Article

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Mechanism of Curcumin Inhibiting NLRP3 Inflammatory Body and Improving Atherosclerotic Endothelial Cell Injury

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Background: Curcumin is a kind of natural hydrophobic polyphenol isolated from the stem of the Curcuma plant. To investigate regulatory curcumin effect on atherosclerotic endothelial cell injury.
Methods: 30 male ApoE$^{-/-}$ mice were selected and divided into the control group, model group, and curcumin group (n = 10). The curcumin group was treated with curcumin by gavage. Body weight, atherosclerotic plaque area, plaque cap thickness, blood lipid levels, total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein cholesterol (LDL-C) content, nitric oxide (NO) content, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) content and circulating endothelial cell number of mice in each group were detected. Western blot detected NACHT, LRR, and receptor family pyrin domain-containing 3 (NLRP3) and Asc-type amino acid transporter protein 1 (ASC) protein level in mice. Human aortic endothelial cells (HAEC) were cultured to establish an atherosclerotic endothelial cell injury model *in vivo*. Cell counting kit-8 (CCK-8) detected the cell viability of each group.
Results: Body weight, atherosclerotic plaque area, plaque cap thickness, TC, TG, and LDL-C content of blood lipid levels of the curcumin group were obviously reduced as compared with the model group ($p < 0.05$), the content of NO and the number of circulating endothelial cells in curcumin group were obviously decreased ($p < 0.05$). The cell viability of the curcumin group was obviously higher than that of the model group ($p < 0.05$). The NO content of the curcumin group was lower than the model group ($p < 0.05$). The content of IL-1β and TNF-α in the curcumin group was obviously lower than in the model group ($p < 0.05$). Compared with the model group, the expression of receptor family pyrin domain-containing 3 (NLRP3) and ASC protein in the curcumin group was decreased obviously ($p < 0.05$).
Conclusion: Curcumin improves endothelial cell injury in atherosclerosis by inhibiting the expression of NLRP3 inflammatory bodies.

Keywords: curcumin; NLRP3 inflammatory body; atherosclerosis; endothelial cell injury

Introduction

Atherosclerosis (AS) is the basic pathological basis for occurrence and cardiovascular disease development. It is a vascular immune inflammatory disease that appears with the growth of people's age [1]. Its plaque stability determines the progress and prognosis of AS [2]. Some studies have proved that plaque rupture and thrombosis caused by AS-vulnerable plaque are the main factors causing acute cardio-cerebrovascular events [3–5]. The current major problem of the global medical community is to prevent AS occurrence and development. AS pathogenesis is related to various regulatory factors and pathological processes, including endothelial injury, lipid infiltration, oxidative stress, etc. [6–9].

At present, the occurrence of AS is related to the activation of inflammatory corpuscles of nucleotide-binding oligomerization domains like receptor family pyrin domain-containing 3 (NLRP3) [10]. After NLRP3 inflammatory corpuscles activation, the precursor of caspase-1 was cut to produce caspase-1, which cut the precursor of inactive interleukin (IL)-1β (pro-IL-1β) into mature IL-1β (maturation IL-1β, m IL-1β), while regulating the secretion of inflammatory factor tumor necrosis factor-α (TNF-α). The secreted inflammatory factors start the immune inflammatory reaction and then promote the development of AS [11–13]. Therefore, interfering with the activation of NLRP3 inflammatory corpuscles may be beneficial to delay the process of AS. Curcumin is a kind of polyphenolic traditional Chinese medicine monomer extracted from turmeric, turmeric, and other ginger plants, which has anti-inflammatory, antioxidant, anti-tumor, and other biological effects [14–16]. Curcumin has a positive repair effect on the damage caused by AS [17]. At the same time, cur-

cumin may inhibit intestinal inflammation by regulating the NLRP3 inflammatory body [18]. However, the mechanism of curcumin improving endothelial cell injury in atherosclerosis has not been reported yet.

To explore curcumin's mechanism in improving damage of atherosclerotic endothelial cells by constructing mice and cell models *in vivo* and *in vitro*.

Materials and Methods

Animals

Thirty specific pathogen-free (SPF)-grade male *ApoE*$^{-/-}$ mice, aged 6 to 7 weeks (21.03 $\pm$ 2.86 g) and a genetic background of C57BL/6 mice (Experimental Animal Center of Xi'an Jiaotong University (SCXK (Shaanxi) 2003-0007), Xi'an, China). The mice were housed in SPF with a constant temperature (22~25 °C), constant humidity (60% relative humidity), and a 12 h/12 h light and dark cycle. They had free access to food and water (This study was approved by the Medical Ethics Committee of Jiangxi Provincial People's Hospital, Ethical and ethical approval numbers: KT054).

Animal Model Construction and Treatment

The mice were divided into Control, Model, and curcumin groups using the random number table method (n = 10). The feed for model group mice contained 21% fat, 0.25% cholesterol, and 78.75% basic feed. After one week of adaptive feeding, mice began to make models. Mice in the curcumin group were fed a high-fat diet continuously for 16 weeks, and curcumin was given gavage every 24 hours on the same day. According to the preliminary study, we set the dosage of curcumin at 100 mg/Kg/d. Model group mice were given 100 mg/Kg/d of sterile physiological saline every 24 h starting from the same day for gastric lavage treatment. The control group mice were fed basic feed and given 100 mg/Kg/d sterile physiological saline every 24 hours starting from the same day for gavage treatment.

Cell Culture

Human aortic endothelial cells (HAEC) (MZ-1234, Mingzhoubio, Ningbo, China) cultured in Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum, mycoplasma testing and short tandem repeat testing both met the experimental requirements. Inoculate it on a 24-well culture plate. After 24 h, replace it with a low-serum endothelial cell culture medium extracellular matrix (ECM) culture medium for 24 h. After that, it can be used in the experiment. HAEC cells were stimulated by 50 μg/mL oxidized low-density lipoprotein (ox-LDL) for 48 h, and oil red O staining showed that the cell volume was increased, round, short spindle-shaped or irregular, with a large number of red or dark red round lipid droplets in the cytoplasm, and significant cell foaming.

HE (Hematoxylin-Eosin) Staining

In the 12th week of modeling, mice were euthanized using sodium pentobarbital and cervical dislocation for subsequent experiments. Pentobarbital sodium is prepared as a 3% solution in sterile saline and given at the usual dose of 1 mL/kg body weight. The drug should not be injected rapidly at one time, but slowly and intermittently, and more slowly after 3/4 of the intended dose has been injected. While injecting, observe the corneal reflex, muscle relaxation and pain response of the animals, and stop the drug injection immediately when the required anesthesia state is reached, and monitor the blood pressure and pulse of the animals at any time. Four rat vascular tissues were randomly removed, fixed with 4% paraformaldehyde (24 h), dehydrated with ethanol and n-butanol, and placed in a 60 °C for paraffin immersion. After embedding, the sections were sliced (5 μm). Spread slices at 45 °C, fish the slices, and bake at 60 °C for 1 h before dewaxing with xylene. Routine hematoxylin-eosin staining after hydration. Firstly, prepared slices were subjected to routine dewaxing and gradient alcohol hydration, followed by staining with Su Mu semen (H8070, Beijing Solarbio Technology Co., Ltd., Beijing, China) for 2 min, rinsing for 10 seconds, and then separating with 1% hydrochloric acid ethanol (10 seconds). Wash with distilled water for 1 min and stain with eosin solution for 1 min. After washing with distilled water for 10 seconds, dehydrate with gradient alcohol, and seal the sections with neutral gum after transparent xylene. After the film was sealed, the histological changes of blood vessels in rats were observed under an optical microscope (XP-330, Shanghai Bingyu Optical Instrument Co., Ltd., Shanghai, China). Each group has 3 samples, and the experiment is repeated independently 3 times. After routine hematoxylin-eosin (HE) staining, the pathological changes of vascular tissue were observed using a light microscope (BX51-P, Olympus Corporation, Tokyo, Japan), and neointima thickness and the ratio of intima to media area were calculated. The single-blind method is used for measurement and calculation, and the measurer is not related to this experiment.

Detection of Serum Index

Venous blood was collected from the tail vein of mice, centrifuged at 3000 r/min (5 min), and the upper layer of plasma was drawn. Enzyme-Linked immunosorbent assay (ELISA) detected serum TNF-α (ml002095, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China), IL-1β (ml098416, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China), interleukin 6 (IL-6, ml098430, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China), interleukin 10 (IL-10, mIC50274-1, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China), vasoactive endothelial growth factor (VEGF) (ml058424, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China), hypersensitive-c-reactive-protein

(hs-CRP) (ml002010, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China), malondialdehyde (MDA) (KCW20347, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China), lactate dehydrogenase (LDH) (ml002267, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China), and superoxide dismutase (SOD, ml001998, Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China) levels. The colorimetric method was used to detect nitric oxide (NO) content, and the operation was strictly carried out in accordance with the corresponding kit instructions.

Circulating Endothelial Cell Count

Flow cytometry was used to count circulating endothelial cells [19]. CD31-positive cells percentage in the total number of mononuclear cells represents the number of circulating endothelial cells.

Cell Counting Kit-8 (CCK-8)

The logarithmic growth phase cells were divided into 3×10^4/well and inoculated in a 96-well plate. They were cultured for 24 hours and replaced with serum-free ECM for another 24 hours. Next, 10 µL of CCK-8 solution was added per well and cultured for 2 hours. 450 nm measured absorbance (A) value and calculated cell survival rate. The CCK-8 kit was purchased from MedChemExpress (HY-K0301, MedChemExpress, Jersey, NJ, USA).

Western Blot

The proteins were taken from the vascular endothelial tissue of each group, the lysate containing benzene sulfonyl fluoride to ground, extract the protein, and detect the protein concentration. Protein samples were transferred to the polyvinylidene fluoride (PVDF) membrane. After sealing, NLRP3 (ab263899, 1:1000, Abcam Trading Co., Cambridge, England), Asc-type amino acid transporter protein 1 (ASC) (ab309497, 1:1000, Abcam Trading Co., Cambridge, England) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ab8245, 1:5000, Abcam Trading Co., Cambridge, England) antibodies were added respectively, and incubated overnight at 4 °C; After adding the second Goat Anti-Rabbit IgG H&L (HRP) for incubation (ab6721, 1:10,000, Abcam Trading Co., Cambridge, England) for 1 h, add enhanced chemiluminescence (ECL, HR0338, Beijing Baiao Leibo Technology Co., Ltd., Beijing, China) luminescent solution for development, and Image J software (version V1.8.0, ThermoFisher Scientific, Waltham , MD, USA) was used for analysis.

Statistical Analysis

All data were analyzed by SPSS software (version 26.0, IBM SPSS Inc., Chicago, IL, USA). Chi-square test and Student's *t*-test analysis compare data between two groups; One-Way ANOVA analysis compares the continuous variable data between multiple groups, and post hoc tests were conducted using the Dunnett-t. $p < 0.05$ means statistically significant.

Results

Atherosclerosis Model Successfully Constructed

After 16 weeks of feeding mice in different ways, the model group mice's body weight was significantly higher than the control group (Fig. 1A, $p < 0.05$). The model group showed a significant increase in endothelial damage degree, a significant increase in the ratio of neointimal area to the media area, orderly cell arrangement, and discontinuous smooth muscle layer. The ratio of neointimal area to media area in the control group significantly decreased, and the cells were arranged neatly (Fig. 1B,C, $p < 0.05$). The comparison of blood lipid levels of total cholesterol (TC), triacylglycerol (TG), and low-density lipoprotein cholesterol (LDL-C) in each group of mice also showed that the TC, TG, and LDL-C levels in the model group were significantly higher than the control group (Fig. 1D–F, $p < 0.05$). The levels of IL-6, IL-10, TNF-α, VEGF, hs-CRP, MDA, and LDH of the model group were significantly increased, while the SOD content was significantly reduced (Table 1, $p < 0.05$).

Curcumin Improves Atherosclerosis

We compared the formation of atherosclerosis in mice in each group. The area and cap thickness of atherosclerotic plaque in the thoracic aorta of the curcumin group were significantly lower than the model group (Fig. 2A,B, $p < 0.05$). The comparison of blood lipid levels, TC, TG, and LDL-C content in each group of mice also showed that the content of TC, TG, and LDL-C in the curcumin group was significantly lower than in the model group (Fig. 2C–E, $p < 0.05$). The levels of IL-6, IL-10, TNF-α, VEGF, hs-CRP, MDA, and LDH of the model group were significantly increased, while the SOD content was significantly reduced (Table 1, $p < 0.05$).

Curcumin Improves Atherosclerosis by Repairing Endothelial Cell Damage

We further detected the endothelial cell damage index NO and the number of circulating endothelial cells in the serum in each group. Nitric oxide (NO) content and the number of circulating endothelial cells in the model group were obviously increased compared with the control group. Compared with the model group, the content of NO and the number of circulating endothelial cells in the curcumin group were decreased obviously (Fig. 3, $p < 0.05$).

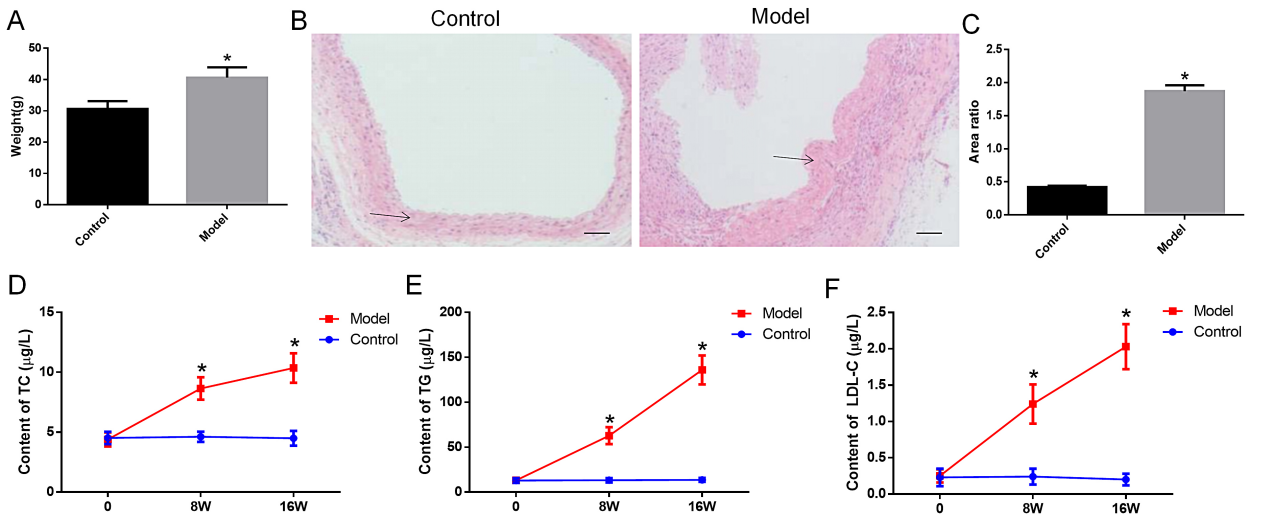

Fig. 1. The mouse atherosclerosis model was successfully constructed. (A) Comparison of mouse body weights. (B) Hematoxylin-Eosin (HE) staining of mouse tissue (the area indicated by the arrow is the vascular endothelium). Scale bar, 250 μm. (C) The ratio of neointima to media area in mice. (D) Comparison of total cholesterol (TC) content of blood lipid indicators at different time periods in mouse modeling. (E) Comparison of blood lipid index triacylglycerol (TG) content in different time periods of mouse modeling. (F) Comparison of low-density lipoprotein cholesterol (LDL-C) levels in blood lipid indicators at different time periods during mouse modeling. *, vs Control, $p < 0.05$.

Table 1. Inflammatory factors, MDA, SOD, and LDH levels in serum of each group.

Group	IL-6 (pg/mL)	IL-10 (pg/mL)	VEGF (pg/mL)	TNF-α (pg/mL)	hs-CRP (pg/mL)	MDA (mmol/L)	SOD (U/mL)	LDH (U/L)
Control	1.62 ± 0.09	13.67 ± 1.02	0.96 ± 0.16	28.61 ± 2.24	4.02 ± 0.39	1.07 ± 0.21	25.25 ± 3.42	48.23 ± 11.02
Model	4.84 ± 0.42*	17.56 ± 1.54*	6.27 ± 0.28*	56.33 ± 5.34*	25.53 ± 2.12*	10.28 ± 2.80*	3.76 ± 1.18*	87.29 ± 8.90*
Curcumin	3.18 ± 0.59#	15.63 ± 1.23#	3.64 ± 0.53#	44.95 ± 5.63#	18.39 ± 2.57#	6.23 ± 0.86#	14.68 ± 2.05#	63.81 ± 9.08#

Note: MDA, malondialdehyde; SOD, superoxide dismutase; LDH, lactate dehydrogenase; VEGF, vasoactive endothelial growth factor; hs-CRP, hypersensitive-c-reactive-protein; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; IL-10, interleukin 10. *, vs Control, $p < 0.05$. #, vs Model, $p < 0.05$.

In Vitro Experiment to Verify Effect of Curcumin on Endothelial Cell Injury and Repair

To further verify whether curcumin has a good repair effect on vascular endothelial cell damage in atherosclerosis. We cultured HAEC *in vitro* and detected the cell viability of different groups. The cell viability of the model group was lower than the control group, while that of the curcumin group was obviously higher than the model group (Fig. 4A, $p < 0.05$). The content of NO in cell supernatant also showed that the NO content in the model group was higher than in the control group, while the curcumin group was lower than the model group (Fig. 4B, $p < 0.05$).

Curcumin Repairs the Injury of Atherosclerotic Endothelial Cells in Mice by Inhibiting NLRP3 Inflammatory Bodies

IL-1β and TNF-α content in mice of the model group was obviously higher than the control group, while the curcumin group was obviously lower than the model group (Fig. 5A, $p < 0.05$). NLRP3 and ASC protein expression in inflammatory bodies of mice and cells in the model group was obviously higher than the control group. Compared with the model group, NLRP3 and ASC protein expression of the curcumin group was obviously decreased (Fig. 5B,C, $p < 0.05$).

Discussion

Atherosclerosis (AS) can easily affect coronary and carotid arteries, etc. The adverse events of the heart and brain caused by AS have become the primary cause of death in humans [20]. Many risk factors causing AS (such as smoking, hypertension, diabetes, lipid metabolism disorders, changes in blood rheology, hypoxia, infection, etc.) can cause inflammation and oxidative stress and may lead to endothelial damage and dysfunction [21–24]. Several significant characteristics of AS are inflammation, oxidative stress, and vascular endothelial damage. Vascular endothelium plays an important role in maintaining vascular homeostasis. The vascular endothelium is a barrier and

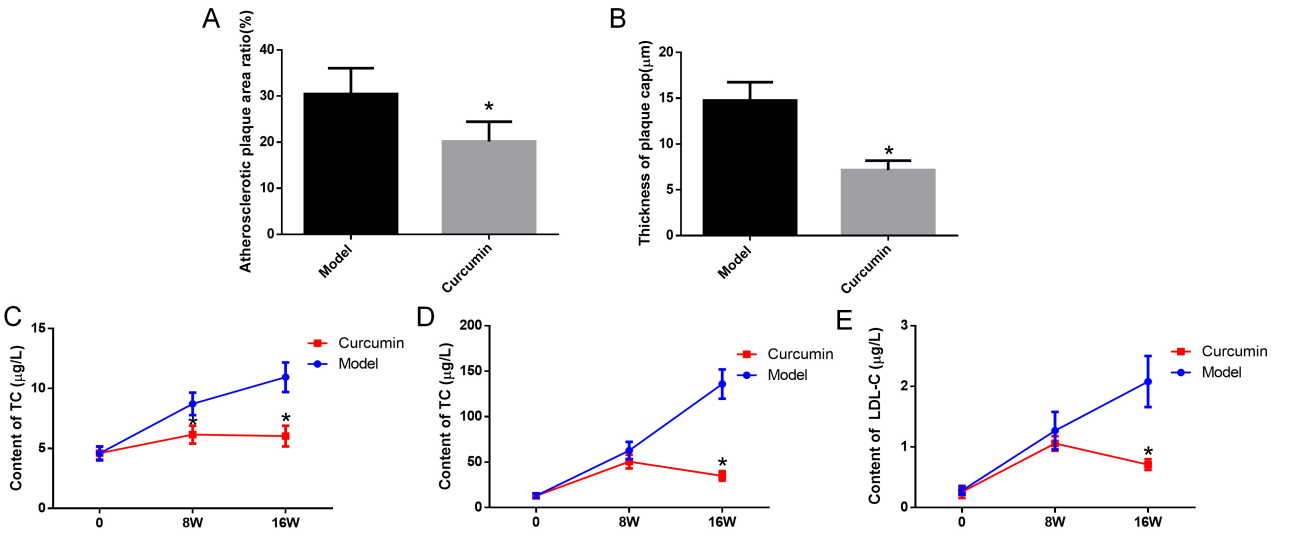

Fig. 2. Curcumin improves atherosclerosis. (A) Comparison of plaque area ratio in mice with arteriosclerosis. (B) Comparison of plaque cap thickness. (C) TC content of blood lipid indicators at different time periods in mouse modeling. (D) Comparison of blood lipid index TG content in different time periods of mouse modeling. (E) Comparison of LDL-C levels in blood lipid indicators at different time periods during mouse modeling. *, vs Model, $p < 0.05$.

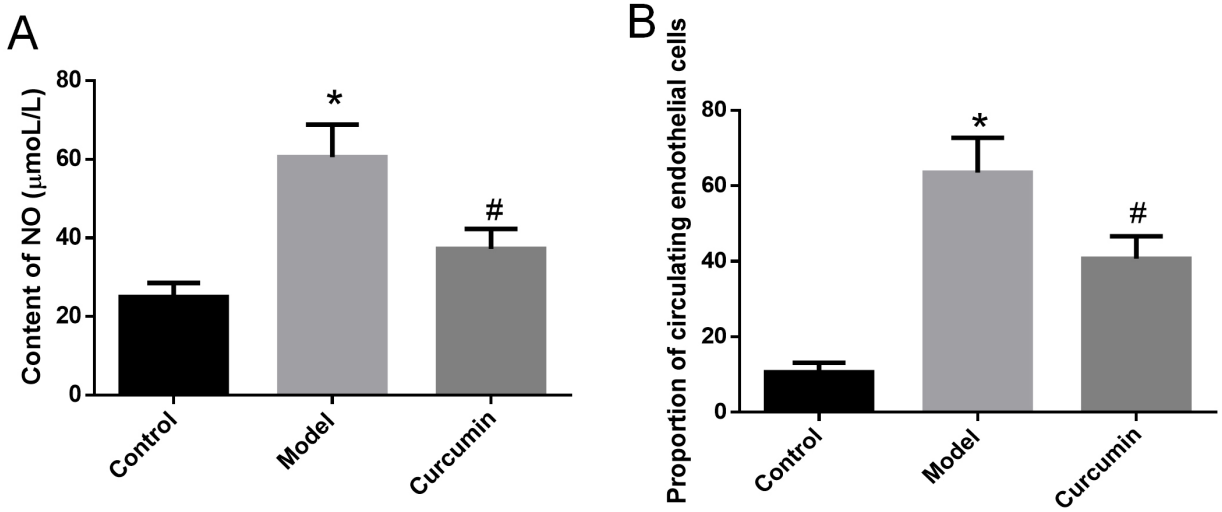

Fig. 3. Effect of curcumin on endothelial cell injury in mice. (A) Comparison of nitric oxide (NO) content in serum. (B) Comparison of the number of circulating endothelial cells in mice. *, vs Control, $p < 0.05$. #, vs Model, $p < 0.05$.

a complex paracrine organ which regulates cell growth, vascular tension, and the interaction between platelets and leukocytes and the vascular wall. Endothelial injury can cause LDL-C to deposit in a large amount under the intima [25–27]. After oxidized and modified into ox LDL, LDL-C is engulfed by monocyte macrophages to form foam cells, thus starting the AS process [28]. It can also lead to dysfunction of vasodilation and contraction and promote thrombosis, thus triggering cardiovascular events [29–31]. It can be seen that inhibiting inflammation and oxidative stress and maintaining the integrity and function of vascular endothelium are important measures to prevent and treat AS [31–33]. The weight of atherosclerotic mice increased obviously, the area of atherosclerotic plaque and the thickness of plaque cap in thoracic aorta increased obviously, TC, TG, LDL-C in blood lipid level content also increased obviously, and the injury of endothelial cells aggravated. This suggests significant inflammatory and oxidative stress responses in AS mice, accompanied by vascular endothelial damage and dysfunction. At the same time, the obvi-

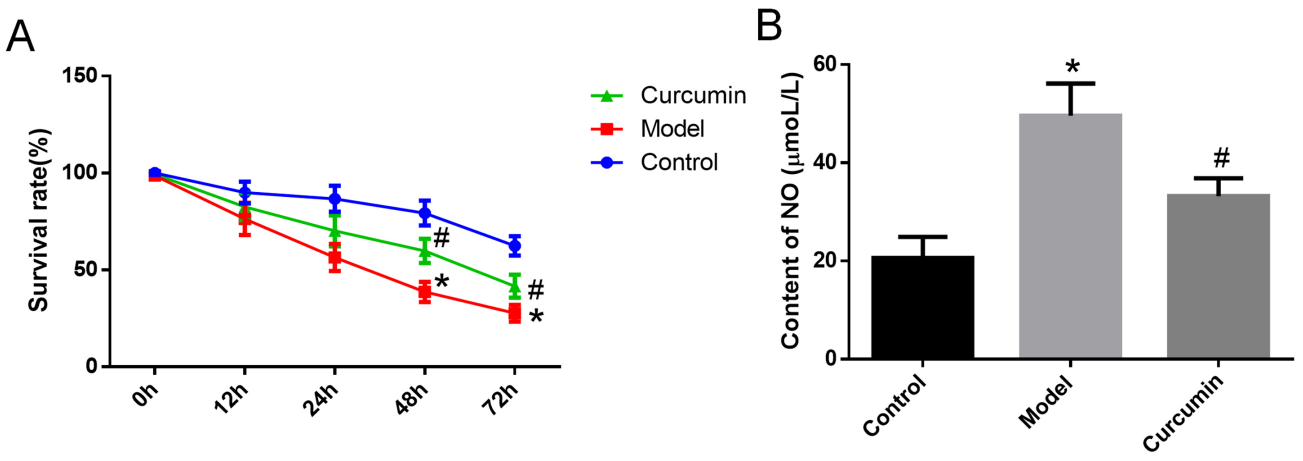

Fig. 4. Effect of curcumin on endothelial cell proliferation (A) and NO content (B). *, vs Control, $p < 0.05$. #, vs Model, $p < 0.05$.

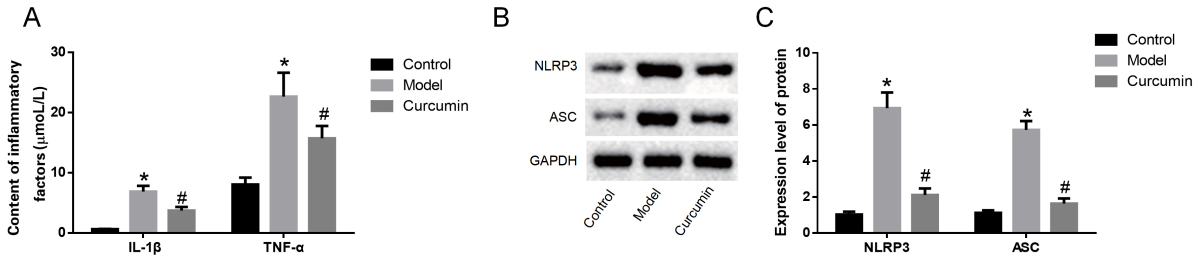

Fig. 5. Curcumin can repair the damage of atherosclerotic endothelial cells in mice by inhibiting receptor family pyrin domain-containing 3 (NLRP3) inflammasome. (A) The serum content of inflammatory factors. (B) Protein banding. (C) NLRP3 and Asc-type amino acid transporter protein 1 (ASC) protein expression in inflammatory bodies of mice in each group. *, vs Control, $p < 0.05$. #, vs Model, $p < 0.05$.

ous increase in body weight of AS mice also indicates that body mass index (BMI) may be one of the risk factors for atherosclerosis. This is similar to inflammation, oxidative stress, vascular endothelial damage, and dysfunction in patients with atherosclerosis. In view of the close correlation between endothelial injury and AS and related cardiovascular adverse events, it is of reference significance to explore effective methods to reverse vascular endothelial injury and dysfunction in AS model rats for clinical prevention and treatment of atherosclerosis.

Curcumin is a kind of polyphenol compound extracted from the rhizome of the curcuma genus. Its pharmacological mechanism is complex and has multiple cellular protective effects, including anti-inflammatory, antioxidant, antiviral, and inhibition of AS [34]. It has been reported that curcumin can effectively fight inflammation by regulating pro-inflammatory cytokines and related signaling pathways, such as inhibiting NF-κB-mediated inflammation [35]. The atherosclerotic plaque area and plaque cap thickness of thoracic aorta vessels were significantly improved in mice treated with Curcumin administration, and lipid indexes TC, TG, and LDL-C contents were stably controlled. This showed that curcumin effectively played the anti-inflammatory, antioxidant and vascular endothelial protection role.

Curcumin can inhibit NLRP3 inflammasome activation. NLRP3 inflammasome is activated by various types of pathogen-related molecular patterns and intracellular danger signals, and it is the most intensively studied inflammasome involving the most diseases [36]. NLRP3 inflammasome is important for the pathogenesis of inflammatory bowel disease (IBD) [37]. Activation of the NLRP3 inflammasome goes through two stages. In the first stage, pathogen-associated molecular patterns (PAMP, such as LPS) or endogenous signaling molecules recognize membrane surface pattern recognition receptors (PRRs, such as TLR), which up-regulate NLRP3 and pro-IL-1β gene expression. In the second stage, various stimulators activate the NLRP3 inflammasome and caspase-1, promoting the cleavage of pro-IL-1β into biologically active mature IL-1β and its secretion into the extracellular. Therefore, we further investigated NLRP3 inflammasome-related protein expression. NLRP3 and ASC protein. Expression and the contents of IL-1β and TNF-α were significantly decreased

in mice treated with curcumin. This suggests that curcumin may ameliorate the injury of AS endothelial cells by inhibiting NLRP3 inflammasome.

Conclusion

The study has concluded that curcumin can improve damage to atherosclerotic endothelial cells by inhibiting the expression of NLRP3 inflammatory bodies.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author HR, upon reasonable request.

Author Contributions

HR, XZ and SS designed the research study and been involved in drafting the manuscript or revising it critically for important intellectual content. HR, XZ and SS contributed to the concept. HR, XZ and SS performed the research. HR contributed to the analysis and interpretation of the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal experiments in this study comply with the relevant guidelines of the revised Animals (Scientific Procedures) Act 1986 in the UK and Directive 2010/63/EU in Europe. This study was approved by the Medical Ethics Committee of Jiangxi Provincial People's Hospital (KT054).

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Conflict of Interest

The authors declare no conflict of interest.

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