Article

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Curcumin Promotes the Proliferation, Migration, and Angiogenesis of HUVECs to Improve Atherosclerosis through the Wnt/β-Catenin Pathway

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Background: Atherosclerosis is a chronic inflammatory disease that leads to ischemic cerebrovascular and cardiovascular diseases. Curcumin, known for its anti-inflammatory properties, may influence the development of atherosclerosis. This study aims to elucidate the effects of curcumin and its mechanisms on atherosclerosis progression.

Methods: The proliferative ability, the angiogenesis capacity, and cell migration rate were assessed using cell counting kit-8 (CCK-8), 5-ethynyl-29-deoxyuridine (EdU), tube formation assays, and wound healing, respectively. Furthermore, the protein expression levels of proliferating cell nuclear antigen (PCNA), glycogen synthase kinase 3β (GSK3β), p-GSK3β, β-catenin, and c-myc were determined utilizing western blot analysis.

Results: Oxidized low-density lipoprotein (ox-LDL) significantly triggered cell damage by inhibiting cell proliferation, reducing the migration rate, and angiogenesis capacity in human umbilical vein endothelial cells (HUVECs) ($p < 0.05$). Curcumin treatment significantly alleviated ox-LDL-induced HUVECs injury ($p < 0.05$), as evidenced by elevating the proliferative ability ($p < 0.05$), cell migration ($p < 0.05$), and angiogenesis ($p < 0.05$). Moreover, the Wnt/β-catenin pathway was substantially boosted following ox-LDL treatment ($p < 0.05$), which was suppressed by curcumin ($p < 0.05$). Additionally, SKL2001 significantly increased the levels of β-catenin and c-myc ($p < 0.05$). The inhibitory effects of curcumin treatment on the Wnt/β-catenin signaling pathway were reduced by SKL2001 ($p < 0.05$). Furthermore, the promoting effects of curcumin on ox-LDL-induced cell damage were hampered following SKL2001 treatment in HUVECs ($p < 0.05$).

Conclusion: Curcumin elevated cell proliferation, migration, and angiogenesis of HUVECs to inhibit the development of atherosclerosis through inactivating the Wnt/β-catenin pathway.

Keywords: atherosclerosis; curcumin; Wnt/β-catenin pathway; SKL2001

Introduction

Atherosclerosis, characterized by the accumulation of lipid droplets and an increased inflammatory response, has become a leading cause of mortality worldwide [1,2]. Atherosclerosis leads to thrombogenesis or luminal stenosis resulting in blood flow obstruction to vital organs such as the brain, heart, and lower extremities, leading to ischemic stroke, coronary heart disease, and peripheral vascular diseases, respectively [3,4]. Previous studies revealed that dysregulation of endothelial cells is an early indicator of the initiation of atherosclerosis and contributes to the development of atherosclerosis [5,6]. The injury of vascular endothelial cells compromises barrier function and the integrity of the endothelium, thereby facilitating lipid deposition and promoting atherogenesis [7]. It has been reported that oxygenated low-density lipoprotein (ox-LDL) plays a pivotal role in atherosclerosis by promoting the expression of adhesion molecules and chemokines, which could facilitate the pro-inflammation responses and result in endothelial cell dysregulation [8]. Thus, ox-LDL is widely used to stimulate endothelial damage and induce atherosclerosis cell model *in vitro* [9,10]. Therefore, exploring the complex mechanisms underlying ox-LDL-induced endothelial cell damage is crucial for identifying potential therapies for atherosclerosis treatment.

Accumulating evidence indicated that natural compounds have been widely applied for treating varieties of diseases because of the anti-inflammation and antioxidant actions, including atherosclerosis [11,12]. Curcumin, a bioactive compound isolated from *Curcuma longa*, possesses excellent pharmacological properties. Several studies have demonstrated that curcumin possesses a variety of biological properties, including anti-inflammatory, anti-malignant, antioxidant, and anti-fibrotic activities in diseases through the modulation of a range of molecular targets such as chemokines, transcription and growth factors, cytokines and enzymes [13–16]. Moreover, the

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role of curcumin in preventing atherosclerosis has been documented. Previous studies indicated that curcumin exerted anti-atherosclerosis impact by improving dyslipidemia, suppressing intestinal cholesterol absorption, promoting cholesterol efflux, and mitigating atherosclerotic lesions [17–20]. Therefore, this study investigated the role of curcumin in an ox-LDL-induced cell model and its potential mechanisms related to endothelial cell damage in the progression of atherosclerosis.

Endothelial cell injury induced by ox-LDL is regarded as an early indicator of atherosclerosis [21], which is helpful for explaining atherosclerosis pathogenesis [22,23]. This study explored the influence of curcumin on cell metastasis, angiogenesis, and proliferation in human umbilical vein endothelial cells (HUVECs) treated with ox-LDL. It has been reported that Wnt/β-catenin signaling plays a vital role in the development of atherosclerosis by regulating endothelial dysfunction [24]. Whether curcumin modulated the Wnt/β-catenin to regulate cell damage after ox-LDL treatment in HUVECs still needs to be investigated. These explorations hold significance in improving our understanding of the complex cellular and molecular mechanisms regulating atherosclerosis and may contribute to identifying potential therapeutic targets for atherosclerosis.

Materials and Methods

Cell Culture and Treatment

The HUVECs (BNCC360874) were obtained from the Bena Culture Collection (Suzhou, China). The cells were maintained in the RPMI 1640 medium (11879020; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% antibiotics (15140122; Gibco, Carlsbad, CA, USA) in a 37 °C incubator under the condition of 5% CO_2. The cells underwent short tandem repeat (STR) identification and mycoplasma testing before further analysis, and no mycoplasma has been found. Ox-LDL was obtained from Solarbio (IO1300; Beijing, China) with a purity of 98.0%. HUVECs were treated with varying concentrations of ox-LDL (25, 50, or 100 μg/mL) for 24 hours to establish an *in vitro* atherosclerosis cell model [25]. Curcumin (purity >95%) was sourced from Sangon Biotech (A600346-0005; Shanghai, China). Dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was utilized to dissolve curcumin with a storage concentration of 30 mM. Before cell treatment, the storage solution was diluted in the medium with <0.1% DMSO to achieve 10, 20, and 30 μM concentrations of curcumin. Subsequently, the cells were exposed to these different concentrations of curcumin for 24 hours [26]. Additionally, the Wnt/β-catenin pathway agonist SKL2001 (40 μM; Selleck Chemicals, Houston, TX, USA) was used to treat HUVECs for 30 minutes before ox-LDL and curcumin treatment [27].

Cell Counting Kit-8 (CCK-8) Assay

HUVECs were seeded into a 96-well plate at a density of 5000 cells per well. Following ox-LDL, curcumin, or SKL2001 treatment, 10 μL of CCK-8 solution (CK04; Dojindo, Kumamoto, Japan) was added into each well and incubated for 4 hours. After incubation, the absorbance at 450 nm was evaluated employing a microplate reader (Multiskan MK3; Thermo Labsystems, Waltham, MA, USA), and further calculated using the ImageJ Pro Plus 6.0 software (NIH, Bethesda, MD, USA).

5-Ethynyl-29-Deoxyuridine (EdU) Staining Assay

Following ox-LDL, curcumin, or SKL2001 treatment, HUVECs underwent exposure to 10 μM of the EdU reagent (C0071S, Beyotime, Shanghai, China) for 2 hours. The fluorescence intensity was observed utilizing an inverted fluorescence microscope (TE-2000E; Nikon, Shanghai, China), and EdU-positive cells were determined using the ImageJ Pro Plus 6.0 software (NIH, Rockville, MD, USA).

Western Blot

The isolated proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto membranes. The membranes were incubated overnight with primary antibodies, including anti-proliferating cell nuclear antigen (anti-PCNA; ab18197, 1:1500, Abcam, Cambridge, UK), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; ab181602, 1:1000, Abcam, Cambridge, UK), anti-glycogen synthase kinase 3β (anti-GSK3β; ab32391, 1:5000, Abcam, Cambridge, UK), anti-c-myc (ab32072, 1:1000, Abcam, Cambridge, UK), anti-p-GSK3β (ab75814, 1:10,000, Abcam, Cambridge, UK), and anti-β-catenin (ab223075, 1:1000, Abcam, Cambridge, UK). The next day, the membranes were probed with goat anti-rabbit secondary antibody (ab205718, 1:10,000, Abcam, Cambridge, UK) at room temperature for 2 hours. Finally, immunoblots were observed using an Enhanced chemiluminescence system (WBULS0100; Millipore, Billerica, MA, USA) and then quantified utilizing ImageJ Pro Plus 6.0 software (NIH, Rockville, MD, USA).

Wound Healing Assay

HUVECs were cultured in the 6-well plates at a density of 2×10^5 cells per well. A 10 μL pipette tip was used to create a uniform scratch in the cells from the center of each well. After this, the remaining cells were cultured in a serum-free medium. The cells were observed using an inverted microscope (IX73, Olympus, Tokyo, Japan), and the images of the migrated cells were captured at 0 hours and 24 hours. Finally, the ImageJ Pro Plus 6.0 software (NIH, Rockville, MD, USA) was employed to quantify the migrated distance to the wound.

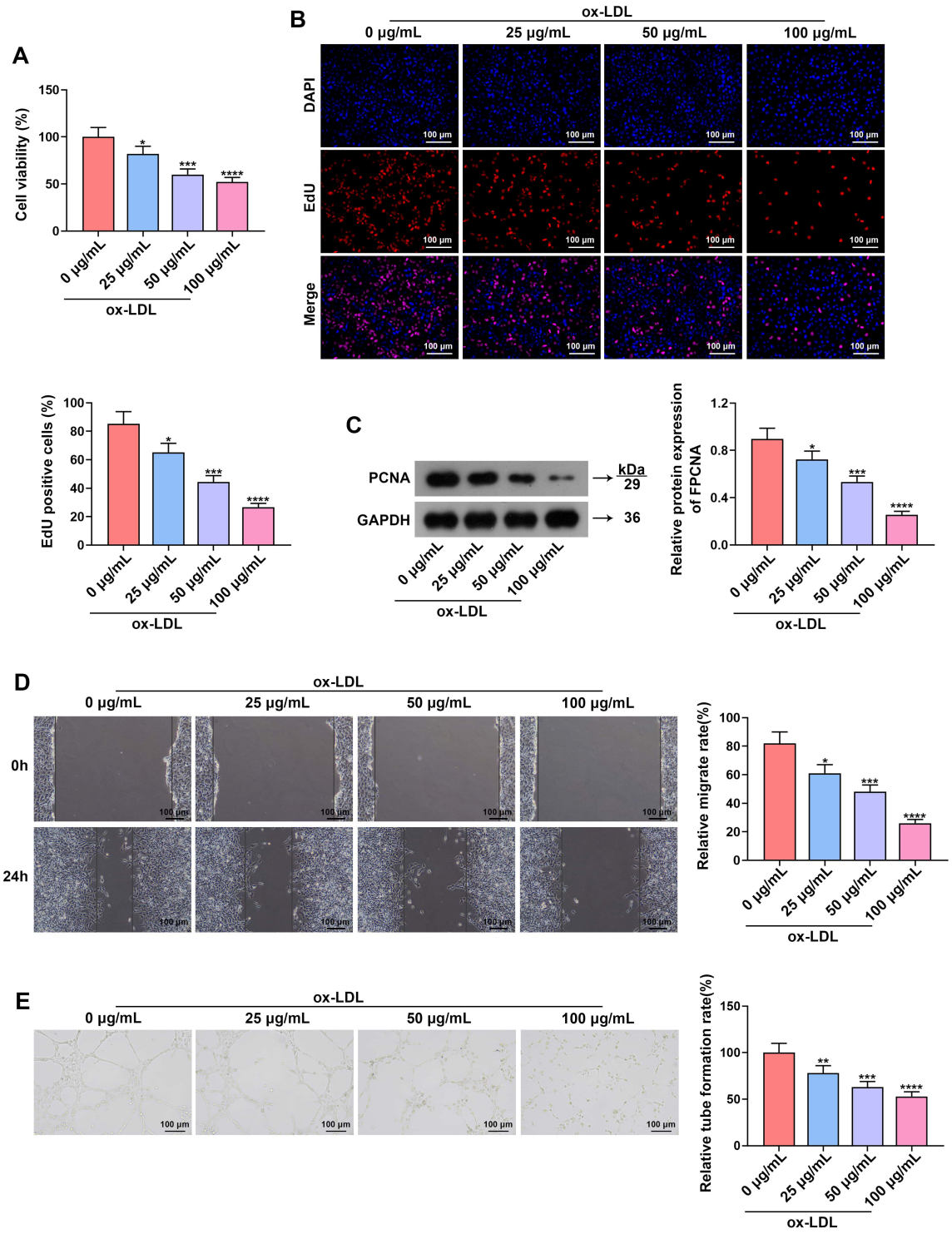

Fig. 1. Ox-LDL markedly repressed proliferation, migration, and the angiogenesis capacity of HUVECs. (A) Cell viability of ox-LDL-treated HUVECs (25, 50, and 100 μg/mL) was evaluated using CCK-8 assay (n = 3). (B) The proliferative ability of HUVECs was determined through EdU assay (Scale bar: 100 μm; n = 3). (C) PCNA protein expression in ox-LDL-treated HUVECs (25, 50, and 100 μg/mL) was tested using western blot analysis (n = 3). (D) Wound healing assay was performed to assess the proportion of migrated cells following ox-LDL treatment (25, 50, and 100 μg/mL) (Scale bar: 100 μm; n = 3). (E) The ability of ox-LDL-treated HUVECs (25, 50, and 100 μg/mL) to form a capillary-like structure was assessed utilizing tube formation assay (scale bar: 100 μm; n = 3). Note: ox-LDL, oxidized low-density lipoprotein; CCK-8, cell counting kit-8; PCNA, proliferating cell nuclear antigen; HUVECs, human umbilical vein endothelial cells; EdU, 5-ethynyl-29-deoxyuridine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

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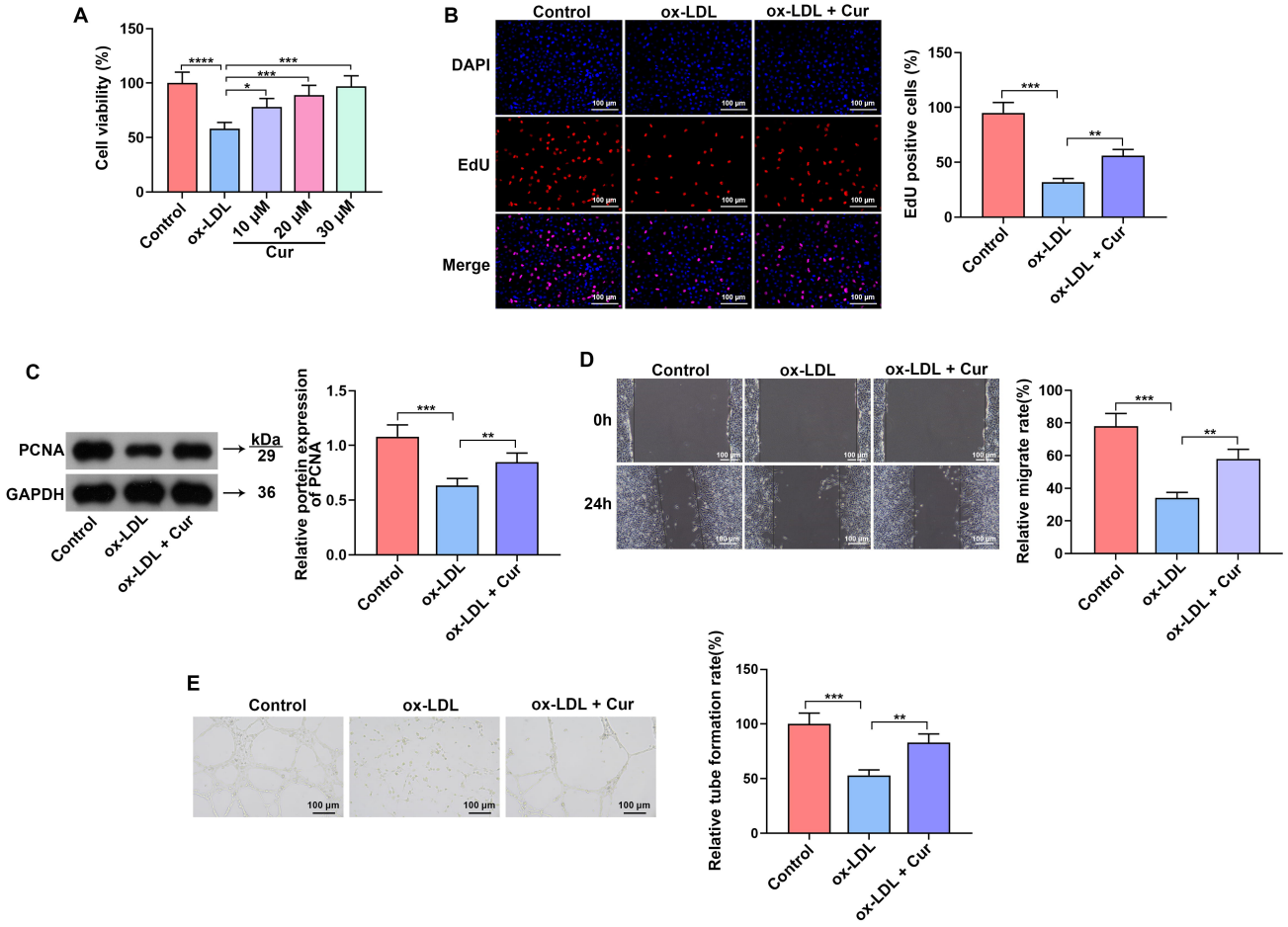

Fig. 2. Curcumin ameliorated ox-LDL-induced cell damage in HUVECs. (A) Cell viability of HUVECS treated with ox-LDL (50 μg/mL) and different concentrations of curcumin (10, 20, and 30 μM) was assessed using CCK-8 assay (n = 3). (B) EdU-positive HUVECs were determined through EdU assay (Scale bar: 100 μm; n = 3). (C) Western blot analysis was used to assess PCNA protein levels (n = 3). (D) Cellular migration rate of HUVECs was assessed through wound healing assay (Scale bar: 100 μm; n = 3). (E) Angiogenesis of HUVECs treated with ox-LDL or curcumin was assessed using tube formation assay (Scale bar: 100 μm; n = 3). Note: Cur, curcumin; DAPI, 4'6-diamidino-2-phenylindole. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Tube Formation Assay

Following ox-LDL, curcumin, or SKL2001 treatment, HUVECs were seeded in a 96-well plate pre-coated with the Matrigel. After 48 hours of incubation, the tube formation was examined using a light microscope (BX40, Olympus, Tokyo, Japan).

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism software (Version 8.0, GraphPad Inc., La Jolla, CA, USA). The data were presented as the mean ± standard deviation. Measurement data exhibiting normal distribution were compared using a *t*-test between two groups, while Analysis of Variance (ANOVA) following Tukey's post hoc analysis was utilized to compare differences among multiple groups. Statistical difference was determined as a p value lower than 0.05.

Results

Ox-LDL Significantly Repressed Proliferation, Migration, and Angiogenesis of HUVECs

As depicted in Fig. 1A, different concentrations of ox-LDL (25, 50, and 100 μg/mL) significantly reduced the viability of HUVECs ($p < 0.05$). Furthermore, the number of EdU-positive cells was substantially decreased following ox-LDL treatment ($p < 0.05$, Fig. 1B). Western blot analysis revealed that ox-LDL treatment down-regulated the protein expression of PCNA, a cell proliferation-associated protein, in a dose-dependent manner ($p < 0.05$, Fig. 1C). Moreover, the migratory rate of HUVECs was substantially repressed following ox-LDL treatment (Fig. 1D). Additionally, ox-LDL treatment significantly inhibited the angiogenesis capacity of HUVECs in a dose-dependent manner ($p < 0.05$, Fig. 1E). Hence, 50 μg/mL dose of ox-LDL was used for subsequent experiments.

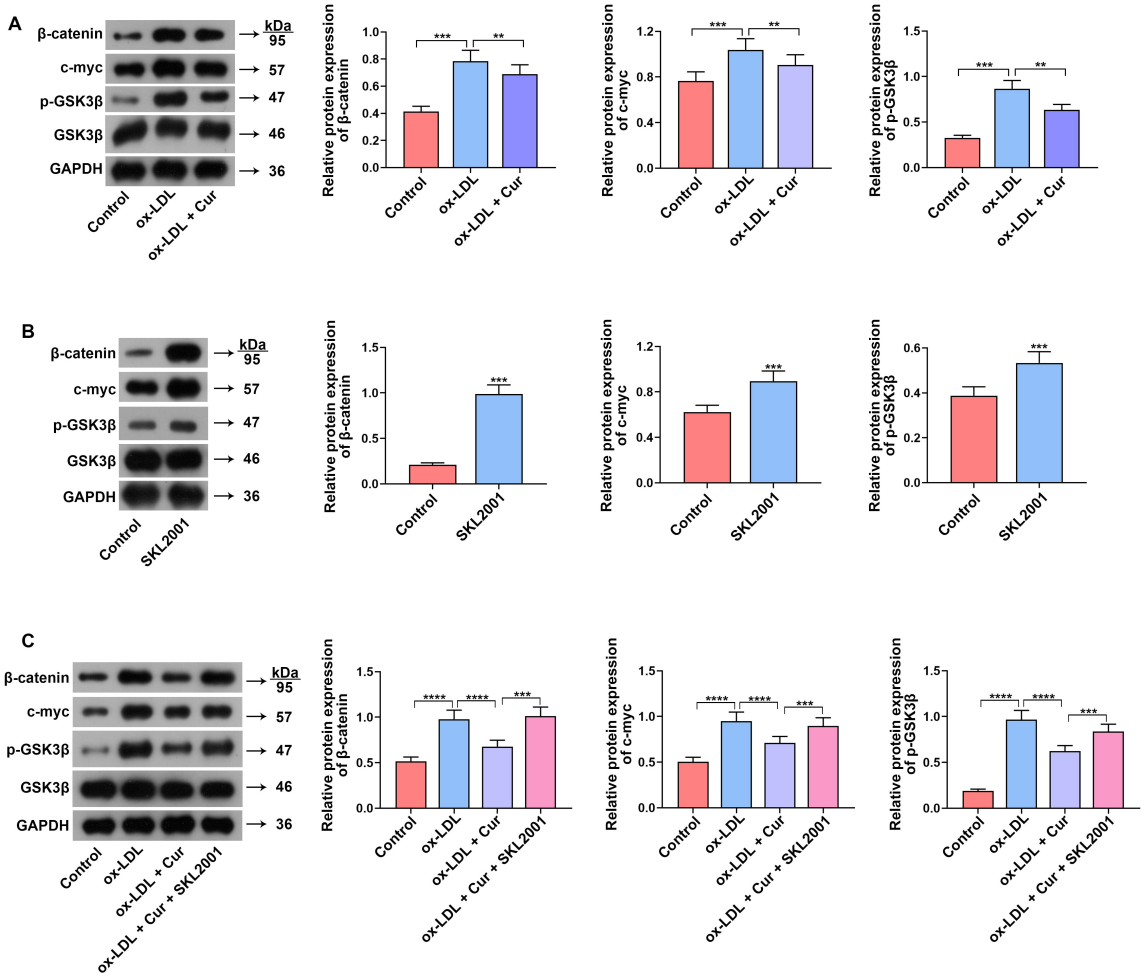

Fig. 3. Curcumin inhibited the Wnt/β-catenin signaling in ox-LDL-stimulated HUVECs. (A,B) The expression levels of β-catenin, p-GSK3β, and c-myc in ox-LDL+curcumin-treated HUVECs (A) or SKL2001-treated HUVECs (B) were determined using western blot analysis (n = 3). (C) The expression levels of β-catenin, p-GSK3β and c-myc in HUVECs pre-treated with SKL2001 for 30 minutes before ox-LDL and curcumin treatment were assessed utilizing western blot analysis (n = 3). Note: GSK3β, glycogen synthase kinase 3β. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Curcumin Ameliorated Ox-LDL-Induced Cell Damage in HUVECs

We observed that ox-LDL-induced inhibition in the viability of HUVECs was rescued following curcumin treatment (10, 20, and 30 μM) ($p < 0.05$, Fig. 2A). Subsequently, 20 μM of curcumin was used in further experiments. EdU assay showed that ox-LDL significantly reduced cell proliferation ($p < 0.05$), which was promoted by curcumin treatment ($p < 0.05$, Fig. 2B). Moreover, the decrease in PCNA protein expression caused by ox-LDL was reversed after curcumin treatment ($p < 0.05$, Fig. 2C). Ox-LDL treatment led to decreased cell migration ($p < 0.05$), while curcumin significantly increased the migration rate ($p < 0.05$, Fig. 2D). Additionally, curcumin treatment substantially elevated the angiogenesis capacity of ox-LDL-triggered HUVECs ($p < 0.05$, Fig. 2E).

Curcumin Inhibited the Wnt/β-Catenin Signaling in Ox-LDL-Stimulated HUVECs

Accumulating evidence indicates the crucial role of the Wnt/β-catenin signaling pathway in atherosclerosis, such as its role in endothelial dysfunction [28]. Therefore, we assessed changes in the Wnt/β-catenin signaling pathway in ox-LDL-stimulated HUVECs. We observed a significant increase in the levels of β-catenin, c-myc, and p-GSK3β following ox-LDL treatment ($p < 0.05$), which were suppressed by curcumin in HUVECs ($p < 0.05$, Fig. 3A). Furthermore, HUVECs were treated with SKL2001, an agonist of Wnt/β-catenin signaling, which resulted in a substantial elevation in the expression levels of β-catenin, c-myc, and p-GSK3β protein ($p < 0.05$, Fig. 3B). Moreover, the inhibitory impacts of curcumin on β-catenin, c-myc, and p-GSK3β levels were blocked by SKL2001 in ox-LDL-triggered HUVECs ($p < 0.05$, Fig. 3C).

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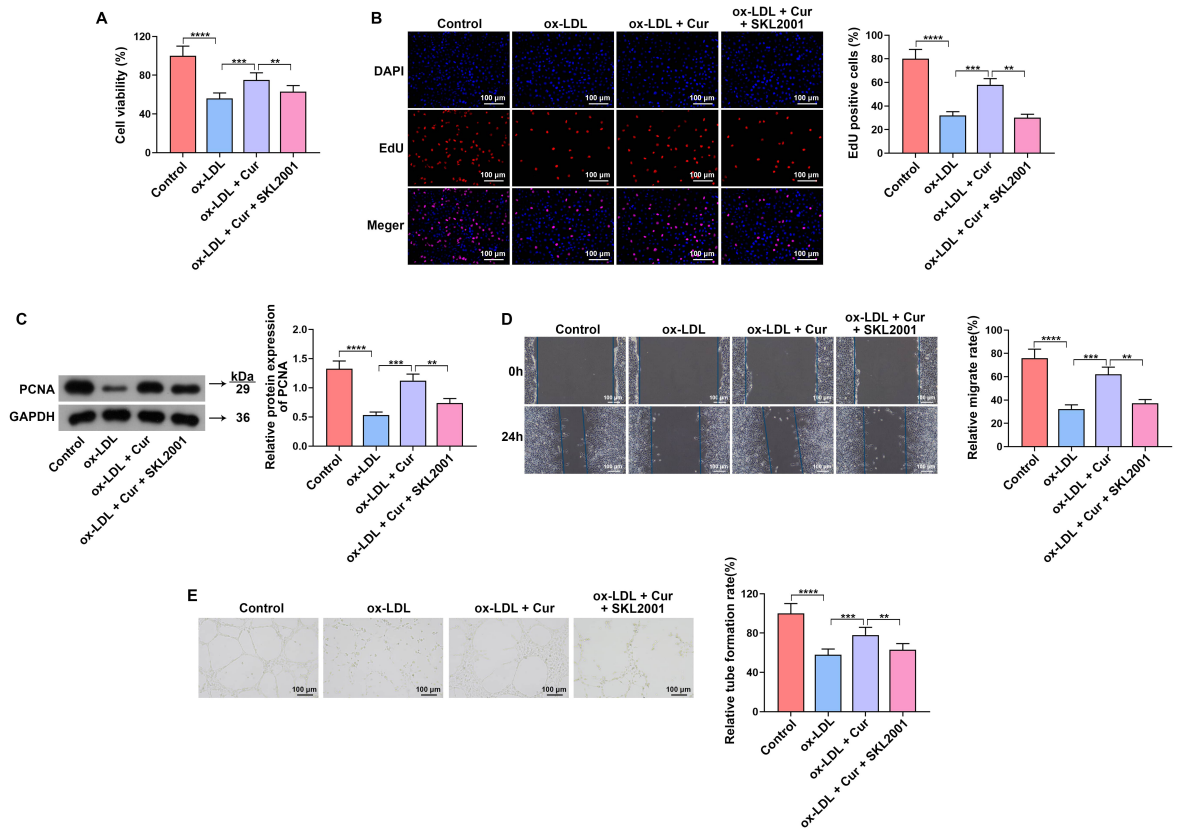

Fig. 4. Curcumin alleviated ox-LDL-induced HUVECs injury by inhibiting the Wnt/β-catenin pathway. HUVECs were treated with SKL2001 for 30 minutes before ox-LDL and curcumin treatment. (A,B) Cell proliferation of HUVECs was evaluated through CCK-8 and EdU assays (Scale bar: 100 μm; n = 3). (C) The PCNA protein levels in HUVECs were assessed using western blot analysis (n = 3). (D) Wound healing assay was used to determine the number of migratory HUVECs (Scale bar: 100 μm; n = 3). (E) Tube formation assay was utilized to evaluate the angiogenesis capacity of HUVECs (Scale bar: 100 μm; n = 3). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Curcumin Alleviated Ox-LDL-Induced HUVECs Injury by Inactivating the Wnt/β-Catenin Signaling Pathway

The promoting effects of curcumin treatment on cell proliferation (Fig. 4A–C) were reversed by SKL2001 in ox-LDL-stimulated HUVECs ($p < 0.05$). Similarly, curcumin significantly elevated the number of migrated cells ($p < 0.05$), but this effect was hampered by SKL2001 treatment in ox-LDL-treated HUVECs ($p < 0.05$, Fig. 4D). Furthermore, curcumin treatment significantly promoted the angiogenesis capacity of HUVECs ($p < 0.05$), while inactivation of the Wnt/β-catenin pathway using SKL2001 hindered this effect in ox-LDL-triggered HUVECs ($p < 0.05$, Fig. 4E). These findings demonstrated that curcumin could inhibit the Wnt/β-catenin signaling pathway, thereby elevating the growth, angiogenesis, and metastasis of HUVECs under ox-LDL treatment, suggesting that suppression of the Wnt/β-catenin signaling is involved in the curcumin-mediated protective effects against ox-LDL-induced damage in HUVECs.

Discussion

Despite considerable improvements in therapeutic methods of atherosclerosis, it continues to pose a significant threat to the health of patients due to its high mortality and morbidity [29]. Therefore, identifying new effective approaches to mitigate endothelial cell dysfunction and damage is useful for preventing and treating atherosclerosis. In the current study, we successfully established an atherosclerosis cell model, as evidenced by inhibition of angiogenesis capacity, metastasis potential, and proliferation abilities, along with decreased PCNA protein expression caused by ox-LDL in HUVECs. Moreover, we found that curcumin significantly alleviated cell damage by promoting proliferation, migration, and angiogenesis capacity. Additionally, the Wnt/β-catenin pathway was elevated following ox-LDL stimulation, while curcumin could decrease the levels of p-GSK3β, β-catenin and c-myc in ox-LDL-triggered HUVECs. These findings indicate the promoting effects of curcumin on atherosclerosis development.

Curcumin possesses many properties, including anti-inflammatory, lipid-lowering, anti-infective, and anti-

oxidative attributes [30–32]. Growing evidence indicates that curcumin can modulate the expression of different signaling molecules to impede atherosclerosis development. For example, curcumin has been found to repress NF-κB levels, thereby regulating inflammatory responses within atherosclerotic plaques in domestic rabbit aortic walls and alleviating the severity of atherosclerosis [33]. Similarly, our findings show the therapeutic effects of curcumin on atherosclerosis by alleviating cellular damage through enhancing cell migration, proliferation, and angiogenesis of ox-LDL-triggered HUVECs.

Furthermore, SKL2001, an agonist of the Wnt/β-catenin pathway, was used to treat ox-LDL-triggered HUVECs to investigate the underlying mechanisms of curcumin in improving atherosclerosis. The highly conserved classical Wnt/β-catenin pathway has been confirmed to participate in multiple physiological processes [34,35]. Recently, several reports have demonstrated the close association between Wnt/β-catenin signaling, endothelial cell dysregulation, and atherosclerosis progression [36–39]. For instance, the upregulated Wnt-5a protein expression has been observed during the development of atherosclerosis, indicating a positive correlation with the severity of atherosclerosis in both human endarterectomy samples and animal experiments [40,41]. Moreover, the Wnt/β-catenin pathway, regulated by pigment epithelium-derived factor, has been involved in endothelial injury and oxidative stress [42]. We found that curcumin significantly repressed the Wnt/β-catenin signaling, and this effect was reversed by SKL2001. Furthermore, SKL2001 also reduced the inhibitory effects of curcumin on cell growth, angiogenesis, and metastasis in ox-LDL-triggered HUVECs, proving that curcumin inactivates Wnt/β-catenin to alleviate ox-LDL-triggered HUVECs injury.

However, the current study has some limitations. This research primarily focused on investigating the effects of curcumin at the cellular level in HUVECs, and therefore, results from the *in vivo* atherosclerosis animal model would enhance the reliability of these findings in ox-LDL-triggered HUVECs. Moreover, we intend to evaluate the bioavailability and metabolic stability of curcumin using an *in vivo* atherosclerosis animal model in future studies, which may provide insight into the therapeutic potential and safety profile of curcumin as a treatment for atherosclerosis patients.

Conclusion

In summary, our findings demonstrate that curcumin enhances cell growth, angiogenesis, and metastasis in ox-LDL-triggered HUVECs, thereby contributing to the inhibition of atherosclerosis progression through the downregulation of Wnt/β-catenin signaling. These findings provide novel insights into the therapeutic potential of curcumin in the treatment of atherosclerosis.

Availability of Data and Materials

The dataset analyzed during the current study are available upon request by contact with the corresponding author.

Author Contributions

JW, JML, ZHL, XKD and LW designed the research study. JML performed the research. ZHL and XKD made data collection. JW analyzed the data and wrote the manuscript. LW helped perform the analysis with constructive discussions. 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

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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