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Allicin alleviates coronary atherosclerosis of mice via endothelial nitric oxide synthase(eNOS)/nuclear factor erythroid 2-related factor(Nrf2)/heme oxygenase-1(HO-1) signaling pathway

Jingyu Yang[1], Hailong Si[1], Bo Dong[1] and Qin Qin[1*]

Abstract

Purpose Endothelial progenitor cells (EPCs) have been revealed to interventions in atherosclerosis (AS) progressions. Traditional Chinese medicines (TCMs) have been discovered to modulate the functions of EPCs. Herein, effects of allicin on EPCs were explored in coronary atherosclerosis (CAS).

Methods Allicin (5 or 10 mg/kg/d) was used to treat the ApoE−/− mice fed with high-fat diet (HFD. TC, TG, LDL-C, and HDL-C were examined. HE staining was applied for observation of CAS lesions. In vitro, EPCs were induced by ox-LDL and then treated with allicin and an eNOS inhibitor, L-NAME. Thereafter, the cell viability, apoptosis and migration were examined using CCK-8, flow cytometry and Transwell methods. Western blot was applied for evaluating eNOS, Nrf2 and HO-1 protein expression. NO production, MDA content, and SOD activity were also measured.

Results Allicin inhibited CAS progression, decreased serum levels of TC, TG, and LDL-C but increased HDL-C. Moreover, counts of circulating EPCs, and the protein levels of eNOS, Nrf2 and HO-1 were increased by allicin treatment in mice fed with HFD. Allicin suppressed MDA contents but enhanced SOD activities. In vitro, allicin reversed the impacts of ox-LDL induction in EPCs, facilitating cell mobility and NO production, and decreasing apoptosis. L-NAME treatment reversed effects of allicin.

Conclusion Allicin alleviated CAS progressions in mice, modulating the cell apoptosis and migration of EPCs via eNOS/ Nrf2/HO-1 pathway.

Keywords Allicin, eNOS, Endothelial progenitor cells, Coronary atherosclerosis, Nrf2/HO-1

Introduction

Atherosclerosis (AS), a chronic inflammatory disorder in vessels, can cause heart attack and ischemic stroke [1]. Coronary atherosclerosis (CAS) is one type of AS that occurs in coronary arteries, causing coronary artery disorders, thereby leading to heart failure eventually [2]. Vascular endothelium can maintain and modulate vascular tension, structure and homeostasis, which can also prevent vessels from damages of immune response, inflammation and thrombogenesis [3]. Unfortunately, exposure of endothelial cells (ECs) in various damaging stimuli can cause endothelial injuries and dysfunctions [4]. Additionally, oxidative stress also stimulates overproductions of proinflammatory cytokines, resulting

*Correspondence:
Qin Qin
uyb33651@126.com
[1] The Second Department of Cardiology, Tianjin Chest Hospital, 261 Taierzhuang Nan Lu, Jinnan District, Tianjin 300222, China

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in persistent inflammatory state [5]. Then, these damages and inflammation accelerate AS progression.

Endothelial progenitor cells (EPCs) derived from bone marrows are precursor cells of ECs, which can repair endothelial damages to restrain the onset and development of AS [6]. However, evidence has revealed that hypercholesterolemia may hamper functions of EPCs, thereby reducing vascular repair [7]. Beyond that, oxidized low-density lipoprotein (ox-LDL) has been verified to discourage EPC mobilization through activating nuclear factor kappa-B (NF-κB) pathway [8]. Hypertriglyceridemia has been discovered to disturb the binding of stromal cell-derived factor-1 with C-X-C chemokine receptor type 4 and NO production, leading to EPC dysregulation and further endothelial dysfunction and injuries [9]. On the contrary, high-density lipoprotein (HDL) has been found to be positively correlated with EPCs progression via promoting eNOS [10].

Nowadays, therapeutic approaches that focus on EPCs have been widely explored. Garlic, an important food for human, is also a well-known medicinal plant [11]. Allicin is a sulfur-containing defensive molecule produced by garlics with pharmacological activity [12, 13]. Allicin has been applied for treating different disorders due to its anti-inflammation effect, anti-tumor activity and immune-protective effect [14–16]. Allicin alleviated renal injury in rats via inhibiting oxidative stress, cell apoptosis and inflammatory responses [17]. Evidence has indicated that allicin decreased the apoptosis in human umbilical vein vascular endothelial cells (HUVECs) induced by ox-LDL and inhibited the reactive oxygen species (ROS) production [18]. Moreover, allicin has been discovered to elevate serum HDL-C, superoxide dismutase (SOD) and suppress contents of serum TC, LDL-C and malondialdehyde (MDA), according to a previous study in rabbits with hypercholesteremia, which also exerted anti-inflammatory effects of allicin via suppressing tumor necrosis-alpha (TNF-α) and NF-κB [19]. Hence, allicin might be a promising mediator to treat CAS. In this research, we evaluated effects of allicin on blood lipid levels, oxidative stress, and biological functions of EPCs in mice to explore whether allicin inhibited CAS progression via mediating EPCs.

Materials and methods
Animal experiments
Male ApoE−/− mice(n=15) were obtained from animal center and accommodated for 2 weeks before experiments. Mice were maintained at 22 ± 2 °C, $55\pm5\%$ relative humidity, with a 12 h light/dark period. Afterwards, the mice were divided into 5 groups randomly: control (normal diet), control with allicin (10 mg/kg/d), high fat diet (HFD) group, HFD with allicin (5 mg/kg/d) group

and HFD with allicin (10 mg/kg/d) group. Treatment of allicin was started with the 16th weeks using oral gavage. HFD was acquired from Beijing Keao Xieli feed CO., Ltd. Animal experiments were approved by the Medical Ethics Committee of Tianjin Chest Hospital (Ethical approval number: 2020YS-089–01) and performed strictly as per the Guide for the Care and Use of Laboratory.

Blood samples collection and lipid level detection
Mice were euthanized using with pentobarbital sodium (50 mg/kg of body weight) after fasting overnight. Thereafter, blood samples were taken from posterior orbital venous plexus and collected using EDTA tubes. Then, collected plasma was centrifugated at 4 °C, 1500 rpm for 20 min. Micro Total Cholestenone (TC) and Triglyceride (TG) Content Assay Kit were obtained from Solarbio (Beijing, China). CheKine™ LDL-C and HDL-C Colorimetric Assay Kits were purchased from Abbkine (Wuhan, China). TC, TG, LDL-C, and HDL-C levels were examined.

Coronary arteries collection and atherosclerotic lesions analysis
Coronary arteries were collected and fixed using 4% paraformaldehyde for 24 h. Thereafter, coronary arteries were embedded into paraffin followed by sectioned into 5 μm. After dewaxing, hematoxylin, and eosin (HE) was used for staining. Zeiss Axiolab 5 (ZEISS, Germany) was used to take images.

Endothelial progenitor cells (EPC) separation and counting
Peripheral blood samples (20 mL) of mice in different groups were collected followed by Ficoll-density gradient centrifugation. Thereafter, mononuclear cells were separated and incubated in Endothelial Cell Growth Basal Medium-2 (EBM-2, Lonza, USA) added with 5% fetal bovine serum (FBS), human vascular endothelial growth factor (hVEGF), human basic fibroblast growth factor (b-FGF), human epidermal growth factor (EGF), human insulin-like growth factor-1 (IGF-1), ascorbic acid and GA-1000 (Lonza). The medium was replaced every 2 days for 14 days. After that, cells were incubated with dil-acLDL and UEA-1 (Maokangbio, China) and then observed under the lab fluorescence microscope from 4 different fields (Nikon, Japan). The cells positive with both dil-acLDL and UEA-1 were considered as EPCs.

Cell culture and pre-treatments
After EPCs were isolated, EPCs in control group were chosen for following experiments. DMEM/F12 medium with 10% FBS and 1% penicillin−streptomycin was used to cultivate EPCs at 37 °C, 5% CO_2 (Gibco). To simulate atherosclerotic environment, ox-LDL (20 μg/mL) was

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applied to induce EPCs for 24 h. Beyond that, Allicin (0.3, 0.5, 1.0, 5.0 and 10.0 μM, Solarbio) was used to treat cells for 12 h. Additionally, N omega-Nitro-L-arginine methyl ester hydrochloride (L-NAME, Abcam, UK), an eNOS inhibitor, was applied to inhibit eNOS expression. EPCs treated by allicin (10 μM) were cultivated for another 2 h with or without L-NAME (200 μM).

CCK-8
CCK-8 assay was used to evaluate viabilities of EPCs. After EPCs (1×10^4 cells/well) were plated into a 96-well plate and cultivated for 24 h. Ox-LDL, Allicin or L-NAME were added to treat cells for 12 h. Next, EPCs in wells were added with 10 μL CCK-8 (Beyotime) and cultivated for another 1 h. Using the Sunrise absorbance microplate reader (Tecan, Switzerland), the absorbance was detected at 450 nm. The experiment was run in triplicate.

Flow cytometry
To measure EPCs apoptosis, cells after treatment were first resuspended by Annexin V-FITC binding buffer (Beyotime). Later, cell suspension was mixed with 5 μL Annexin V-FITC and 10 μL PI (Beyotime) following by incubation for 20 min at 23 ℃ in darkness. Thereafter, Accuri C6 Plus (BD Biosciences, USA) was used to detect apoptosis of EPCs.

EPCs migration assays
Transwell assays were performed to observe the migration of EPCs after treatment. We conducted the experiments as described previously [20].

MDA and SOD detection
MDA assay kit (Solarbio) and SOD assay kit (Solarbio) were applied for detecting MDA and SOD, respectively. EPCs were lysed by cell lysis buffer and samples were quantified by BCA protein kit (Beyotime). In accordance with manufacturer's protocols, MDA and SOD contents were examined. Absorbance of MDA was examined at 532 nm and absorbance of SOD were evaluated at 560 nm using Sunrise absorbance microplate reader (Tecan).

NO content detection
After EPCs were treated, NO content assay kit (Solarbio) was used to detect NO levels. Supernatant was added with Griess Reagent I (50 μL) and Griess Reagent II (50 μL) at 25 ℃. Thereafter, the absorbance was examined by Sunrise absorbance microplate reader (Tecan) at 540 nm.

Western blot
After EPCs were harvested, cell lysis buffer (Beyotime) was used to segregate total protein followed by separation using SDS-PAGE and shifting onto PVDF membranes. Then, membranes were blocked by 5% non-fat milk powder and incubated with anti-eNOS (1:1000, ab76198, Abcam, UK), anti- Nrf2(1:1000, ab62352, Abcam), anti-HO-1(1:1000, ab52947, Abcam), and anti-GAPDH (1:2000, ab9484, Abcam) overnight at 4 ℃. Then, goat Anti-Rabbit IgG (HRP) (1:800, ab97051, Abcam) was added to incubate membranes at 23 ℃ for 1 h. BeyoECL Star (Beyotime) was applied for developing and protein bands were checked by Image J.

Statistical analysis
Data analysis was conducted on GraphPad Prism 9 (GraphPad). Kruskal–Wallis test and Dunn's multiple comparison was used to analyze the statistical significance between the groups where there were multiple groups. Student's t-test examined differences between two groups. Two-way ANOVA was used in analysis of the cck8 results. **$P < 0.032$.

Results
Allicin alleviated coronary atherosclerosis development in mice
To evaluate effects of allicin on coronary atherosclerosis (CAS), ApoE−/− mice were fed with normal diet, normal diet with allicin (10 mg/kg/d), HFD or HFD with allicin (5 mg/kg/d and 10 mg/kg/d). Serum TC, TG, and LDL-C levels in mice were elevated and the serum HDL-C level was reduced in HFD group compared to the control group with normal diet(Fig. 1A–D). The allicin treatment of 5 mg/kg/d reduced LDL-C and increased HDL-C significantly but didn't change the serum TC and TG levels in mice fed with HFD (Fig. 1A–D). Moreover, serum TC, TG, and LDL-C levels were decreased while the serum HDL-C level was elevated with significant difference after allicin treatment of 10 mg/kg/d in mice fed with HFD (Fig. 1A–D). No significant change was detected in the control group with allicin treatment (10 mg/kg/d) in comparison with the control group; no significant changes were found in the mice fed with normal diet and then treated with allicin (10 mg/kg/d) group (Fig. 1A–D).Furthermore, CAS lesions showed that HFD increased lesions of atherosclerosis in animals while allicin treatment alleviated this (Fig. 1E). Hence, allicin treatment could alleviate CAS development in ApoE−/− mice.
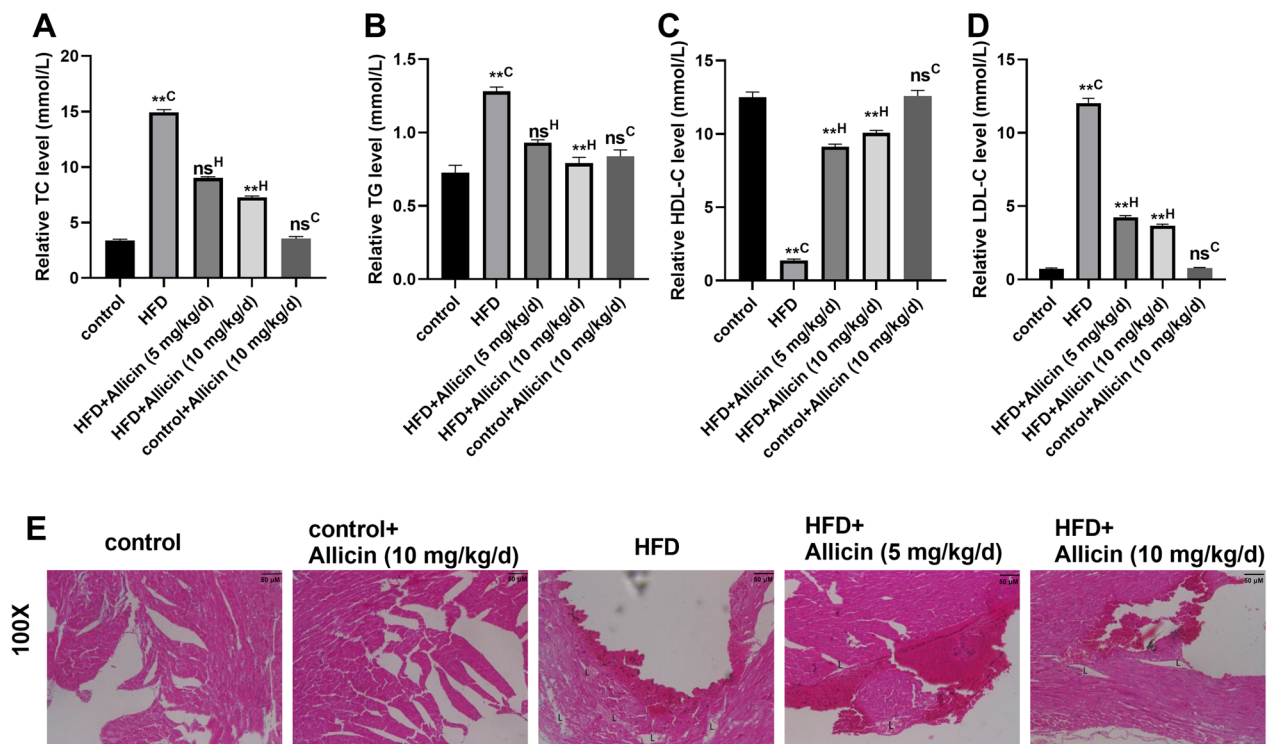
Fig. 1 Allicin alleviated CAS development in ApoE−/− mice. **A**–**D** TC, TG, HDL-C and LDL-C levels of mice in control, HFD and HFD with allicin (5 μM and 10 μM) groups were examined, n = 3 in each group. vs HFD group, $^{**}P < 0.032$, ns: not significant. ^{**C}: significant vs control; ^{**H}: significant vs HFD group. **B** CAS lesions were evaluated using HE staining

Allicin suppressed CAS progression via eNOS/Nrf2/HO-1 signaling pathway

Circulating EPC counts were elevated in mice fed with HFD, suggesting that circulating EPCs were triggered to repair CAS lesions (Fig. 2A). After allicin treatment for 4 weeks, circulating EPC counts in mice were further elevated, indicating that allicin might facilitate the repair functions of EPCs (Fig. 2B). Moreover, the protein levels of eNOS, Nrf2 and HO-1 in EPCs were all downregulated in HFD-fed mice while allicin treatment recovered their protein expressions (Fig. 2C). Furthermore, MDA level was increased by HFD in mice but was decreased after allicin treatment (Fig. 2E). However, HFD reduced NO production and SOD activity in mice, which were recovered by allicin treatment (Fig. 2D, F).

Suppression of eNOS reversed protective effects of allicin on ox-LDL-induced injuries in EPCs

To further explore effects of allicin on EPCs in CAS, different concentrations of allicin were applied to treat EPCs that were isolated from mice in normal group. Results of CCK-8 showed that allicin had no toxicity to EPCs at a concentration of 0.3, 0.5, 1, 5 and 10 μM (Fig. 3A).

Thereafter, ox-LDL (20 μg/mL) was applied to induce injuries of EPCs, inhibiting eNOS, Nrf2, and HO-1 protein expressions (Fig. 3B). We used allicin (10 μM) to treat EPCs. Moreover, L-NAME, an eNOS inhibitor, was used. Western blot results indicated that allicin treatment upregulated eNOS, Nrf2, and HO-1 protein expressions while L-NAME treatment suppressed their protein expressions (Fig. 3B). Additionally, MDA level was elevated by ox-LDL induction, which was inhibited with allicin treatment but promoted by L-NAME treatment (Fig. 3C). SOD activity and NO production were suppressed in ox-LDL-induced EPCs while allicin treatment increased their levels, which were inhibited by L-NAME treatment (Fig. 3D, E).

Allicin treatment restored the viabilities and migration in ox-LDL-induced EPCs via eNOS/Nrf2/HO-1 regulation

Furthermore, results of CCK-8 and migration assays indicated that ox-LDL induction decreased EPCs viabilities and migration, which were recovered after allicin treatment, but L-NAME treatment could revert this in EPCs (Fig. 4A, C). Beyond that, ox-LDL-induced apoptosis of EPCs was suppressed by allicin treatment and then further reverted by L-NAME treatment (Fig. 4B).

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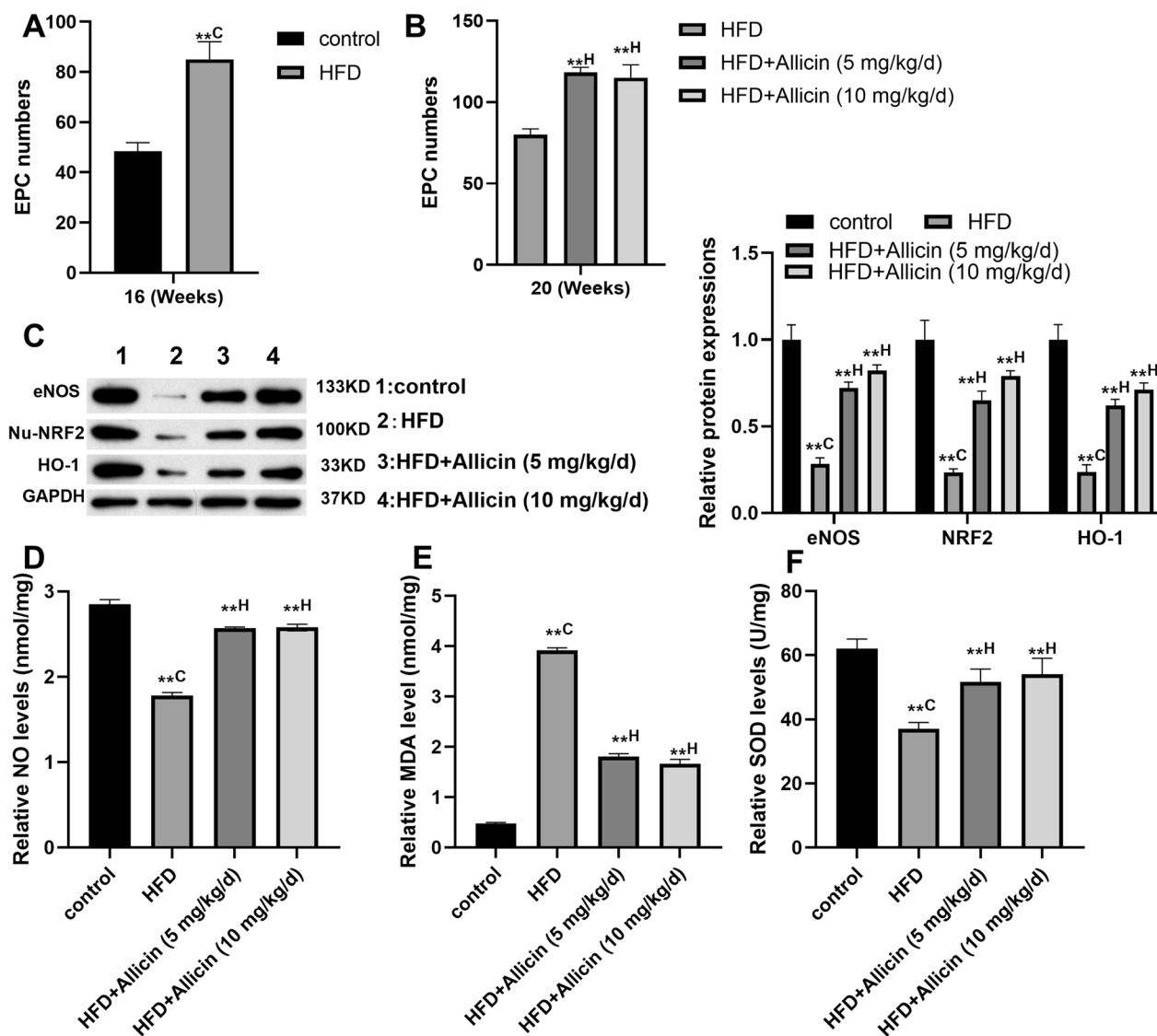
Fig. 2 Allicin suppressed oxidative stress in Apoe−/− mice. **A**, **B** Circulating EPCs from mice groups, control, HFD and HFD with allicin (5 μM and 10 μM) groups were counted. **C** eNOS, Nrf2 and HO-1 protein expressions in EPCs from mice in different groups were validated using western blot, n = 3 in each group. **D**–**F** MDA, SOD and NO levels from mice in different groups, **P < 0.032, ns: not significant. **C: significant vs control; **H: significant vs HFD group

Discussion

Recently, Cardiovascular diseases (CVDs) have become a prevalent problem in modern society, in which AS is an essential contributor to the increasing mortality and disability in patients with CVDs [21]. Allicin was revealed to activate SOD and glutathione peroxidase and reduce levels of 8-hydroxy-desoxyguanosine and ROS to inhibit Acrylamide-caused oxidative stress and DNA injuries [22]. Nrf2/HO-1 is a well-known signaling pathway against oxidative stress. Nrf2 was detached from its endogenous inhibitor, Kelch Like ECH Associated Protein 1, and translocated to the nucleus to protect from oxidative stress [23]. Elevated Nrf2 caused by Se yeast consolidated antioxidant defense system and restrained apoptosis and inflammatory responses [24]. Downregulation of Nrf2 and HO-1 could weaken antioxidant defense, causing myocardial cell apoptosis [25].According to Ma, L., et al., allicin decreased the apoptosis of ischemia/hypoxia-induced H9C2 cell via elevating eNOS, Nrf2 and HO-1 protein expressions and NO production and suppressing oxidative stress [26]. Allicin has been discovered to reduce viabilities and lipid accumulation of foam cells derived from THP-1 macrophage by upregulating ABCA1 expressions via activating PPARγ/LXRα

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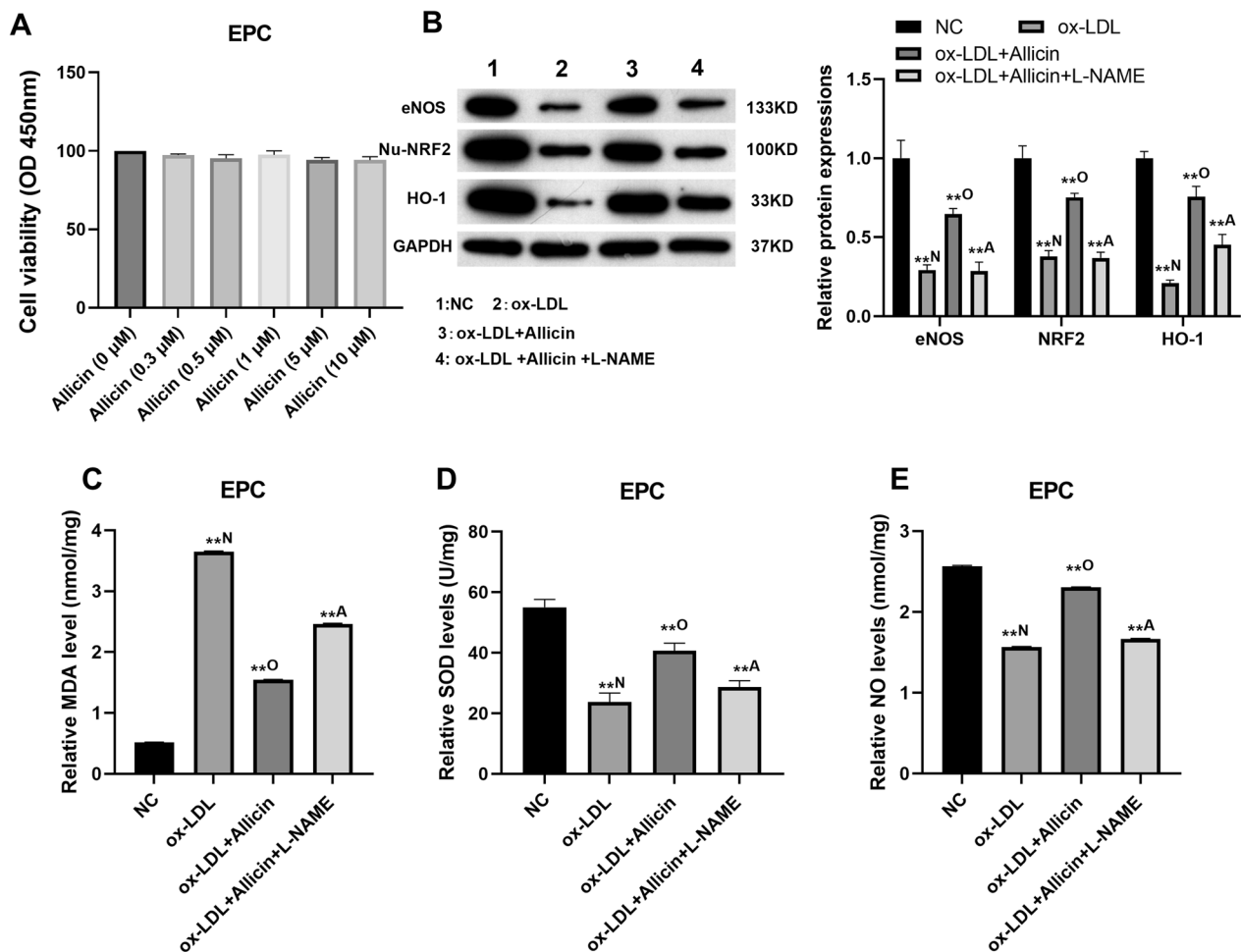
Fig. 3 Allicin alleviated ox-LDL induction in EPCs in vitro. **A** CCK-8 was applied for examining cytotoxicity of allicin in EPCs in vitro. **B** eNOS, Nrf2 and HO-1 protein expression after ox-LDL induction(24 h), allicin treatment(12 h) and L-NAME treatment (2 h) were evaluated using western blot. **C**–**E** MDA, SOD and NO level were detected after ox-LDL induction, allicin treatment and L-NAME treatment in vitro. **P < 0.032, **N: significant vs NC; **O: significant vs ox-LDL group; **A: significant vs ox-LDL + Allicin

signaling pathway [27]. In our study, TC, TG, and LDL-C levels were decreased after HFD-fed mice were treated by allicin while the HDL-C level was increased. Moreover, CAS lesions were also reduced after allicin treatment. Allicin elevated eNOS, Nrf2 and HO-1 protein expressions and promoted SOD activities and inhibited MDA contents in HFD-fed mice. These results suggested that allicin treatment could inhibit CAS development by suppressing oxidative stress via activating eNOS/Nrf2 and HO-1 signaling pathway.

According to previous studies, allicin could prevent the AS progression, decreasing plasma homocysteine, TC and TG levels and carotid artery intima-media thickness [28]. Allicin could reduce AS plaques through the modulation of gut microbiota and trimethylamine-N-oxide [29].Allicin accelerated migratory and angiogenic capacities of cardiac microvascular endothelial cells

to protect hearts via activating platelet endothelial cell adhesion molecule-1 [30]. Therefore, we have analyzed impacts of allicin related to EPCs in vivo and in vitro. Based on the mice model, we counted the circulating EPCs, and found that the circulating EPC counts in the HFD mice group were increased compared to the normal control, which might suggest that EPCs were mobilized to repair the damaged endothelium in mice with CAS lesions. In addition, the circulating EPCs were increased after 4-week allicin treatment, suggesting that allicin treatment promoted the EPCs function to facilitate the repair of CAS lesions. This indicated that allicin might alleviate the CAS progression in mice through EPC regulations via eNOS/Nrf2/HO-1 pathway. We used allicin (0.3 μM-10 μM) to treat normal EPCs from mice, only to find that cytotoxicity of allicin (10 μM) on EPCs could be excluded. Considering the role of ox-LDL

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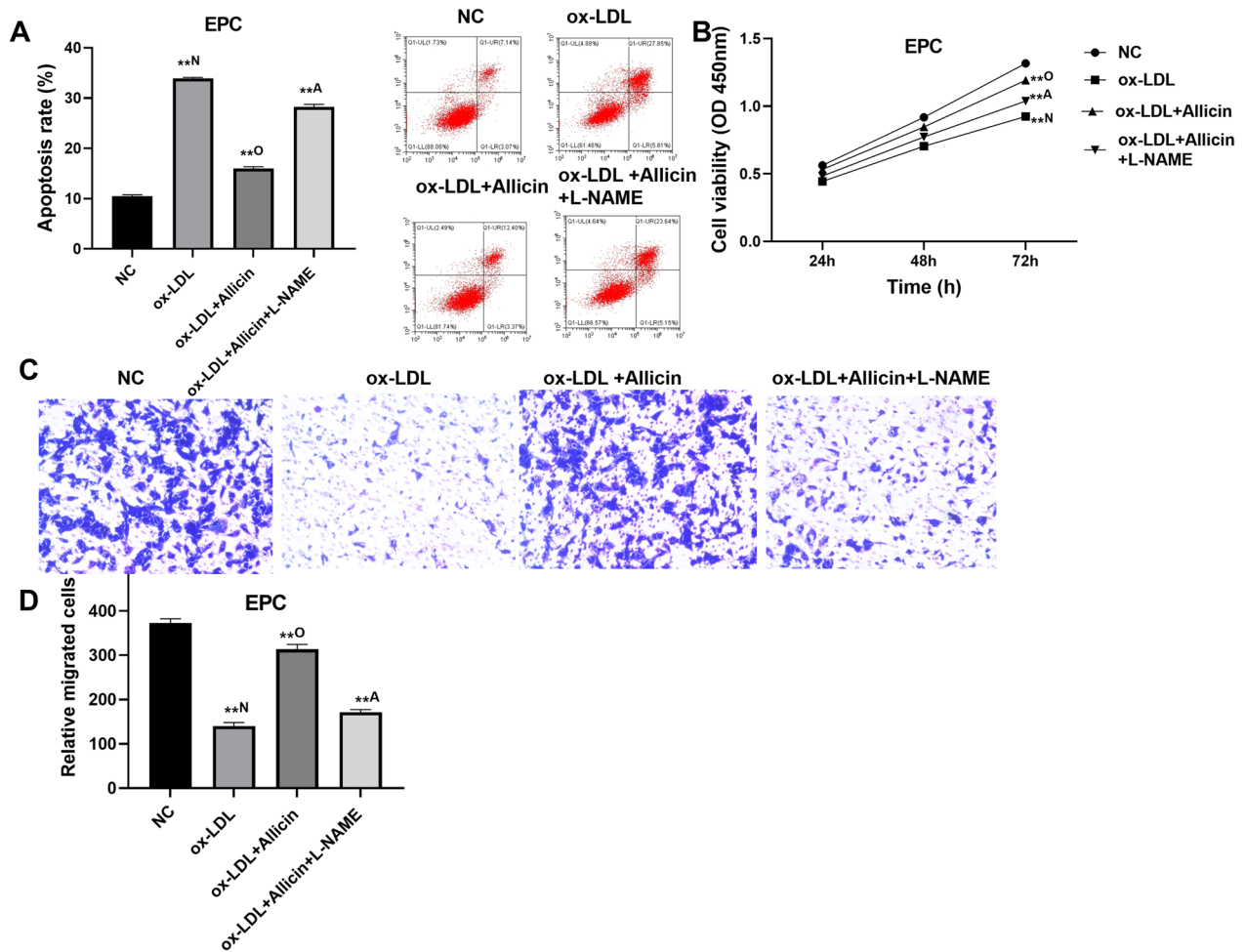
Fig. 4 Allicin facilitated EPC viabilities and migratory abilities but hampered EPC apoptosis. **A** CCK-8 was applied to evaluated EPCs viabilities after ox-LDL induction (24 h), allicin treatment(12 h) and L-NAME treatment (2 h). **B** Flow cytometry was used for detecting EPCs. **C** Transwell was used to analyze EPCs migratory. **P < 0.032, **N: significant vs NC; **O: significant vs ox-LDL group; **A: significant vs ox-LDL + Allicin

in AS progression and endothelial dysfunction [31], ox-LDL was applied to induce EPCs to establish a cellular model. Hu, Z., et al., have reported that EPCs mobilization was associated with eNOS elevation [32]. Jiang, Q., et al., have discovered that ox-LDL suppressed Nrf2 expressions and inactivation of Nrf2 enhanced ROS production in EPCs [20]. According to the study of Shen, X., et al., the activation of HO-1 inhibited ROS levels and facilitated endothelial repair ability of EPCs [33]. In this study, allicin elevated eNOS, Nrf2 and HO-1 expressions in ox-LDL-induced EPCs and elevated NO production. Moreover, allicin treatment decreased MDA levels but increased SOD levels in EPCs induced by ox-LDL. Therefore, allicin might alleviated ox-LDL-induced injury via eNOS/Nrf2/HO-1 signaling pathway and suppressing

ROS generation in EPCs. Furthermore, we used L-NAME to inhibit eNOS, and results showed that L-NAME could reverse the protective effect of allicin in EPCs.

Abbreviations

CAS	Coronary atherosclerosis
Nrf2	Nuclear factor erythroid 2-related factor
HO-1	Heme oxygenase-1
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
AS	Atherosclerosis
TCM	Traditional Chinese medicine
HFD	High-fat diet
TC	Total cholesterol
TG	Triglyceride
HDL-C	High-density lipoprotein cholesterol
LDL-C	Low-density lipoprotein cholesterol
ox-LDL	Oxidized low-density lipoprotein

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MDA	Malondialdehyde
SOD	Superoxide dismutase
NO	Nitric oxide
HE	Hematoxylin–Eosin
ApoE	Apolipoprotein E
L-NAME	N (gamma)-nitro-L-arginine methyl ester
EC	Endothelial cell
NF-κB	Nuclear factor kappa-B
HUVEC	Human umbilical vein vascular endothelial cell
ROS	Reactive oxygen species
FBS	Fetal bovine serum
EGF	Epidermal growth factor
hVEGF	Human vascular endothelial growth factor
b-FGF	Basic fibroblast growth factor
IGF-1	Insulin-like growth factor-1

Declarations

Competing interests
The authors declare that they have no competing interests.

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