Endothelial stem cells attenuate cardiac apoptosis via downregulating cardiac microRNA-146a in a rat model of coronary heart disease

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Abstract. Coronary artery disease (CAD) is one of the main causes of hospitalization worldwide and has high morbidity. It has previously been demonstrated that stem cells serve an important role in improving myocardial function. MicroRNA (miRNA)-146a downregulation has been reported to inhibit vascular smooth muscle cell apoptosis in a rat model of coronary heart disease. The aim of the present study was to investigate the mechanisms underlying the effects of endothelial stem cell (ESC)-derived paracrine factors and cardiac miRNAs in CAD. Acute myocardial infarction was induced in 20 rats. Autologous ESCs (n=10; experimental group) or PBS (n=10; control group) were injected in the border zone. Reverse transcription-quantitative polymerase chain reaction, ELISA and immunohistochemistry assays were performed to analyze the therapeutic effects of ESCs in rats with coronary heart disease rats. Serum interleukin (IL)-1, IL-17 and tumor necrosis factor-α were reduced in the experimental group compared with control rats, as was the number of circulating proatherogenic cells. The results demonstrated that ESC transplantation markedly downregulated miRNA-146a expression and decreased apoptosis in the myocardium compared with the control group. Rats in the experimental group also had higher levels of vascular endothelial growth factor compared with the control group. In addition, it was demonstrated that miRNA-146 knockdown reduced cardiac apoptosis and increased VEGF expression. Furthermore, the infarct area in the border zone or rats with CAD was reduced in the experimental group compared with the control group. In conclusion, these results suggest that ESC transplantation may improve cardiac function via downregulating miR-146a, which may be have potential as a treatment for CAD.

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Introduction

Coronary artery disease (CAD) is typically caused by the development of atherosclerotic lesions and results in myocardial ischemia (1,2). CAD is associated with inflammation and thrombosis, which can cause luminal stenosis or occlusion (3). Prospective trials have indicated that the morbidity and mortality of myocardial infarction is increasing worldwide since 1990 (2,4). A review of the available clinical data has suggested that cardiovascular interventions, including stent implantation and radiofrequency ablation may help to reduce the mortality of myocardial infarction (5). A large number of proteins have been reported to protect the host against myocardial ischemia and reperfusion injury by modulating myocardial apoptosis and inflammation (6,7). Research has demonstrated that early apoptotic myocardial vascular disease may increase the incidence rate of myocardial infarction (8,9).

The potential use of stem cell transplantation to treat human diseases has been widely investigated (10-12). Currently, stem cell transplantation is a therapeutic protocol used to treat CAD, as stem cells are able to migrate to damaged cardiac tissue, repair the myocardial infarction area and ultimately reduce infarct-associated mortality (13). A previous study reported that bone marrow stem cell mobilization is able to decrease left ventricular ejection fraction, stroke volume and cardiac output for patients with coronary heart disease (14). Ghem *et al* (15) demonstrated that endothelial stem/progenitor cells (EPCs), mesenchymal stem/progenitor cells (MSCs), and hematopoietic stem/progenitor cells (HSCs) decreased left ventricular dysfunction, diabetes and intermediate risk in patients with cardiovascular diseases. However, the role and potential mechanism of endothelial stem cells (ESCs) in cardiac cells is not well understood.

In the present study, the therapeutic effects of ESCs in a rat model of CAD were assessed, as well as the underlying mechanism. The effects of ESCs on serum cytokine levels and circulating proatherogenic cells were also investigated.

Materials and methods

Animal study. The present study was approved by the Ethics Committee of Shenzhen Nanshan People's Hospital (Shenzhen, China). A total of 20 female Sprague-Dawley rats (6-8 weeks of age, body weight: 200-220 g) were purchased from the Chinese Academy of Sciences (Shanghai, China). All rats were housed at 23˚C with 50% humidity and a 12 h light/dark cycle. Rats were provided with free access to food and water. Rats were injected with vitamin D3 once every 30 days ($2x10^6$ U/kg) and received a high-fat diet containing 2% cholesterol, 3% lard oil, 0.5% sodium cholate, 0.2% propylthiouracil and 94.3% basic diet supplemented with vitamin D3 ($1.25x10^6$ U/kg) to establish acute myocardial infarction. Rats were then divided into the experimental (n=10) and negative control groups (n=10). Rats were anesthetised with 35 mg/kg pentobarbital intravenously, following which they received an intra-myocardial injection of $1x10^4$ ESCs (Tehran Heart Center, Tehran University of Medical Sciences, Tehran, Iran) in 100 μl PBS (experimental group) or an equal volume of PBS (control group). At 10 days after treatment, rats were sacrificed and heart tissues were harvested. Tissues were either snap-frozen in liquid nitrogen or fixed in 10% formalin solution (Ph 7.4) for 15 min at 37˚C for further analysis. The total experimental period was 42 days.

Cardiac function analysis. Rats were fed with the aforementioned diet for 30 days, injected with PBS or ESCs, left for 10 days and then sacrificed. Rats were fed with the specified diet for 30 days. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using Blood Pressure Monitors (BP786N; Omron, Kyoto, Japan) for experimental rats between ESCs and control groups on day 42. Heart rate of experimental rats was measured using a heart rate detector (Prince 180D; Heal Force Bio-meditech Holdings, Ltd., Shanghai, China).

ELISA. Central venous blood samples (5 ml) were collected from rats in each group on day 42. Following centrifugation at 3,000 x g for 15 min at 4˚C, serum levels of aspartate transaminase (AST; E-EL-M0160), lactate dehydrogenase (LDH; cat. no. TWp001252; Shanghai Jianglai Biological Technology Co., Ltd., Shanghai, China), Troponin T (TnT; cat. no. 0-113685, JiangLai Biology, Shanghai Jianglai Biological Technology Co., Ltd.), TnI (cat. no. YBE-0070Q; Shanghai Jianglai Biological Technology Co., Ltd.) interleukin (IL)-1β (RLB00; R&D Systems, Inc., Minneapolis, MN, USA), IL-17a (M1700; R&D Systems, Inc.) and tumor necrosis factor (TNF)-α (RTA00; R&D Systems, Inc.) were measured using commercial ELISA kits according to the manufacturer's protocol. The results were read using an ELISA microplate reader at 450 nm (Spectra Max 190, Molecular Devices LLC, Sunnyvale, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from heart tissues (1.0 g) using an RNAeasy Mini kit (Qiagen AB, Sollentuna, Sweden). The concentration of RNA samples were measured using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total RNA (1 μg) was reverse-transcribed to cDNA using a Transcriptor First Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.). Micro (mi)RNA expression was assessed using an miRNA microarray analysis with the miRCURY LNA™ miRNA Array (Qiagen AB) according to the manufacturer's protocol. All miRNA primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) Primers used were as follows: miRNA-146a forward, AUGCCAUUGCGAGGGAUUUCG-3'; and reverse, 5'-ATACCTTCAGAGCCTGAGACTCTGCC-3'; RNU6B (U6) forward, 5'-CGCAAGGAUGACACGCAAAUU CGUGAAGCGUUCCAUAUUUUU-3' and reverse, 5'-GAT ATCCCAGCTGAAGAACTGAATTTGAC-3'. RT-qPCR reaction was performed using a TaqMan™ MicroRNA Reverse Transcription kit (cat. no. 4366596; Invitrogen; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 2 min at 95˚C; followed by 40 cycles of 10 sec at 95˚C, annealing for 20 sec at 55˚C and a primer extension for 30 sec at 72˚C and then for 10 min at 72˚C. The microarray data was validated using real-time PCR with TaqMan probes and relative miRNA expression levels were normalized using the RNU6B (U6) small non-coding RNA as control. Expression was calculated using the $2^{-\Delta\Delta Cq}$ method (16).

MiRNA-146a overexpression. Myocardial cells ($1x10^5$ cells/well) from experimental rats were cultured in 6-well plates until they reached 90% confluence, following which the medium was discarded. Cells were subsequently transfected with 10 pmol plentivirus-miRNA-146a (pmiRNA-146a, 5'-UACGCCCUUUUAACAUUGCAU CG-3') or 10 pmol miRNA-146a-mimic control (pControl, 5'-ACGUACUUUUGUGUAGUACC-3') using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in Opti-MEM medium according to the manufacturer's protocol. Cells were used for further analysis following 72 h transfection.

Knockdown of miRNA-146a. Myocardial cells ($1x10^5$ cells/well) were seeded in 6-well plates for 12 h at 37˚C. Cells were washed with PBS three times. Small interfering (si)RNR-miRNA-146a (miRNA-146aKN) and siRNR-mimic (Control) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Sequences were as follows: miRNA-146aKN, 5'-CCUGCUGGAUUGAGCUAC ACCUGAA-3' and siRNR-mimic, 5'-CUCGUCUCAUUG ATGACAGTT-3'. SiRNAs (100 pmol) were transfected into myocardial cells using RNAi MAX (Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol. Further experiments were performed at 72 following transfection.

Viability of myocardial cells. Myocardial cells ($2x10^3$ cells/well) were seeded in 96-well plates and cultured at 37˚C for 12 h. A CCK-8 detection kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to measure cell viability according to the manufacturer's protocol.

Western blotting. Myocardial tissues (5 μg) were homogenized in lysate buffer containing protease-inhibitor (Sigma-Aldrich;

Merck KGaA) and centrifuged at 8,000 x g for 10 min at 4˚C. The protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Protein samples (20 μg) were separated by 15% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. After blocking for 1 h at 37˚C with 10% Bovine Serum Albumin (cat. no. 10735108001; Roche Applied Science, Penzburg, Germany), the membrane was incubated with primary antibodies against the following: Vascular endothelial growth factor (VEGF; 1:1,000; ab32152; Abcam, Cambridge, UK), IL-6 (1:1,000; ab9324; Abcam), intercellular adhesion molecule 1 (ICAM-1; 1:1,000; sc-7891, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), FAS (1:1,000; ab82419; Abcam), TNF-α (1:1,000; ab6671, Abcam) and β-actin (1:2,000; cat. no. ab8226; Abcam) for 12 h at 4˚C. The membrane was next incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2,000; PV-6001, OriGene Technologies, Inc., Rockville, MD, USA) for 24 h at 4˚C. The results were visualized by using a chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.).

Immunological staining. Immunohistochemistry was performed as previously described (17). Cardiac tissues were prepared, fixed in 4% paraformaldehyde for 2 h at 37˚C, embedded in paraffin and cut into 4 μm sections. Epitope retrieval was performed using Tris-HCl buffer (AP-9005-050; Thermo Fisher Scientific, Inc.). The paraffin sections were quenched with hydrogen peroxide (3%) for 15 min at 37˚C, and subsequently blocked with 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 15 min at 37˚C. Cardiac tissue sections were rinsed with PBS and incubated with rabbit anti-rat primary antibodies against VEGF (1:1,000), sFAS (1:1,000) and TNF-α (1:1,000) for 12 h at 4˚C. Sections were washed with PBS for 30 min at room temperature and incubated with Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488; 1:2,000; ab150077; Abcam) for 2 h at 37˚C. The results were visualized by using an enhanced chemiluminescence kit (Pierce; Thermo Fisher Scientific, Inc.). Densitometry was performed using Quantity-One software 1.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

TUNEL analysis. A TUNEL assay was performed to assess the apoptosis of myocardial cells in rats. Cardiac tissue sections were stained using TUNEL with an *In Situ* Cell Death Detection kit (Roche Applied Science) according to the manufacturer's protocol. Images of tissue sections were captured using a ZEISS LSM 510 confocal microscope at 488 nm (ZEISS AB, Oberkochen, Germany). Images were analyzed using ImageJ software 2.0 (National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. All data are expressed as the mean ± standard deviaton of three independent experiments. Data were cmpared using Student's t test with SPSS version 19. (IBM, Corp., Armonk, NY, USA) and $P<0.05$ was considered to indicate a statistically significant difference.

Results

ESCs therapy improves cardiac function in a rat model of CAD. The therapeutic effects of ESCs on cardiac function were investigated in a rat model of CAD. The results revealed that ESCs therapy decreased SBP and DBP in CAD rats compared with the control group (Fig. 1A). ESCs therapy also decreased serum AST, LDH (Fig. 1B), TnT and TnI (Fig. 1C) compared with the control group. The results also demonstrated that ESCs therapy improved rats' heart rate compared with the control group (Fig. 1D). ESCs therapy improved the atherosclerosis indices compared with the control group (Table I). These data suggest that ESCs therapy is able to improve cardiac function in a rat model of CAD.

Table I. Plasma total cholesterol, HDL-cholesterol, LDL-cholesterol and triacylglycerides indices.

	Concentration (mg/dl)		
Plasma component	Baseline	Control group	ESCs group
Total cholesterol	172.25±8.82	163.58±7.54	81.10±5.42[a,b]
HDL-cholesterol	64.66±6.28	62.46±5.12	41.36±3.06[a,b]
LDL-cholesterol	110.24±7.20	112.85±6.85	70.54±6.40[a,b]
Triacylglycerides	42.15±4.32	43.25±5.24	30.22±3.06[a,b]

Values are expressed as the mean ± standard deviation. [a]$P<0.01$ vs. baseline and [b]$P<0.0.1$ vs. control group. ESCs, endothelial stem cells; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

ESCs therapy decreases serum cytokine levels and circulating proatherogenic cells in a rat model of CAD. The anti-inflammatory effects of ESCs were investigated in a rat model of CAD. The results demonstrated that ESCs treatment decreased IL-1, IL-17 and TNFα serum levels compared with the control group (Fig. 2A). Rats treated with ESCs had fewer progenitor cells, including stem cells antigen-1-negative progenitors (SCA1P), common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP) (Fig. 2B). These results suggest that ESCs therapy could decrease serum cytokine levels and circulating proatherogenic cells in rats with CAD.

ESCs therapy decreases myocardial infarction in a rat model of CAD. Myocardial infartctions and cardiac apoptosis were analyzed on day 30 after ESCs therapy. Rats treated with ESCs therapy exhibited smaller infarct areas in the border zone compared with control rats (Fig. 3A). The results demonstrated that ESCs therapy increased VEGF expression compared with the control group (Fig. 3B). Markers of myocardial apoptosis, sFas and TNFα, were decreased by ESCs therapy compared with the control group (Fig. 3C). These results suggest that ESCs therapy may decrease myocardial infarction size in a rat model of CAD.

ESCs therapy regulates cardiac apoptosis via downregulating miRNA-146a. As presented in Fig. 4A, miRNA-146a expression was decreased in myocardial cells isolated from rats treated with ESCs compared to those from the control

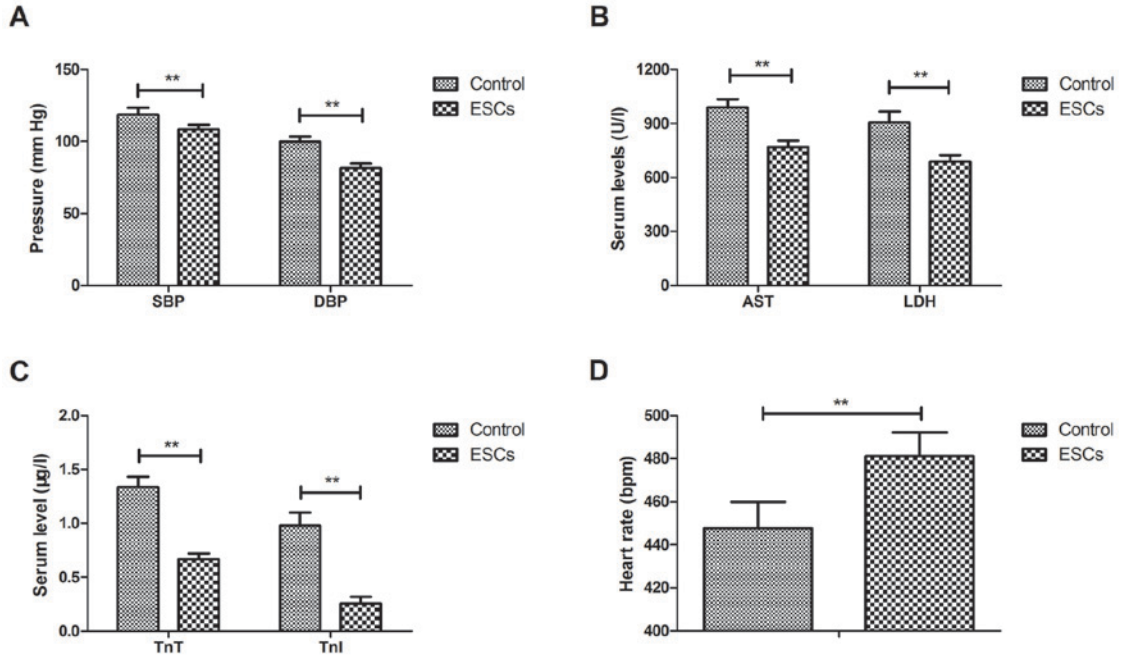

Figure 1. ESCs therapy improves cardiac function in a rat model of coronary heart disease. (A) Effects of ESCs therapy on (A) SBP and DBP, (B) serum AST and LDH, (C) serum TnT and TnI and (D) heart rate in a rat model of CAD. **P<0.01. ESCs, endothelial stem cells; SBP, systolic blood pressure; DBP, diastolic blood pressure; AST, aspartate transferase; LDH, lactic dehydrogenase; Tn, Troponin.

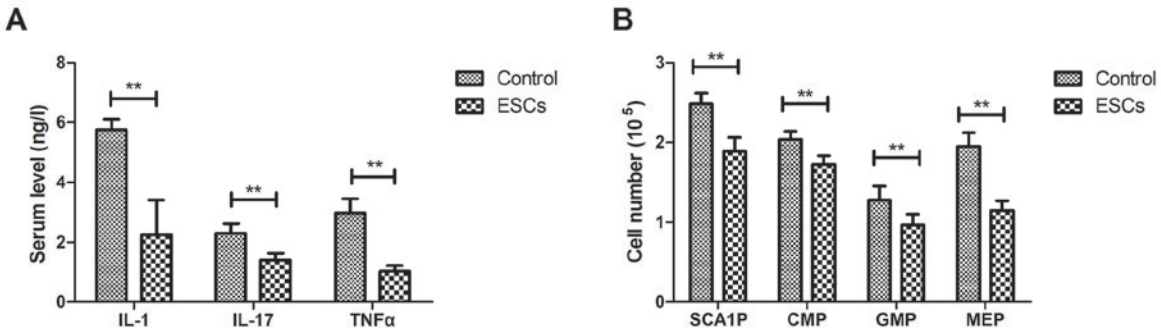

Figure 2. ESCs therapy decreases serum cytokine levels and circulating proatherogenic cells in a rat model of coronary heart disease. (A) Serum IL-1, IL-17 and TNF-α levels and (B) stem cells antigen-1 negative progenitors, common myeloid progenitors, granulocyte-macrophage progenitors and megakaryocyte-erythroid progenitors were decreased in rats treated with ESCs compared with the control group. **P<0.01. ESCs, endothelial stem cells; IL, interleukin; TNF, tumor necrosis factor.

group. Transfection with miRNA-146aKN decreased VEGF, IL-6 and sICAM-1 protein expression in myocardial cells compared with cells transfected with miRNA-mimic (Fig. 4B). Transfection with pmiRNA-146a the decreased viability of myocardial compared with cells transfected with miRNA-mimic (Fig. 4C). sFas and TNFα expression was also downregulated by miRNA-146a knockdown compared with the miRNA-mimic group (Fig. 4D). These results suggest that ESCs therapy regulates cardiac apoptosis by downregulating miRNA-146a expression.

Discussion

CAD is associated with an increase in the expression of inflammatory cytokines (18). It has previously been reported that miR-146a serves an essential role in inflammatory signaling pathways and the formation of atherosclerotic plaques (19). Advancements in stem cell technology have provided novel strategies for the treatment of heart disease (20). In the present study, the therapeutic effects of ESCs transplantation in a rat model of CAD were assessed. The results revealed that ESCs therapy decreased serum IL-1, IL-17 and TNFα levels and apoptosis in the myocardium of rats with CAD. ESCs therapy was demonstrated to regulate cardiac apoptosis via downregulating miRNA-146a expression in myocardial cells.

The current understanding of atherosclerosis pathophysiology emphasizes the role of inflammatory mediators in the development of CAD (21). IL-1 signaling may be regarded as an essential mediator in the pathogenesis of heart failure as it inhibits cardiac contractility, promotes myocardial hypertrophy and induces cardiomyocyte apoptosis (22). The results of the present study suggest that ESCs therapy decreases serum IL-1 levels in a CAD rat model. Bujak *et al* (22) reported that

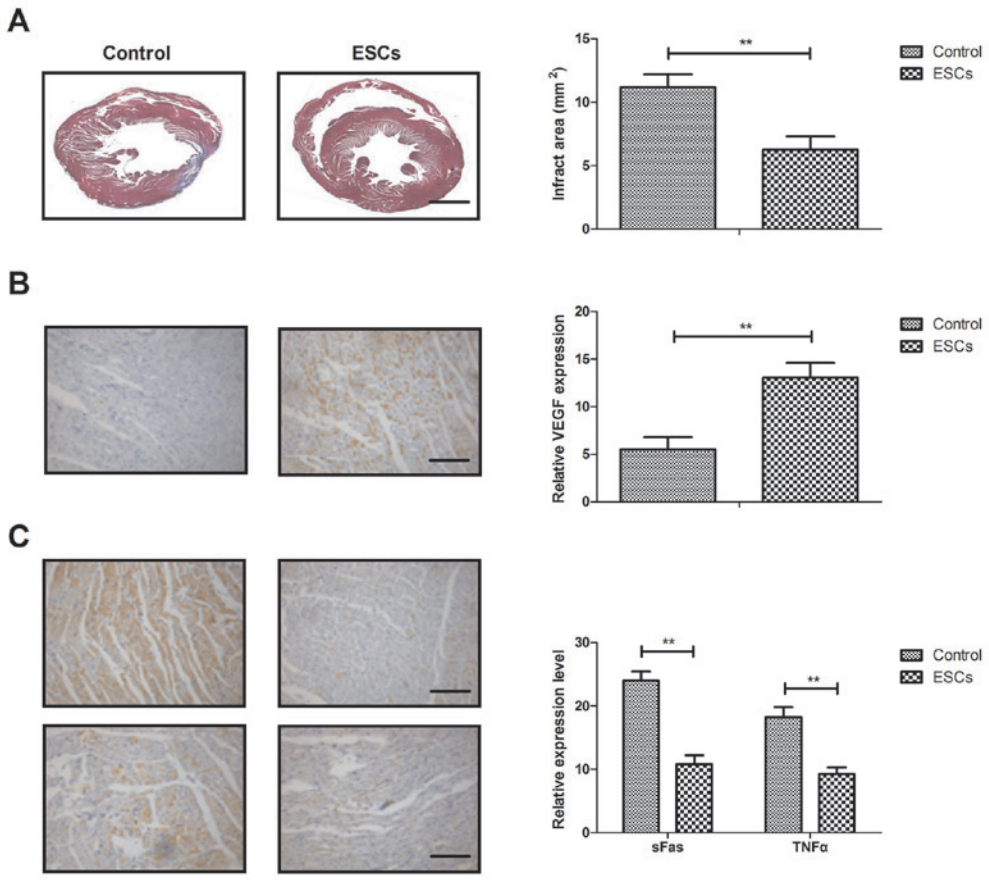

Figure 3. ESCs therapy decreased myocardial infarction in a rat model of coronary heart disease. ESCs treatment (A) decreased the infarct area in the border zone (scale bar=2 mm). (B) increased VEGF expression (scale bar=50 mm) and (C) decreased sFas and TNFα expression compared with the control group (scale bar=50 mm). **P<0.01. ESCs, endothelial stem cells; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor.

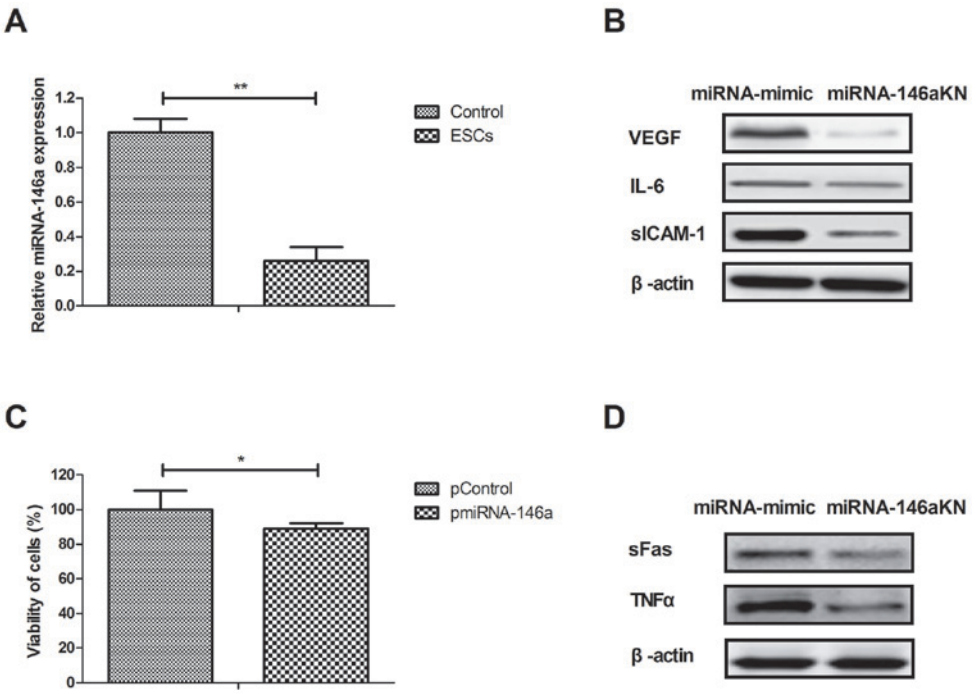

Figure 4. ESCs therapy regulates cardiac apoptosis via downregulating miRNA-146a expression. (A) MiRNA-146a expression in myocardial cells isolated from rats treated with ESCs. (B) Transfection with miRNA-146aKN decreases VEGF, IL-6 and sICAM-1 protein expression in myocardial cells. (C) Transfection with pmiRNA-146a decreases the viability of myocardial cells compared with transfection with miRNA-mimics. (D) Transfection with miRNA-146aKN decreases sFas and TNFα expression in myocardial cells. *P<0.05 and **P<0.01. ESCs, endothelial stem cells; miRNA, microRNA; miRNA-146aKN, miRNA-146a knockdown; VEGF, vascular endothelial growth factor; IL, interleukin; ICAM-1, intercellular adhesion molecule 1; TNF, tumor necrosis factor. Control, miRNA-mimic for consistency.

serum IL-17 levels may be associated with predictive or prognostic values of ischemic heart disease. Li *et al* (23) revealed that serum TNFα levels transiently declined after 24 h of cardiopulmonary bypass and may be an important biological indicator for monitoring the efficacy of cardiopulmonary bypass in children with congenital heart disease. The results of the present study suggest that IL-17 and TNFα upregulation in CAD may be inhibited by ESCs therapy. However, further investigation is required to confirm the anti-inflammatory effects of ESCs in CAD.

It has previously been reported that ESCs are a valuable source of cell therapy for angiogenesis induction as a treatment for myocardial ischemia (23). The results of the present study indicate that the therapeutic effects of ESCs improve the atherosclerosis indices in a rat model of CAD compared with control rats. Wu *et al* (24) reported that miRNA-146a induced vascular smooth muscle cell apoptosis via the NF-κB signaling pathway. In the present study, ESCs therapy decreased miRNA-146a expression and increased VEGF expression in the myocardium of rats with CAD. The results indicated that miRNA-146 inhibition reduced cardiac apoptosis and increased VEGF expression. Furthermore, it was demonstrated that ESCs therapy decreased serum AST, LDH, TnT and TnI levels in a rat model of CAD, as well as improving heart rate.

In conclusion, the results of the present study demonstrate the therapeutic effects of ESCs in decreasing inflammatory cytokines and the number of apoptotic cells in heart tissue. It was also demonstrated that miR-146a downregulation mediated by ESCs ameliorates the apoptosis of cardiac cells, suggesting that ESCs may have potential applications in CAD treatment.

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YF performed the experiments. SC, ZL, WA, XH, LW, PX and BJ prepared and analyzed experimental data. HF designed the experiments.

Ethics approval and consent to participate

This experiment was approved by the Ethics Committee of Shenzhen Nanshan People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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