotometer to measure the absorbance at a wavelength of 450 nm. Several common Inflammatory cytokines including IL-6, MCP-1, and ICAM-1 were measured to confirm the effect of downregulating NEAT1 on endothelial inflammatory.

2.8. TUNEL and DAPI Staining. To determine the effect on apoptosis after downregulating NEAT1, we performed TUNEL assay on the endothelial cells described above using the In Situ Cell Death Detection kit (Roche, Mannheim, Germany) according to the product's instructions. The cell slide was fixed in 4% paraformaldehyde at room temperature for 20 mins and washed using PBS for 3 times. After that, we used 1% Triton X-100 to increase cell permeability. The fixed cells were incubated with terminal deoxynucleotide transferase recombinant (rTdT)-catalyzed reaction mixture for 30 min at room temperature. Then, we used Streptavidin-FITC to label the apoptosis cells and DAPI to illustrate the nuclei. Apoptotic cells were photographed under a fluorescence microscope at an excitation wavelength of 450 nm and emission wavelength of 515 nm.

2.9. Statistical Analysis. We used SPSS version 23.0 and R version 3.6 to analyze the sequencing data, Student's t-tests

Table 2: Top 40 differently expressed miRNAs in sequencing analysis.

Gene symbol	P	FC	Average expression	Regulation
hsa-miR-151a-5p	0.00830	4.967587	11.843500	Up
hsa-miR-92a-3p	0.00552	4.717289	13.551630	Up
hsa-miR-148b-3p	0.0498	4.493670	12.211111	Up
hsa-miR-21-5p	0.00539	4.433062	16.684564	Up
hsa-let-7i-5p	0.00758	4.169530	11.920628	Up
hsa-miR-30a-5p	0.00624	4.151897	12.066384	Up
hsa-miR-10a-5p	0.00737	3.555440	14.343647	Up
hsa-miR-99b-5p	0.01085	3.496305	14.620504	Up
hsa-let-7f-5p	0.00700	2.995222	11.353220	Up
hsa-miR-222-3p	0.01295	2.850281	10.920330	Up
hsa-miR-4284	0.00777	2.654383	0.863570	Up
hsa-miR-374a-5p	0.01244	2.405461	5.723251	Up
hsa-miR-27b-3p	0.00747	2.125160	11.039688	Up
hsa-miR-18a-5p	0.00708	1.873955	3.569991	Up
hsa-miR-96-5p	0.01107	1.783202	7.315302	Up
hsa-miR-651-5p	0.0435	1.776173	2.523913	Up
hsa-miR-20a-5p	0.01147	1.774804	7.238996	Up
hsa-miR-4521	0.0355	1.773481	7.092690	Up
hsa-miR-210-5p	0.00520	1.502487	2.287306	Up
hsa-miR-10a-3p	0.01273	1.431455	7.732293	Up
hsa-miR-100-5p	0.00749	−4.78274	17.89078	Down
hsa-miR-223-3p	0.00605	−4.61353	0.45163	Down
hsa-miR-144-3p	0.03854	−4.05538	0.32115	Down
hsa-miR-142-5p	0.04626	−3.43605	0.83274	Down
hsa-miR-4772-5p	0.00895	−3.15733	0.86166	Down
hsa-let-7a-5p	0.00921	−2.71715	13.60388	Down
hsa-miR-199a-5p	0.03156	−2.67627	1.37259	Down
hsa-miR-451a	0.04254	−2.37644	2.03836	Down
hsa-miR-3617-5p	0.01273	−2.00633	0.13456	Down
hsa-miR-4443	0.00648	−1.68771	0.85807	Down
hsa-miR-23a-5p	0.00518	−1.54230	1.53357	Down
hsa-miR-3940-3p	0.00762	−1.47825	1.12245	Down
hsa-miR-143-3p	0.00738	−1.47642	4.87572	Down
hsa-miR-191-3p	0.00607	−1.44144	1.31566	Down
hsa-miR-148a-5p	0.00701	−1.33610	1.32972	Down
hsa-miR-7976	0.00962	−1.29245	4.13996	Down
hsa-miR-185-3p	0.01159	−1.27379	1.12633	Down
hsa-miR-27b-5p	0.00107	−1.26108	3.56518	Down
hsa-miR-105-5p	0.01158	−1.25366	1.08368	Down
hsa-miR-576-3p	0.00686	−1.16215	4.20788	Down

were adopted to compare the difference between two groups, and repeated measures were tested by one-way analysis. $P < 0.05$ was the threshold to be statistically significant. In addition, we use fold changes and Student's t-tests to identify specific RNAs in the sequencing results. The ncRNAs and mRNAs with FC ≥ 1.5 and $P < 0.05$ were considered as differentially expressed.

3. Results

3.1. Altered lncRNA, miRNA, and mRNA Expression in the Endothelial Cells. The expression of lncRNAs, miRNAs, and mRNAs in the endothelial cells under cyclic stretch for 24 hours was profiled using RNA sequencing. After processing the raw data, we identified a total of 32978 lncRNAs, 1046 miRNAs, and 31958 mRNAs in 6 samples of endothelial cells compared with the established databases. We adopted FC ≥ 1.5 and P value ≤ 0.05 as selective criteria; after screening, we got 155 different expressed lncRNAs, 74 different expressed miRNAs, and 960 different mRNAs, among them, 39 lncRNAs, 35 miRNAs, and 568 mRNAs were upregulated, while 116 lncRNAs, 39 miRNAs, and 392 mRNAs were downregulated. The most upregulated were AL031282.2, hsa-miR-151a-5p, and SSUH2; by contrast, the most downregulated were AC092162.2, hsa-miR-100-5p, and RAD21L1. Expression profiles are depicted as heatmap (Figure 1) and dot plot (Figure 2) after normalization; additionally, the top 20 upregulated and downregulated members of each kind of RNAs are listed in Tables 1–3. These results suggest that circumferential stress may vary transcriptional regulation in human endothelial cells.

3.2. Reconstruction of lncRNA-Associated ceRNA Network. To evaluate whether ceRNA is involved in endothelial cells after stretched, we combined the data acquired in the online database with the data above and constructed a lncRNA-miRNA-mRNA network (Figure 3) based on the ceRNA

Table 3: Top 40 differently expressed mRNAs in sequencing analysis.

Gene ID	Gene symbol	P	FC	FDR	Regulation
ENSG00000125046	SSUH2	0.00164	4.34497	0.02918	Up
ENSG00000187134	AKR1C1	0.00012	2.44192	0.00543	Up
ENSG00000135373	EHF	0.00016	2.31835	0.00644	Up
ENSG00000231924	PSG1	0.00105	2.31706	0.02229	Up
ENSG00000145358	DDIT4L	0.00070	2.10851	0.01696	Up
ENSG00000182957	SPATA13	0.00221	2.04019	0.03561	Up
ENSG00000147872	PLIN2	$1.22E-07$	1.67389	$3.22E-05$	Up
ENSG00000004799	PDK4	$1.35E-07$	1.63004	$3.35E-05$	Up
ENSG00000173237	C11orf86	0.00016	1.62141	0.00633	Up
ENSG00000100292	HMOX1	$1.34E-06$	1.60182	0.00017	Up
ENSG00000178150	ZNF114	$2.22E-05$	1.55896	0.00162	Up
ENSG00000109846	CRYAB	$3.12E-07$	1.54027	$6.14E-05$	Up
ENSG00000114268	PFKFB4	$3.11E-07$	1.50759	$6.14E-05$	Up
ENSG00000087086	FTL	$9.60E-10$	1.46587	$1.04E-06$	Up
ENSG00000120738	EGR1	$1.11E-07$	1.35264	$3.07E-05$	Up
ENSG00000167996	FTH1	$8.01E-13$	1.33776	$6.52E-09$	Up
ENSG00000163347	CLDN1	$6.03E-09$	1.31604	$3.27E-06$	Up
ENSG00000132196	HSD17B7	0.00027	1.24930	0.00902	Up
ENSG00000128510	CPA4	$8.09E-05$	1.23659	0.00410	Up
ENSG00000104419	NDRG1	$1.08E-09$	1.20327	$1.10E-06$	Up
ENSG00000244588	RAD21L1	$5.43E-05$	−6.29595	0.00305	Down
ENSG00000268434	AC011530.1	0.00276	−4.12341	0.04191	Down
ENSG00000198049	AVPR1B	0.00341	−3.74115	0.04818	Down
ENSG00000269955	C7orf55	0.00108	−3.40469	0.02263	Down
ENSG00000140807	NKD1	0.00221	−3.15605	0.03561	Down
ENSG00000112139	MDGA1	0.00029	−2.51469	0.00953	Down
ENSG00000118557	PMFBP1	0.00058	−2.43974	0.01516	Down
ENSG00000162496	DHRS3	$4.02E-14$	−2.41244	0.00000	Down
ENSG00000141469	SLC14A1	0.00097	−2.10327	0.02102	Down
ENSG00000102174	PHEX	0.00054	−2.06964	0.01443	Down
ENSG00000214279	SCART1	0.00039	−1.83248	0.01167	Down
ENSG00000065320	NTN1	0.00145	−1.81924	0.02707	Down
ENSG00000260851	AC010542.3	0.00214	−1.80247	0.03497	Down
ENSG00000273167	AL359736.1	0.00024	−1.78956	0.00822	Down
ENSG00000175093	SPSB4	0.00012	−1.78920	0.00528	Down
ENSG00000054219	LY75	0.00072	−1.68978	0.01736	Down
ENSG00000171189	GRIK1	0.00063	−1.66405	0.01580	Down
ENSG00000104728	ARHGEF10	0.00280	−1.64612	0.04222	Down
ENSG00000134955	SLC37A2	$1.42E-06$	−1.64513	0.00018	Down
ENSG00000135205	CCDC146	0.00046	−1.63602	0.01302	Down

hypothesis. We identified a total of 13 lncRNAs, 44 miRNAs, and 115 mRNAs as targets in the ceRNA network. In the network, lncRNAs and miRNAs were binding with mRNAs competitively, the cyclic stretch downregulated lncRNAs, thus increased miRNAs and targeted mRNAs indirectly. For example, lncRNA ASCC3 was downregulated, while miR-4728-5p was upregulated, resulting in the decline of Notch2. These data indicated that lncRNA could regulate the expression of mRNA by interacting with miRNA competitively and plays a regulatory role in transcription signals under circumferential stress.

3.3. GO and KEGG Analysis of Differentially Expressed mRNAs. To annotate functions of the mRNAs in the ceRNA network, we performed GO and KEGG enrichment using David tools and WEB-based gene set analysis. GO analysis indicated that most of the genes were involved in biological regulation, metabolic process, and response to stimulus and located in nucleus and membrane; furthermore, the majority of genes were related to protein binding as molecular function (Figure 4), indicating their pivotal role in transcriptional regulation. Meanwhile, KEGG pathway analysis showed that several pathways were associated with the differentially expressed mRNAs including longevity regulating, cell cycle, mTOR signal, FoxO signal, and MAPK signal (Figure 5), which suggests that they may be involved in proliferation and inflammation in endothelial cells.

3.4. Identification of lncRNA NEAT1-Associated Subnetwork. To identify the hub RNAs and their related networks, we calculated the degrees of each node in the ceRNA network using Cytoscape plugin cytoHubba, and the top 20 nodes were ranked by degrees as shown in Table 4. It showed that lncRNA NEAT1 is among the top-ranked nodes, indicating

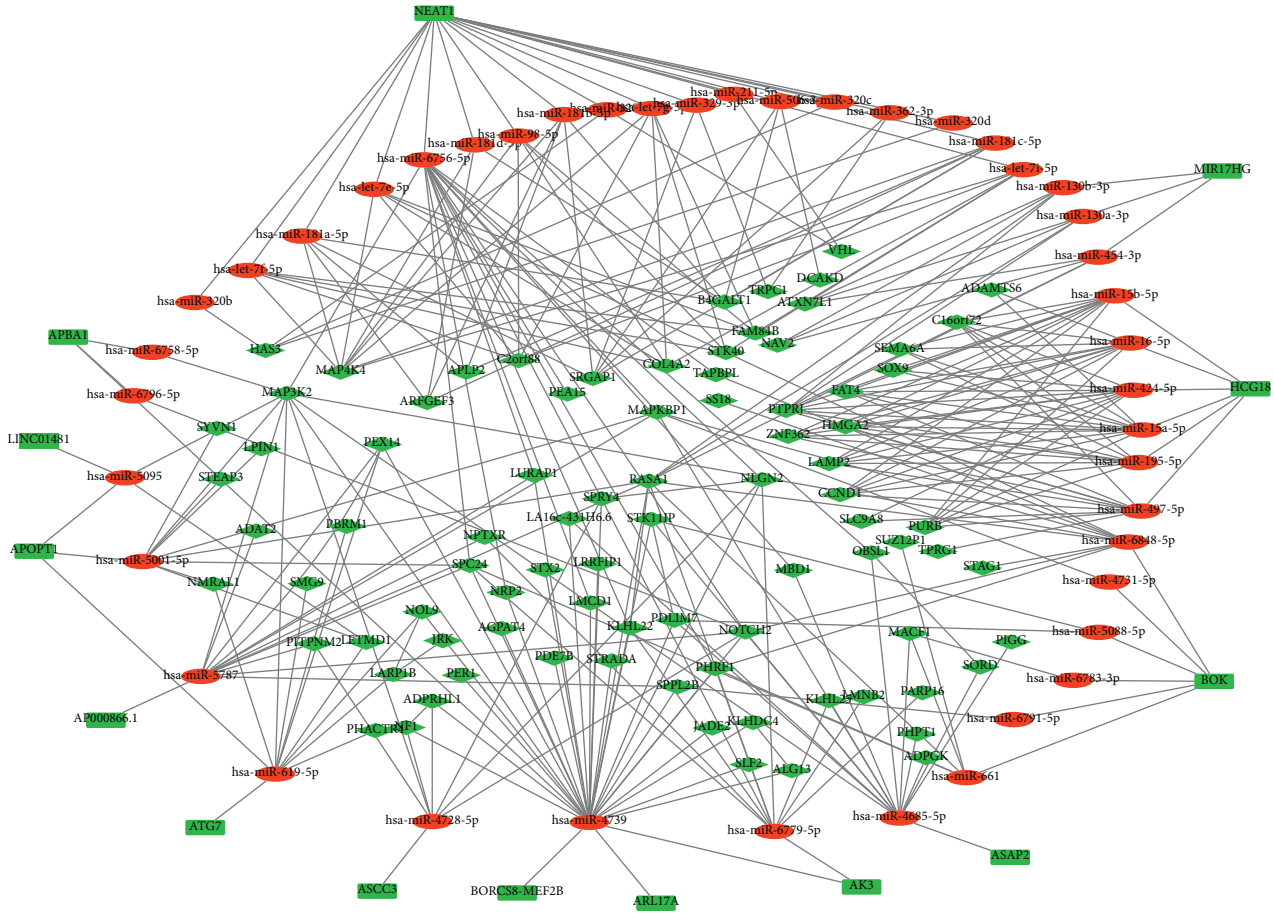

Figure 3: The lncRNA-miRNA-mRNA competing endogenous RNA network in HUVECs stimulated by cyclic stretch. The rectangles indicate lncRNAs in green, ellipses represent miRNAs in red, and diamonds represent mRNAs in light red.

its important role in transcriptional regulation. Therefore, we extracted a subnetwork of lncRNA NEAT1-miRNA-mRNA out of the ceRNA network, which was composed of 1 lncRNA node, 17 miRNA nodes, 13 mRNA nodes, and 67 edges (Figure 6), and it may be the center of the whole ceRNA network. Several RNAs that had been broadly studied such as miR-let7 cluster and MAP4K4 are involved in the subnetwork, suggesting that the subnetwork may regulate biological processes through various pathways.

3.5. Downregulated NEAT1 Validation. In order to validate the expression of NEAT1 in endothelial cells under cyclic stretch, quantitative real-time polymerase chain reaction (qRT-PCR) was performed in the 6 samples above. As shown in Figure 7, lncRNA NEAT1 was significantly downregulated in endothelial cells after being stretched for 24 hours, which was consistent with the RNA-seq data. These results indicated that NEAT1 may play an essential role in the ceRNA network.

3.6. Secretion of Inflammatory Cytokines. To further investigate the effect on inflammatory response after NEAT1 downregulation, we transduced si-NEAT1 into endothelial cells for 24 h and performed ELISA to measure the level of IL-6, MCP-1, and ICAM-1 in the culture medium. After decreasing NEAT1 for 24 h, IL-6 and MCP-1 were significantly increased by about 1 hold (Figure 8), indicating reducing NEAT1 can worsen endothelial inflammatory and may contribute to vein graft failure.

3.7. Endothelial Apoptosis Induction. Apart from inflammatory response, we also adopted TUNEL assay to determine the apoptotic effect after downregulating NEAT1. As depicted in Figure 9, fewer TUNEL-positive cells were found in cells treated with si-NEAT1 for 24 h than those with negative control. NEAT1 inhibition leaded to significant endothelial apoptosis, which may result in the aggregation and adhesion of platelet and lead to vein graft failure eventually.

4. Discussion

Our results indicate that the lncRNA-associated ceRNA network may function as a transcriptional regulation factor in endothelial cells under cyclic stretch, and we further identified that lncRNA NEAT1 may be a target for further investigation of mechanisms in endothelial cells; by triggering inflammatory response and inducing apoptosis,

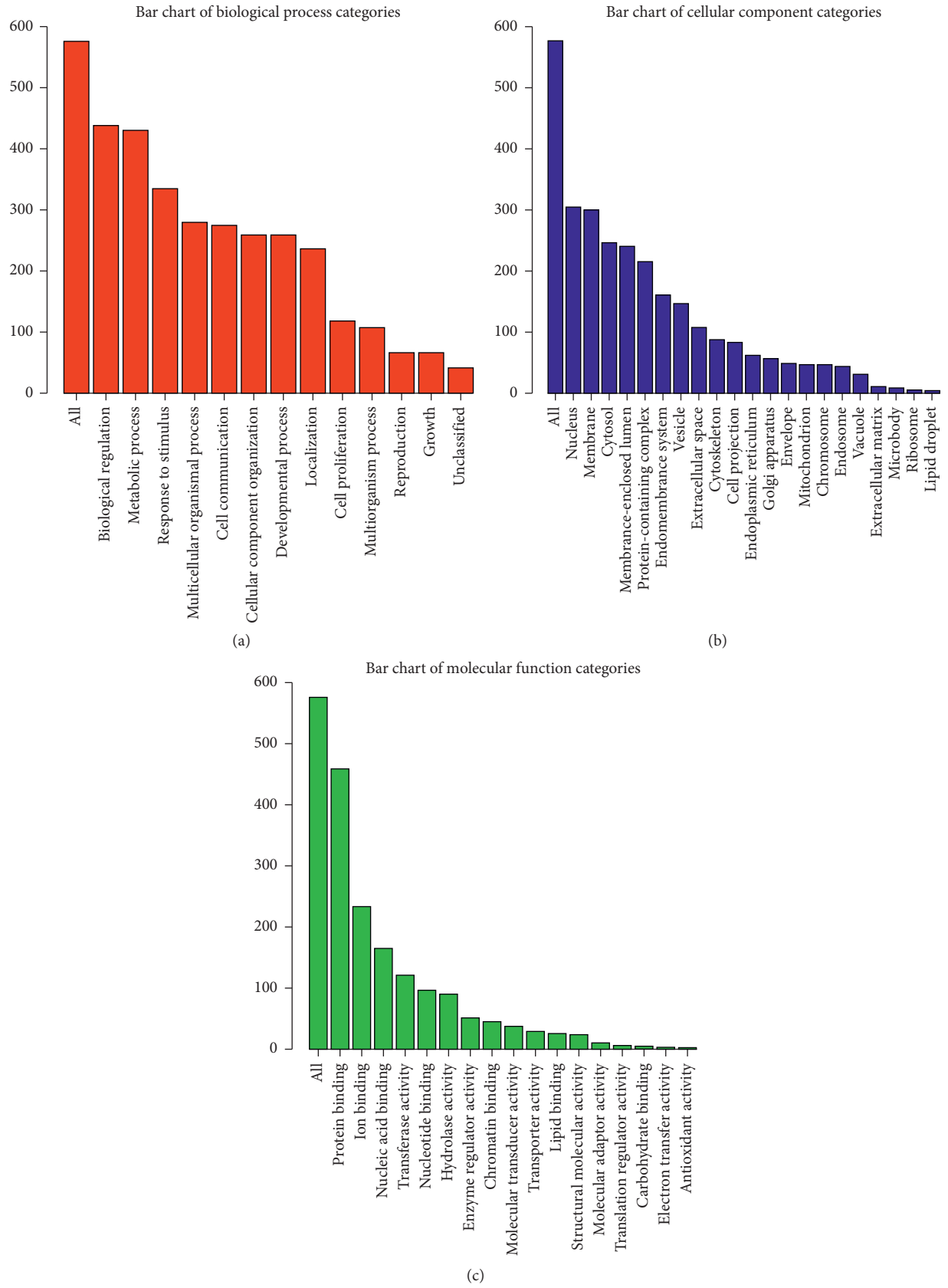

Figure 4: GO enrichment analysis of differentially expressed genes of cyclic stress vein genes (Top 10). (a) Red bar plot of biological process. (b) Blue bar plot of cellular component. (c) Green bar plot of molecular function.

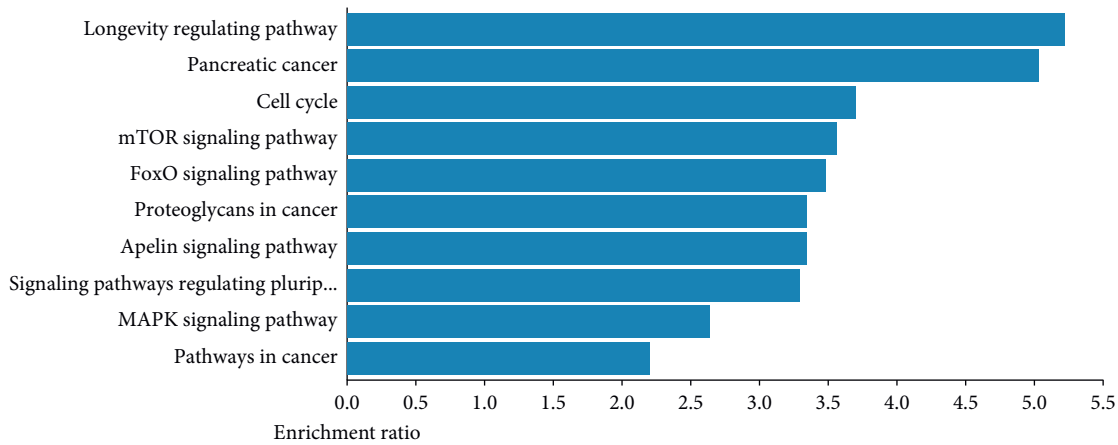

Figure 5: The top 10 enriched KEGG pathways of cyclic stretch mRNAs in the ceRNA networks.

NEAT1 may play an integral role in vein graft failure and could be a target for RNAi therapy.

Vein graft failure, which was characterized as narrow or occlusion of the saphenous vein grafting from the aorta to the coronary artery, limits the long-term effect of CABG. The velocity of the grafted vein reduced to 80% only 1 year after grafting and then to 40% within 10 years [23], resulting in the long-term patency of vein grafts are merely 60%–65% [24] and causing angina recurrence and revascularization [25]. The mechanotransduction of a grafted vein involves the response to shear stress, cyclic stress, and normal stress. Bondareva reported that oscillatory shear stress (OSS) stimulation for 6 hours could activate the EGR1 and YAP/TAZ complex in human endothelial cells, based on ChIP-seq and luciferase assays [26]. Moreover, Moonen et al. suggested that disturb laminar shear stress (LSS) could aggravate endothelial-to-mesenchymal transition (EndMT), targeting MEK5 signaling, eventually leading to neointimal hyperplasia [27]. Similarly, certain progress has been made in the studies on its effect under circumferential stress. For example, high-cyclic stretch significantly elevated miR-124-3p, downregulating lamin A/C and inducing VSMC apoptosis [28]. Cyclic stretch is an initial factor of vein graft failure; after CABG, the stretch rate of the saphenous vein will be 10–15% due to the pulsating pressure from the aorta [29], which causes over deformation of the endothelium. Endothelial cells, located on the inner layer of the saphenous vein, are sensible to the hemodynamic changes; thus, overstretching them will initiate inflammation and proliferation [30]. As a result, cyclic stretch caused by the aorta triggers endothelial dysfunction, which is a key process of vein graft failure [31]. Furthermore, cyclic stretch induces a large number of growth factors releasing from VSMCs, aggregating endothelial dysfunction, and intimal hyperplasia, which eventually results in vein graft failure [32]. Take together, cyclic stretch stimulation to endothelial cells and its inflammation is the early response of vein graft failure. However, the transcriptional regulation in the endothelial dysfunction remains unclear.

Noncoding RNA is a cluster of RNAs that regulate transcription without expressing into protein, mainly including miRNA, lncRNA, and circRNA [33]. miRNAs are single-stranded and endogenously expressed small noncoding RNAs molecules with lengths of 22 to 24 nucleotides [34]. MiRNA plays a regulatory role in numerous cellular activities, including growth, differentiation, metabolism, apoptosis, and migration [35]. Recently, accumulated studies have shown the role of lncRNAs in various biological processes. Aberrantly expressed lncRNA has been observed in coronary artery disease [36]; furthermore, it was found that lncRNA can regulate mRNA expression by interacting with miRNA, leading to the introduction of ceRNA hypothesis [37]. Further studies have shown that ceRNA genes were mediated by miRNAs which are interacting with increasingly complicated ceRNA networks [38]. The ceRNA network has been found to be involved in the pathogenesis in several diseases; however, very little was found on the association between ceRNA and vein graft failure.

Table 4: The list of differentially expressed genes (node degree >5).

Number	Gene type	Gene symbol	Degree
1	miRNA	hsa-miR-4739	35
2	lncRNA	NEAT1	18
3	miRNA	hsa-miR-6756-5p	17
4	miRNA	hsa-miR-4685-5p	16
5	miRNA	hsa-miR-5787	14
6	miRNA	hsa-miR-15a-5p	12
7	miRNA	hsa-miR-619-5p	12
8	mRNA	MAP3K2	11
9	miRNA	hsa-miR-424-5p	11
10	miRNA	hsa-miR-15b-5p	11
11	miRNA	hsa-miR-497-5p	11
12	miRNA	hsa-miR-16-5p	11
13	miRNA	hsa-miR-6779-5p	11
14	miRNA	hsa-miR-6848-5p	10
15	mRNA	RASA1	10
16	miRNA	hsa-miR-195-5p	10
17	miRNA	hsa-miR-5001-5p	10
18	mRNA	MAP4K4	9
19	mRNA	PTPRJ	9
20	miRNA	hsa-miR-4728-5p	8

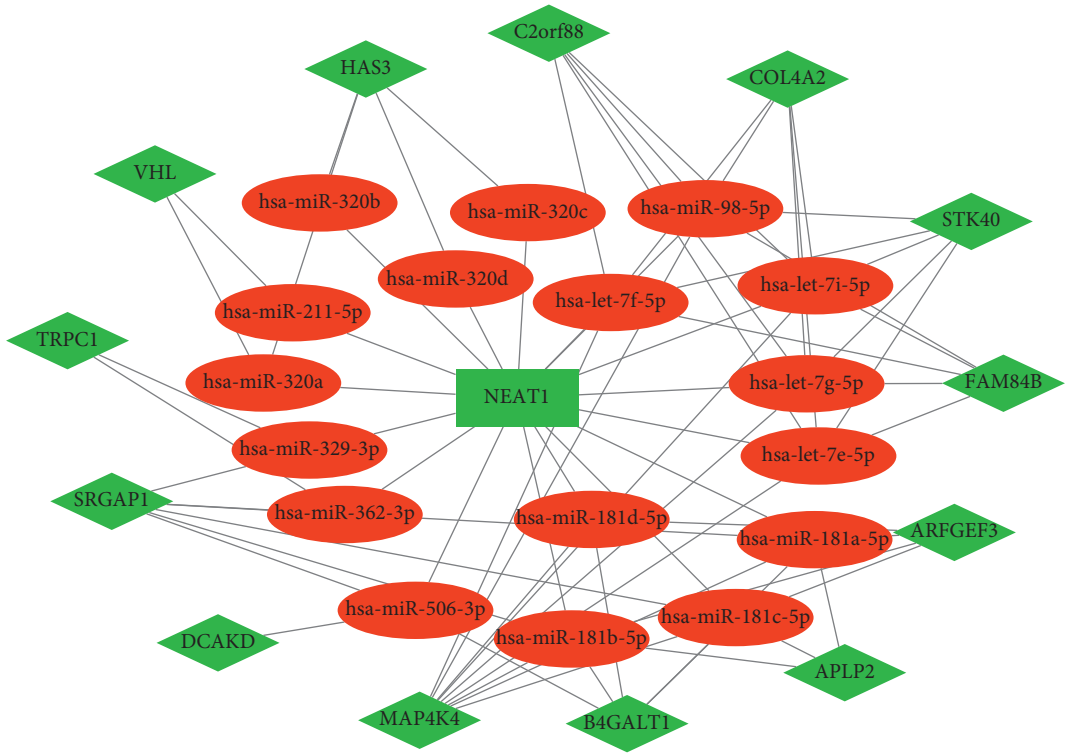

Figure 6: The subnetwork of lncRNA NEAT1 interaction network. The rectangles indicate lncRNAs in green, ellipses represent miRNAs in red, and diamonds represent mRNAs in green.

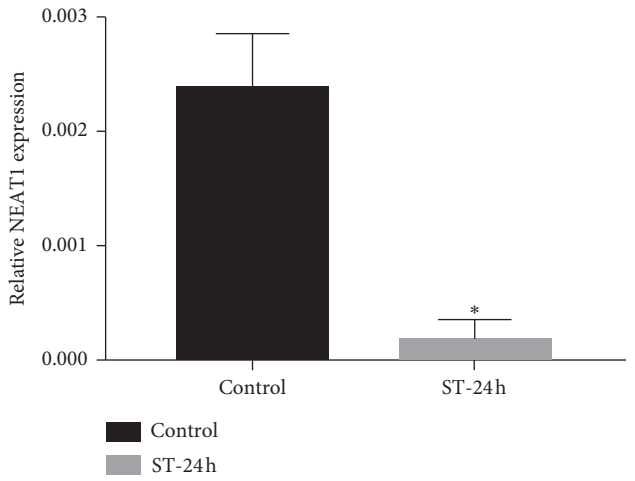

Figure 7: The relative miRNA expression of NEAT1 (normalized to GADPH). NEAT1 level was significantly downregulated in cells of the ST-24 group, in comparison with those of the control group ($^*P < 0.05$).

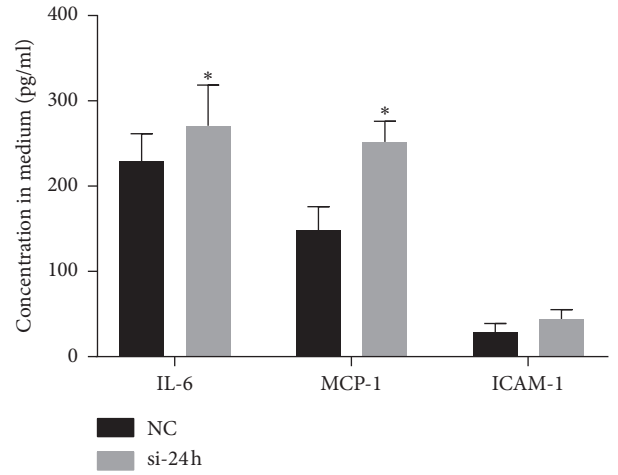

Figure 8: The secretion of inflammatory cytokines measured by ELISA. After si-NEAT1 was transduced into endothelial cells for 24 h, inflammatory cytokines including IL-6 and MCP-1 were significantly increased, comparing to the control group ($^*P < 0.05$).

To identify the regulatory role of lncRNA-associated ceRNA network in endothelial dysfunction, we constructed a ceRNA network based on RNA-sequencing data of endothelial cells under cyclic stretch for 24 hours. With FC $\geq$ 1.5 and P value <0.05 threshold, 39 upregulated and 116 downregulated lncRNAs, 35 upregulated and 39 downregulated miRNAs, and 568 upregulated and 392 downregulated mRNAs showed differential expression between two groups. Among them, several genes have been found to be associated with the pathogenesis of vein graft failure. For instance, our previous study found that egr1 was upregulated under cyclic stretch, targeting ICAM-1, leading to vein graft failure [39]. Furthermore, NF-κB is proved to play an integral role in vascular inflammation, and it has been demonstrated that inhibition of NF-κB signaling can reduce the inflammatory response in endothelial cells [40]. On the other hand, activation of the NF-κB pathway is found to impede recovery in the carotid artery injury model [41].

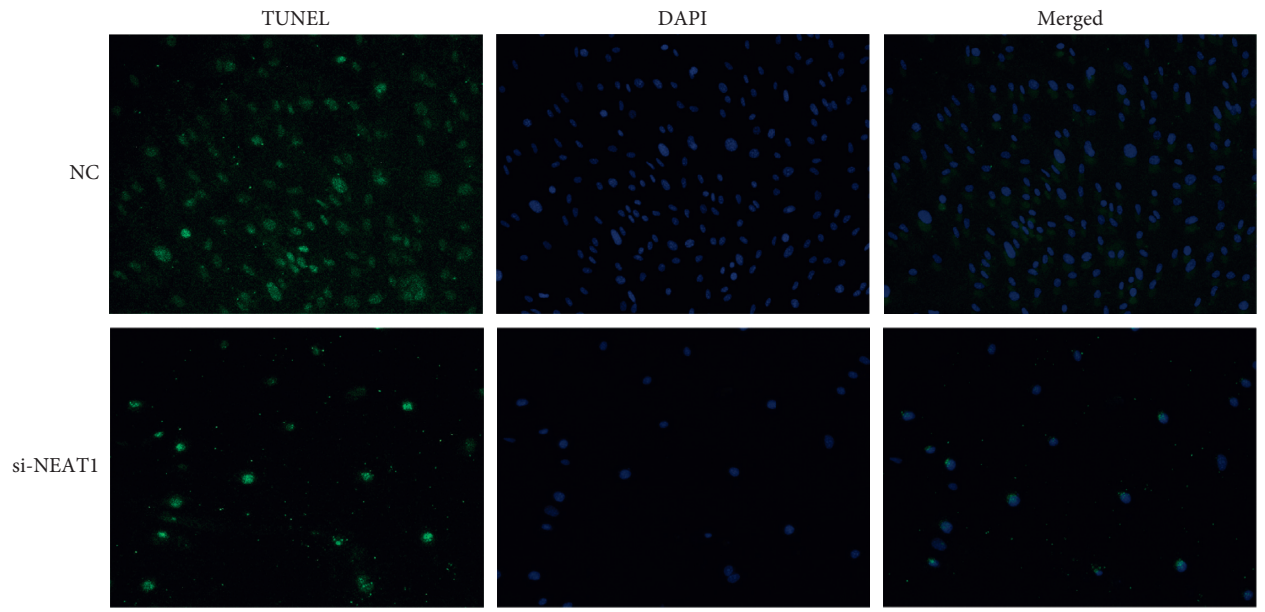

Figure 9: The endothelial cell apoptosis induced by transduction of si-NEAT1. Endothelial cell apoptosis was increased after NEAT1 silencing, and TUNEL-positive cells appeared less in the si-NEAT1 group.

In order to better annotate the biological functions of the downstream mRNAs, we enriched GO and KEGG pathways. Among the most enriched GO terms, biological regulation and response to stimulus have been reported to be involved in endothelial dysfunction. Remarkably enriched KEGG pathways are mTOR, FoxO, and MAPK pathways, and they are known to play an essential role in endothelial dysfunction [42–44], causing inflammation and proliferation of the tunica intima.

Our bioinformatic analysis has also determined that the lncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) is the core of the mechanical stress factor. NEAT1 has been reported as a cancer biomarker [45]. In the current study, the total RNA was extracted after stretching for 24 h and we found that NEAT1 expression was significantly decreased, which was consistent with the result of bioinformatics analysis. NEAT1 is a nuclear-enriched lncRNA located on chromosome 11q13.1 and is considered to promote carcinogenesis and metastasis [46]. The upregulation of NEAT1 has been documented in kidney cancer, ovarian cancer, lung cancer, breast cancer, and glioma cancers, contributing to the accurate prediction of clinical outcomes [47, 48]. Recently, NEAT1 is found to be related to the inflammation response to stimuli [49], regulating the expression of several chemokines and cytokines, including IL-6 and CXCL10, via the MAPK pathway [50]. Our present study indicated that NEAT1 expression was downregulated in endothelial cells after mechanical stretch, subsequently leading to upregulated expression of miRNAs and eventually triggering inflammation response to give rise to vein graft failure. NEAT1 is a potent inflammatory regulator; to the best of our knowledge, our study is the first to report its role in ceRNA.

According to the bioinformatic enrichment and PCR validation, we investigated the endothelial inflammation and apoptosis after NEAT1 was repressed by performing ELISA and TUNEL assays. Similar to the results of previous studies, after transduction of si-NEAT1 for 24 h, inflammatory cytokines were significantly increased in the culture medium. Due to the structural difference between the vein and artery, the endothelial layer of the saphenous vein is prone to cyclic stretch, and the high pressure of the aorta after grafting leads to EC activation and loss, initiating the complex networks and leading to rapid expression of a cascade of adhesion such as MCP-1 and ICAM-1 [51]. Apart from that, endothelial cells are essential parts of vascular homeostasis to maintain a anticoagulant and anti-inflammation environment [52]. TUNEL staining showed that inhibiting NEAT1 can induce endothelial cells apoptosis, which may lead to the exposure of smooth muscle cells, leading to phenotype switch, aggregating intimal hyperplasia, and resulting vein graft failure [53].

Nevertheless, more studies should be carried out to clarify the role of NEAT1 during vein graft failure. Different cyclic stress times and frequencies should be further investigated in future studies in the ceRNA network. In addition, further studies should be made on validation of the downstream genes; moreover, the various reasons to explain the failure of vein graft should be elucidated.

In conclusion, we constructed a lncRNA-associated ceRNA network based on the sequencing data and identified lncRNA NEAT1 is an essential fraction in endothelial dysfunction. We found that cyclic stress in the endothelial cells gives rise to inflammatory response and promotes cell apoptosis by downregulating NEAT1 and its related ncRNAs. Our study provided a novel insight into the transcriptional regulation of vein graft failure.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Clinical Study

Plasma Choline as a Diagnostic Biomarker in Slow Coronary Flow

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Aim. The slow coronary flow (SCF) phenomenon was characterized by delayed perfusion of epicardial arteries, and no obvious coronary artery lesion in coronary angiography. The prognosis of patients with slow coronary flow was poor. However, there is lack of rapid, simple, and accurate method for SCF diagnosis. This study aimed to explore the utility of plasma choline as a diagnostic biomarker for SCF. *Methods*. Patients with coronary artery stenosis <40% evaluated by the coronary angiogram method were recruited in this study and were grouped into normal coronary flow (NCF) and SCF by thrombolysis in myocardial infarction frame count (TFC). Plasma choline concentrations of patients with NCF and SCF were quantified by Ultra Performance Liquid Chromatography Tandem Mass Spectrometry. Correlation analysis was performed between plasma choline concentration and TFC. Receiver operating characteristic (ROC) curve analysis with or without confounding factor adjustment was applied to predict the diagnostic power of plasma choline in SCF. *Results*. Forty-four patients with SCF and 21 patients with NCF were included in this study. TFC in LAD, LCX, and RCA and mean TFC were significantly higher in patients with SCF in comparison with patients with NCF (32.67 ± 8.37 vs. 20.66 ± 3.41, $P < 0.01$). Plasma choline level was obviously higher in patients with SCF when compared with patients with NCF (754.65 ± 238.18 vs. 635.79 ± 108.25, $P = 0.007$). Plasma choline level had significantly positive correlation with Mean TFC ($r = 0.364$, $P = 0.002$). Receiver operating characteristic (ROC) analysis showed that choline with or without confounding factor adjustment had an AUC score of 0.65 and 0.77, respectively. *Conclusions*. TFC were closely related with plasma choline level, and plasma choline can be a suitable and stable diagnostic biomarker for SCF.

1. Introduction

The slow coronary flow (SCF) phenomenon was an angiographic observation characterized by angiographically normal or near-normal coronary arteries with delayed opacification of the distal vasculature [1]. The SCF phenomenon was first proposed by Tambe et al. in 1972. Studies have found that the prevalence of SCF patients was about 1% of those who underwent coronary angiography. Previous researches have shown an increased risk of major adverse cardiovascular events (MACE) in patients with SCF, such as atherosclerosis [2], acute coronary syndrome [3], non-obstructive myocardial infarction [4], malignant arrhythmia [5], and metabolic syndrome, which was explained by vascular endothelial dysfunction, microangiopathy, inflammation, atherosclerosis, platelet activity and dysfunction, and insulin resistance [2, 6–10].

Choline, a saturated quaternary amine in all types of cells, participates in the lipid components of cell membranes [11]. Plasma choline level is considered to be related with several detrimental outcomes, including incidence of MACE and all-cause death, atherosclerosis, acute coronary syndrome, arrhythmia, and heart failure [12–16], and the main mechanism of choline-induced cardiovascular events is to promote a progressive inflammatory response which is attributed to aggregation of macrophages and CD36 receptors and enhance oxidative stress, endothelial dysfunction, and disorder lipid metabolism [17–19]. Therefore, SCF and

choline-induced MACE may share a common underlying pathwayinflammation-related endothelial cell dysfunction during the individual pathological process.

Although patients with SCF often suffer a high morbidity and poor prognosis, it is generally regarded as a rare disease which can be ignored or overlooked easily. Moreover, coronary angiography is the gold standard for SCF diagnosis, but it is an expensive and invasive procedure with a certain degree of death, stroke, and myocardial infarction risk [20, 21]. Therefore, a rapid, simple, noninvasive, and accurate diagnostic method for SCF is highly desirable. Increasing number of studies has been providing crucial evidences that plasma choline can be employed as a stable and exact diagnostic indicator in the area of cancer, cardiac vascular disease, and acute ischemic stroke progression [22–24]. To our knowledge, the role of plasma choline in SCF has not been previously evaluated. In our study, we detected the plasma choline level of patients with normal coronary flow (NCF) and SCF and analyzed the relationship between choline and SCF to determine whether plasma choline can be served as a good diagnostic biomarker of SCF.

2. Methods

2.1. Study Population. A total of 1395 blood samples were randomly collected from patients who underwent coronary angiography between 2014 and 2017 in Xiangya Hospital. The inclusion criteria of our study were patients who were characterized by (1) having chest pain symptom or ischemic evidence on electrocardiogram, treadmill exercise test, or myocardial scintigraphy and (2) the detection of coronary angiography were normal (artery stenosis degree <40%) [25, 26]. The exclusion criteria of our study was confirmed coronary heart disease, coronary artery dissection, coronary artery spasm, obvious dilation of coronary artery, heart valves, cardiomyopathy, and cardiac insufficiency (ejection fraction <50%). Echocardiography showed left ventricular insufficiency and left ventricular hypertrophy, autoimmune diseases, tumors, systemic infectious diseases, renal insufficiency (serum creatinine >1.5 mg/dl), uncontrolled hypertension: systolic pressure >160 mmHg or diastolic pressure >105 mmHg, poor image quality of coronary angiography, patients with dysphagia, intestinal dysfunction, gastrointestinal surgery history, vitamin B supplementation, and antibiotics or probiotics in recent 6 months [26]. There were 65 patients included in our study based on inclusion and exclusion criteria. The study protocol was approved by the Ethics Committee of Xiangya Hospital, and informed consent was obtained from each patient.

2.2. Assessment of Thrombolysis in Myocardial Infarction (TIMI) Frame Count (TFC). All patients underwent coronary angiography (CAG) with the standard Judkins method. Coronary flow rates were assessed by TFC, which was a classical method described by Gibson et al. [27], and standard images were obtained at 30 frames per second. The first frame used for TIMI frame counting is the frame in which the contrast agent enters both sides of the wall at the beginning of coronary artery and moves forward smoothly. The final frame is defined as the frame when the contrast agent enters the anatomical landmark of the distal vessels. The anatomical landmarks are defined as distal apical "eight-character" bifurcation in the left anterior descending (LAD), the furcation of the distal blunt margin branch in the left circumflex (LCX), and the first posterior branch of the left ventricle in the right coronary artery (RCA). Because the LAD is generally longer than LCX and RCA, the TIMI of LAD was divided by 1.7 to obtain corrected TIMI frame count (cTFC). The mean TIMI frame count for each subject was obtained by adding the corrected TFC of the LAD to the LCX and the RCA and then dividing the obtained value by 3 [27, 28].

2.3. Grouping Criteria for This Study. All patients in this study were grouped into NCF and SCF by the following criteria: patients with corrected TFC less than 27 frames per second in at least one of the LAD, LCX, and RCA were classified into the NCF group; patients with corrected TFC greater than 27 frames per second were diagnosed as SCF.

2.4. Laboratory Analysis. Blood samples were obtained from antecubital vein for analysis of biochemical and hematological data in the morning after an overnight fasting. For measurement of choline, TMAO, betaine, and L-carnitine, blood samples were obtained during coronary angiography and were centrifuged immediately with 3000 rpm for 10 minutes to obtain plasma samples. All samples were stored at −80°C until assayed. Plasma choline, TMAO, betaine, and L-carnitine were measured by ultra-high-performance liquid chromatography electrospray ionization mass spectrometry (RP-UHPLC-ESI-MS), and the internal standards choline-trimethyl-d9 (d9-choline), TMAO trimethyl-d9 (d9-TMAO), L-carnitine-trimethyl-d9 (d9- L-carnitine), and betaine-trimethyl-d9-methylene-d2 (d11-betaine) were added into plasma samples after protein precipitation, oscillated for 10 second, and centrifuged with 13200 rpm for 10 min, 200 μl supernatant for detection by mass spectrometry system. The liquid phase system was ACQUITY UPLC I-Class (Waters, USA), and the mass spectrometry system was SCIEX 6500+ (SCIEX, USA).

2.5. Statistical Analysis. Continuous variables were expressed as mean ± standard error of the mean (SEM), and categorical variables were expressed as percent. Kolmogorov Smirnov test was used to evaluate the normal distribution of continuous variables. Student's t-test or Mann–Whitney U-test were used to compare the difference of continuous variables. The chi-square test was used to compare the difference of categorized variables. The correlations between plasma choline levels and mean TIMI frame count were assessed by the Pearson or Spearman correlation test. The diagnostic power of choline in SCF was assessed by receiver operating characteristic (ROC) curve. P value <0.05 was considered statistically significant. All statistical analyses

Table 1: Clinical characteristic of the study subject.

	NCF group ($n = 21$)	SCF group ($n = 44$)	P value
Clinical and hemodynamic data			
Age (years)	58 ± 10	56 ± 8	0.448
Male, n (%)	4 (19)	21 (47.7)	0.026
BMI (kg/m^2)	24.03 ± 1.86	24.24 ± 3.09	0.785
Smoking, n (%)	4 (19)	17 (38.6)	0.114
Drinking, n (%)	2 (9.5)	15 (34.1)	0.035
SBP (mmHg)	128 ± 20	127 ± 16	0.883
DBP (mmHg)	75 ± 9	77 ± 10	0.425
HR (bpm)	70 ± 12	71 ± 11	0.650
Hypertension, n (%)	10 (47.6)	18 (40.9)	0.609
DM, n (%)	4 (19)	3 (6.8)	0.137
Hyperlipidemia, n (%)	3 (14.4)	14 (31.8)	0.133
Baseline medication			
ARB/ACEI, n (%)	10 (47.6)	24 (54.5)	0.601
BB, n (%)	11 (52.4)	32 (72.7)	0.105
CCB, n (%)	7 (33.3)	16 (36.4)	0.811
Nitrates, n (%)	4 (19)	12 (27.3)	0.472
Statins, n (%)	17 (81)	38 (86.4)	0.572
Antiplatelet agent, n (%)	17 (81)	40 (90.9)	0.253
Anticoagulant, n (%)	3 (14.3)	11 (25)	0.326
Biochemical and hematological data			
WBC (10^9/L)	5.38 ± 1.63	6.16 ± 1.22	0.035
TG (mmol/L)	1.30 ± 0.62	1.68 ± 1.15	0.167
TC (mmol/L)	4.22 ± 0.69	4.16 ± 0.97	0.151
HDL (mmol/L)	1.43 ± 0.41	1.26 ± 0.26	0.087
LDL (mmol/L)	2.40 ± 0.65	2..42 ± 0.80	0.900
FPG (mmol/L)	5.41 ± 0.80	5.16 ± 0.84	0.261
Creatinine (mmol/L)	83.38 ± 30.19	79.94 ± 13.04	0.521
Hs-CRP (mg/L)	2.26 ± 2.30	2.30 ± 3.20	0.956

Data were expressed as mean ± standard error of the mean (SEM) or the number (%) of patients. NCF, normal coronary flow; SCF, slow coronary flow; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; DM, diabetes mellitus; ARB, angiotensin receptor blocker; ACEI, angiotensin converting enzyme inhibitor; BB, beta-blocker; CCB, calcium channel blocker; WBC, white blood cell; TG, triglyceride; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FPG, fasting plasma glucose; Hs-CRP, high-sensitivity C-reactive protein;

were performed using SPSS for Windows version 11.5 (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Clinical Characteristics. A total of 65 subjects were recruited into our study, according to the grouping criteria, 21 of them were classified as patients with NCF (TFC < 27), and 44 patients were diagnosed as SCF (TFC < 27). The baseline demographic and clinical characteristics of the patients with SCF and NCF are presented in Table 1. There were no significant differences in the basic indexes of age, body mass index, heart rate, and systolic and diastolic blood pressure. In addition, there were also no obvious differences between patients with SCF and NCF in the terms of CAD risk factors, medication history, and basic biochemical indicators, such as serum triglyceride, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, fasting glucose, creatinine, and high-sensitivity C-reactive protein. However, when compared with patients with NCF, patients with SCF showed significant predominance for male sex, alcohol consumption, and white blood cells (WBC) ($P = 0.026$, $P = 0.035$ and $P = 0.035$, respectively).

3.2. Coronary Angiographic Findings between NCF and SCF Group. In order to evaluate TFC of all 3 epicardial coronary artery in patients with NCF and SCF. We assessed the TFC through coronary angiographic analysis of every patient. As shown in Figure 1, TFC of LAD, LCX, and RCA in patients with SCF was significantly higher compared with patients with NCF (53.88 ± 16.04 vs. 34.57 ± 5.90, $P < 0.001$, Figure 1(a); 39.95 ± 12.71 vs. 23.90 ± 2.71, $P < 0.001$, Figure 1(b); 32.67 ± 8.37 vs. 20.66 ± 3.41, $P < 0.001$, Figure 1(c), respectively). Furthermore, the mean of TFC in patients with SCF was also significantly higher than patients with NCF (32.67 ± 8.37 vs. 20.66 ± 3.41, $P < 0.001$, Figure 1(d)).

3.3. Plasma Choline Levels in NCF and SCF Group. To explore the role of choline in slow coronary flow, we performed RP-UHPLC-ESI-MS assay to determine the plasma choline concentration. As shown in Figure 2 plasma choline level was significantly higher in patients with SCF than those with NCF (754.65 ± 238.18 vs. 635.79 ± 108.25 mmol/L, $P = 0.007$).

3.4. Associations between Plasma Choline and SCF. In order to further investigate the relationship between TFC value

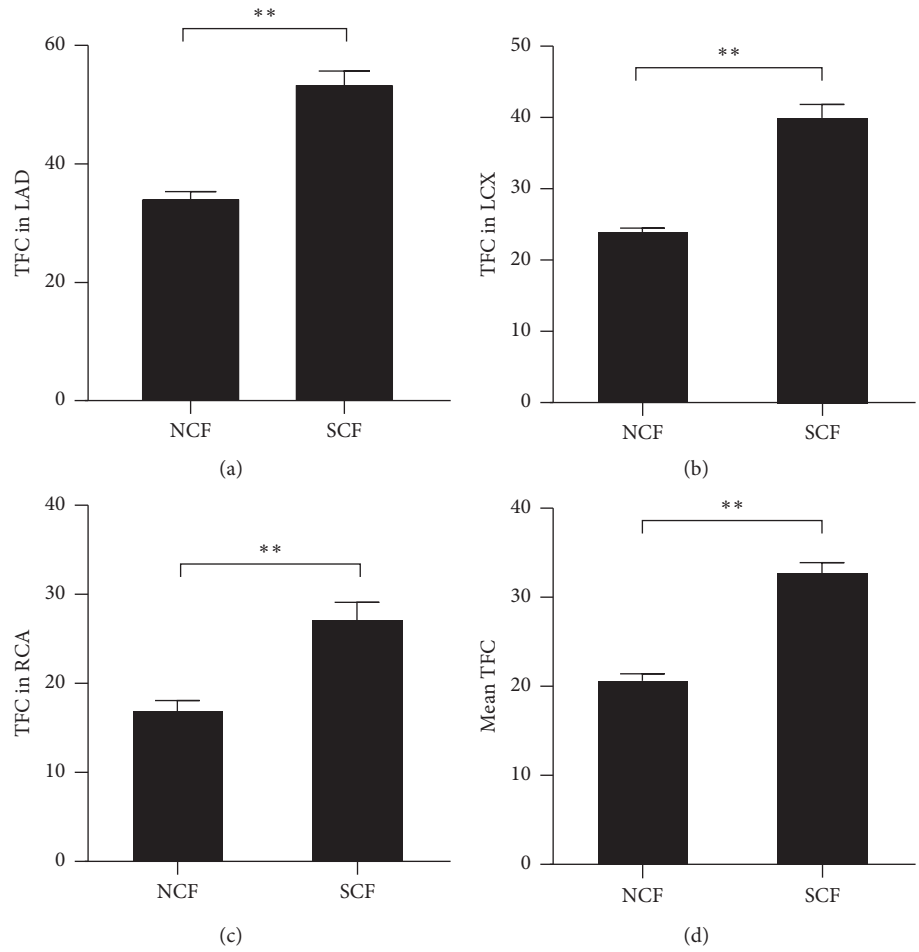

Figure 1: Thrombolysis in Myocardial Infarction (TIMI) frame count (TFC) in patients with NCF and SCF. (a) TFC in LAD between patients with NCF and SCF. (b) TFC in LCX between patients with NCF and SCF. (c) TFC in RCA between patients with NCF and SCF. (d) Mean TFC of LAD, LCX, and RCA between patients with NCF and SCF. LAD, left anterior descending; LCX, left circumflex; and RCA, right coronary artery. Data were expressed as mean ± SEM. $^{**}P < 0.01$.

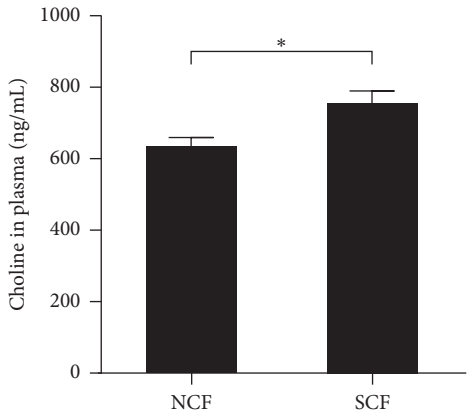

Figure 2: Plasma Choline levels in patients with NCF and SCF. Data were expressed as mean ± standard error of the mean.

and plasma choline level, we applied the correlation analysis method for TFC value and plasma choline concentration in all subjects. Figure 3 suggested that plasma choline level was positively correlated with mean TFC ($r = 0.364$, $P = 0.002$).

3.5. Diagnostic Power of Choline in SCF. We further performed receiver operating characteristic (ROC) analysis to determine whether choline can serve as an independent diagnostic indicator of SCF. As shown in Figure 4(a), the ROC analysis indicated a cutoff value of 673.5 ng/mL for plasma choline level to diagnose SCF with 56.8% sensitivity and 85.7% specificity, and the area under the ROC curve (AUC) was 0.6548 (95% CI: 0.523–0.786, $P = 0.044$). In order to get rid of the effect of confounding factors in the diagnosis process, we adjusted the factors which increased significant differences between patients with NCF and SCF, including sex, drinking, and number of white blood cell. We found that diagnostic ability of plasma choline with 79.5% sensitivity and 76.2% specificity was significantly enhanced after adjusting with confounding factors (cut-off value, 1030 ng/mL; AUC, 0.767; 95% CI: 0.642–0.892 $P = 0.0005$, Figure 4(b)).

3.6. Plasma Trimetylamine-Oxide, L-Carnitine, and Betaine in the NCF and SCF Group. Choline, carnitine, betaine, and their metabolite Trimetylamine-oxide (TMAO) are closely

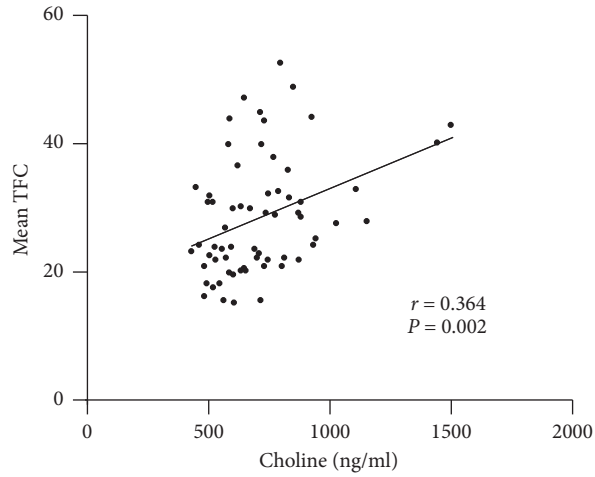

Figure 3: The relationship analysis between plasma choline level and mean TFC. *r*, Pearson correlation coefficient.

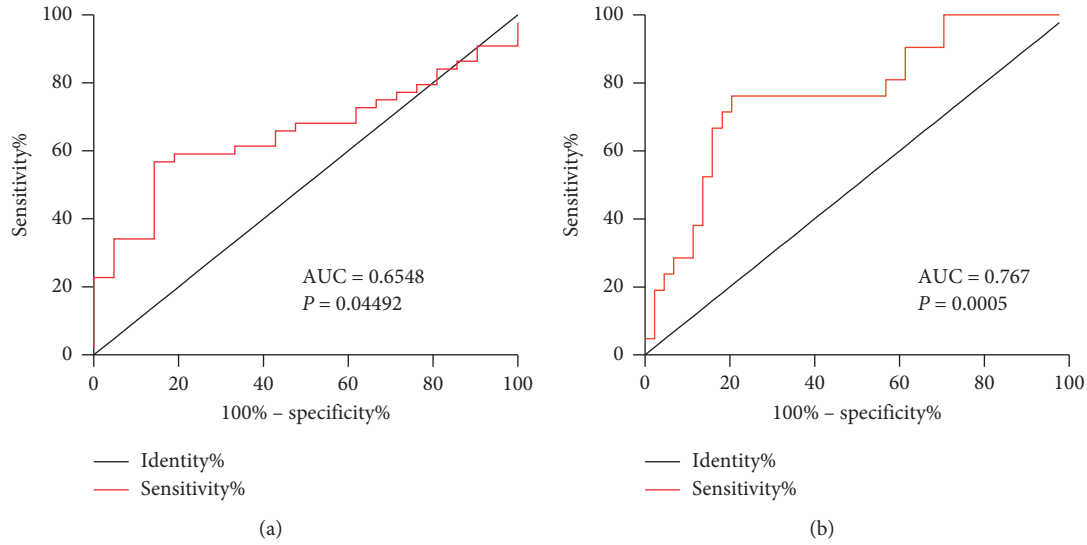

Figure 4: The diagnostic power of plasma choline in SCF. (a) Receiver operating characteristic (ROC) curve analysis of plasma choline level in patients with NCF and SCF. (b) Receiver operating characteristic (ROC) curve analysis of plasma choline level in patients with NCF and SCF after adjustment with sex, drinking, and white blood cell numbers. AUC, area under the curve.

related to adverse cardiovascular events, and we further studied the level of carnitine, betaine, and TMAO in plasma of patients with NCF and SCF by using RP-UHPLC-ESI-MS assay. The results showed that there was no significant difference in plasma TMAO, L-carnitine, and betaine between patients with NCF and SCF (Figure 5).

4. Discussion

As shown in the result, we concluded the following findings: (i) patients with SCF showed a significant predominance for male sex, alcohol consumption, and white blood cell numbers compared with patients with NCF; (ii) TFC of LAD, LCX, RCA, and mean TFC in patients with SCF were significantly higher compared with patients with NCF; (iii) the plasma choline level was obviously increased in the SCF group compared with the NCF group; (iv) the plasma choline level was positively correlated with mean TFC; and (v) plasma choline showed a significant diagnostic power in patients with SCF.

Prior studies have noted that inflammation is an important pathogenesis of SCF, and Li et al. showed that plasma c-reactive protein and interleukin-6 levels were significantly increased in the SCF group compared with the control group (all $P < 0.01$) [28]. Turhan et al. found that plasma ICAM-1, VCAM-1, and E-selectin levels were higher in the slow coronary flow group than in the control group (all $P < 0.01$), and there was a positive correlation between TFC and inflammatory mediators ($P < 0.01$) [29], indicating that patients with SCF might be characterized by vascular endothelial activation and response to inflammation. Coincidentally, in our study, we found that the number of white blood cells was significantly higher in the SCF group than in the NCF group, which was consistent with previous studies. However, multicenter and larger sample size will be needed to further verify the correlation between SCF and inflammation.

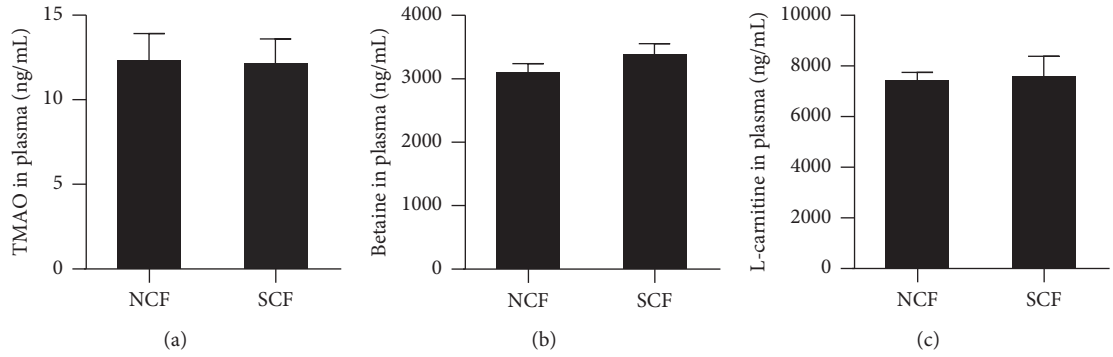

Figure 5: Plasma Trimetylamine-oxide (TMAO), L-carnitine, and betaine levels in the NCF group and SCF group. (a) Plasma TMAO concentration in patients with NCF and SCF, (b) plasma betaine level in patients with NCF and SCF, and (c) plasma L-carnitine level in patients with NCF and SCF. Data were expressed as mean ± standard error of the mean.

Choline is a water-soluble nutrient chemically defined as 2-hydroxyethyl-trimethyl ammonium hydroxide, which is an important element of B vitamins. Dietary choline mainly comes from red meat such as beef, pork, and eggs [30]. Several studies explored the role of choline in cardiovascular diseases, for example, Danne et al. proposed that whole blood choline level was related to cardiac death and nonfatal cardiac arrest [12], choline activated in leukocytes and platelets, secretion of matrixmetalloproteinases by macrophages, activation of macrophages by oxidized low-density lipoprotein, and represented a major feature of unstable or ruptured coronary plaque [31]. Zuo et al. proposed that plasma choline increased atrial fibrillation risk (AF), choline determined the methylation state of liver, and increased the susceptibility to AF [16, 32]. In our study, plasma choline was significantly upregulated in patients with SCF compared with patients with NCF, and a positive correlation between choline levels and TFC has been further confirmed. However, the underlying mechanism still remains uncertain.

It was reported that endothelial dysfunction was closely related to slow coronary flow [26]. Pohl and Busse indicated endothelium-derived NO is an important regulator of slow coronary flow with potent vasodilatory effects [33]. Ren et al. found that choline has been shown to decrease both the production and bioavailability endothelium-derived NO, increase ET-1 level, and accelerate oxidative stress. It reduces vascular compliance, accelerates vascular remodeling, increases vascular resistance, and limits blood flow [17]. Thus, choline may play an important role in regulating coronary flow. Wang et al. found choline can increase aortic macrophage and scavenger receptor CD36 content and the diffuse atherosclerotic may increase epicardial resistance and reduce coronary flow [19]. Besides, Jia et al. demonstrated that choline would dramatically increase the TNF-α and CRP level, promoting inflammatory response and inducing SCF [18]. These findings apparently support the concept that choline is closely related with SCF and elevated in patients with SCF.

Choline, as an organic compound, is an important component of the cell membrane, which is widely distributed, easily detected, and has good stability [34]. Previous studies showed plasma choline could serve as a stable and exact diagnostic indicator in the area of cancer. Yang et al. identified that choline may be a valuable potential diagnostic biomarker for neoplasm progression, recurrence, chemoradiotherapy, and prognosis [22]. Yu et al. verified plasma choline as a reasonable biomarker to diagnose the progression of acute ischemic stroke in terms of selectivity, linearity, sensitivity, precision, accuracy, carryover effect, and stability [35]. Besides, Danne et al. found whole blood choline can be a diagnostic biomarker of ACS in future clinical studies [12]. Although our study showed a similar biomarker role of plasma choline in SCF, there were some differences between our study and Danne's study: (1) there were different inclusion criteria, and compared with their 342 patients with ACS in the United States, we included 65 Chinese patients with acute or chronic chest pain and ischemia evidence; (2) Danne detected the plasma concentration in whole blood instead of plasma; and (3) their cut-off value was 28.2 μmol/L, while our cut-off value was 1030 ng/ml. Plasma choline showed a significant diagnostic power with and without confounding adjustment. Therefore, plasma choline could be served as a reliable, convenient, and noninvasive diagnostic biomarker of SCF with a high specificity.

Choline metabolizes trimethylamine (TMA) by intestinal microorganisms, and then TMA is oxidized to TMAO by flavin-containing monooxygenase 3 (FMO3) in liver [36]. Trimethylamine-N-oxide (TMAO), as an intestinal metabolite of choline, plays an important role in mediating choline-induced cardiovascular disease [37–39]. TMAO is in turn derived from the diet red meat, L-carnitine or betaine, and some mechanisms connect between TMAO, choline, and cardiovascular disease [40–43]. Trøseid et al. observed that TMAO and choline levels were elevated in patients with chronic heart failure [44]. However, there was no significant difference of plasma TMAO and red meat-L-carnitine or betaine between patients with NCF and SCF in our study, and the following reasons could be considered: (1) the choline metabolite TMAO was affected by other precursors L-carnitine and betaine and (2) the individuals with differences of intestinal microorganisms and FMO3 activity.

5. Conclusion

In summary, the level of plasma choline was positively correlated with TFC and plasma choline can be a novel diagnostic biomarker for SCF.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Additional Points

A multicenter and larger sample size study will be required in our future work.

Ethical Approval

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Z.-Y. Li and C.-C. Li conceived and supported this study. Y.-T. Zhu and L.-P. Zhu designed the experiment, analyzed the data, and prepared the article. Z.-Y. Wang and C.-C. Li provided study materials for patients. X.-T. Qiu, W.-Z. Wu, W.-W. Liu, Y.-Y. Feng, W.-K. Xiao, and X.-Luo collected and assembled the sample.

Acknowledgments

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Research Article

Reduced Circulating Endothelial Progenitor Cells and Downregulated GTCPH I Pathway Related to Endothelial Dysfunction in Premenopausal Women with Isolated Impaired Glucose Tolerance

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Background. Individuals at a prediabetic stage have had an augmented cardiovascular disease (CVD) risk and CVD-related mortality compared to normal glucose tolerance (NGT) individuals, which may be attributed to the impaired vascular endothelial repair capacity. In this study, circulating endothelial progenitor cells' (EPCs) number and activity were evaluated, and the underlying mechanisms in premenopausal women with impaired glucose regulation were explored. *Methods*. Circulating EPCs' number and activity and flow-mediated dilation (FMD) were compared in premenopausal women with NGT, isolated impaired fasting glucose (i-IFG), or isolated impaired glucose tolerance (i-IGT). Plasma nitric oxide (NO), EPCs-secreted NO, and intracellular BH4 levels were also measured. The key proteins (Tie2, Akt, eNOS, and GTPCH I) in the guanosine triphosphate cyclohydrolase/tetrahydrobiopterin (GTPCH/BH4) pathway and Tie2/Akt/eNOS signaling pathway were evaluated in these women. *Results*. It was observed that the i-IGT premenopausal women not i-IFG premenopausal women had a significant reduction in circulating EPCs' number and activity as well as reduced FMD when compared to NGT subjects. Plasma NO levels or EPCs-secreted NO also decreased only in i-IGT women. The expression of GTCPH I as well as intracellular BH4 levels declined in i-IGT women; however, the alternations of key proteins' expression in the Tie2/Akt/eNOS signaling pathway were not observed in either i-IGT or i-IFG women. *Conclusions*. The endothelial repair capacity was impaired in i-IGT premenopausal women but was preserved in i-IFG counterparts. The underlying mechanism may be associated with the downregulated GTCPH I pathway and reduced NO productions.

1. Introduction

In the last decade, type 2 diabetes mellitus (T2DM) is regarded as one of the most popular metabolic diseases in China, which leads to an increased risk of diabetes-related morbidity or mortality due to its concomitant macrovascular and microvascular complications [1]. As a prelude to the development of T2DM, prediabetes is an intermediate state, characterized by isolated impaired fasting glucose (i-IFG), isolated impaired glucose tolerance (i-IGT), and combined IGT/IFG [2]. Researchers have suggested that prediabetes itself could be of a harmful state in which an

increased risk of macrovascular and microvascular complications related with T2DM is already present [3–5].

The underlying mechanism of why some individuals with prediabetes are susceptible to microvascular and macrovascular complications is unclear yet. Multiplier effects may involve in the pathophysiology, wherein genetic susceptibility, activated protein kinase C (PKC), alterations in nitric oxide (NO) synthase, vascular endothelial growth factor, as well as a group of inflammation regulators may together contribute to such consequences [6–9]. Of them, more evidences have indicated that endothelial progenitor cells (EPCs), deriving from the bone marrow and attending vascular endothelium restoration, could modulate the cardiovascular (CV) function and angiogenesis and maintain endothelial homeostasis, thus contributing to vascular endothelial repair after the successional or concurrent blows of CV risk factors, for instance, hypertension, dyslipidemia, smoke, and hyperglycemia [10–12].

Indeed, in previous studies, the recruitment of EPCs was suppressed in both prediabetic patients [13] and women in gestational alterations of glucose tolerance [14], EPCs' declining number correlated with diminished vascular repair capability in both prediabetic [15] and diabetic patients [16]. However, so far, no study has been reported relating to the underlying mechanism of why the inability of circulating EPCs is present in prediabetic individuals. The question of whether or not there is a difference in the inability of circulating EPCs between i-IGT and i-IFG subjects is still left behind since the two different prediabetic status are regarded as having different pathophysiological characteristics.

The nitric oxide (NO), vascular endothelial growth factors (VEGFs), and granulocyte macrophage colony stimulating factor (GM-CSF) are important regulatory factors of circulating EPCs [17–19]. However, of them, only plasma NO and EPCs-secreted NO were observed to be associated with the alternations of endothelial improve capability in prehypertensive premenopausal women in our previous studies [20, 21].

Tie2/Akt/eNOS signaling pathway is a major upstream regulatory pathway of endothelial nitric oxide synthase (eNOS) [21]. We wonder if this pathway could involve the process of circulating EPCs' number reduction and dysfunction in prediabetic individuals. Previous studies have demonstrated that guanosine triphosphate cyclohydrolase (GTPCH I) deactivation could lead to tetrahydrobiopterin (BH4) insufficiency following with subsequent eNOS uncoupling [22–24], which may be attributable to the reduced eNOS-mediated NO production [17]. Thus, investigating the key protein expressions in the GTPCH/BH4 signaling pathway was also covered in the present study.

As previously studied, sexual difference in circulating EPCs' number and function was usually present among the middle-aged individuals because of the estrogen-related protective effect [20]. To exclude gender difference, the present study was conducted only in premenopausal women.

In summary, the following issues still need further investigation: (1) if the inability of circulating EPCs is present in premenopausal i-IGT or i-IFG women? (2) If that is, what is the possible underlying mechanism? Whether or not NO production will also be responsible for the deleterious changes of circulating EPCs in the targeted population just as it was in diabetic and hypertensive patients? Attempting to elucidate the abovementioned questions, the present study evaluated circulating EPCs' number and function, endothelial function in premenopausal NGT, i-IFG, or i-IGT women. In addition, plasma NO and EPCs-secreted NO were evaluated, and the key proteins in the guanosine triphosphate cyclohydrolase/tetrahydrobiopterin (GTPCH/BH4) pathway and Tie2/Akt/eNOS signaling pathway were evaluated meanwhile.

2. Materials and Methods

2.1. Characteristics of Subjects. Sixty-one premenopausal women were recruited and divided into three groups including NGT ($n = 20$), i-IFG ($n = 21$), and i-IGT ($n = 20$) according to their oral glucose tolerance test (OGTT) results. On the basis of Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [25], subjects with fasting plasma glucose (FPG) < 100 mg/dl and the 2 h plasma glucose (2-h PG) after 75 g OGTT < 140 mg/dl are diagnosed with NGT, with 100 mg/dl ≤ FPG < 125 mg/dl and 2 h PG < 140 mg/dl were diagnosed with i-IFG, and with FPG < 100 mg/dl and 140 mg/dl ≤ 2 h PG < 200 mg/dl were diagnosed with i-IGT. The participants were excluded if they had the following conditions: established CVD, malignant disease, infectious or inflammatory disorders, smoke, polycystic ovary syndrome, previous hysterectomy or irregular menstrual cycles, pregnant or breastfeeding, with hormone replacement therapy, or any other use of E2/progesterone administration. The experimental protocol was approved by the Ethical Committee of our hospitals, and all the participants had signed the written informed consent for participation as enrolling in the present study.

Seventy-five-gram glucose solution was taken orally within 5 minutes after at least 8 hours of overnight fasting. Blood samples for glucose were collected before and at 30 min and 120 min postchallenge.

At the menstrual periods of the participants' menstrual cycles (day 2 to day 5 after the first day of menstrual bleeding), peripheral venous blood samples were drawn after overnight fasting. Serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), hyper-sensitivity C-reactive protein (hsCRP), glycosylated hemoglobin (HbA1c), and estradiol were also measured.

2.2. Evaluation of Circulating EPCs' Number. Flow cytometry and cell culture assay were used to assess the number of circulating EPCs. The detailed protocol has been written in the previous studies in assessing circulating EPCs' number [18, 26]. In brief, after the participants' peripheral blood mononuclear cells were separated by Ficoll density gradient centrifugation, they were cultured in endothelial cell basal medium-2 (EBM-2, Lonza Group, Ltd., Basel, Switzerland).

Following 7 days of culture, endothelial marker proteins were examined by flow cytometry. Peripheral blood (100 μl) was incubated for 40 min at 4°C with phycoerythrin- (PE-) labeled monoclonal mouse anti-human antibodies recognizing cluster of differentiation (CD) 31, von Willebrand factor, and kinase-insert domain receptor or corresponding immunoglobulin G isotype control. Following this, erythrocytes were lysed, and the remaining cells were washed with PBS and fixed in 2% paraformaldehyde at 37°C for 10 min prior to further analysis using an ACEA NovoCyte™. Cells were then incubated with monocytic lineage marker CD14, fluorescein isothiocyanate (FITC) anti-human CD45, and PE-Cy7 anti-human CD34 antibodies for 40 min at 4°C. NovoExpress software™ was used to analyze the results.

The EPCs' counts were assessed by the ratio of CD34+KDR+ cells per 100 peripheral blood mononuclear cells (PBMNCs). The circulating EPCs were cultured for one week after isolation and quantified using DiI-acLDL uptake and FITC-labeled Ulex europaeus agglutin (lectin) staining which was similar as previous studies reported [26]. Two independent staff were assigned to count the cultured EPC which were identified as differentiating EPCs with DiI-acLDL/lectin double positive cells.

2.3. Migration and Proliferation Assay of EPCs. Circulating EPCs' migration was evaluated by employing a modified Boyden chamber which had been written detailly in our other studies [18, 20, 27].

In short, after being cultured for one week, 2×10^4 EPCs were laid in the upper chamber of a modified Boyden chamber. DAPI was used to stain cell nuclei for quantification in 24 h. Cells which migrated into the lower chamber were counted manually in 3 random microscopic fields. EPCs' proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 g/l; Fluka; Honeywell International, Inc., Shanghai, China) assay after being nurtured for 7 days.

2.4. Measurement of Flow-Mediated Dilation. Brachial artery FMD was measured according to the previous studies [28]. In short, high-resolution ultrasonography was used with a 5–12 MHz linear transducer on an HDI 5000 system (Washington, USA). The participants were supine for at least 15 min, and then their brachial arteries were scanned longitudinally 2 to 10 cm proximal to the antecubital fossa. After increasing the pressure in an upper-forearm sphygmomanometer cuff to 250 mmHg for 5 min and monitoring electrocardiogram after cuff deflation for 90 s, FMD was taken as the percentage augment in mean diastolic diameter after reactive hyperemia 55 to 65 s following deflation to baseline.

2.5. Measurement of NO, VEGF, and GM-CSF Levels in Plasma and Secretion by EPCs. Levels of NO, VEGF, and GM-CSF in plasma and secretion by cultured EPCs were measured as the previous study reported [18, 20, 26].

The present study measured nitrite in plasma with the Greiss method and presented the results as μmol NOx of NO_3^-/NO_2^- per liter of medium. Plasma levels of VEGF and GM-CSF were determined by high-sensitive ELISA assays (R&D Systems, Wiesbaden, Germany).

The cultured EPCs were transferred to Dulbecco's Modification of Eagle's Medium/20%-fetal bovine serum (Sigma-Aldrich; Merck KGaA) for 48 h, and the NO, VEGF, and GM-CSF levels were measured in the condition media with the similar methods as in the plasma.

2.6. Measurement of Intracellular Tetrahydrobiopterin and Western Blot Analysis. High-performance liquid chromatography with florescence detection was used to assess intracellular BH4 concentrations, which were calculated by subtracting BH2 and oxidized biopterin from total biopterins and expressed as p mol/mg protein [24, 29].

Measurements of the expressions of Tie2, Akt, eNOS, and GTPCH I were previously described [26]. Total proteins of EPC were harvested by cell lysis buffer (Cell Signaling Technology Inc, Danvers, MA, USA). Protein extracts were subjected to SDS-PAGE and transferred to polyvinylidene fluoride membranes (Cell Signaling Technology Inc.). The antibodies including rabbit anti-phosphorylated Tie2, anti-Tie2, anti-phosphorylated Akt, anti-Akt, anti-phosphorylated eNOS, anti-eNOS (1 : 1000; Cell Signaling Technology Inc.), anti-GTPCH I (1 : 1000; Santa Cruz Technology Inc.), and β-actin (1 : 1000; Santa Cruz Technology Inc.) were used. Proteins were visualized with HRP-conjugated anti-rabbit IgG (1 : 2000; Cell Signaling Technology Inc.), followed by use of the ECL chemiluminescence system (Cell Signaling Technology Inc.). The results were presented by the ratio of specific phosphorylated proteins to total proteins or total GTPCH I, Tie2, Akt, and eNOS proteins to β-actin and were statistically compared relative to those of premenopausal women with NGT.

2.7. Statistical Analysis. SPSS V11.0 statistical software (SPSS Inc., Chicago, Illinois, USA) was applied in statistical analysis. Quantitative variables of normal distribution were expressed as mean ± standard deviation (SD). ANOVA was applied to evaluate statistical significance, and the least significant difference post hoc test was used to compare the difference between each two groups (NGT vs. i-IFG, NGT vs. i-IGT, and i-IFG vs. i-IGT). Univariate correlations were assessed with Pearson's coefficient (r). It was considered as statistical significance when P values < 0.05.

3. Results

3.1. Baseline Characteristics. The demographic characteristics of individuals in this research are shown in Table 1. Age, BMI, lipid profile, hsCRP, and estradiol were comparable in the 3 groups. Compared with the NGT group, i-IFG or i-IGT women had an obviously increased HbA1c (both $P < 0.05$), i-IFG women had an increased FPG ($P < 0.05$), and i-IGT women had an increased 2 h PG ($P < 0.05$). I-IGT women had a higher HbA1c than i-IFG women ($P < 0.05$). FMD in

Table 1: Clinical and biochemical characteristics.

Characteristics	NGT ($n = 20$)	i-IFG ($n = 21$)	i-IGT ($n = 20$)
Age (years)	40.1 ± 5.3	41.2 ± 4.8	43.9 ± 3.6
Height (cm)	162.0 ± 7.6	163.1 ± 6.5	161.2 ± 5.9
Weight (kg)	61.2 ± 5.0	60.6 ± 6.9	61.9 ± 4.9
Body mass index (kg/cm^2)	23.4 ± 2.1	22.8 ± 2.5	23.9 ± 1.8
Systolic blood pressure (mmHg)	121.2 ± 11.4	123.4 ± 7.8	121.9 ± 10.0
Diastolic blood pressure (mmHg)	71.7 ± 6.5	73.2 ± 5.4	70.7 ± 7.2
Heart rate (beats/min)	71.0 ± 6.3	73.8 ± 8.1	75.7 ± 8.7
Aspartate transaminase (mmol/L)	27.2 ± 6.1	25.5 ± 4.7	23.2 ± 4.6
Alanine aminotransferase (mmol/L)	25.6 ± 8.0	23.7 ± 4.6	22.6 ± 4.8
Blood urea nitrogen (mmol/L)	5.2 ± 1.3	5.0 ± 1.0	5.5 ± 0.9
Creatinine (mmol/L)	62.2 ± 19.0	60.6 ± 14.5	65.2 ± 15.7
Low-density lipoprotein (mmol/L)	2.80 ± 0.46	2.87 ± 0.44	2.99 ± 0.45
Total cholesterol (mmol/L)	4.80 ± 0.54	4.90 ± 0.56	5.04 ± 0.51
High-density lipoprotein (mmol/L)	1.43 ± 0.25	1.39 ± 0.24	1.35 ± 0.24
Triglyeride (mmol/L)	1.37 ± 0.22	1.42 ± 0.21	1.45 ± 0.20
Fasting plasma glucose (mmol/L)	4.81 ± 0.45	6.18 ± 0.38*	4.96 ± 0.35
2-hour plasma glucose (mmol/L)	6.51 ± 0.62	7.14 ± 0.45	9.53 ± 1.04$^{*\#}$
Glycosylated hemoglobin A1c (%)	5.30 ± 0.60	5.76 ± 0.49*	5.91 ± 0.42$^{*\#}$
Hypersensitive C-reactive protein (mmol/L)	1.15 ± 0.66	1.32 ± 0.71	1.44 ± 0.86
Estradiol (pmol/L)	237.4 ± 33.5	215.9 ± 37.4	202.1 ± 23.6
Flow-mediated brachial artery dilatation (%)	11.0 ± 1.41	9.63 ± 1.33	8.09 ± 1.36$^{*\#}$

Abbreviations. NGT, normal glucose tolerance; i-IFG, isolated impaired fasting glucose; i-IGT, isolated impaired glucose tolerance. *Notes.* Data are given as mean ± standard deviation. $^*P < 0.05$ vs. NGT; $^\#P < 0.05$ vs. i-IFG.

i-IGT women was evidently lower than that in NGT or i-IFG counterparts (both $P < 0.05$). The difference in FMD was comparable between women with NGT and i-IFG ($P > 0.05$).

3.2. Circulating EPCs' Number and Activity in Three Groups. Premenopausal women with i-IGT had a significantly reduced number in circulating EPCs when compared to NGT or i-IFG women. The NGT group had a comparable EPCs' number with the i-IFG group (Figures 1(a) and 1(b)).

In a similar, EPCs' migratory and proliferative function were evidently reduced in the i-IGT group in comparison with the NGT or the i-IFG group (both $P < 0.05$). No difference in activity of circulating EPCs was noted between i-IFG and NGT women (Figures 1(c) and 1(d)).

3.3. FMD, NO, VEGF, and GM-CSF in the Plasma Levels and Secretion by EPCs in the Three Groups. Difference between different glucose tolerant status concerning plasma NO levels was discovered, presenting that i-IGT women had a lower plasma NO levels compared to NGT women or i-IFG women (Figure 2(a)). No difference of the index was noted between i-IFG and NGT women. The differences in plasma VEGF and GM-CSF levels appeared to be insignificant between groups (Figures 2(b) and 2(c)).

EPCs-secreted NO had a similar trend as plasma NO levels had concerning the difference between the i-IGT and NGT or i-IFG group. NO secretion by EPCs only in i-IGT women was significantly lower than NGT women (Figure 2(d)). VEGF or GM-CSF secretion by cultured EPCs were comparable between these groups (Figures 2(e) and 2(f)).

3.4. Correlation between Circulating EPCs or NO Level and FMD. Significantly positive correlations were observed between circulating EPCs' number and FMD (flow cytometry analysis, $r = 0.46$, $P < 0.05$, Figure 3(a)) (cell culture, $r = 0.51$, $P < 0.05$, Figure 3(b)), and FMD. The migration or proliferation of circulating EPCs also correlated with FMD ($r = 0.42$, $P < 0.05$, and $r = 0.62$, $P < 0.05$, respectively, Figures 3(c) and 3(d)). In addition, plasma NO levels ($r = 0.41$, $P < 0.05$, Figure 3(e)) as well as EPCs-secreted NO ($r = 0.43$, $P < 0.05$, Figure 3(f)) correlated with FMD.

3.5. Expression in GTCPH I Signaling Pathway and the Tie2/Akt/eNOS Signaling Pathway. Since the malfunction of circulating EPCs of premenopausal women with i-IGT was presented, the key proteins' expressions both in GTCPH/BH4 signaling pathway or in Tie2/Akt/eNOS signaling pathway were assessed to explore its related mechanism. As shown in Figures 4(a) and 4(b), the expressions of GTCPH I and intracellular BH4 were lower in the i-IGT group than that in the NGT or i-IFG group but were at a similar level between the i-IFG group and NGT group.

No differences of the expressions of the phosphorylation or total tie2, Akt, and eNOS were observed between these 3 groups (Figures 4(c)–4(e)).

4. Discussion

The present results demonstrated that it was i-IGT premenopausal women that presented declined number and impaired function in circulating EPCs, which related with endothelial dysfunction. As another status of prediabetes,

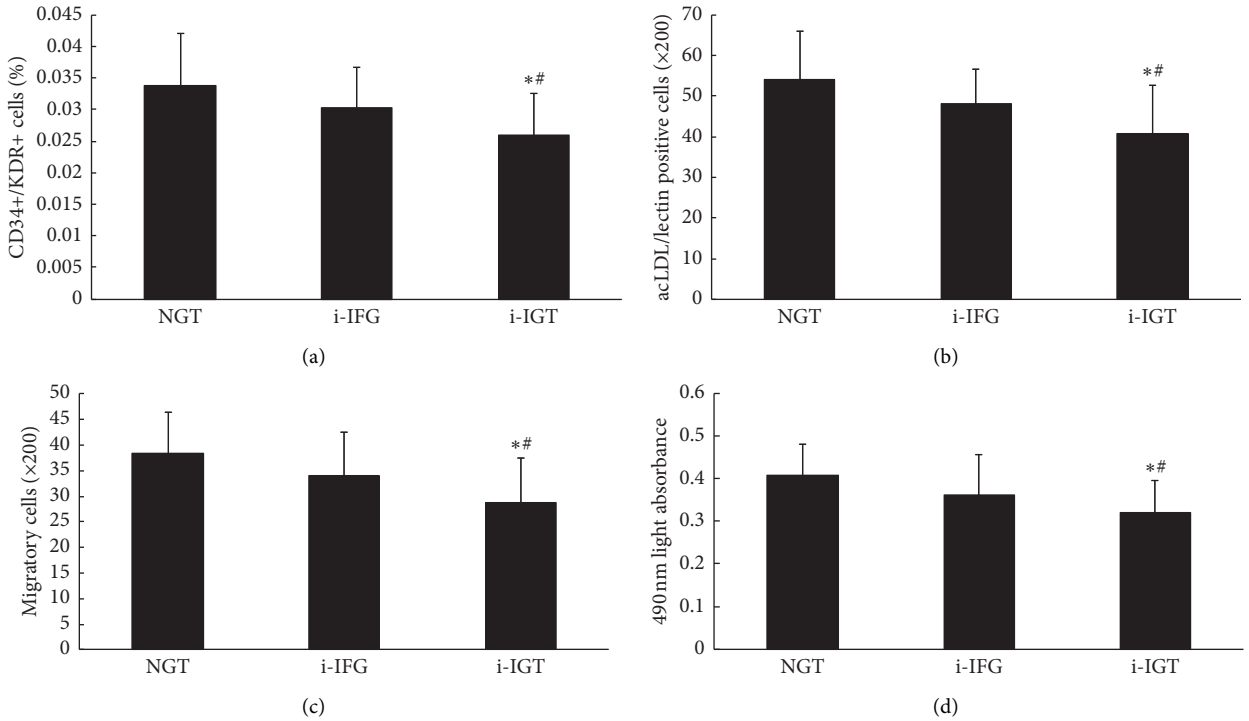


Figure 1: Number and activity of circulating EPCs in the three groups. Circulating EPCs were evaluated by (a) flow cytometric analysis and (b) cell culture assay. The number of circulating EPCs in premenopausal women with i-IGT was significantly lower than that in NGT and i-IFG women (both $P < 0.05$). No difference of the number of circulating EPCs was observed between NGT and i-IFG women. The migratory (c) and proliferative (d) activities of circulating EPCs in premenopausal women i-IGT were significantly decreased compared to NGT and i-IFG women (all $P < 0.05$). No difference of the activity of circulating EPCs was observed between NGT and IFG women. Data are given as mean ± SD ($^*P < 0.05$ vs. NGT; $^\#P < 0.05$ vs. IFG, $n = 20$ for the NGT group and i-IGT group and $n = 21$ for the IFG group). NGT: normal glucose tolerance; i-IFG: isolated impaired fasting glucose; i-IGT: isolated impaired glucose tolerance; acLDL, acetylated low-density lipoprotein; CD, cluster of differentiation; KDR, kinase-insert domain receptor; EPCs, endothelial progenitor cells.

i-IFG premenopausal women had a well preservation of endothelial function and endothelial repair capacity relying on their comparable counts and function of circulating EPCs with NGT women.

Another interesting point in this study is the alterations in NO production that may at least partly account for the inability of circulating EPCs in i-IGT premenopausal women. Moreover, we further investigated the possible underlying mechanism for the reduced NO production, which was presented that downregulation of the GTCPH I/BH4 signaling pathway may contribute to the occurrence of this event. Therefore, we confirmed the previous results that the harmful effects of i-IGT on endothelial function as well as circulating EPCs in the certain population further explored its possibly underlying mechanism. The results could shed light on the cause of the increased risk of CVD in i-IGT population and highlight the need of taking effective actions to improve endothelial function in i-IGT premenopausal women.

Prediabetes is a particular metabolic state between the normal glucose metabolism and diabetic hyperglycemia. There is a high risk for the progression of prediabetes to diabetes with an estimated 10% of annual conversion rate [30]. In addition, patients in the prediabetic state, especially in the i-IGT state, have had already increased risk for a scope of microvascular and macrovascular complications [5, 6].

Many evidences have concentrated on the impairment in circulating EPCs of patients with diabetes [31]; however, a few of studies were conducted to explore the changes in circulating EPCs in prediabetes. Nathan et al. observed attenuated circulating EPCs in Asian Indian prediabetic men [32]. Fadini et al. observed an obvious reduction of circulating EPCs in IGT subjects and newly diagnosed T2DM patients [33]. Similar results were also observed in obese/overweight IGT subjects [34] and gestational women with IGT [14]. As was consistent with these previous studies, we also noted the reduction and impaired functional activity of circulating EPCs in i-IGT although in a different population that is relatively younger and premenopausal, which confirmed the inability of circulating EPCs preceded overt diabetes that cut across different populations and ages.

Endothelial dysfunction is one of the key indicators and contributors for CVD, also involving in the pathogeneses of macrovascular complications of T2DM [35]. FMD, which is usually used to assess vascular endothelial dilation function [28], was lower in i-IGT premenopausal women than age-matched NGT women and positively correlated with circulating EPCs' number as well as function. Thus, it could be

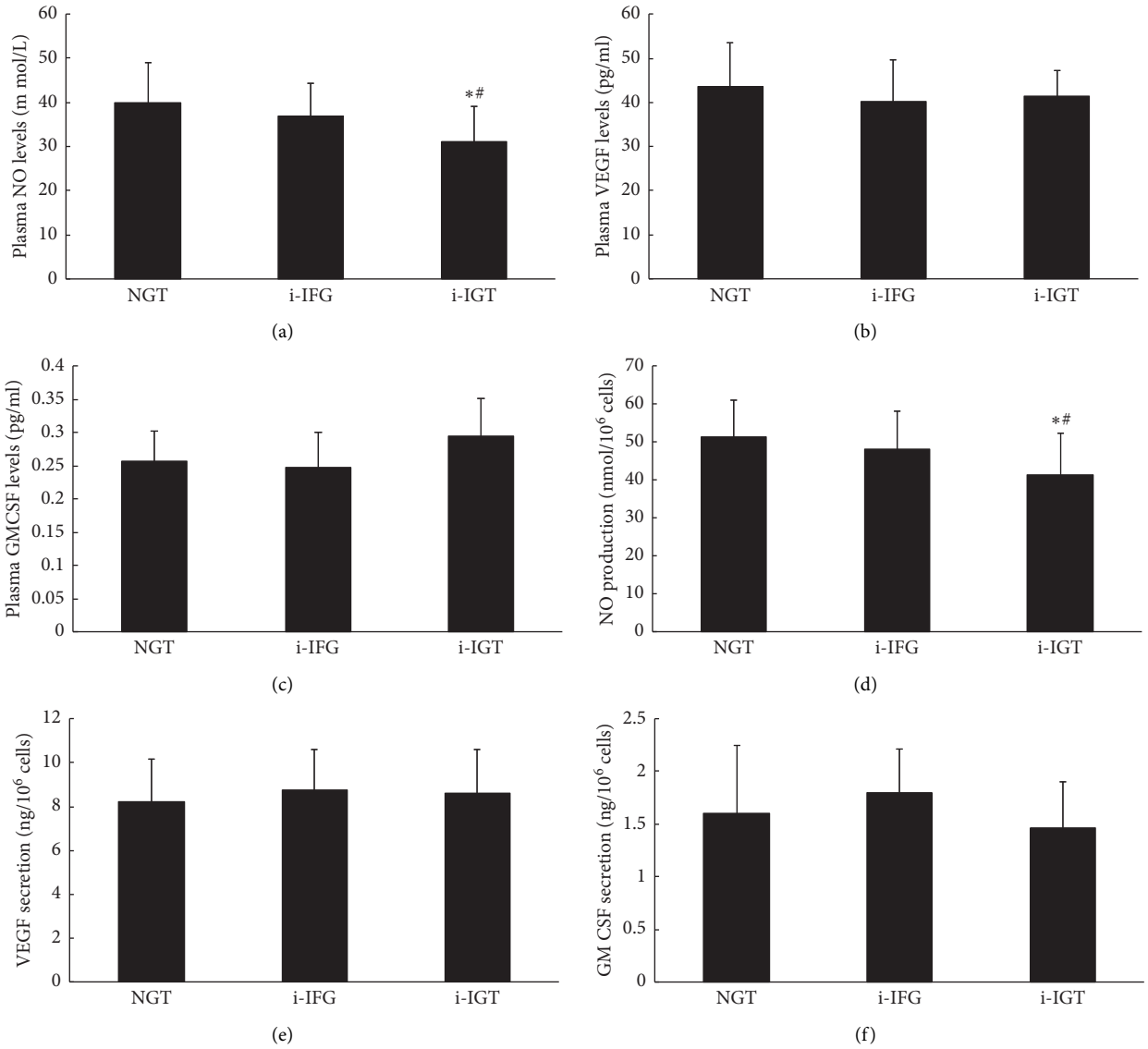


Figure 2: The NO, VEGF, and GM-CSF levels in plasma and secretion by EPCs in the three groups. Both the plasma NO level (a) and NO secretion by EPCs (d) in premenopausal women with i-IGT was significantly lower than NGT and i-IFG women (all $P < 0.05$). No difference of NO levels either in plasma or in culture media was observed between NGT and i-IFG women (a and b). No significant difference of VEGF or GM-CSF was found either in plasma level (b and c) or in secretion by EPCs (e and f) between the three groups. Data are given as mean ± SD ($^{*}P < 0.05$ vs. NGT; $^{\#}P < 0.05$ vs. IFG, $n = 20$ for the NGT group and i-IGT group and $n = 21$ for IFG group). NGT: normal glucose tolerance; i-IFG: isolated impaired fasting glucose; i-IGT: isolated impaired glucose tolerance; GM-CSF, granulocyte macrophage-colony stimulating factor; NGT, normal glucose tolerance; NOx, nitric oxide; VEGF, vascular endothelial growth factor.

hypothesized that the inability of circulating EPCs had a deleterious effect on endothelial function and may augment CVD-related risk in prediabetes.

Although i-IFG as well as i-IGT are classified into impaired glucose regulation preceding overt diabetes, there is a controversy over their risk of causing CVD [2, 36]. In fact, it seems to be that CVD may be more related with post-challenge glucose than fasting glucose, which may be explained by differences in metabolic traits, for example, insulin secretion and insulin sensitivity and other CVD risk factors between i-IGT and i-IFG subjects [37]. It is usually acknowledged that i-IGT, characterized by the elevation of postload glucose during OGTT and normal FPG, is largely related with defect of the first-phase insulin secretion and peripheral insulin resistance (IR), while i-IFG, characterized by moderately increased FPG and normal postload glucose, is mainly caused by increased hepatic glucose production resulting from hepatic IR [37].

In previous studies, there seemed to have a contradictory phenomenon about the alternations of endothelial function and circulating EPCs in i-IFG individuals. Subjects with i-IFG seemed to maintain a normal level of circulating EPCs in the study of Fadini et al. [33]; however, in other studies, endothelial dilated function was reported to be declined, which could be improved by regular aerobic exercise training [38, 39]. We did not observe either the inability of circulating

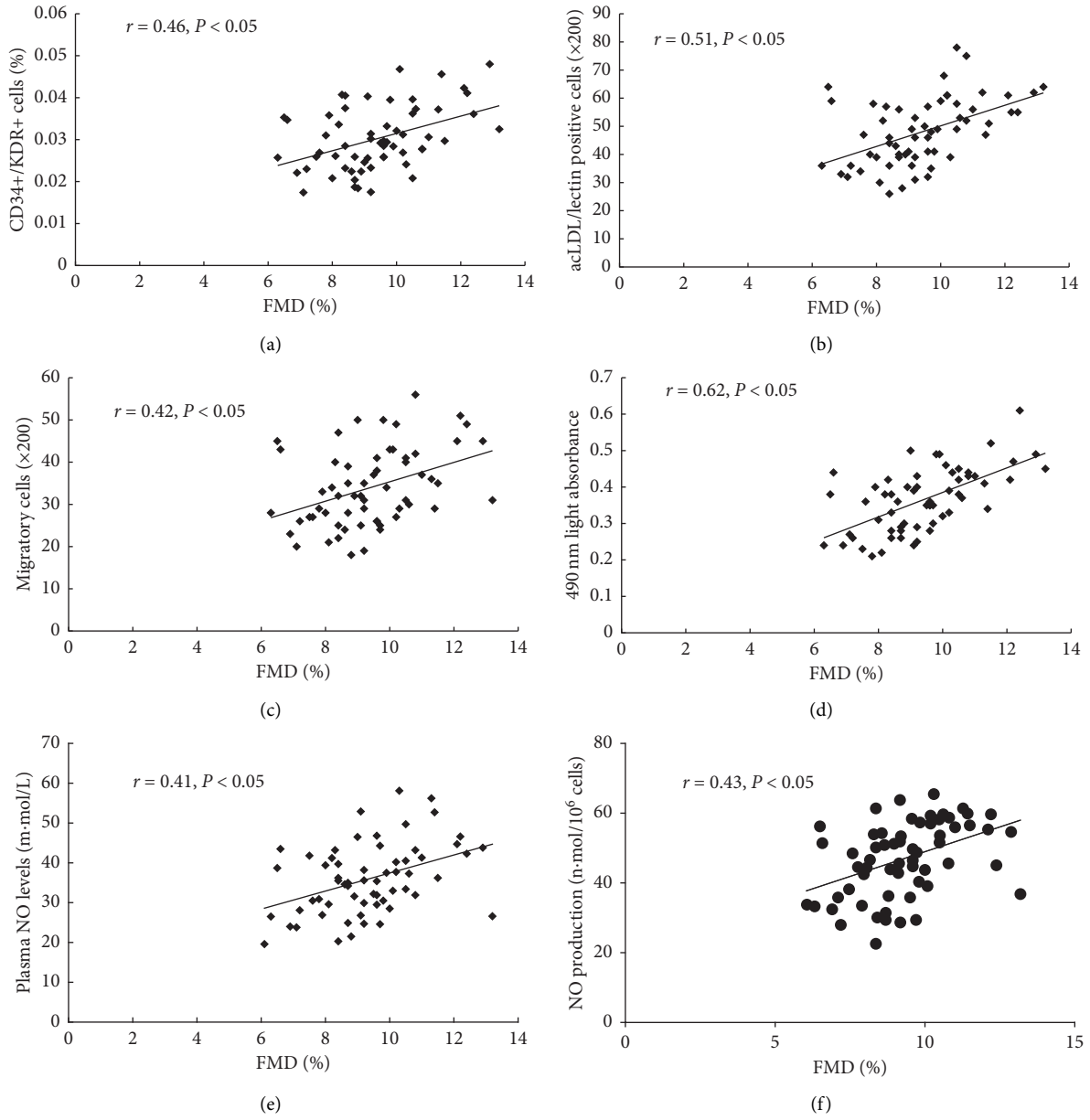


Figure 3: The correlations between circulating EPCs or NO production and FMD. The number of circulating EPCs evaluated by flow cytometric analysis (a) or by cell culture (b) strongly correlated with FMD. The migratory (c) or proliferate (d) activity of circulating EPCs also correlated with FMD. Both plasma NO level (e) and NO secretion by EPCs (f) correlated with FMD. LDL, low density lipoprotein; CD, cluster of differentiation; KDR, kinase-insert domain receptor; FMD, flow-mediated brachial artery dilatation; EPCs, endothelial progenitor cells; NO, nitric oxide.

EPCs or impairment of endothelial dilated function in i-IFG premenopausal women. The divergence may be able to be explained by the enrolled populations with an obvious age difference (with an average 65 years in previous studies vs. 42 years in the current study) and only in premenopausal state in the present study. So it could be speculated that relatively high estradiol levels in premenopause may exert a protective effect on endothelial function in women with IFG although the relationship of the estradiol level and FMD in these patients needed to be further elucidated.

The reason of why there is a difference in the alternations of circulating EPCs between i-IFG and i-IGT premenopausal women is unclear yet. We speculate that the relatively constant postload glucose in i-IFG subjects could be counted on for the difference since glucose excursion could lead to a greater degree of reduced EPCs compared to constantly high glucose concentrations in some vitro experiments [40]. We might hypothesize that the preservation of endothelial function could be partly attributable to the relatively low risk for CVD in i-IFG population.

Though many studies had observed the inability of circulating EPCs in i-IGT population, the exploration of mechanism about this phenomenon does not go far enough for now. Many experiments have suggested that hyperglycemia

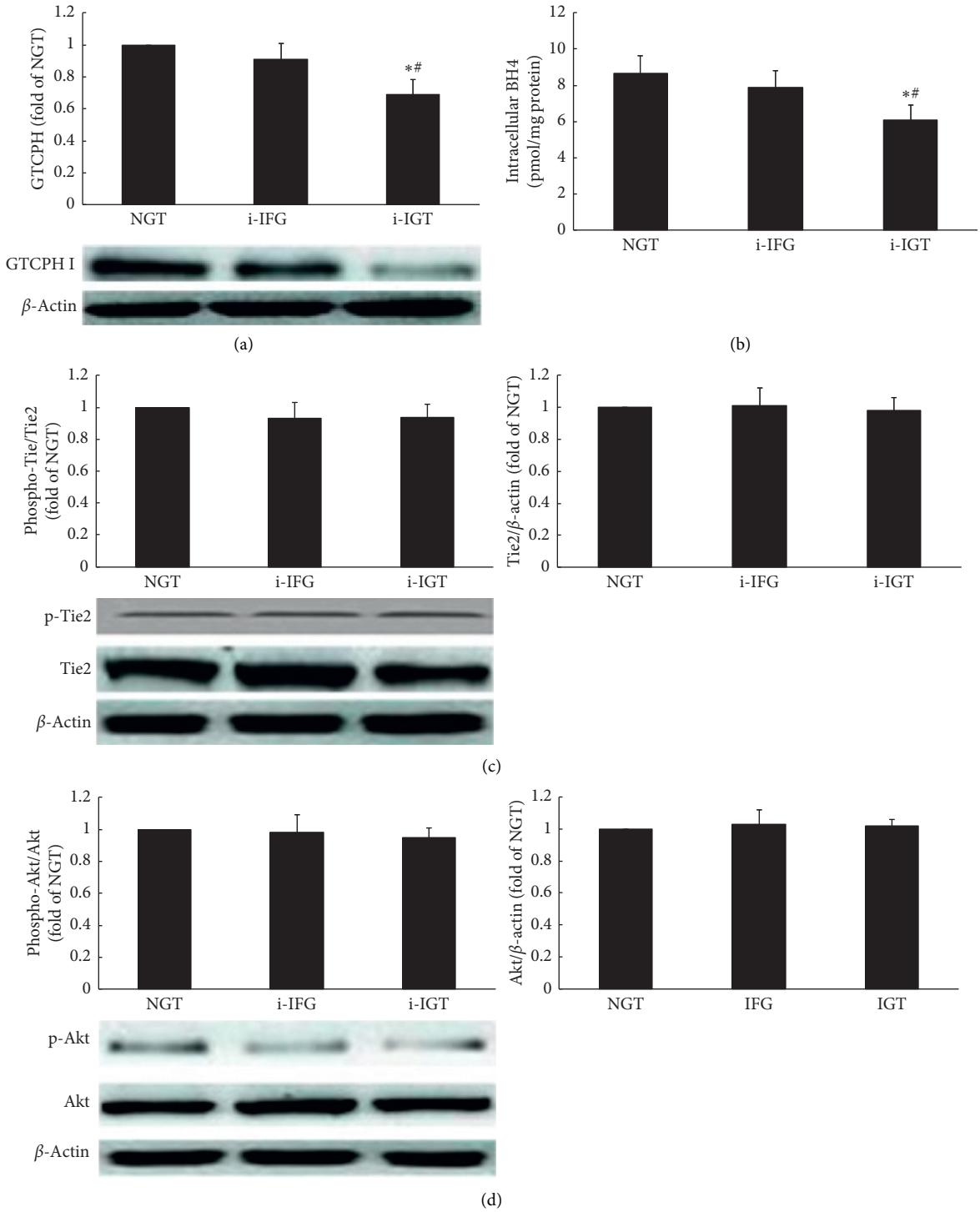
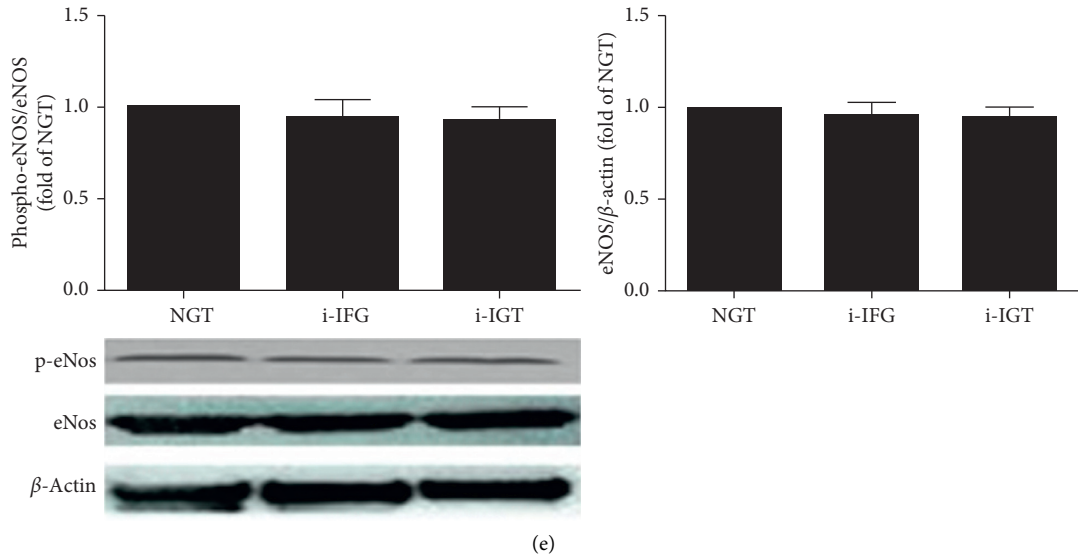


Figure 4: Continued.



(e)

Figure 4: The CTCPH I/BH4 pathway and the Tie2/Akt/eNOS signaling pathway of circulating EPCs in the three groups. Both GTCPH I expression (a) and intracellular BH4 (b) levels were lower in premenopausal women with i-IGT than that in NGT and IFG women (all $P < 0.05$). No difference of the two indexes was observed between NGT and i-IFG women. Either the phosphorylation forms or the total protein of tie2 (c), Akt (d), and eNOS (e) of circulating EPCs had not exhibited significant difference between each two groups. Data are given as mean ± SD ($^{*}P < 0.05$ vs. NGT; $^{\#}P < 0.05$ vs. IFG, $n = 20$ for the NGT group and i-IGT group and $n = 21$ for IFG group). NGT: normal glucose tolerance; i-IFG: isolated impaired fasting glucose; i-IGT: isolated impaired glucose tolerance; GTPCH: guanosine triphosphate cyclohydrolase; BH4: tetrahydrobiopterin; Tie2: tyrosine kinase with immunoglobulin and epidermal growth factor homology domain-2; Akt: protein kinase B; eNOS: endothelial nitric oxide synthase.

could lead to dysfunction of circulating EPCs by acting against their production and promoting their removal from the circulation [35, 41, 42]. Among these classical regulators of the insufficiency and/or dysfunction of circulating EPCs such as NO, VEGF, and GM-CSF [42, 43], and only NO production seems to be more likely to be responsible for the changes in circulating EPCs in many clinical or subclinical situations including obesity, smoke, prehypertension, and diabetes [11, 21, 24, 44]. As expected, NO production could also be attributed to the changes in circulating EPCs in i-IGT premenopausal women. Decreased NO levels both in plasma and secretion by EPCs in this certain population were observed, and positive correlation between NO production and the number or functional indexes of circulating EPCs in all populations was found in the present study.

Similarly, the underlying mechanism that hypeglycemia could contribute to the depletion or dysfunction of EPCs is closely related with NO production and NO bioavailability [41]. In in vitro experiments, high glucose concentrations could reduce intracellular BH4 cofactor (tetrahydrobiopterin), thus uncoupling endothelial NO synthesis (eNOS) and reducing NO bioavailability [42]. In the current study, we also observed the reduced expression of GTCPH I and decreased intracellular BH4 levels in i-IGT premenopausal women that was in parallel with the reduced NO production, indicating that the reduced expression of the two key proteins may contribute to the insufficiency of NO production either in the plasma or secretion by EPCs. Therefore, based on these results, we could speculate that the insufficiency and/or dysfunction of circulating EPCs in i-IGT premenopausal women may be associated with the decreased NO production through downregulating GTCPH I/BH4 signaling pathway.

Tie2/Akt/eNOS signaling pathway is also important in modulating circulating EPCs' number and function [45]. As previously demonstrated, the expressions of the phosphorylation of several key proteins in this signaling pathway including tie2, Akt, and eNOS, in circulating EPCs, were reduced in prehypertensive premenopausal women with DM [21]. However, in the present study, we did not observe any reduced expression of either the abovementioned proteins themselves or their phosphorylation forms in i-IGT premenopausal women, indicating that decreased NO secretion by EPCs could be independent of the Tie2/Akt/eNOS pathway in i-IGT premenopausal women.

Several implications can be taken from the current results. First, in premenopausal women with i-IGT but not with i-IFG, circulating EPCs' number and function declined, which was related to endothelial dysfunction. The results may partly explain the difference of the risk of CVD in the two prediabtic status. On the contrary, proper intervention to enhance vascular repair capacity should be considered in premenopausal women when they are at a prediabetic (especially IGT) stage. Second, we did not observe the difference in endothelial function and circulating EPCs between i-IFG and NGT premenopausal women, indicating that impaired fasting glucose metabolism alone may be not enough to impair endothelial function. Third, the reduced number or activity of circulating EPCs may be associated with the decreased NO production, which was at least partly mediated by the GTPCH I/BH4 signaling pathway. The clinical interventions aiming to increase the NO production

such as regular exercise, quitting smoking, and statins usage may be more effective in improving endothelial dysfunction of i-IGT premenopausal women.

5. Conclusions

The present investigation demonstrated the unfavorable effects of i-IGT on circulating EPCs and endothelial function in premenopausal women, which could correlate with NO production reduction as well as downregulation in the GTPCH I/BH4 signaling pathway. Endothelial function and circulating EPCs' number or activity were preserved in i-IFG premenopausal women, indicating that moderately increased fasting glucose in premenopausal women may not exert much negative effect on endothelial function. Our results provided new vision to vascular preservation in i-IGT premenopausal women, pointing out that the GTPCH I/BH4 signaling pathway could be a latent point for improving endothelial improve capacity.

Data Availability

The datasets during the current study are accessible from the corresponding author on reasonable request.

Ethical Approval

The protocol for the research project was approved by the ethics committee of the Sixth Affiliated Hospital, Sun Yat-Sen University (Guangzhou, People's Republic of China), and the ethics committee of the Guangzhou Economic Development Zone Hospital (Guangzhou, People's Republic of China).

Consent

The written informed consent was acquired from all participants.

Conflicts of Interest

The authors declare they have no conflicts of interest.

Authors' Contributions

Shaohong Wu and Haitao Zeng directed design of the study and the manuscript's writing and provided the final approval of the version to be published. Juan Liu and Xiangbin Xing completed the study, wrote and revised the manuscript, and contribution equally to the publication of this manuscript. Xinlin Wu, Xiang Li, Shun Yao, and Ren Zi involved in the experimental work and the writing of the manuscript in the present study. Juan Liu and Xiangbin Xing contributed equally to this work.

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Research Article

Low Expression of *FFAR2* in Peripheral White Blood Cells May Be a Genetic Marker for Early Diagnosis of Acute Myocardial Infarction

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Objective. To find molecular markers for the diagnosis of acute myocardial infarction (AMI), this research further verified the relationship between the expression level of *FFAR2* gene and AMI by expanding the sample size based on the previous gene chip results. *Methods*. Peripheral venous leukocytes were collected from 113 patients with AMI and 94 patients with non-coronary artery disease as the experimental group and the control group, respectively. Real-time fluorescence quantitative polymerase chain reaction was used to detect the expression of the *FFAR2* gene. Western blot analysis was applied to detect the relative expression of the *FFAR2* gene at the level of protein. Furthermore, the relationship between gene expression and clinical data was also analyzed and compared. *Results*. The level of expression of *FFAR2* gene in peripheral blood of patients with AMI was significantly lower than that of the control group (0.33 [0.04–1.08], 0.62 [0.07–1.86], respectively; $p < 0.05$), which was 0.53 times that of the control group. Western blot results presented that the *FFAR2* protein level in the peripheral blood of the AMI group was lower than that of the control group (0.114; $p = 0.004$). Analyzing clinical data of the subjects indicated that the average age of the AMI group was significantly higher than the age of control group ($p < 0.01$). Also, the fasting blood glucose level was higher ($p < 0.01$), and the high-density lipoprotein cholesterol (HDL-C) level was lower ($p = 0.03$). The *FFAR2* mRNA level correlated positively with the HDL-C level ($p < 0.01$). Logistic regression analysis suggested that the low expression of the *FFAR2* gene in peripheral blood may be a risk factor for AMI independent of age, family history of diabetes, fasting blood glucose level, and HDL-C level ($p = 0.025$). Compared with the high *FFAR2* expression group, the risk of AMI in the low *FFAR2* expression group was 6.308 times higher. *Conclusion*. The expression level of the *FFAR2* gene in peripheral blood of patients with AMI was significantly lower than that in the control group. Low expression of the *FFAR2* gene in peripheral blood is an independent risk factor for AMI. Hence, it may also be a potential biomarker to predict AMI.

1. Introduction

Acute myocardial infarction (AMI) is a serious consequence of coronary atherosclerotic heart disease [1]. AMI is also one of the cardiovascular diseases with high morbidity and mortality [2,3]. Nearly half of patients with cardiovascular diseases die from AMI [4]. By 2020, cardiovascular disease is expected to be the leading cause of death in both developed and developing countries [5], accounting for 36% of all deaths worldwide [6]. In the United States, the death toll from AMI is more than 2.4 million [7]. According to China's cardiovascular disease report, 11 million patients are suffering from coronary heart disease. The incidence of cardiovascular diseases in China is on the rise, accounting for more than 40% of deaths from diseases.

AMI is a polygenic disease and occurs as a result of interaction between genetic and environmental factors.

Therefore, it is very important to find molecular markers for early diagnosis to alert physicians of the possibility of AMI.

Eighty percent of genes in peripheral blood cells are expressed in other tissues. Studies have shown that based on the how different genes in white blood cells were expressed from peripheral blood, gene expression can be used as molecular markers for the diagnosis and prognosis of systemic lupus erythematosus, solid malignant tumors, organ transplantation, and other diseases [8]. It is essential in the prediction of cardiovascular diseases and other complex diseases. The aim of this research was to find the diagnostic markers of AMI from peripheral blood leukocytes.

Previous results of gene chip research showed that *FFAR2* gene expression in peripheral blood was lower than that in the control group. Therefore, this research aims to verify the results of the *FFAR2* gene chip from peripheral blood of a large cohort of patients. Furthermore, the research examined whether the *FFAR2* gene can be used as a molecular marker for the diagnosis of AMI.

2. Subjects and Methods

2.1. Research Subjects. A total of 113 patients with AMI hospitalized in the Department of Cardiovascular Medicine, China-Japan Union Hospital, Jilin University, from March 2018 through May 2018, were selected as cases. The diagnosis of AMI was based on the global definition of myocardial infarction issued by the European Heart Association in 2017 [9], that is, the presence of definite angiopathy confirmed by coronary angiography and severely narrowed and occluded main coronary arteries (left main coronary artery and right coronary artery, etc.) and main branches (circumflex and anterior descending branches, etc.). The exclusion criteria for AMI were as follows: (1) myocardial infarction secondary to ischemic imbalance; (2) myocardial infarction when serum biochemical markers (troponin and myoglobin) were not available; (3) myocardial infarction associated with percutaneous coronary intervention or stent thrombosis; or (4) coronary artery bypass grafting-related myocardial infarction. Ninety-four patients with noncoronary heart disease were treated as the control group. The criteria for inclusion of subjects in the control group were as follows: hospitalized patients with chest pain, coronary angiography showing degree of coronary artery stenosis less than 50%, no secondary changes of pathological Q-wave, T-wave, and ST-segment in ECG, except acute pneumonia, pleurisy, intercostal neuritis, etc.

Informed consent was obtained from all patients before the collection of test samples and sample information. The study was conducted in accordance with the principles and guidelines laid down in the Declaration of Helsinki. Age, sex, history of smoking, hypertension, and diabetes, levels of fasting blood sugar, serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were recorded in detail.

2.2. Research Methods

2.2.1. Acquisition of Peripheral Blood Lymphocytes. In the morning, 6 ml of fasting peripheral blood of the study subjects was stored in an EDTA tube at 4°C, and lymphocytes were extracted within 4 hour. The reagent used was peripheral blood lymphocyte separating solution. The details of the steps that were followed are (1) fresh anticoagulant was mixed with an equal volume of 0.9% sodium chloride injection evenly; (2) the above mixture was carefully added to an equal volume of human peripheral blood lymphocyte separating solution, followed by centrifugation for 20 minutes at 3000 r/minute; and (3) after centrifugation, the four layers from top to bottom were plasma, milky white lymphocyte layer, transparent separation layer, and erythrocyte layer. The milky white lymphocyte layer was aspirated for use in subsequent experiments.

2.2.2. Synthesis of cDNA of Peripheral Blood Lymphocytes. (1) The Blood Total RNA Kit (Xinjing Biological Reagent Development Co., Ltd., Hangzhou, China) was used to extract total RNA from lymphocytes. In order to avoid RNA degradation or contamination, the extraction process was carried out in strict accordance with the kit instructions. The quality of RNA solution was detected by polyacrylamide gel electrophoresis. 28S and 18S rRNA bands were visible, and the brightness of the 28S rRNA band was about two times that of the 18S rRNA. The concentration and absorbance of the standard samples were determined by an enzyme-labeling instrument. Reverse transcription was performed after meeting the requirements. (2) According to the instructions of the reverse transcription kit (FastKing gDNA Dispelling RT SuperMix, Tiangen Biochemical Technology Co., Ltd., Beijing, China), reverse transcription of the total RNA that met the requirements of the experiment was carried out, and a consistent concentration of RNA was added to each sample. The DNA samples obtained were stored at −80°C for the subsequent fluorescence, quantitative, polymerase chain reaction detection.

2.2.3. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) Detection. After diluting the obtained DNA samples 20 times, the SYBR fluorescence quantitative kit (biochemical fluorescence quantitative kit, Taq qPCR synthetic premix, Shanghai, China) was used for PCR amplification. *GAPDH* was used as internal reference gene and *FFAR2* as target gene. The specificity of amplification conditions was determined by software dissolution curves using the ABI-FAST7500 instrument. The sequence of RT-PCR primers used is shown in Table 1.

2.2.4. Western Blotting. Peripheral white blood cells were collected by radioimmunoprecipitation and centrifuged. The supernatant was then collected and placed in water at 98°C for 10 minutes. Buffer (5x) and 30 μg sample protein were added. The voltage in the laminated gel was set at 60 V.

Table 1: RT-PCR primer sequence.

Genes		Genes primer sequence (5′–3′)
FFAR2	F[a]	CTTCGGACCTTACAACGTGTC
	R[b]	CTGAACACCACGCTATTGAC
GAPDH	F[a]	TGTGGGCATCAATGGATTTGG
	R[b]	ACACCATGTATTCCGGGTCAAT

F[a]: upstream primers. R[b]: downstream primers. RT-PCR = reverse-transcriptase polymerase chain reaction.

When the protein band was straight and reached the boundary between the stacking gel and the separation gel, the voltage was adjusted to 110 V until the end of electrophoresis. According to the instructions of BDTM semidry ink–absorbing paper, the protein was transferred to polyvinylidene fluoride film and incubated overnight with the primary antibody at 4°C. The secondary antibody was incubated at room temperature for 2 hours and analyzed by a chemiluminescence imaging system.

2.3. Statistical Analysis. All the data were analyzed by SPSS 25.0 software. A normality test was used to test the measurement data; $\overline{X} \pm S$ was used to describe data obeying a normal distribution ($p > 0.1$). Two independent samples t tests were used to compare the differences between the groups. Median and quartile intervals were used for statistical analysis for the data not obeying a normal distribution ($p \leq 0.1$). The nonparametric rank sum test of two independent samples was used to compare the differences between the groups. Frequency analysis was used to describe the counting data, and a χ^2 test was used to analyze the differences between the groups. Bivariate logistic regression analysis was used to analyze the risk factors related to AMI. The results were statistically significant with a bilateral $p \leq 0.05$.

3. Results

3.1. Baseline Data Analysis. Clinical data of the research subjects showed that there were no significant differences in gender, history of hypertension, smoking history, serum TG level, TC level, and LDL-C level between the AMI group and the control group ($p > 0.05$). However, compared with the patients in the control group, those in the AMI group were significantly older ($p < 0.01$). More people had type 2 diabetes ($p = 0.02$). Fasting blood sugar level was significantly higher ($p < 0.01$). HDL-C level was lower ($p = 0.03$) (Table 2). The proportion of hypoglycemic drugs used in the AMI group was significantly higher than that in the control group ($p = 0.01$) (Table 3).

3.2. Analysis of the FFAR2 Gene

3.2.1. Identification of RT-PCR Products. In this research, the amplification curves of the internal reference and *FFAR2* genes were significantly smooth "S" shaped curves and the dissolution curves had a single peak, without multiple peaks, which showed that the amplified primers had strong specificity, suitable reaction conditions, and no nonspecific amplification.

3.2.2. Analysis of Expression Level of the FFAR2 Gene. RT-PCR was repeated for 3 times for each sample, and the standard deviation was consistent with RT-PCR requirements. Independent sample t-test was carried out for the AMI group and the control group, meeting the requirement of $p < 0.05$. The results showed that the relative expression of the *FFAR2* gene in the AMI group (i.e., the $2^{-\Delta Ct}$ value quantitatively measured from PCR) was 0.33 (0.04–1.08) and 0.62 (0.07–1.86) in the control group. There was a significant difference between the two groups ($p < 0.05$). The relative expression of the *FFAR2* gene in peripheral blood of patients with AMI was significantly lower (0.53 times) than that in the control group (Figure 1). In this study, beta-actin was taken as the internal reference protein. The protein test was repeated for 3 times for each group to detect the peripheral blood protein level of the research subjects. Western blot results showed that there was no significant difference in the expression of beta-actin between the AMI and control groups, whereas *FFAR2* gene expression was statistically significant between the two groups ($p = 0.004$). The expression of the *FFAR2* gene at protein level in the AMI group was 0.114 times of that in the control group (Figures 2(a) and 2(b)).

3.3. Correlation Analysis. The baseline data revealed differences between the groups in age, history of diabetes, fasting blood glucose level, and HDL-C. Further analysis was applied to examine whether the relative expression of the *FFAR2* gene correlated with these factors.

All subjects were divided into an elderly group (≥65 years) and a younger group (<65 years), a type 2 diabetes group and a non–type 2 diabetes group, a high fasting blood glucose group (≥5.6 mmol/l) and a normal fasting blood glucose group (<5.6 mmol/l), and a low HDL-C group (<1.04 mmol/l) and the high HDL-C group (≥1.04 mmol/l). The relative expression level of the *FFAR2* gene in each subject was expressed by $2^{-\Delta Ct}$. The correlation between the relative expression levels of the *FFAR2* gene in the elderly group and the younger group, between the type 2 diabetes mellitus group and the non–type 2 diabetes mellitus group, between the high fasting blood glucose group and the normal fasting blood glucose group, and between the low HDL-C and high HDL-C groups were statistically analyzed and compared.

The results found no correlation between *FFAR2* gene expression and age ($p = 0.121$). The *FFAR2* mRNA level was not correlated with type 2 diabetes mellitus ($p = 0.836$), nor with the level of fasting blood sugar ($p = 0.339$). However, it correlated with the level of HDL-C ($p < 0.001$). The results are summarized in Table 4.

3.4. Logistic Regression Analysis. Based on the cutoff value of relative expression of the *FFAR2* gene, all subjects were divided into a low-expression group ($2^{-\Delta Ct} < 2.850$) and a

Table 2: Baseline data (which showed no differences between the test and the control groups).

Data category	AMI group ($n = 113$)	Controls ($n = 94$)	$t/x^2/z$	p value
Gender				
Male (%)	80 (70.80)	46 (48.94)		
Female (%)	33 (29.20)	48 (51.06)		
Hypertension (%)	55 (48.67)	41 (43.62)	0.53	0.47
Smoking (%)	52 (46.02)	40 (42.55)	1.58	0.66
TG (mmol/l)	1.57 (1.12–2.50)	1.29 (1.03–1.97)	−1.81	0.07
TC (mmol/l)	4.47 ± 1.26	4.57 (3.81–5.36)	−0.40	0.69
LDL-C (mmol/l)	2.99 ± 0.98	2.92 ± 0.77	0.51	0.61

AMI = acute myocardial infarction; LDL-C = low-density lipoprotein cholesterol; TC = total cholesterol; TG = triglyceride.

Table 3: Baseline data (which showed differences between the test and the control groups).

Data category	AMI group ($n = 113$)	Control ($n = 94$)	$t/x^2/z$	p value
Age (years)	64.10 ± 11.23	57.88 ± 10.42	4.10	0.00
Type 2 diabetes mellitus (%)	26 (29.89)	12 (15.38)	5.07	0.02
Fasting blood glucose (mmol/l)	6.56 (5.32–9.43)	5.39 (5.03–6.18)	−4.24	0.00
HDL-C (mmol/l)	0.95 (0.81–1.13)	1.05 (0.92–1.24)	−2.24	0.03
Hypoglycemic drug (%)	25 (22.12)	8 (8.51)	7.695	0.01

AMI = acute myocardial infarction; HDL-C = high-density lipoprotein cholesterol.

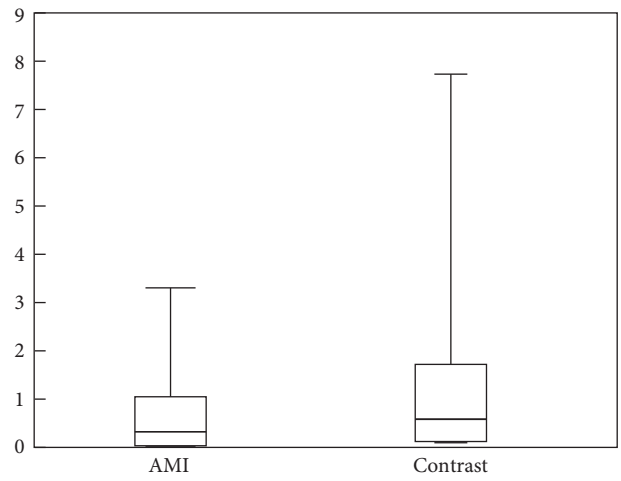

Figure 1: Relative expression of the *FFAR2* gene. AMI = acute myocardial infarction.

high-expression one ($2^{-\Delta Ct} \geq 2.850$). The sum of sensitivity and specificity is at its peak when using the 2.850 as the cutoff value. According to baseline data, all subjects were divided into a high fasting blood sugar group and a normal fasting blood sugar group, an elderly group and a younger group, a type 2 diabetes mellitus group and a non–type 2 diabetes group, and a low HDL-C group and a high HDL-C group.

The results were further analyzed using binary logistic regression analysis. The results showed that low expression of the *FFAR2* gene was an independent risk factor for AMI ($p = 0.025$) (Table 5). The risk of AMI in the group with low expression of the *FFAR2* gene was 6.308 times higher than that in the group with high expression of *FFAR2* gene. In addition, high fasting blood sugar was an independent risk factor for AMI ($p = 0.008$), and high fasting blood sugar increased the risk of AMI to 3.132 times (Figure 3).

4. Discussion

This study showed that *FFAR2* gene expression in the peripheral blood of patients with AMI was lower than that in the control group at the level of gene and protein.

FFAR2, also known as *GPR43*, is located in a set of standard intron-free genes on chromosome 19q13.1. It encodes a member of the GP40 family of G protein–coupled receptors [10], which belongs to the largest known receptor family [11]. Many molecules, including biogenic amines, amino acids, proteins, fatty acids, lipids, nucleotides, and ions, are activated ligands of G protein–coupled receptors [12]. Short chain fatty acids are mainly produced by intestinal microflora through fermentation of undigested carbohydrates and dietary fibers, which further activates *FFAR2* [10]. The *FFAR2* protein contains seven hydrophobic regions, which are consistent with transmembrane helix (tm). Sequence analysis revealed that *FFAR2* protein belongs to class A of G protein–coupled receptors. The receptors encoded by the *FFAR2* gene contain cysteine residues in the first and second extracellular rings, which may control the structure by forming intramolecular disulfide bonds [13]. *FFAR2*, as a signaling molecule, plays an important role in regulating blood glucose, inflammation, and serum lipid [14]. Abnormal blood glucose, inflammation, and lipid levels increase the risk of AMI in healthy people. The low expression of *FFAR2* may be a potential biomarker to predict the occurrence of AMI by affecting the above pathways.

The results showed that the number of patients with type 2 diabetes mellitus in the AMI group was higher than that in the control group, and the level of fasting blood sugar was higher. Abnormal blood sugar is a recognized risk factor for coronary artery disease [15]. Diabetic patients taking insulin-stimulating drugs show reduced mortality and risk of cardiovascular events [16]. In type 2 diabetes mellitus, the effect of incretin decreased [17,18], and the morbidity and

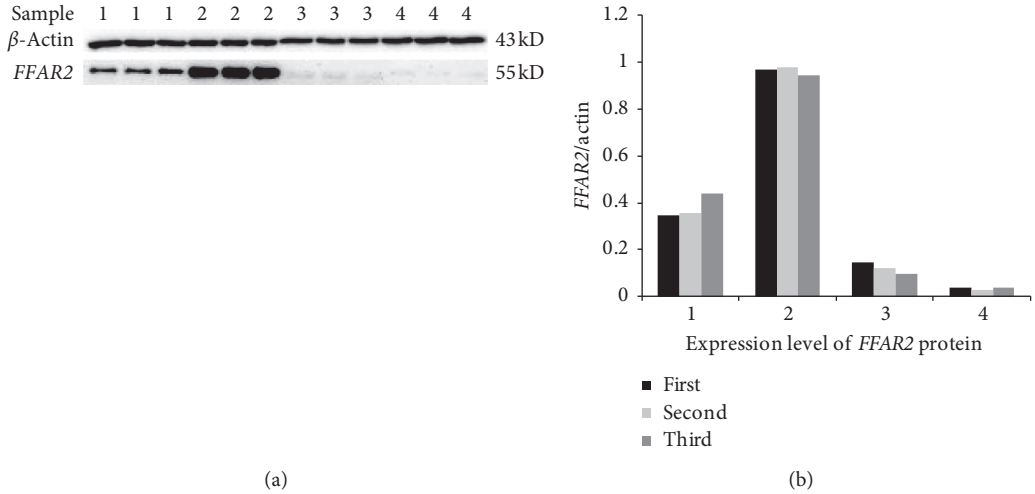

Figure 2: Expression level of *FFAR2* protein. In the control group, the sample numbers are 1 and 2, and in the AMI group, the sample numbers are 3 and 4. AMI = acute myocardial infarction.

Table 4: Correlation analysis of *FFAR2* mRNA level with age, history of diabetes, fasting blood glucose, and HDL-C level.

Group	*N*	Relative expression of *FFAR2*	*Z*	*p* value
Younger age	121	0.50 (0.06–1.51)		
Older age	86	0.34 (0.03–1.02)	−1.552	0.121
Type 2 diabetes mellitus-free group	126	0.24 (0.02–1.13)		
Type 2 diabetes mellitus group	38	0.26 (0.05–0.86)	−0.207	0.836
High fasting blood glucose group	71	0.39 (0.02–1.45)		
Normal fasting blood glucose group	89	0.23 (0.02–1.08)	−0.956	0.339
Low HDL-C group	103	0.21 (0.02–1.02)		
High HDL-C group	83	0.70 (0.15–1.79)	−3.508	0.000

HDL-C = high-density lipoprotein cholesterol.

Table 5: Logistic regression analyses of independent risk factors for AMI.

	B	Standard variation	Wald	Degree of freedom	*p* value	OR	95% CI
Low *FFAR2* gene expression	1.842	0.824	5.001	1	0.025	6.308	1.256–31.694
High fasting blood glucose group	1.142	0.428	7.115	1	0.008	3.132	1.354–7.248
Older age	0.610	0.405	2.264	1	0.132	1.840	0.832–4.069
Type 2 diabetes mellitus	0.593	0.528	1.262	1	0.261	1.810	0.643–5.093
Low HDL-C group	0.277	0.412	0.452	1	0.502	1.319	0.588–2.955

AMI = acute myocardial infarction; CI = confidence interval; HDL-C = high-density lipoprotein cholesterol; OR = odds ratio.

mortality of cardiovascular diseases increased [19]. This was mainly due to the decrease of glucagon-like peptide-1 response associated with diet. Diabetes mellitus can be used as an independent predictor of mortality and new cardiovascular events in hospitalized patients with AMI [20], which may play a role in promoting inflammation in AMI [21].

FFAR2 and other free fatty acid receptors are considered to be key components of human nutritional sensing mechanism. Studies on these receptors considered them as new therapies for diabetes and other metabolic disorders [22]. The activation of *FFAR2* is coupled with intracellular signals, such as increased IP3 production, increased intracellular Ca^{2+}, and the activation of erk1/2 pathway, which contribute to the stimulation of GSI in islets [23]. As a signal molecule of short chain fatty acids, *FFAR2* is coupled with Gαq and Gαi, resulting in the activation of phospholipase C and increase in intracellular calcium levels, or the inhibition of cAMP production by adenylate cyclase, respectively. Thus, it is clear that activation of *FFAR2* contributes to the expansion of pancreatic beta cell clusters and insulin secretion to maintain normal glucose homeostasis [24]. *FFAR2* maintains fasting blood glucose level through the FA signaling pathway [25]. *FFAR2* agonists can be used as a new insulin sensitizer for type 2 diabetes mellitus, with therapeutic potential in this disease [26]. Sodium butyrate, the metabolite of microorganism, can significantly improve the level of *FFAR2*, increase the storage of glycogen, and play a good role in maintaining blood glucose homeostasis, in

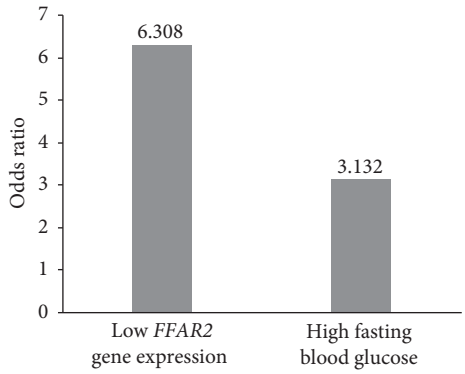

Figure 3: Independent risk factors for AMI. AMI = acute myocardial infarction.

which *FFAR2*-Akt-Gsk3 pathway may be involved. *FFAR2* is involved in the role of short chain fatty acids in colon cells, adipocytes and immune cells in promoting the secretion of gut hormone, reducing fat decomposition, and regulating immune mediators. The beneficial effect of short chain fatty acids on islets may be secondary to the indirect stimulation and protection of these endocrine cells on islet β cells. It can be determined that short chain fatty acids can resist apoptosis of islet cells in a *FFAR2*-dependent manner. When *FFAR2* was reduced or deleted, islet quality and beta cell survival were impaired [14,27], and the conversion of glucagon-like peptide into insulin was reduced [28]. In theory, low expression of the *FFAR2* gene and promotion of blood sugar may be one of the mechanisms of low expression of the *FFAR2* gene promoting AMI. However, the correlation between fasting blood glucose and *FFAR2* gene expression was not observed in this study; However, the baseline data analysis showed that the proportion of hypoglycemic drugs used in the AMI group was higher ($p = 0.01$). Hence, we speculated that as more subjects in the hyperglycemic group used hypoglycemic drugs, there was no correlation.

FFAR2 was originally cloned from white blood cells, and its highest level was detected in neutrophils, monocytes, and other immune cells. The tissue distribution of *FFAR2* indicates that it has a potential role in the activation and differentiation of immune cells [29]. *FFAR2* protein is considered to be the mediator of short chain fatty acids on immune cells. Many studies have confirmed the role of the interaction between *FFAR2* and short chain fatty acids in regulating inflammatory response [30]. *FFAR2* is involved in short chain fatty acids that inhibit histone deacetylase expression and hypermethylation of inflammatory inhibitors [31]. *FFAR2* reduces the production of inflammatory mediators by inhibiting the expression of cytokines and chemokines, and it participates in the regulation of neutrophil activation, affecting inflammatory leukocyte migration [32]. Therefore, decreased expression of the *FFAR2* gene leads to the enhancement of the downstream camp-pka-creb pathway, histone deacetylase (HDAC) overexpression, and inflammation inhibition [31]. It attenuates the inhibition of inflammatory response and increases the activation of circulating inflammatory cytokines, chemokines, and immune cells, which play an important role in AMI.

This research showed that the level of HDL-C in the AMI group was significantly lower than that in the control group ($p = 0.03$), and the low expression of the *FFAR2* gene in peripheral blood of the AMI group was associated with a lower level of HDL-C ($p < 0.001$). *FFAR2*, similar to gpr109a (a nicotinic acid receptor), can bind to the gastrointestinal signaling pathway in adipocytes to inhibit fat dissolution, reduce plasma-free fatty acids, and increase the level of HDL-C [33]. In the past few years, epidemiological studies have shown that low concentrations of HDL-C are associated with increased risk of coronary artery disease and cardiovascular events [34]. As an important component of lipid metabolism disorders, abnormal level of HDL-C has increasingly attracted attention. A large number of experiments have proved that HDL-C has a protective effect on atherosclerosis regardless of sex and race. It mainly plays an antiatherosclerotic role by reversing the transport of cholesterol from peripheral tissues. In addition, HDL-C may have antioxidant and anti-inflammatory effects and inhibit cytokine-induced expression of transduced endothelial cell adhesion molecules, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1, and it can also inhibit thrombosis [35]. The relatively low expression of the *FFAR2* gene, which decreases HDL-C level, is one of the mechanisms by which *FFAR2* promotes AMI.

Logistic regression analysis showed that low expression of the *FFAR2* gene in peripheral blood was a risk factor for AMI, independent of age, history of diabetes mellitus, fasting blood glucose level, and HDL-C level ($p = 0.025$). Compared with high expression of the *FFAR2* gene, low expression of the *FFAR2* gene increased the risk of AMI to 6.308 times. Theoretically, the low expression of *FFAR2* may be related to the high fasting blood glucose, but the above correlation was not seen in this research. It may be further verified by expanding the sample size due to the use of hypoglycemic drugs in the research subjects. At the same time, high fasting blood glucose level is an independent risk factor for AMI. Compared with normal fasting blood glucose level, the risk of AMI increased to 3.132 times.

5. Conclusion

The expression level of the *FFAR2* gene in peripheral blood of patients with AMI was significantly lower than that in the control group. Low expression of the *FFAR2* gene in peripheral blood is an independent risk factor for AMI. One of the mechanisms may be that low expression of the *FFAR2* gene reduces the level of HDL-C and promotes the occurrence of AMI. Low expression of the *FFAR2* gene in peripheral blood may be a potential biomarker in predicting the risk of AMI.

Data Availability

The data used to support the findings of this study have been deposited in the Figshare repository.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Figure S1: RT-PCR curve. (a) RT-PCR amplification curve of the *FFAR2* gene. (b) RT-PCR dissolution curve of the *FFAR2* gene. (*Supplementary Materials*)

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Research Article

Elevated GTP Cyclohydrolase I Pathway in Endothelial Progenitor Cells of Overweight Premenopausal Women

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Background/Aims. Sexual differences exist in endothelial progenitor cells (EPCs), and various cardiovascular risk factors are associated with the preservation of endothelial function in premenopausal women. However, it is unclear whether differences in endothelial function and circulating EPCs exist between overweight premenopausal women and age-matched men. *Methods*. We compared EPC counting and functions in normal-weight and overweight premenopausal women and men, evaluated endothelial function in each group, and detected the expression of the guanosine triphosphate cyclohydrolase I (GTPCH I) pathway. *Results*. The number of EPCs was lower in the male group than in the female group, regardless of normal-weight or overweight status, and there was no significant difference between the different weight groups among females or males. Endothelial function and EPC migration and proliferation were preserved in overweight premenopausal women compared with overweight men as were nitric oxide (NO) levels in plasma and secreted by EPCs. Endothelial function, the circulating EPC population, and NO levels were not different between normal-weight and overweight premenopausal women. Flow-mediated dilatation was significantly correlated with EPC function, plasma NO levels, and EPC-secreted NO. *Conclusions*. This investigation provides the first evidence for sex-based differences in EPC activity and endothelial function in overweight middle-aged individuals; these differences are associated with alterations in NO production and may partly occur through downregulation of the GTPCH I pathway. The present results provide new insights into the mechanism underlying the preserved endothelial function in overweight premenopausal women and may uncover a potential therapeutic target for endothelial repair in overweight population.

1. Introduction

Epidemiological studies have shown an increasing prevalence of overweight and obesity in adults worldwide, resulting in a high incidence of cardiovascular disease (CVD), such as coronary heart disease and peripheral arteriosclerosis, and constituting a major health threat [1, 2]. Body mass index (BMI, calculated as weight in kilograms/(height in meters)^2), a reasonable and practical estimate of general adiposity, is used routinely in the diagnosis of

overweight and obesity. Both increased body weight and increased BMI are correlated with CVD and its risk factors, such as hypertension, diabetes, dyslipidemia, and insulin resistance [3–5]. The established noninvasive flow-mediated endothelium-dependent vasodilatation (FMD) method is widely used to assess endothelial function. Evidence supports the concept that endothelial dysfunction is persistent in overweight and obese individuals, contributing to the pathogenesis and progression of atherosclerotic vascular disease [6–8]. Accordingly, restoration of endothelial dysfunction may be an effective means to maintain vascular homeostasis and prevent obesity-associated cardiovascular complications. Endothelial progenitor cells (EPCs), a group of immature cells derived from the bone marrow, play prominent roles in endothelialization at sites of vascular injury and in the maintenance of endothelial homeostasis, and disorders in EPCs are associated with the progression of atherosclerosis [9–11]. Both the number and the function of EPCs are compromised in the presence of various CVD risk factors, including hyperlipidemia, hypertension, diabetes, and smoking, which may contribute to the progression of endothelial injury and dysfunction [12–14]. A previous study revealed that the numbers of circulating EPCs were reduced in both overweight and obese subjects, with an inverse correlation between EPC counts and BMI [15], and EPC deficits were reversible after significant weight loss [16, 17]. A clinical study further demonstrated that EPC dysfunction is correlated with impaired endothelial function and the associated phenomenon of atherosclerosis in overweight and obesity, suggesting a significant protective effect of EPCs on the endothelium in vivo [18, 19]. Moreover, weight loss can elevate the levels of EPCs in circulating and promote their function, subsequently improving endothelial function [15, 16].

Accumulating evidence has revealed that premenopausal women have a lower risk than postmenopausal women and men of similar age of suffering a broad range of CVDs [20, 21], which is partly associated with the sex-related attenuation of endothelial injury and dysfunction [22, 23]. In healthy middle-aged adults and those with prehypertension, there are sex-based differences in the number or activity of EPCs [24–26], indicating that the alteration of endogenous endothelial repair capacity may conserve endothelial function in premenopausal women. Research has shown that endothelium-dependent vasodilatation is significantly decreased in obese premenopausal women, indicating adiposity-induced endothelial dysfunction in these women [8]. However, it is not clear whether there are sex-based differences in circulating EPCs and endothelial function in overweight middle-aged adults.

Cytokines such as nitric oxide (NO), vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) are important factors that regulate circulating EPCs [27–32]. Impairment of endothelium-dependent vasodilatation secondary to decreased NO bioavailability is one of the early deleterious effects of obesity [33], and reduced NO levels may result from increased oxidative stress [34] or proinflammatory cytokine levels, which are predisposing risk factors for CVD. The guanosine triphosphate cyclohydrolase I (GTPCH I)/tetrahydrobiopterin (BH_4) pathway was shown to potentially regulate NO production and impair EPC mobilization and function by endothelial nitric oxide synthase (eNOS) uncoupling in diabetes [35], and it was shown to be involved in declined EPC function in postmenopausal women with overweight [36]. Additionally, VEGF and GM-CSF are potent cytokines that mobilize EPCs from the bone marrow into circulation [29, 30]. Both TNF-α and IL-6 are acute inflammatory response mediators that play important roles in systemic inflammation in obesity and are associated with future atherosclerosis [37, 38]; these cytokines have been shown to impair the proliferative, migratory, and tube-forming capacities of EPCs in a dose-dependent manner [31, 32].

Accordingly, we measured EPC number and activity as well as FMD in normal-weight and overweight premenopausal women and men; evaluated the levels of NO, VEGF, GM-CSF, TNF-α, and IL-6 circulating in plasma or secreted by EPCs; and investigated the possible underlying mechanism.

2. Materials and Methods

2.1. Characteristics of Subjects. Twenty overweight premenopausal females (BMI $27.5 \pm 2.3\,kg/m^2$) and 20 overweight age-matched males (BMI $27.7 \pm 2.5\,kg/m^2$) were recruited. Forty age-matched subjects of normal weight (20 premenopausal females, BMI $22.6 \pm 2.1\,kg/m^2$, and 20 males, BMI $23.2 \pm 1.5\,kg/m^2$) were also included in the control groups. Normal weight was defined as a BMI of 18.5–24.9 kg/m^2, and overweight was defined as a BMI of over 23 kg/m^2, according to the weight classifications in the American Heart Association (AHA) Scientific Statement [39]. All enrolled subjects were evaluated through an extensive medical history, routine clinical screening, and laboratory tests to exclude the following statuses and conditions in order to prevent their potential impacts on EPC levels and activity: diabetes mellitus, diagnosed CVD, malignant disease, infection or inflammatory diseases, smoking, irregular menstrual cycles, polycystic ovary syndrome, and previous hysterectomy. Pregnant or breastfeeding women were also excluded. The intake of medications such as antiplatelet, anti-inflammatory, or hypolipidemic agents or sex hormone therapy was not allowed for any of the subjects because these medications have additional effects on circulating EPCs or may weaken gender differences. All the participants refrained from ingesting alcohol or caffeine for 12 h before the study. The experimental protocol was approved by the Ethics Committee of our hospital, and all the participants signed an informed consent form prior to participation in the study.

The baseline characteristics of all the subjects are shown in Table 1. Details regarding peripheral venous blood collection and laboratory tests are provided in a previous study [40].

2.2. Evaluation of Circulating EPC Number and Function. EPC counts were evaluated by fluorescence-activated cell sorting (FACS) and cell culture assays as previously described [12, 27, 41, 42]. EPC migratory activity was

Table 1: Clinical and biochemical characteristics.

Characteristics	Normal-weight women ($n = 20$)	Overweight women ($n = 20$)	Normal-weight men ($n = 20$)	Overweight men ($n = 20$)
Age (years)	46.4 ± 3.2	47.3 ± 2.78	48.1 ± 4.2	45.9 ± 3.8
Height (cm)	161.8 ± 5.2	162.8 ± 5.9	167.2 ± 6.1$^{\#}$	168.3 ± 6.5$^{\#}$
Weight (kg)	59.2 ± 5.8	66.3 ± 6.5*	64.8 ± 3.9$^{\#}$	72.1 ± 5.4$^{*,\#}$
BMI (kg/cm^2)	22.6 ± 2.1	27.5 ± 2.3	23.2 ± 1.5	27.7 ± 2.5
Systolic blood pressure (mmHg)	124.5 ± 9.7	148.8 ± 4.1	122.7 ± 5.5	149.3 ± 4.9*
Diastolic blood pressure (mmHg)	76.4 ± 7.2	89.3 ± 6.5	75.4 ± 6.7	89.6 ± 5.0*
Heart rate (beats/min)	76.8 ± 9.4	78.5 ± 7.5	79.3 ± 8.1	80.7 ± 8.4
AST (mmol/L)	27.2 ± 6.8	25.6 ± 5.1	24.7 ± 6.3	24.6 ± 6.2
ALT (mmol/L)	24.8 ± 7.8	24.0 ± 5.4	22.8 ± 5.1	21.9 ± 5.9
BUN (mmol/L)	5.0 ± 0.9	4.9 ± 1.0	5.2 ± 0.8	5.4 ± 0.9
Cr (mmol/L)	65.6 ± 15.4	63.2 ± 14.5	67.6 ± 15.8	72.5 ± 15.5
LDL (mmol/L)	3.03 ± 0.48	2.89 ± 0.43	2.87 ± 0.43	2.78 ± 0.40
TC (mmol/L)	5.08 ± 0.43	4.83 ± 0.51	4.81 ± 0.65	4.72 ± 0.63
HDL (mmol/L)	1.36 ± 0.26	1.42 ± 0.25	1.38 ± 0.18	1.44 ± 0.21
TG (mmol/L)	1.51 ± 0.19	1.46 ± 0.23	1.44 ± 0.16	1.40 ± 0.22
FPG (mmol/L)	4.55 ± 0.56	4.76 ± 0.51	4.67 ± 0.61	4.36 ± 0.46
Estradiol (pmol/L)	211.4 ± 33.0	200.4 ± 37.6	97.7 ± 31.3$^{\#}$	101.9 ± 15.1$^{\#}$
FMD (%)	9.56 ± 1.80	8.61 ± 1.64	8.17 ± 1.759$^{\#}$	6.51 ± 1.81$^{*,\#}$

Abbreviation: BMI, body mass index; LDL, low-density lipoprotein; TC, total cholesterol; HDL, high-density lipoprotein; TG, triglyceride; FPG, fasting plasma glucose; hrCRP, hypersensitive C-reactive protein; FMD, flow-mediated brachial artery dilatation. Notes: data are given as mean ± SD. $^{*}P < 0.05$ vs. the same gender group of normal-weight; $^{\#}P < 0.05$ vs. the same weight class of premenopausal women.

determined using a modified Boyden chamber, and the proliferative potential was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

2.3. FMD Measurement. Brachial artery FMD was measured by high-resolution color ultrasonography with a 5–12 MHz linear transducer on an HDI 5000 system (Washington, USA), as in our previous study and other reports [40, 43]. A baseline image was recorded, and the pressure in an upper-forearm sphygmomanometer cuff was then raised to 250 mmHg for 5 min. The electrocardiogram was monitored continuously for 90 s after cuff deflation. FMD was recorded as the percentage increase in the mean diastolic diameter after reactive hyperemia 55 to 65 s after deflation to baseline.

2.4. Measurement of NO, VEGF, GM-CSF, IL-6, and TNF-α Levels in Plasma and Secreted by EPCs. NO, VEGF and GM-CSF levels in the plasma and secreted by EPCs were assessed as in our previous study [27]. The plasma concentration of TNF-α was evaluated by a high-sensitivity immunoassay, and the IL-6 level was measured by an enzyme-linked immunoassay.

2.5. Western Blot Analysis of eNOS and GTPCH I and Measurement of BH_4. Total protein was harvested from EPCs by cell lysis buffer (Cell Signaling Technology Inc., Danvers, MA, USA). Protein extracts were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Cell Signaling Technology Inc.). Rabbit antiphosphorylated eNOS and anti-eNOS (1 : 1000; Cell Signaling Technology Inc.) and anti-GTPCH I and β-actin (1 : 1000; Santa Cruz Biotechnology Inc.) were used to evaluate eNOS and GTPCH I expression as previously described [40, 44, 45]. Intracellular BH_4 concentrations were measured according to previous reports and calculated by subtracting BH_2 plus oxidized biopterin from total biopterins; the results are presented in pmol/mg protein [44, 46, 47].

2.6. Statistical Analysis. SPSS V11.0 statistical software (SPSS Inc., Chicago, Illinois) was used for statistical analysis. All data are presented as mean ± SD. Comparisons among the four groups were analyzed by two-factor analysis of variance (sex and weight classification). When indicated by a significant F value, the post hoc Newman–Keuls method was used to identify significant differences among the mean values. Statistical significance was evaluated by ANOVA. Univariate correlations were assessed using Pearson's coefficient (r). $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Baseline Characteristics. The clinical and biochemical characteristics of all the subjects are detailed in Table 1. Height and weight were significantly greater in normal-weight men (167.2 ± 6.1 and 64.8 ± 3.9) and overweight men (168.3 ± 6.5 and 72.1 ± 5.4) than in normal-weight premenopausal women (161.8 ± 5.2 and 59.2 ± 5.8) or overweight premenopausal women (162.8 ± 5.9 and 66.3 ± 6.5) ($P < 0.05$ and $P < 0.05$ for normal-weight and overweight men, respectively, compared to women of the same weight class). Both systolic and diastolic blood pressure were higher in overweight men than in normal-weight men but were equal in the two weight classes of premenopausal women. Estradiol levels were higher in normal-weight and overweight premenopausal women (211.4 ± 33.0 and 200.4 ± 37.6) than in normal-weight or overweight men (97.7 ± 31.3 and 101.9 ± 15.1) ($P < 0.05$ and < 0.05,

respectively). FMD was preserved in normal-weight and overweight premenopausal women compared with normal-weight and overweight men ($P < 0.05$ and $P < 0.05$, respectively) but was decreased in overweight men compared with normal-weight men ($P < 0.05$); however, there was no difference in FMD between normal-weight and overweight premenopausal women in our study ($P > 0.05$). There were no differences in blood pressure or in fasting plasma glucose (FPG), low-density lipoprotein (LDL), total cholesterol (TC), triglycerides (TG), or high-density lipoprotein (HDL) levels among the four groups ($P > 0.05$).

3.2. EPC Numbers and Activity in the Four Groups. The difference in EPC number between normal-weight and overweight individuals was not statistically significant ($P > 0.05$, regardless of sex), as evaluated by FACS analysis and cell culture assays (Figures 1(a) and 1(b)). Both the number and function of EPCs were markedly lower in the male group than in the female group ($P < 0.05$, irrespective of weight classification) (Figures 1(a) and 1(b)). In addition, EPC function was impaired in overweight men compared with normal-weight men but was similar between normal-weight and overweight premenopausal women (Figures 1(c) and 1(d)).

3.3. Plasma NO, VEGF, GM-CSF, IL-6, and TNF-α Levels in the Four Groups. Plasma NO levels were significantly increased in the premenopausal female group compared with the male group ($P < 0.05$ for normal-weight comparison and $P < 0.05$ for overweight comparison) and were higher in normal-weight men than in overweight men ($P < 0.05$). Nevertheless, plasma NO levels were almost equal in normal-weight and overweight premenopausal women ($P > 0.05$). In contrast, the differences in plasma VEGF, GM-CSF, TNF-α, and IL-6 levels among the four groups were not statistically significant ($P > 0.05$) (Figures 2(a)–2(c)).

3.4. Levels of NO, VEGF, and GM-CSF Secretion by EPCs in the Four Groups. NO secretion by EPCs was significantly increased in normal-weight and overweight premenopausal women compared with normal-weight and overweight men ($P < 0.05$ and $P < 0.05$, respectively) and was higher in normal-weight men than in overweight men ($P < 0.05$). However, NO secretion was nearly equal between normal-weight and overweight premenopausal women ($P > 0.05$). In contrast, no difference in VEGF or GM-CSF secretion by EPCs was observed among the four groups (Figures 3(a)–3(c)).

3.5. Correlation of FMD with EPC Behavior and NO Levels. Positive correlations between FMD and EPC migratory activity ($r = 0.65$, $P < 0.05$) and proliferative potential ($r = 0.51$, $P < 0.05$) were found in this study (Figures 4(a) and 4(b)). Univariate analysis showed significant correlations between FMD and plasma NO level ($r = 0.49$, $P < 0.05$) and NO secretion by EPCs ($r = 0.47$, $P < 0.05$) (Figures 4(c) and 4(d)).

3.6. The GTPCH I/BH_4 Pathway in EPCs from the Four Groups. In this study, GTPCH I expression was lower in overweight men than in normal-weight men ($P < 0.05$) but exhibited no difference between the two weight classes of premenopausal women ($P > 0.05$) (Figure 5(a)). The difference in intracellular BH_4 in circulating EPCs between normal-weight and overweight men was statistically significant ($P < 0.05$), but the difference between normal-weight and overweight women was not significant ($P > 0.05$) (Figure 5(b)). In addition, no difference in eNOS expression or phosphorylation was found among the four groups in our study ($P > 0.05$) (Figures 5(c) and 5(d)). These results indicate that the GTPCH I pathway may be a mechanism for the deficiency in NO secretion by EPCs in overweight men, and it likely affects eNOS uncoupling rather than eNOS expression or phosphorylation.

4. Discussion

The results of this study showed that EPC migratory activity and proliferative potential were preserved in overweight premenopausal women compared with overweight men, consistent with the alterations in endothelial function. In addition, EPC function was correlated with plasma NO levels and NO secretion by EPCs, indicating that changes in circulating EPCs may be due to varied NO production in overweight middle-aged adults. Müller-Ehmsen et al. [15] analyzed the relationship between BMI and EPC counts in overweight and obese populations and showed that circulating EPC counts in overweight and obese individuals were inversely related to BMI, regardless of sex; furthermore, the reduction in EPC number was reversible upon weight loss in overweight subjects. However, that team did not compare EPC counts between overweight and normal-weight populations or based on gender. Additionally, the study did not exclude smokers or those with hyperlipidemia, diabetes, prior cardiovascular events, or potential medication intake, all of which have been shown to affect EPC levels in peripheral blood. In our study, we found that EPC counts were not significantly lower in overweight subgroups than in normal-weight subgroups (whether female or male), consistent with the results of MacEneaney et al. [17], who found that EPC counts were decreased in obese but not overweight subjects, while the colony-forming capacity of EPCs was impaired in overweight and obese adults compared with normal-weight adults [17]. A previous study found that EPC migratory capacity was not influenced by overweight or obesity [48]. On the other hand, Tsai et al. and Campis et al. revealed that the function of EPCs but not their number is reduced in the context of obesity uncomplicated by atherosclerosis [49, 50]. Our results showed that EPC proliferation and migration were slightly decreased in overweight premenopausal women and men compared with those at a normal weight, but the difference was not statistically significant. Our result suggested a gender difference in FMD in overweight patients, in contrast to the findings of Suh et al. [8], who reported that endothelial function was significantly blunted in obese premenopausal women. A possible reason for the different results may be the difference

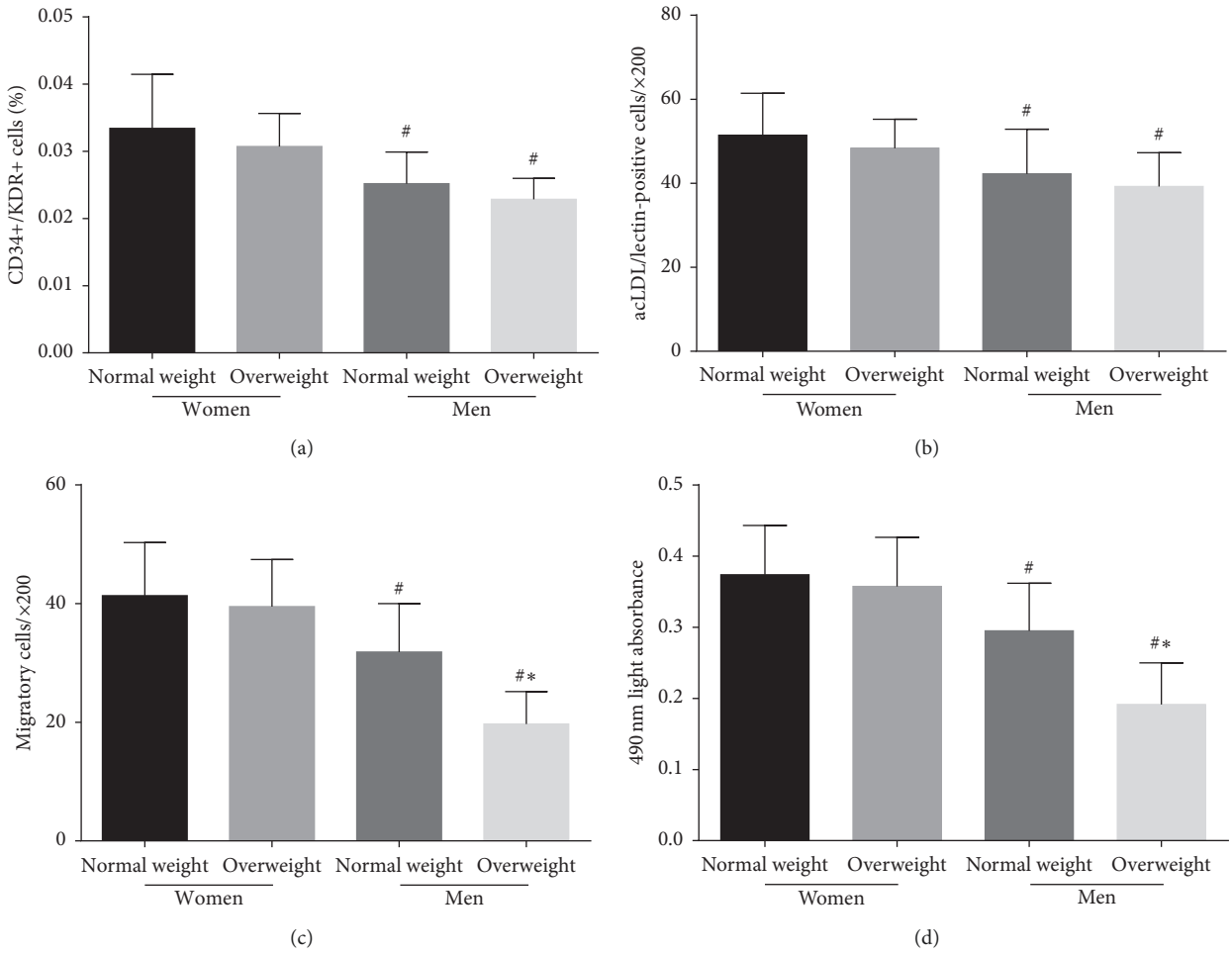

Figure 1: The difference in the number and activity of circulating EPCs in the four groups. Evaluated by (a) FACS analysis and (b) phase-contrast fluorescent microscope, there were no significant difference in the level of circulating EPCs between overweight premenopausal women or men and normal-weight premenopausal women or men. However, the EPC number in normal-weight and overweight men was lower than that in normal-weight and overweight premenopausal women. The migratory (c) and proliferative (d) activity of circulating EPCs in normal-weight and overweight men were lower than those in normal-weight and overweight premenopausal women. There was no difference in the migratory (c) and proliferative (d) activity between normal-weight and overweight premenopausal women. Data are given as mean ± SD. $^{*}P < 0.05$ vs. the same gender group of normal weight; $^{\#}P < 0.05$ vs. the same weight class of premenopausal women.

in the average BMI of the enrolled subjects in the two studies. The BMI range in the previous study was $28.8 \pm 3.6\,kg/cm^2$, compared with $26.5 \pm 1.8\,kg/cm^2$ in the female group in our study, and previous research suggests that EPC counts or activity may be correlated with BMI: the higher a person's BMI is, the more obvious the EPC damage [15]. In conclusion, this study suggests a gender difference in the impairment of endothelial function in overweight, which is related to the downregulation of EPC quantity and quality; this study also indicates that enhancing EPC-regulated endogenous endothelial repair capacity is necessary for maintaining endothelial function in overweight men.

Overweight is an intermediate state between normal weight and obesity and confers an increased risk of numerous adiposity-related comorbidities, such as hypertension, diabetes, disrupted lipid metabolism, insulin resistance, and CVD. Therefore, both overweight and obesity are considered the independent risk factors for CVD and are associated with a high risk of CVD morbidity and mortality. Endothelial dysfunction, as a key and early prognostic indicator for atherosclerosis, is responsible for CVD pathogenesis and promotes its progression. Relevant data have shown that endothelial dysfunction, which presents as decreased FMD and is caused by deregulation of the endothelial NO pathway, is persistent in overweight and obesity and is closely related to elevated CVD risk in these weight classes [6–8]. Our recent study found that endothelial function was preserved in premenopausal women with prehypertension [26], indicating the protective effect of the premenopausal state on endothelial function. The current study suggested that FMD was higher in premenopausal women than in men of the same age, regardless of the weight class, and was decreased in overweight men compared to normal-weight men, indicating that endothelial function is attenuated in overweight men. In contrast, there was no difference in FMD between normal-weight and overweight premenopausal women, suggesting that endothelial function is preserved in overweight

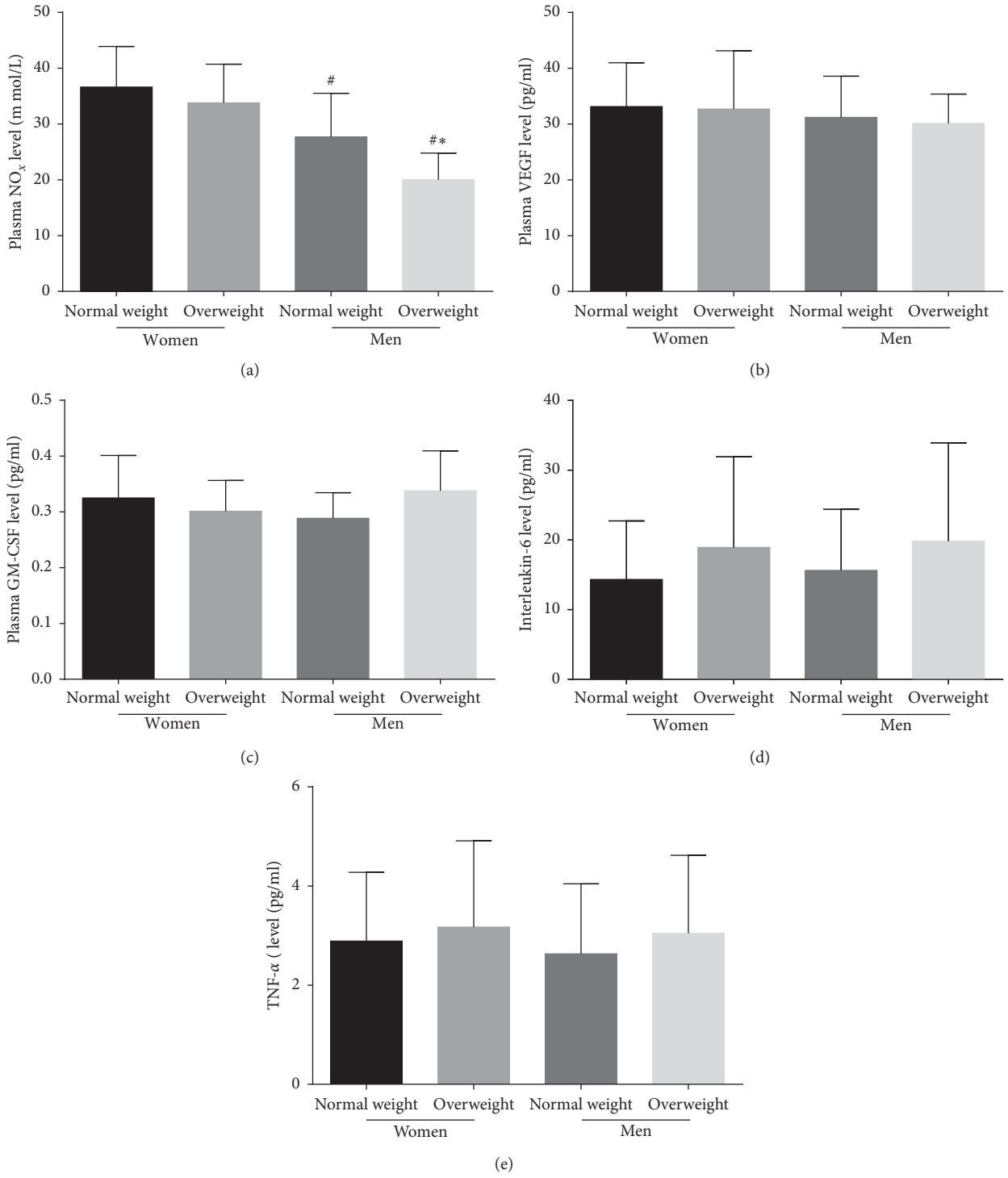

Figure 2: The difference in plasma NO, VEGF, GM-CSF, TNF-α, and IL-6 levels in the four groups. (a) The plasma NO level in normal-weight and overweight men was lower than that in normal-weight and overweight premenopausal women. There was no difference in plasma NO level between normal-weight and overweight premenopausal women. No significant difference was found in plasma VEGF (b), GM-CSF (c), TNF-α (d), and IL-6 (e) level between the four groups. Data are given as mean ± SD. $^{*}P < 0.05$ vs. the same gender group of normal weight; $^{\#}P < 0.05$ vs. the same weight class of premenopausal women.

premenopausal women. The difference in endothelial function between overweight men and premenopausal women may partly explain the reduced risk of CVD in premenopausal women compared to men, suggesting a protective effect of female gender on endothelial function in overweight women and implying that improving endothelial function in overweight individuals may be beneficial for decreasing CVD risk.

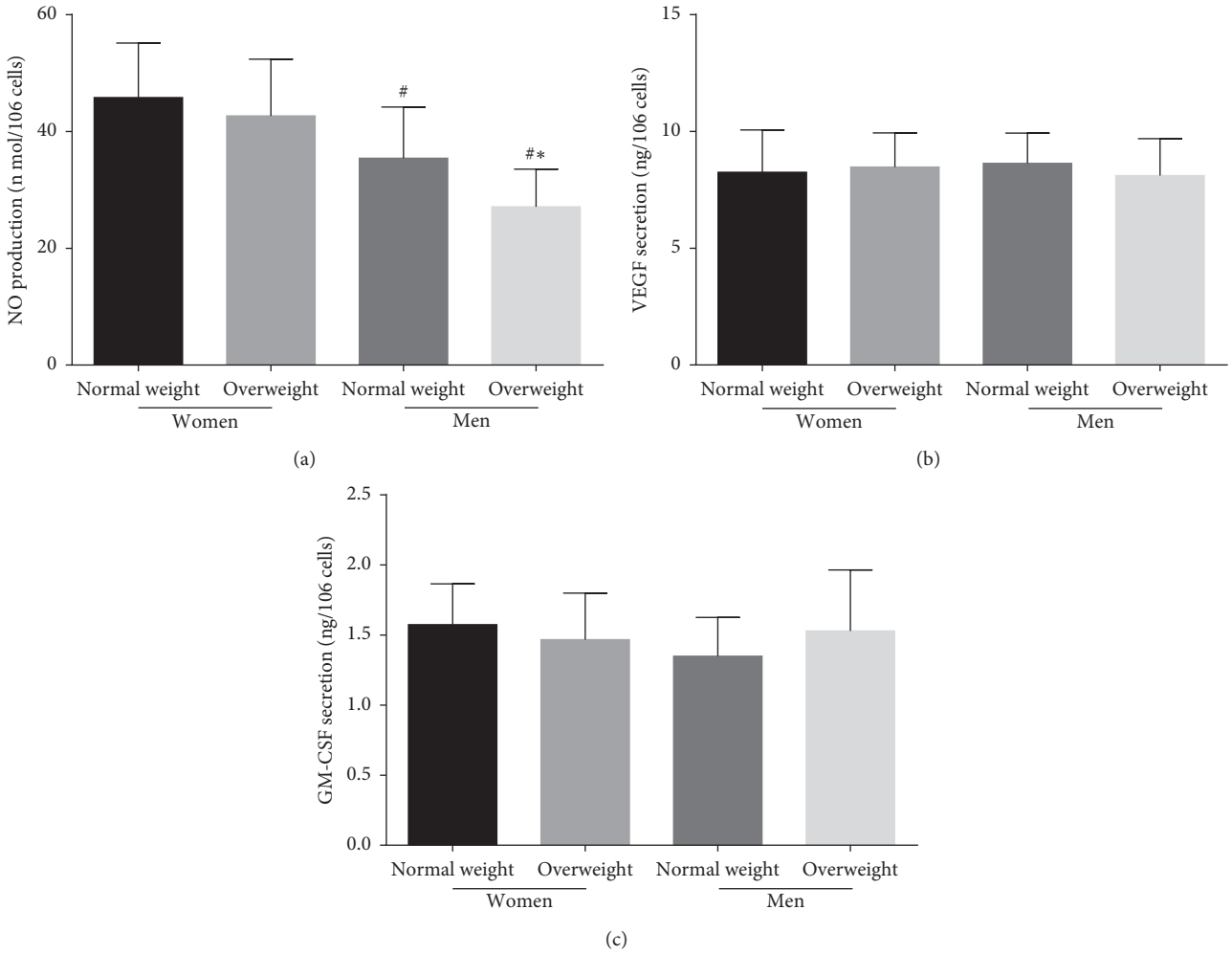

Figure 3: The NO, VEGF, and GM-CSF secretion by EPCs in the four groups. (a) The NO secretion by EPCs in normal-weight and overweight premenopausal women was higher than that in normal-weight and overweight men. There was no difference in NO secretion by EPCs between normal-weight and overweight premenopausal women. (b) No significant difference was found in VEGF secretion by EPCs between the four groups. (c) No significant difference was found in GM-CSF secretion by EPCs between the four groups. Data are given as mean ± SD. $^{*}P < 0.05$ vs. the same gender group of normal weight; $^{\#}P < 0.05$ vs. the same weight class of premenopausal women.

Circulating EPCs that are derived from the bone marrow and mobilized into the peripheral blood adhere to the sites of vascular injury and participate in the repair of endothelial injury and angiogenesis. These cells play a vital role in the occurrence and progression of CVD by regulating the endothelial repair capacity in the context of most vascular risk factors. Previous studies have shown that the number or activity of EPCs is decreased in overweight and obese individuals [15–17], and EPC dysfunction impairs endothelial function in the context of overweight and obesity and the associated phenomenon of atherosclerosis [49, 50], suggesting that the decreased endothelial repair capacity is involved in the pathological process of CVD in overweight and obesity. In middle-aged healthy volunteers and those with prehypertension, sex-based differences exist in the number, proliferative potential and migratory ability of circulating EPCs, and these factors are related to the risk of CVD, suggesting that the vascular protection mediated by EPCs in premenopausal women may be due to improved endothelial repair capacity [24–26, 51, 52]. However, it is unknown whether there are similar gender differences in EPCs in overweight middle-aged adults, and the relationship of any such difference with endothelial also remains uncharacterized. In this study, EPC function was decreased in overweight men and was significantly correlated with endothelial function, revealing that EPC impairment in overweight leads to the loss of endothelial repair function and brings about endothelial dysfunction. Nevertheless, the difference in EPCs between overweight and normal-weight premenopausal women was not significant, indicating that gender differences probably play a protective role against vascular damage in overweight women. Furthermore, the present results revealed a positive relationship between endothelial function and EPC activity, indicating that the attenuated endogenous repair capacity of the vascular endothelium mediated by EPCs leads to endothelial dysfunction and confirming the important role of circulating EPCs in maintaining endothelial function in overweight

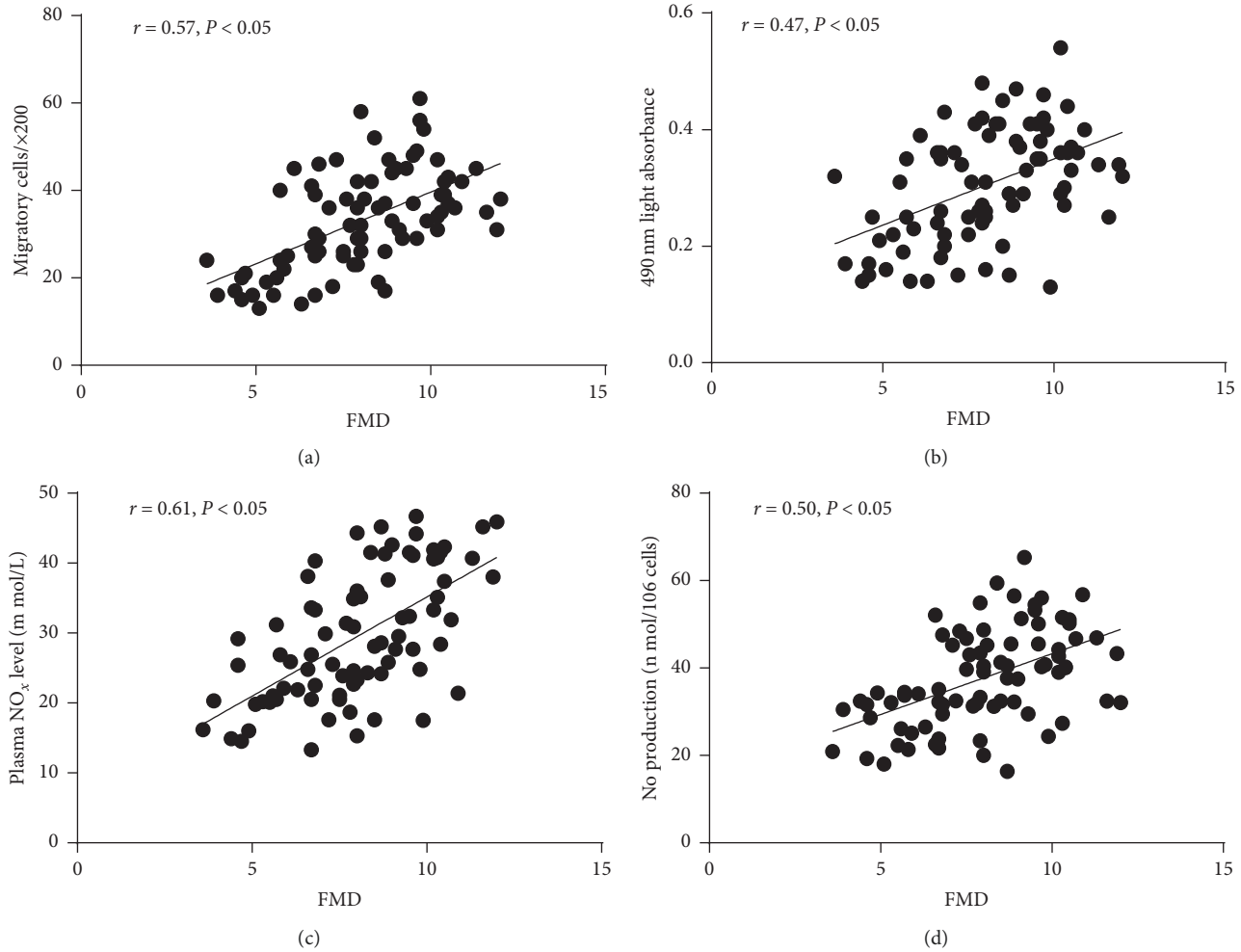

Figure 4: The correlation between circulating EPCs or NO level and FMD. There was a correlation between the EPC proliferatory (a) or migratory (b) and FMD. There was a correlation between the plasma NO level (c) or NO secretion by EPCs (d) and FMD.

individuals. Increasing the number and function of EPCs may be a vasculoprotective therapeutic strategy for improving endothelial function and preventing the initiation and progression of overweight-associated atherosclerosis.

NO, VEGF, GM-CSF, TNF-α, and IL-6 are important for regulating the number and activity of EPC both in healthy subjects and in patients with vascular diseases [27–32], and endogenous NO biosynthesis is required for circulating EPCs to function [53]. In this study, we found that NO levels in plasma and secreted by circulating EPCs were restored in overweight premenopausal women. Furthermore, NO levels in plasma and secreted by circulating EPCs were reduced in overweight men compared with normal-weight men and overweight premenopausal women and paralleled the change in EPC function, highlighting the role of NO in regulating EPC function in overweight middle-aged adults. However, no differences in VEGF, GM-CSF, TNF-α, or IL-6 levels were found among the four groups, suggesting that the impaired EPC-mediated endothelial repair capacity in overweight men may be independent of the modulation of these cytokines. In addition, our study found positive correlations between FMD and NO levels, both in plasma and produced by cultured EPCs, suggesting that reduced systemic NO production and endogenous NO biosynthesis by circulating EPCs may contribute to endothelial dysfunction in overweight.

eNOS, a key enzyme in endogenous NO biosynthesis, is constitutively expressed in endothelial cells and involved in the production of NO, which not only modulates the mobilization of EPCs from the bone marrow but also promotes EPC migration and proliferation [28, 53]. eNOS expression and phosphorylation have been shown to be essential for EPC survival, migration, and angiogenesis [54], and the uncoupling of eNOS may result in eNOS-mediated NO production and induce EPC senescence [55]. The subsequent reduction in EPC levels or impairment of EPC function contributes to the pathogenesis of CVD in obesity [18, 19]. Our previous study and other investigations have reported that GTPCH I deactivation results in BH_4 deficiency and subsequent eNOS uncoupling, which may lead to decreased eNOS-mediated NO production; furthermore, the subsequent reduction in EPC levels and the impairment of EPC function likely contribute to the pathogenesis of vascular disease [24, 44]. In this investigation, we found that GTPCH

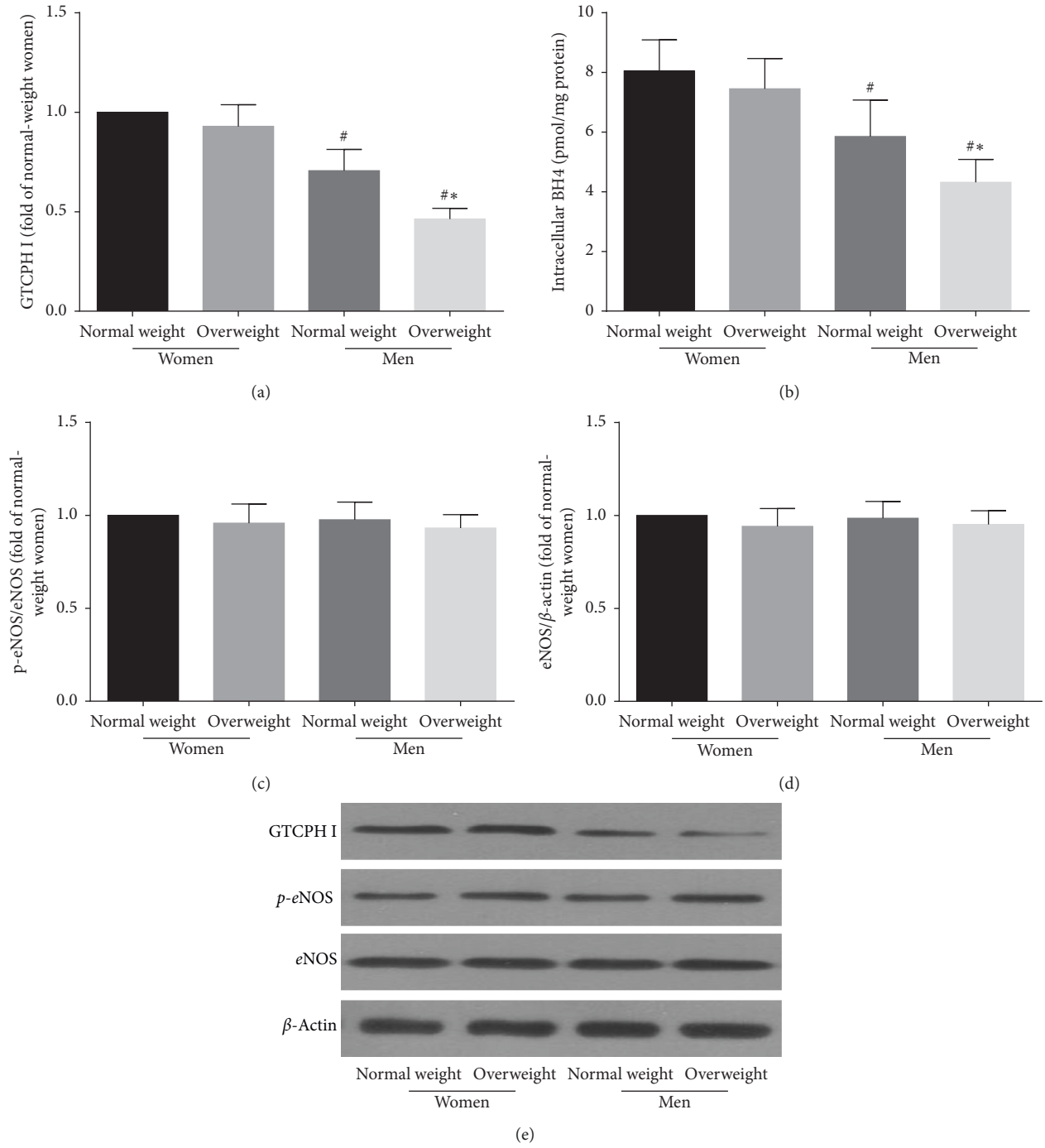

Figure 5: The GTPCH I/BH4 pathway and the phosphorylation of eNOS in circulating EPCs in the four groups. The level of GTPCH I (a) or intracellular BH4 (b) of EPCs in normal-weight and overweight premenopausal women was higher than that in normal-weight and overweight men. There was no difference in the level of GTPCH I (a) or intracellular BH4 (b) of EPCs between normal-weight and overweight premenopausal women. No significant difference was found in either eNOS phosphorylation (c) or eNOS protein expression (d) of EPCs between the four groups. (e) Representative photographs of GTPCH I, phosphorylated eNOS and eNOS expression of EPCs. Data are given as mean ± SD. $^{*}P < 0.05$ vs. the same gender group of normal weight; $^{\#}P < 0.05$ vs. the same weight class of premenopausal women.

I and intracellular BH_4 levels were reduced in overweight men compared with overweight premenopausal women and normal-weight men, but no such significant reduction was found in overweight premenopausal women. GTPCH I and BH_4 were regulated in parallel with the alterations in EPC function and EPC-mediated endothelial function. Meanwhile, eNOS phosphorylation and expression showed differences among the four groups. These results indicated that the GTPCH I pathway may be involved in abnormal NO bioactivity due to eNOS uncoupling but not phosphorylation, leading to a reduction in EPC function and subsequent endothelial dysfunction in overweight people.

The present study has the following implications. First, in overweight men, EPC migration and proliferation are impaired in parallel with attenuated endothelial function. As an independent and valid risk factor for endothelial dysfunction, EPC dysfunction is an early, effective marker of cardiovascular risk in overweight individuals. In addition, early intervention to enhance vascular repair capacity by upregulating EPC function may be an impactful approach to the treatment of overweight-related CVD. Second, we found no difference in FMD or EPCs between normal-weight and overweight premenopausal women in this study, suggesting that premenopausal status exerts a protective effect on EPC and endothelial function in overweight individuals. Third, our study revealed that reduced EPC activity, but not EPC counts, in overweight may contribute to the decreased NO production, at least partly through downregulation of the GTPCH I/BH_4 pathway. The upregulation of GTPCH I and subsequent improvement in the NO synthesis and plasma levels could enable the modulation of EPC migration and proliferation, thus strengthening the endothelial repair capacity in overweight individuals.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon resonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Shaohong Wu and Hao He participated in the design and performance of the study, statistical analysis, and preparation of the manuscript. Gexiu Liu, Xiaopeng Li, and Shun Yao participated in the performance of the study and statistical analysis. Huangxing Su, Xiang Li, and Zi Ren performed experiments. Haitao Zeng and Jinli Liao participated in the design and performance of the study, statistical analysis, and preparation of the manuscript.

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