Original Research Article

Corilagin Alleviates Ang II-Induced Cardiac Fibrosis by Regulating the PTEN/AKT/mTOR Pathway

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Abstract

This research aimed to evaluate the therapeutic effect of corilagin (Cor) against angiotensin II (Ang II)-induced cardiac fibrosis and its underlying mechanisms. C57BL/6 mice (male, 8-10 weeks) received saline or Ang II (2.0 mg/kg/day) via subcutaneous infusion and intraperitoneal injection of Cor (30 mg/kg) for 28 days. Ang II induction increased the fibrotic area, whereas Cor treatment inhibited the fibrotic area significantly. Cor markedly reduced the Ang II-induced cardiac fibroblasts. Cor significantly inhibited Ang II-induced increase in expressions of smooth muscle alpha-actin (α-SMA), collagen I, collagen III, transforming growth factor beta 1 (TGF-β1), fibronectin, and connective tissue growth factor (CTGF). Cor suppressed the intracellular reactive oxygen species (ROS) production. Cor therapy reduced Ang II-induced malondialdehyde (MDA) content, whereas superoxide dismutase (SOD) and catalase (CAT) activities were increased (all, $P < .001$). Moreover, Ang II induction elevated the expression of phosphorylated phosphatase and tensin homolog (p-PTEN), phosphorylated protein kinase B (p-AKT) (Ser473) and phosphorylated mammalian target of rapamycin (p-mTOR) (Ser 2448), whereas Cor reduced their expressions. Cor treatment inhibited the migration ability of the cardiac fibroblast, whereas a PTEN inhibitor, VO-ohpic, increased the migration capability. Cor could have a protective effect against Ang II-induced cardiac fibrosis via inhibition of the PTEN/AKT/mTOR pathway.

Keywords

corilagin, angiotensin II, cardiac fibrosis, reactive oxygen species, cardiac fibroblasts

Introduction

Chronic cardiac stress causes an increase in the formation of extracellular matrix components, which leads to cardiac fibrosis. Cardiac fibrosis can stiffen the left ventricle, alter cardiac conduction, and impair systolic and diastolic function.[1-3] The development of fibrosis has been linked to increased cardiovascular and all-cause mortality.[4] Currently, no evidence-based medicines demonstrate considerable efficacy against fibrotic disorders because the causes of cardiac fibrosis remain unknown. In addition, our understanding of the processes of cardiac fibrosis is primarily acquired from cell culture systems or transgenic mice with cardiomyocyte-specific modification.[5]

Pathological stimulation of the renin-angiotensin system (RAS) plays a significant role in developing cardiovascular disorders. The main RAS effector peptide produced in the circulatory system and local cardiac tissue is Ang II. Ang II has a role in initiating and progressing several cardiac impairs, including myocardial infarction, alcoholic cardiomyopathy, and diabetic cardiomyopathy.[6-8] Ang II interacts with its

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receptor, namely angiotensin II receptor type 1 (AT1), activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and producing ROS.[9] Oxidative stress can occur when ROS generation surpasses the body's scavenging capacity. Oxidative stress induced by Ang II can immediately activate the apoptosis signaling pathway, resulting in cardiomyocyte apoptosis or necrosis and, ultimately, ventricular remodeling and heart failure.[10] High production of ROS can also cause apoptosis, myocardial inflammation, ventricular remodeling, and hypertrophy via the epidermal growth factor receptor, the mitogen-activated protein kinase, and nuclear factor-kappa B (NF-kB).[11,12]

The PI3K/Akt/mTOR signaling pathway regulates intracellular autophagy. Activation of this pathway may regulate cell metabolism and growth, suppress the action of several autophagy-related proteins, and decrease autophagy capacity, which is strongly connected to cell growth, energy metabolism, and the incidence and development of several disorders. The PI3K/Akt/mTOR pathway has been associated with chondrocyte autophagy.[13,14] Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumour suppressor gene that suppresses the PI3K/Akt/mTOR growth signaling cascade.[15] PTEN can directly inhibit the PI3K/Akt/mTOR pathway, enhancing autophagy and decreasing apoptosis.[16] PTEN also regulates PI3K/Akt/mTOR in chondrocyte metabolism. However, the effect of regulating the PTEN/AKT/mTOR pathway on Ang II-induced cardiac fibrosis remains unclear.

Corilagin (Cor) is a tannin-family ellagitannin major bioactive component in many medicinal plants.[17] Previous research found Cor has many biological and pharmacological benefits, including hepatoprotective, anti-inflammatory, antibacterial, antioxidant, anti-hypertensive, anti-diabetic, and anti-tumor properties.[18-20] It can induce apoptosis and prevent cell proliferation, reducing the development of numerous cancer cells.[17] Cor has been demonstrated to have hepatoprotective characteristics in treating various liver-related disorders, including hepatocellular carcinoma, drug-induced liver damage, and liver fibrosis.[21] Recent research has shown Cor to attenuate hepatic fibrosis induced by schistosomiasis by regulating the interleukin-13 (IL-13), GATA binding protein 3 (GATA3), and miR21/smad7/extracellular regulated protein kinases (ERK) signaling pathways.[22-25] However, the potential effect of Cor on Ang II-induced cardiac fibrosis via the PTEN/AKT/mTOR pathway remains unknown. Therefore, the current research evaluated Cor's protective impact against Ang II-induced cardiac fibrosis in mice and examined the underlying procedures precisely.

Materials and Methods

Animals and Treatment

C57BL/6 mice (male, 8-10 weeks) were maintained in the SPF laboratory animal room under temperature-controlled conditions (22 ± 2°C; humidity 40%) with a 12 hours light/dark cycle. Mice were divided into 3 different groups, with 8 mice for each group: (1) Control group: mice received the phosphate-buffered saline injection through inserted a mini pump and received a phosphate-buffered saline injection intraperitoneally. (2) Ang II group: mice received the Ang II injection via a mini pump and received a phosphate-buffered saline injection intraperitoneally. (3) Cor group: mice received the Ang II injection via a mini pump and received Cor injection intraperitoneally. Mice were received saline or Ang II (2.0 mg/kg/day, HY-13948, MedChemExpress) injection subcutaneously using Alzet osmotic mini-pumps (Model 2004, USA) for 28 days. The Cor (30 mg/kg/day, HY-N0462, MedChemExpress) or the same volume of saline was injected intraperitoneally once a day for 28 days. The first injection of Cor was 2 hours before the insertion of osmotic mini-pumps. The mouse systolic blood pressure (SBP) was measured by a tail-cuff system (Softron BP98 A; Softron Tokyo, Japan) on day 28. This study was approved (ZPYYLL-2018-02) by the Animal Care and Use Committee of Shanghai Pudong Zhoupu Hospital (2020-10-23) and confirmed by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Echocardiography

Echocardiography was used to assess the heart function of mice using a Small Animal Ultrasound Imaging System (Vevo3100, VisualSonics, Canada). M-mode images were obtained, and parameters were recorded as follows: ejection fraction (EF), fractional shortening (FS), left ventricular end-systolic diameter (LVESd), and left ventricular end-diastolic diameter (LVEDd).

Sample Collection

All mice were weighed on day 28 after the Ang II infusion, and the body weight (BW) was recorded. Mice were anesthesized, and enough volume of whole blood was collected using a 25-27-gauge needle into a tube. Subsequently, the serum samples were separated by refrigerated centrifugation at 1000-2000 x g for 10 minutes. The heart and tibia were obtained, and heart weight (HW) and tibia length (TL) were measured to measure the ratios of HW/BW and HW/TL. Cardiac tissue was taken and separated into two sections; one was snap-frozen in a solution of liquid nitrogen for RT-qPCR and Western blot analysis, and another was fixed in 4% paraformaldehyde for histological investigation.

Biochemical Analysis

Serum samples were collected 28 days after Ang II infusion to evaluate the biochemical markers cardiac troponin I (cTnI) (SEKM-0153, Solarbio, China), cardiac troponin T (cTnT) (SEKM-0150, Solarbio, China), creatine kinase-myoglobin binding (CK-MB) (SEKM-0152, Solarbio, China), lactate dehydrogenase (LDH) (E-BC-K046-M, Elabscience, China),

atrial natriuretic peptide (ANP) (E-EL-M0166c, Elabscience, China), and B-type natriuretic peptide (BNP) (E-EL-M0204c, Elabscience, China) activity by commercial kits.

Histology

Cardiac fibrosis was assessed by staining cardiac tissue with Masson's trichrome. The quantification of fibrosis was calculated by the ratio of fibrotic area to total area (collagen volume fraction).

Isolation of Rat Cardiac Fibroblasts

The neonatal Sprague-Dawley (SD) rats were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneal injection). Then, the heart was isolated, and the ventricles were cut and put in a sterile petri dish. The heart tissues were cut into pieces (1 mm^3 in size) and were filtered through a filter (200-mesh). Cardiac fibroblasts were obtained through trypsin digestion and centrifugation and cultured in DMEM with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. The culture medium was changed by every 3 days. The cardiac fibroblasts were stained with Vimentin and photographed under the fluorescence microscope.

Cell Culture

Cardiac fibroblasts were obtained from the neonatal SD rats and cultured in the Dulbecco-modified eagle medium (DMEM) containing 10% (v/v %) FBS in a humidified atmosphere at 37°C with 5% CO_2. The cells were starved for 24 hours, then stimulated with Ang II (1 μM) (HY-13948, MedChemExpress) for 48 hours and subsequently treated with Cor (50 μM) for 2 hours. For the inhibitor experiment, VO-ohpic (1 μM) (HY-110067, MedChemExpress) was used to inhibit PTEN activity.

Cell Migration Assay

The Transwell test was used to explore the migration abilities of cardiac fibroblast cells. Cells were placed on the upper portion of Transwell chambers (8 μm, 1×10^5 cells/mL) in 100-L DMEM medium without FBS. The bottom container was filled with 600 μL of 10% FBS DMEM medium. After 72 hours of incubation, the cells in the upper container were washed using a cotton swab. .1% crystal violet was utilized to label the moving cells. To calculate the average number of moved cells, the labelled moving cells were measured from 5 fields underneath a light microscope (magnification, ×200).

Cellular Immunofluorescence

The Immunofluorescence method was performed as previously described.[26] Frozen sections of the atrial tissue from mice were obtained. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (C1086, Beyotime), Vimentin (ab92547, Abcam, UK), α-SMA (ab7817, Abcam, UK), and dihydroergotamine (DHE) (S0063, Beyotime) were applied after three phosphate buffered saline washes. The cells were then counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and examined under an inverted microscope (IX51, Olympus, Japan). TUNEL, Vimentin, α-SMA, and DHE were carried out following the manufacturer's recommendations.

Intracellular ROS Level Evaluation

Rat cardiac fibroblasts were treated with dichloro-dihydro-fluorescein diacetate (DCFH-DA) (1:1000) for 30 minutes in the dark at 37°C. The samples were then washed three times with phosphate buffered saline. Intracellular ROS levels were determined using a microplate reader (IX71, Olympus, Japan) at 485 nm (excitation) and 535 nm (emission).

Measurement of Oxidative Stress Indicators

To obtain cell lysate, the cardiac fibroblasts were homogenized. The cell lysate was then stored at −80°C in the refrigerator. A commercial kit was used to measure the MDA (S0131S, Beyotime, Shanghai, China), SOD (S0109, Beyotime), and CAT (S0051, Beyotime) activities, and a previously published protocol was followed to estimate the amount of oxidative stress.[27]

Reverse Transcription-Quantitative PCR (RT-qPCR)

TRizol (Invitrogen, USA) was used to isolate total RNA from cardiac fibroblasts. Reverse transcription was used to create complementary DNA (cDNA) from total RNA. To amplify the mRNA by Real-Time Quantitative PCR (RT-qPCR), SYBR Green reagent (TaKaRa, Japan) was used in an ABI Prism 7700 Real-Time PCR apparatus (Applied Biosystems, USA). Using the 2-ΔΔCt formula, the relative gene expression was normalised to the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[28] The primers for Mouse α-SMA, Mouse Collagen I, Mouse Collagen III, Rat α-SMA, Rat Collagen I, Rat Collagen III, Rat TGF-β1, Rat Fibronectin, Rat CTGF, Mouse GAPDH, and Rat GAPDH were designed by the NCBI Primer-Blast Tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), which is listed in Table 1.

Western Blotting

The protein samples were collected after lysing the cells using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Protein concentration was determined using a BCA kit (Beyotime). An equivalent volume of protein (40 μg for each well) was mixed with loading buffer (Beyotime) and then denatured in a boiling-water bath for 3 minutes. Once bromphenol blue reached the separation gel, electrophoresis was started for 30 minutes at 80 V and subsequently for 1∼2 hours at 120 V. The proteins were subsequently placed onto membranes in an ice bath at 300 mA for 60 minutes. After rinsing the membranes for 1∼2 minutes with

Table 1. Primer Sequences Used in the Current Study.

Genes	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
Mouse α-SMA	TCCTGACGCTGAAGTATCCGATA	GGCCACACGAAGCTCGTTAT
Mouse collagen I	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Mouse collagen III	TCCCCTGGAATCTGTGAATC	TGAGTCGAATTGGGGAGAAT
Rat α-SMA	CTATTCCTTCGTGACTACT	ATGCTGTTATAGGTGGTT
Rat collagen I	GCCTCAGCCACCTCAAGAGA	GGCTGCGGATGTTCTCAATC
Rat collagen III	CCAGGACAAAGAGGGGAACC	CCATTTCACCTTTCCCACCA
Rat TGF-β1	AACAATTCCTGGCGTTACCT	GCCCTGTATTCCGTCTCCTT
Rat fibronectin	GGATCCCCTCCCAGAGAAGT	GGGTGTGGAAGGGTAACCAG
Rat CTGF	GTGTGCACTGCCAAAGATG	TCGGTAGGCAGCTAGGGC
Rat PTEN	CCAATGGCTAAGTGAAGACGACAA	CATAGCGCCTCTGACTGGGAATA
Rat Akt	ATGGACTTCCGGTCAGGTTCA	GCCCTTGCCCAGTAGCTTCA
Rat mTOR	GCTTATCAAGCAAGCGACATCTCA	TCCACTGGAAGCACAGACCAAG
Mouse GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA
Rat GAPDH	CCCCCAATGTATCCGTTGTG	TAGCCCAGGATGCCCTTTAGT

Abbreviation: α-SMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

washing solution, they were inactivated for 1 hour at room temperature or sealed overnight at 4°C. The membranes received treatment with the primary antibodies against PTEN (1:500, ab267787, rabbit monoclonal, Abcam), p-Akt (Ser473) (1:400, ab81283, rabbit monoclonal, Abcam), t-Akt (1:400, ab8805, rabbit polyclonal, Abcam), p-mTOR (Ser2448) (1:500, sc-293133, mouse monoclonal, Santa Cruz), mTOR (1:500, sc-517464, mouse monoclonal, Santa Cruz), and GAPDH (1:2000, ab9485, rabbit polyclonal, Abcam) on a shaking table for 1 hour at room temperature. The membranes were washed with washing solution three times in 10 minutes before and after 1 hour of incubation with the secondary antibody at room temperature. After adding membranes to the developing liquid, chemiluminescence imaging analysis equipment (Gel Doc XR, Bio-rad) was used for observation.

Statistical Analysis

The mean ± standard deviation (SD) of at least three distinct experiments was used to express all data. Statistical analyses have been conducted using SPSS 20.0 (SPSS, Chicago, IL, USA) or Graph Pad Prism 9.0 software or both. The differences between several groups were examined using one-way ANOVA and the post hoc Tukey test. AF incidence between various groups was analyzed using Fisher's exact test. Statistical significance has been deemed as a P value of $<.05$.

Results

Effects of Cor on Heart Enlargement and Cardiac Dysfunction in Ang II-Infused Mice

Cor (30 mg/kg/day) was administered intraperitoneally into mice two hours before intravenous Ang II (2.0 mg/kg/day) infusion for 28 days to explore the possible effect of Cor in Ang II-induced heart size and cardiac dysfunsction in mice. Cor did not affect mice SBP at 0, 7, 14, 21, or 28 days following the stimulation of SBP by Ang II infusion (Figure 1A). Cor treatment did not significantly attenuate the HW/BW and HW/TL ratios, compared to phosphate buffered saline-treated mice (Figure 1B and C). Echocardiography on day 28 showed that Ang II infusion increased left atrial diameter (LAD) in mice, which was attenuated after Cor treatment (Figure 1D). Cor treatment raised the rate of ejection fraction (EF) and fractional shortening (FS) (Figure 1E and F). Moreover, Cor also attenuated an Ang II-induced increase in left ventricular end-systolic diameter (LVESd) and left ventricular end-diastolic diameter (LVEDd) (Figure 1G and H).

Cor Inhibits Ang II-Induced Cardiac Fibrosis and Regulates Related Molecules in Mice

The effects of Cor on Ang II-induced cardiac fibrosis and regulation-related molecules in mice were determined. Cardiac fibrosis was evaluated by Masson trichrome staining ventricular muscle tissue and the results showed that Ang II injection increased the fibrotic area, whereas Cor therapy significantly inhibited the fibrotic area (Figure 2A and B). The serum levels of creatine kinase isoenzyme (CK)-MB, lactate dehydrogenase (LDH), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), cardiac troponin I (cTnI), and cardiac troponin T (cTnT) were investigated. The results showed that Cor significantly decreased the Ang II-induced increased serum levels of CK-MB, LDH, ANP, BNP, cTnI and cTnT (Figure 2C–E). The mRNA expression of α-SMA, Collagen I (COL1A1), and Collagen III (COL3A1) was assessed by RT-qPCR. We observed that the Ang II injection stimulated the levels of

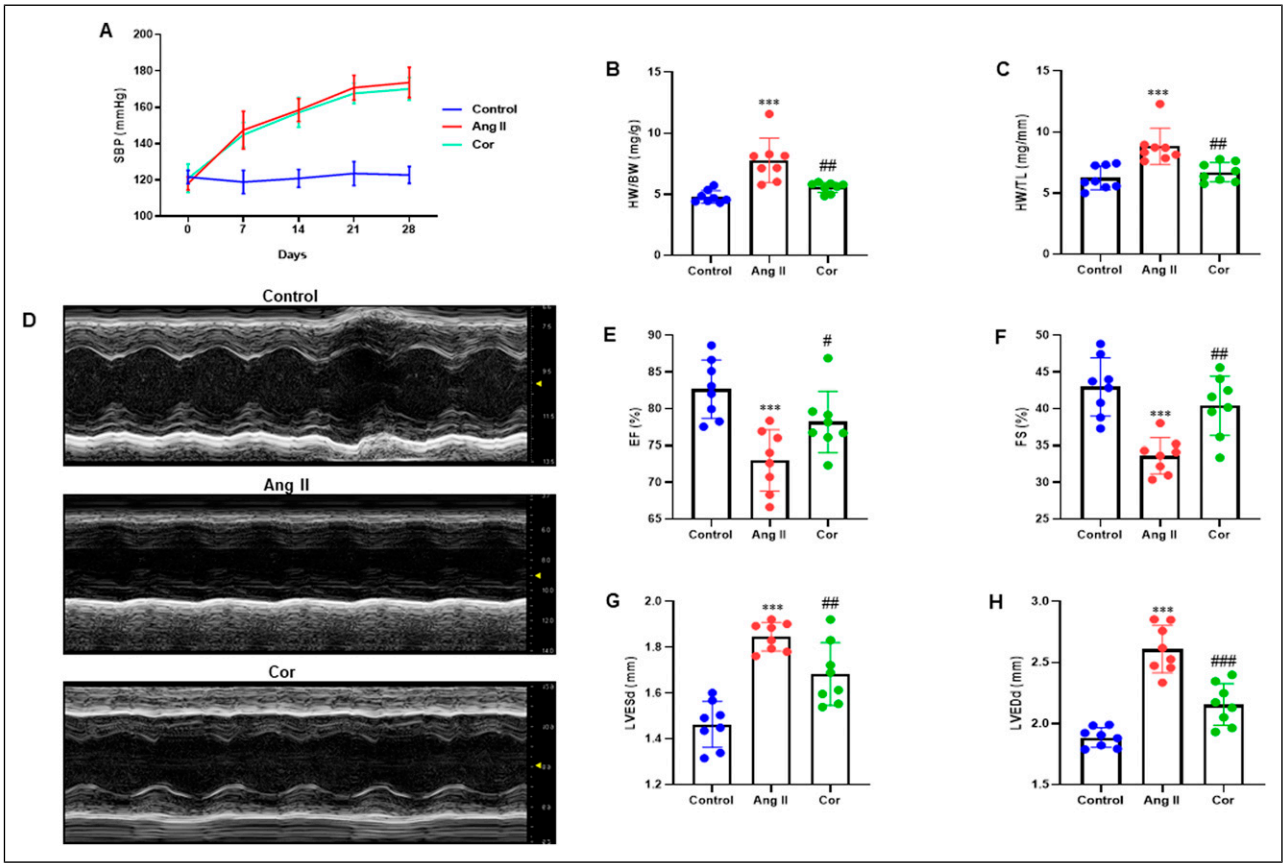

Figure 1. Effects of Cor on heart size and cardiac dysfunction of Ang II-infused mice. Cor was injected intraperitoneally (30 mg/kg/day) in mice 2 hours before subcutaneous infusion of Ang II (2.0 mg/kg/day) for 28 days. (A) Cor did not change the blood pressure of mice at 0, 7, 14, 21, and 28 days after the start of Ang II infusion. (B, C) The HW/BW and HW/TL ratios were used to evaluate the heart size of mice. (D) Representative images of M-mode echocardiography. Echocardiographic parameters are as follows: (E) EF, (F) FS, (G) LVESd, and (H) lLVEDd. Data are presented as mean ± SD (n = 8 in each group). One-way ANOVA with Tuckey's test was used for analysis among three groups. Ang II, angiotensin II; Cor, corilagin; SBP, systolic blood pressure HW, heart weight; BW, body weight; TL, tibia length EF, ejection fraction; FS, fractional shortening; LVESd, left ventricular end-systolic diameter; LVEDd, Left ventricular end-diastolic diameter. ***$P < .001$ vs Control group; #$P < .05$, ##$P < .01$, ###$P < .001$ vs Ang II group.

α-SMA, Collagen I, and Collagen III, whereas Cor therapy markedly inhibited (Figure 2F).

Cor Attenuates Ang II-Induced Migration of Rat Cardiac Fibroblasts

Primary rat cardiac fibroblasts were isolated from neonate SD rats and identified by Vimentin antibody (Figure 3A). Cardiac fibroblasts were stimulated with Ang II (1 μM) infusion and incubated for 48 h and then Cor (50 μM) treated for 2 hours. The results demonstrated that Cor therapy potentially attenuated cardiac fibroblasts (Figure 3B). Transwell assay assessed cell migration. Quantifying cell migration by calculating the number of migrated cells per field from six random fields, we observed that Cor significantly inhibited Ang II-induced increased migrated cells (Figure 3C). We performed RT-qPCR analysis to determine the mRNA expression of α-SMA, Collagen I, Collagen III, TGF-β1, Fibronectin, and CTGF. The results showed that Ang II injection increased the α-SMA, Collagen I, Collagen III, TGF-β1, Fibronectin, and CTGF expressions, whereas Cor therapy significantly inhibited their expressions (Figure 3D and E).

Cor suppresses intracellular ROS generation and oxidative stress in Ang II-induced cardiac fibroblasts

Cells were stained with DHE to assess the intracellular ROS level. Ang II infusion increased cardiac superoxide formation (DHE staining), which was attenuated by Cor treatment (Figure 4A and B). The indicators of oxidative stress, including MDA, SOD, and CAT, were evaluated in the lysate of cardiac fibroblasts. Ang II injection raised levels of MDA content and decreased SOD and CAT activities, whereas these changes were altered by Cor therapy (Figure 4C–E).

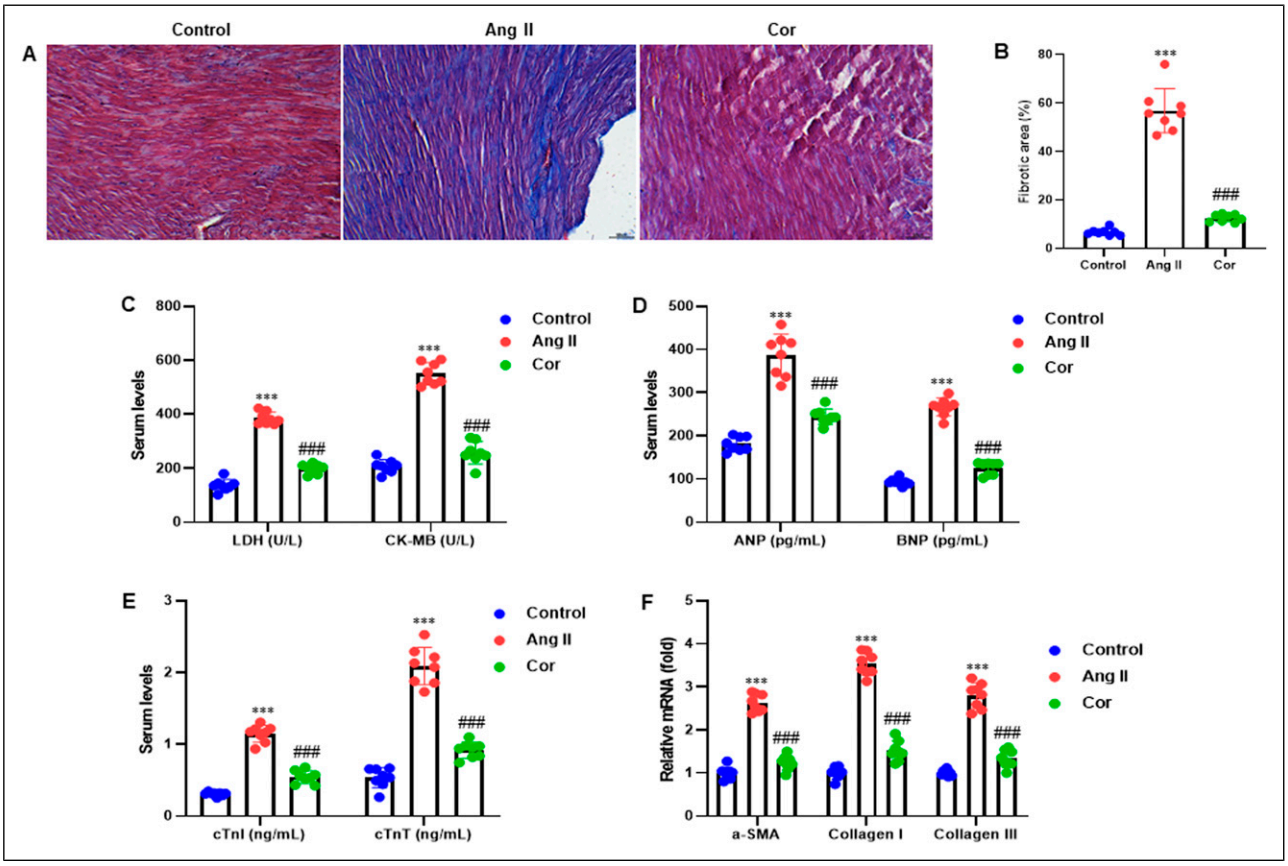

Figure 2. Cor suppresses cardiac fibrosis and regulates related molecules in Ang II-infused mice. (A) Cardiac fibrosis was evaluated by staining ventricular muscle tissue with Masson trichrome, and the representative images are shown (Magnification ×200). (B) Cardiac fibrosis was quantified by calculating the percentage of the fibrotic area (blue area). Colorimetry and ELISA measured (C) Serum levels of LDH and CK-MB, respectively. ELISA was performed to measure mice's serum levels of (D) ANP, BNP, and (E) cTnI, cTnT. (E) The mRNA expression of α-SMA, Collagen I (COL1A1), and Collagen III (COL3A1) was assessed by RT-qPCR. Data are presented as mean ± SD (n = 8 in each group). LDH, lactate dehydrogenase; CK-MB, creatine kinase isoenzyme MB; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; cTnI, cardiac troponin I; cTnT, cardiac troponin T; α-SMA, α-smooth muscle actin. ***$P < .001$ vs control group; ###$P < .001$ vs Ang II group.

Cor Inhibits Ang II-Induced Cardiac Fibroblast Migration via Modulating the PTEN-AKT-mTOR Pathway

We carried out the western blot analysis to investigate the potential effect of Cor on Ang II-induced cardiac fibroblast migration and the involvement of the PTEN-AKT-mTOR pathway. The Ang II injection enhanced the expression of p-PTEN, p-Akt (Ser473) and p-mTOR (Ser 2448), whereas Cor therapy significantly reduced their expressions (Figure 5A and B and Supplemental Figure S1). We performed qPCR to measure the mRNA expression of PTEN, AKT, and mTOR in Ang II-induced cardiac fibroblasts, and the results were consistent with western blot data (Figure 5C). To confirm the involvement of the PTEN-AKT-mTOR pathway, we used a PTEN inhibitor VO-ohpic in Ang II-induced cardiac fibroblasts. As shown in Figure 5D–E, Ang II increased the migration ability of the cardiac fibroblast. Cor treatment significantly inhibited the migration ability, whereas a PTEN inhibitor VO-ohpic significantly increased the migration ability.

Discussion

The current study explored the possible impact of Cor in Ang II-induced cardiac fibrosis. Echocardiography on day 28 showed that Ang II infusion increased LAD in mice, which was attenuated after Cor treatment. Cor treatment raised the rate of EF and FS. Moreover, Cor also attenuated an Ang II-induced increase in LVESd and LVEDd. Cor treatment potentially reduced Ang II-induced cardiac fibrosis, migration of rat cardiac fibroblasts, and intracellular ROS generation and oxidative stress in Ang II-induced cardiac fibroblasts. In addition, Cor inhibits Ang II-induced cardiac fibroblast migration via modulating the PTEN/AKT/mTOR pathway. Our findings suggest that Cor might be protective against Ang II-induced cardiac fibrosis.

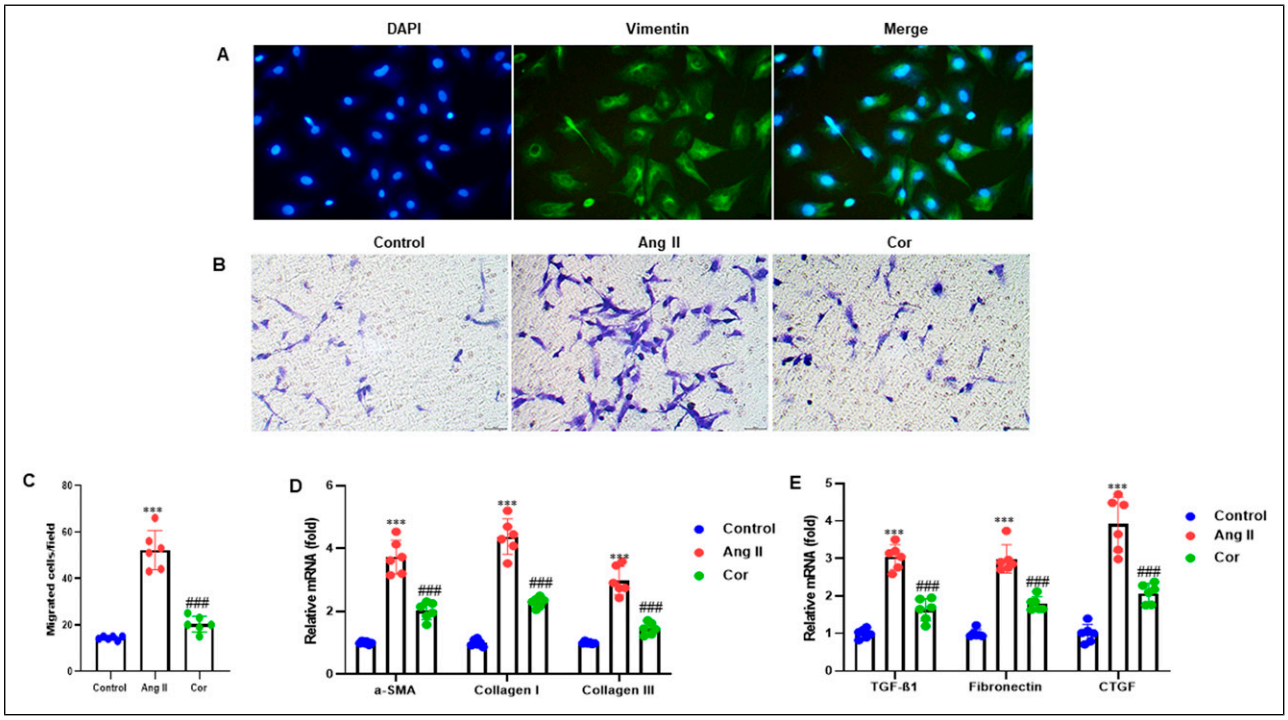

Figure 3. Cor inhibits Ang II-induced migration of rat cardiac fibroblasts. (A) Primary rat cardiac fibroblasts were isolated from neonate SD rats and, identified by Vimentin antibody and photographed under immunofluorescence (400×). (B) Cardiac fibroblasts were stimulated with Cor (50 μM) for 2 hours before being incubated with Ang II (1 μM) for 48 hours. Cell migration was assessed by Transwell assay. (C) Quantification of cell migration by calculating the number of migrated cells per field from 6 random fields. The mRNA expression of (D) α-SMA, Collagen I, Collagen III, (E) TGF-β1, Fibronectin, and CTGF was determined by RT-qPCR. GADPH served as an internal control. Data are shown as mean ± SD (n = 6 per group) from three independent experiments. TGF-β1, transforming growth factor-β1; CTGF, connective tissue growth factor. ***$P < .001$ vs the control group; ###$P < .001$ vs Ang II group.

Atrial fibrillation (AF) is a severe arrhythmia that can lead to death. Multiple factors promote the initiation and progression of AF disorders.[29] Atrial fibrosis is the most common cause of AF patients' structural remodeling and the underlying AF persistence. AF is related to remodeling of the left atrium (LA), including structural, electrical, and autonomic remodeling.[30,31] Atrial fibrosis in the LA promotes AF's development, progression, and maintenance.[32] Cardiac fibrosis is a common feature in both experimental and human AF.[33] In this research, we studied the effect of Cor on Ang II-induced cardiac fibrosis and related molecules in mice. The results showed that the Cor treatment reduced Ang II-induced cardiac fibrosis and regulated its related molecules, such as α-SMA, Collagen I, and Collagen III, which is consistent with the previous report.[34]

Ang II is the major regulator of cardiac oxidative stress, improving the production of ROS in the cardiovascular system by activating membrane-bound NADPH oxidase (NOX), endoplasmic reticulum stress, and mitochondrial oxidative stress.[32] ROS have the ability to cause myocardial fibrosis, which promotes the development and progression of AF. This leads to a profound positive reinforcement cycle, leading to atrial fibrillation and subsequently worsened fibrosis.[35] Earlier studies revealed inhibiting the proarrhythmic response of Ang II in AF by targeting ox-CaMKII loss in oxidation-resistant CaMKII MMVV mice.[36] The researchers suggested that CaMKII serves as an early signal for ROS, which could be activated by NOX.[37] In a previous study, it was found that administering Ang II increased the production of ROS in the atrial tissue. However, when Ang II and Cor were given together, the elevation of ROS was reduced.[34] This finding is consistent with our present study, in which we observed that Cor treatment inhibited the generation of intracellular ROS and oxidative stress in cardiac fibroblasts induced by Ang II.

Cardiac fibrosis is often considered the most significant structural remodeling in heart failure, myocardial infarction, and atrial fibrillation.[38,39] The degenerative process of cardiac fibrosis is primarily caused by excessive deposition of ECM proteins such as collagen and fibronectin. According to early research, ECM contributes to around 25% of the mass in the murine heart.[40] Many different kinds of cells, mainly fibroblasts, produce and maintain ECM. Cardiovascular fibroblasts can be triggered in response to several cardiac stresses or injuries, such as inflammatory cytokines, growth hormones, and physical strain, and eventually convert to myofibroblasts.[41] Myofibroblasts exhibited phenotypic characteristics such as enhanced ECM synthesis, expression of α-SMA, and the capacity to contract. Ang II promotes cardiac fibrosis by

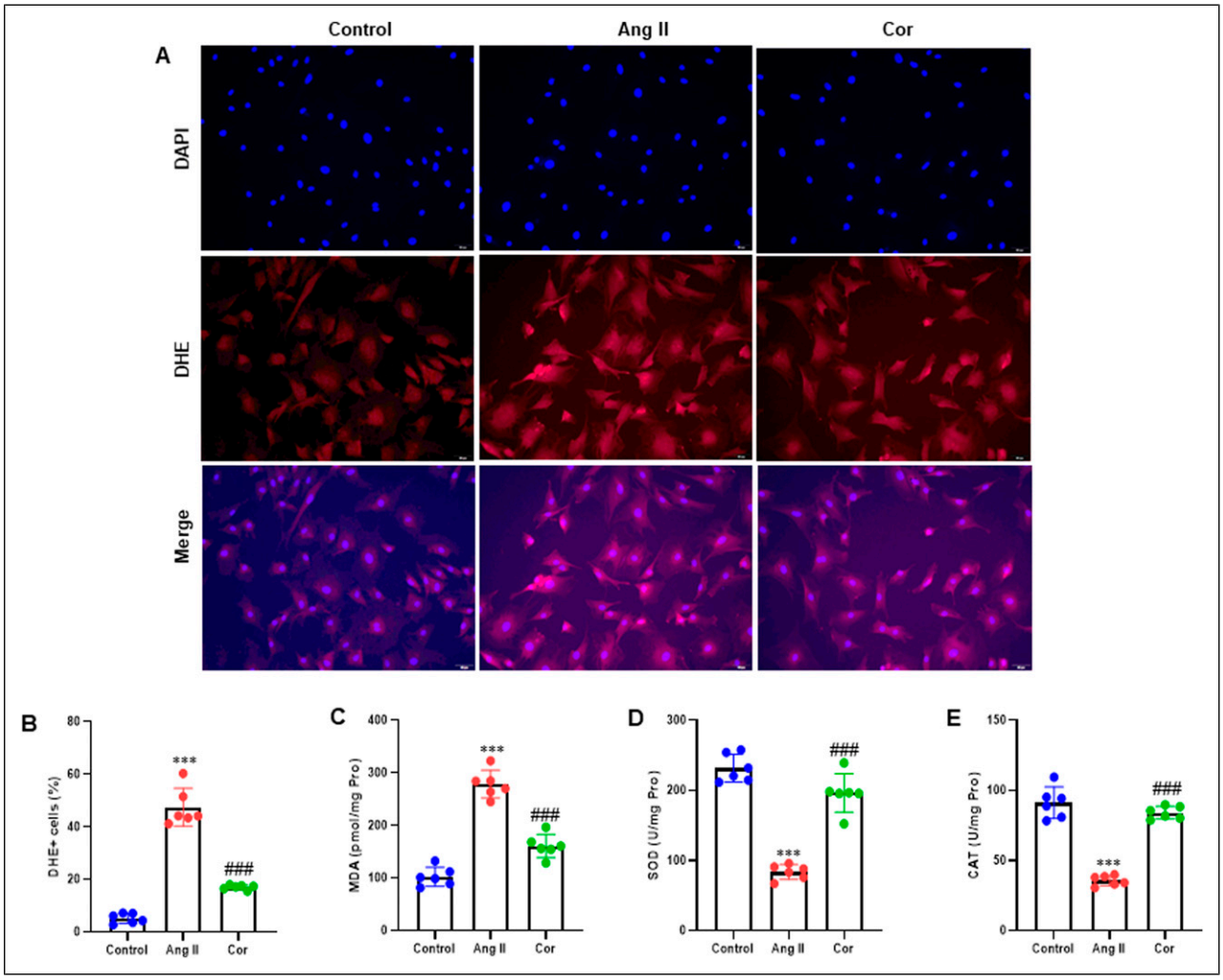

Figure 4. Cor attenuates intracellular ROS generation and oxidative stress in Ang II-induced cardiac fibroblasts. (A) Cells were stained with DHE to assess the intracellular ROS level, and the representative image was shown (200×). (B) Quantification of intracellular ROS by calculating the percentage of DHE-positive cells. The cell lysate of cardiac fibroblasts was collected to determine oxidative stress indicators: (C) MDA, (D) SOD and (E) CAT. Data are shown as mean ± SD (n = 6 per group). DHE, dihydroethidium; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase. ***$P < .001$ vs control group; ###$P < .001$ vs Ang II group.

inducing fibroblast accumulation and fibroblast-to-myofibroblast transition. A previous study found that Cor inhibits the migration of rat cardiac fibroblasts induced by Ang II, preventing excessive movement.[39] However, our present study results demonstrated that the Ang II induction increased the α-SMA, Collagen I, Collagen III, TGF-β1, Fibronectin, and CTGF expressions, whereas Cor therapy inhibited their expressions significantly.

The PI3K/Akt/mTOR signaling pathway plays a significant role in regulating intracellular autophagy, which affects cell metabolism, growth, and energy consumption, and is associated with several impairments. It has been found to be related to chondrocyte autophagy.[13,14] PTEN, a tumor suppressor gene, inhibits the PI3K/Akt/mTOR growth signaling cascade, leading to increased autophagy and reduced apoptosis.[15,16] A previously published study revealed that the PTEN/AKT/mTOR pathway plays a crucial role in the anti-cancer activity of epigallocatechin-3-gallate (EGCG) against ovarian cancer. The study also found that the PTEN inhibitor VO-Ohpic was able to reverse the effects of EGCG on inhibiting cell proliferation, inducing apoptosis, and activating the PTEN/AKT/mTOR pathway in ovarian cancer cells.[42] In the present research, we demonstrated that Ang II induction enhances the expression of p-PTEN, p-Akt (Ser473), and p-mTOR (Ser 2448), while Cor therapy significantly reduces their expressions. Additionally, Cor treatment inhibits migration ability, while a PTEN inhibitor VO-ohpic markedly increases migration ability.

Limitations

This current study has several limitations. (1) In the efficacy of the Cor, no dose-dependent impact was studied. In the further

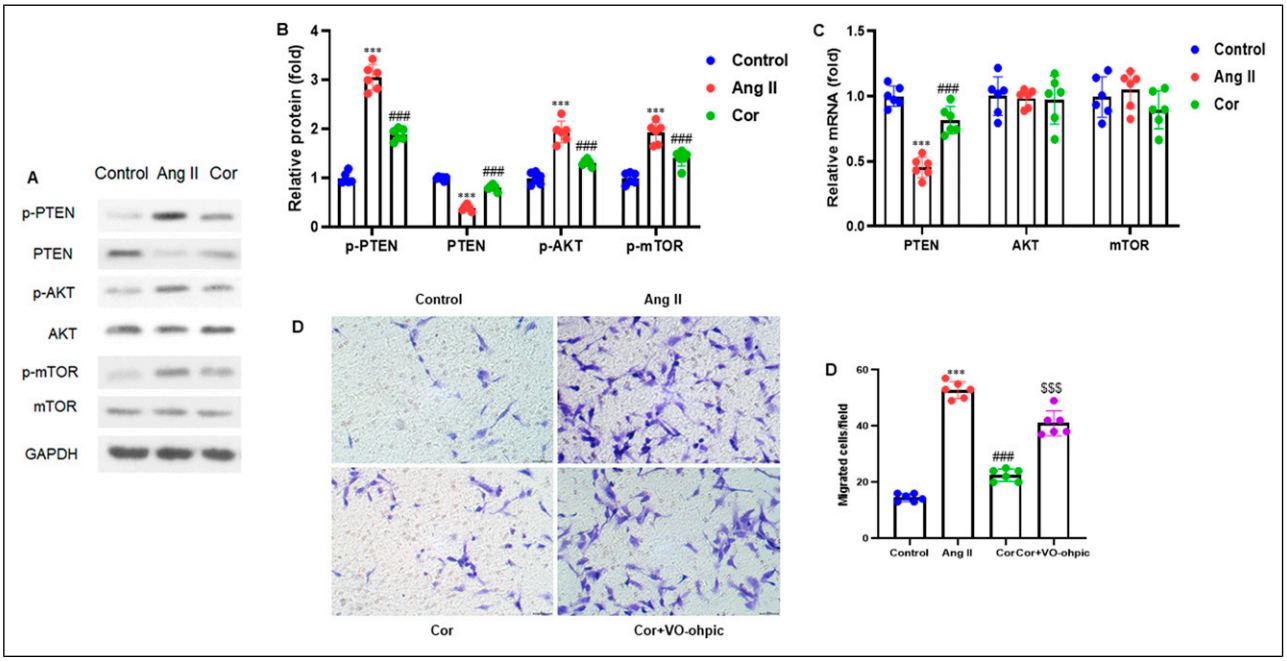

Figure 5. Cor modulates the PTEN-AKT-mTOR pathway for its inhibitory effects on Ang II-induced cardiac fibroblast migration. (A) Representative images of Western blot of p-PTEN (Ser380), PTEN, p-Akt (Ser473) and p-mTOR (Ser 2448) are shown. (B) Quantitative analysis of protein bands that are normalized to GAPDH (p-PTEN and PTEN), Akt (p-Akt) or mTOR (p-mTOR). The cardiac fibroblasts were stimulated with Ang II (1 μM) infusion, incubated for 48 hours, and then treated with Cor (50 μM) for 2 hours. Then, the PTEN inhibitor VO-ohpic (1 μM) was treated for 2 hours. (C) The mRNA expression of PTEN, AKT and mTOR was determined by RT-qPCR. GADPH served as an internal control. (D) The cell migrative capability of cardiac fibroblasts was determined by Transwell assay. (E) Quantification of the average number of migrated fibroblasts in each field. Data are shown as mean ± SD (n = 6 per group). Cor group cells were cotreated with Ang II and Cor; Cor+VO-ohpic group cells were cotreated with Ang II, Cor, and VO-ohpic. ***$P < .001$ vs control group; ###$P < .001$ vs Ang II group; $$$ $P < .001$ vs Cor group.

study, we will analyze the dose-dependent curve of the Cor to assess its impact. (2) Adult male C57BL/6 mice were not included in the in vivo tests to investigate the underlying mechanism of Cor's protective effect against Ang II-induced cardiac fibrosis. In the future, we will use young and adult male C57BL/6 mice to validate our results in the experiment. (3) The study's underlying outcomes have not been confirmed in preclinical or clinical settings. These findings could be confirmed through clinical and preclinical trials. (4) Further research is needed to establish the underlying mechanism of Cor's protective effects against Ang II-induced heart fibrosis. However, our study provides significant aspects of Cor's protective effects against cardiac fibrosis.

Conclusion

In conclusion, this study examined the protective effect of Cor against Ang II-induced cardiac fibrosis in model mice. Our growing evidence shows that the Cor treatment potentially decreased Ang II-induced cardiac fibrosis by inhibiting the PTEN-AKT-mTOR signaling pathway. Therefore, our data demonstrated that Cor could be a potential therapeutic agent for treating cardiac fibrosis.

Author Contributions

ZP designed the project, supervised the project, and revised the manuscript. XZ and BT performed experiments and wrote the first draft of the manuscript. XC helped perform the experiments, collect data, analyzed the data and performed statistical analysis.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Statement

Ethical Approval

This study was approved (ZPYYLL-2018-02) by the Animal Care and Use Committee of Shanghai Pudong Zhoupu Hospital (2020-10-

23) and confirmed by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). All methods carried out in this study were in accordance with ARRIVE guidelines.

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Data Availability Statement

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

Supplemental Material

Supplemental material for this article is available online.

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