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RESEARCH ARTICLE

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Angiopoietin-like protein 4 treated bone marrow-derived mesenchymal stem cells alleviate myocardial injury of patients with myocardial infarction

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Abstract

Bone marrow-derived mesenchymal stem cells (BMSCs) and their exosomes are of great significance for the recovery of cardiac function in patients with myocardial infarction (MI). However, the underlying mechanisms of BMSCs applied to MI treatment remain unclear. Fluorescence-activated cell sorting (FACs) are performed to assess the apoptosis, reactive oxygen species levels and glucose uptake capacity of BMSCs. Reverse transcription polymerase chain reaction is conducted to detect the levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), transforming growth factor-beta 1, connective tissue growth factor, and platelet-derived growth factor. The levels of apoptosis-related proteins were detected by Western blot. The levels of VEGF, bFGF, HGF, and IGF were assessed by enzyme-linked immunosorbent assay. The biochemical kits are applied to detect the levels of malondialdehyde, superoxide dismutase, and adenosine triphosphate/adenosine diphosphate. 2,3,5-triphenyltetrazolium and Masson staining and immunofluorescence are performed to assess myocardial function of rats. Angiopoietin-like protein 4 (ANGPTL4) alleviates apoptosis and oxidative stress of BMSCs induced by serum deprivation and hypoxia; ANGPTL4 activates paracrine and accelerate metabolic energy of BMSCs; and ANGPTL4 treated-BMSCs alleviate myocardial injury of rats with MI. ANGPTL4 treated-BMSCs alleviate myocardial injury in rats with MI, indicating the combination therapy of ANGPTL4 and BMSCs may alleviate myocardial injury in rats with MI.

KEYWORDS
ANGPTL4, BMSCs, cardiac, myocardial infarction, myocardial injury

Key Points

- Angiopoietin-like protein 4 alleviates apoptosis and oxidative stress of bone marrow-derived mesenchymal stem cells induced by hypoxia/serum deprivation.
- Angiopoietin-like protein 4 activates paracrine and accelerates metabolic energy of bone marrow-derived mesenchymal stem cells.
- Angiopoietin-like protein 4 treated-bone marrow-derived mesenchymal stem cells alleviate myocardial injury of rats with myocardial infarction.

1 | INTRODUCTION

Myocardial infarction (MI) is defined as sudden ischemic death of myocardial tissue, which is the main cause of death worldwide, usually the cause of sudden death (Kuriachan & Sumner, 2015). An increasing number of studies have shown that the therapeutic potential of bone marrow-derived mesenchymal stem cells (BMSCs) is multipotent (Li & Zhen, 2016). BMSCs can differentiate into endothelial cells, vascular smooth muscle cells, and even muscle cells after transplantation into ischemic hearts (Orlic & Kajstura, 2001). Therefore, BMSCs have the potential to repair and regenerate heart tissue. However, the underlying mechanism remains unclear.

BMSCs play an important role in the formation and regulation of hematopoietic microenvironment (Robey, 2017). BMSCs may secrete a variety of adhesion molecules and cytokines that are involved in regulating immune response and promoting endogenous regeneration. Intravascular injection of BMSCs has been proved to be an effective strategy for the treatment of autoimmune diseases, vascular diseases, and diabetes (Hare & Fishman, 2012; Shi & Hu, 2010; Shi & Wang, 2018). In addition, BMSCs and their exosomes are of great significance for the recovery of cardiac function in patients with MI (Xu & Zhang, 2019).

Angiopoietin like protein 4 (ANGPTL4) is a protein that inhibits lipoprotein lipase. It controls the absorption of fatty acids in fat and oxidized tissues, regulates the circulating tag rich lipoprotein, and plays an important role in the regulation of cardiovascular disease (Aryal & Singh, 2018). It has been reported that ANGPTL4 has a crucial regulatory role in cell bioactivities. As an endocrine regulator, ANGPTL4 is the target of the receptor activated by peroxide proliferators (Kersten & Mandard, 2000; Oike & Akao, 2005). Under the condition of serum deprivation (SD) and hypoxia, the level of ANGPTL4 is abnormal in various types of cells, such as fat cells, cardiomyocytes, and liver cells (Belanger & Lu, 2002; Shibata & Nakayama, 2010). Furthermore, ANGPTL4 has a decisive anti-apoptotic effect on human vascular endothelial cells and colorectal cancer cells (Kim & Park, 2011). It supports the survival of BMSCs in SD environment (Akhter & Rahman, 2013). Therefore, ANGPTL4 may protect BMSCs from hypoxia/SD induced apoptosis.

This study suggests that exogenous ANGPTL4 can reduce hypoxia/SD induced apoptosis of BMSCs and improve cell survival rate. The survival of BMSCs are critical for cardiac function recovery in patients with MI.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Adult Wistar rats (250–300 g) were purchased from the experimental animal center of Guangzhou University and fed with standard rat feed and free drinking water. The suitable temperature for raising rats is 18–25°C, and the relative temperature is 40%–60%. The room requires warm winter, no wind, cool and ventilated summer, and shade. It is required to be ventilated at least 20 times per h in order to effectively remove moisture and heat from the house and give it no less than 10 h of light every day. This study was approved by the institutional animal protection and utilization committee of HB2002019.

2.2 | Modeling

Under aseptic conditions, rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg). Following skin preparation and disinfection, the neck skin was cut open and bluntly separated. For visual intubation for mechanical ventilation, mechanical ventilation parameters are 7 ml/kg tidal volume, respiratory ratio 1:2, frequency 50 beats/min. The skin and subcutaneous tissue were cut along the ribs to expose the intercostal space. Mosquito forceps were used to tear the third and fourth intercostal muscles and the intercostal space homeopathically. The rat heart was exposed and squeezed. Under direct vision, the coronary artery of the heart was quickly ligated. After the anterior descending branch was ligated, the heart was put into the chest cavity, sutured, and the operation was completed.

2.3 | Intervention process

Using the random block method, 50 rats were divided into control group, sham group, MI model group (model group), model + BMSCs group, and model + ANGPTL4 treated-BMSCs group with 10 in each group.

1. The control group was given normal saline every day.
2. The sham operation group was given normal saline every day.
3. The MI model group was given the same amount of normal saline every day.
4. In the model + BMSCs group, following the MI model was successfully constructed, the rats were injected with BMSCs suspension which contained 1×10^6 cells from coronary artery.
5. In the model + ANGPTL4 treated-BMSCs group, BMSCs were treated first with ANGPTL4 (100 ng/ml) for 24 h. Subsequently, 1×10^6 ANGPTL4 treated-BMSCs were injected into model rats through the coronary artery.

2.4 | BMSCs culture

BMSCs were isolated from rat femur and tibia. Red blood cells were lysed with ack lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA), washed and resuspended, and cultured in Dulbecco's modified eagle's medium (DMEM)/F12 (Carlsbad, CA, USA) containing 40% MCDB201, 100 U/ml penicillin, 100 U/ml streptomycin, and 2% fetal bovine serum (FBS). BMSCs were cultured in DMEM (Roche,

Basel, Switzerland). One percent penicillin streptomycin solution (Solarbio, Beijing, China) and 10% FBS were added to the medium. The cells were cultured in a moist incubator containing 5% CO_2 at 37°C.

2.5 | Cell counting kit-8 (CCK-8) assay

When BMSCs converged, medium was removed, and the holes of the plate were washed three times with 0.1 m PBS. CCK-8 assay kits were used to measure cell viability according to the manufacturer's instructions. The absorption of 450 nm was measured using a microplate reader (PerkinElmer, Waltham, MA, USA).

2.6 | Fluorescence-activated cell sorting (FACs)

A certain dose of ANGPTL4 was used to treat 1×10^5 BMSCs, cultured in 12 well plate with serum-free DMEM for 24 h, centrifuged at 1500 g for 5 min, and washed twice with 1 × PBS. The cells were fixed with 70% ethanol (Solarbio, Beijing), placed at 20°C for 15 min, centrifuged at 1500 g for 5 min, and washed twice by precooling 1 × PBS. Subsequently, the cells were incubated with 10 μg/μl Dnase-free RnaseA (Sigma, St. Louis, MO, USA) to remove RNA, and washed twice with precooled 1 × PBS. Finally, after centrifugation at 150 g for 5 min, the cells were incubated with 1 mg/ml iodide (Sigma) in the dark at 4°C for 12 min. Flow cytometry and ModiFit software (Olympus, Tokyo, Japan) were used to quantify the reactive oxygen species (ROS) level and glucose uptake of BMSCs. In addition, according to the instructions, annexin FITC/PI apoptosis detection kit (Beyotime, Nanjing, China) was combined with flow cytometry to detect apoptosis (Dong & Cui, 2017). ModiFit software (Olympus, Tokyo, Japan) was performed to analyze the data.

2.7 | Western blot

The total proteins of BMSCs treated with a certain dose of ANGPTL4 were isolated by cell lysis buffer (Beyotime, Nanjing, China). Western blot was performed as described previously (Cui & Dong, 2019). All the antibodies used in this study were from Abcam (Cambridge, UK), including Bax (ab32503, 1; 1000), Bcl-2 (ab182858, 1:1000), cleaved casapase3 (ab32042, 1:1000), and cleaved casapase9 (ab2324, 1:1000). β-actin (ab8226, 1:1000) was used as internal reference. The optical density of protein bands was quantified by Image J Software (ImageJ Software Inc., USA).

2.8 | Reverse transcription polymerase chain reaction (RT-qPCR)

TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA) was used to extract total RNA. The cDNA was synthesized with M-MLV Reverse Transcriptase (Rnase H) kit (Takara, Kusatsu, Japan). SYBR Green PCR Master Mix (Takara, Kusatsu, Japan) was used for RT-qPCR. For mRNA detection, β-actin served as negative control. $2^{-\Delta\Delta Ct}$ methods were performed to calculate the relative expression. The primers used in this study are shown in Table 1.

2.9 | ELISA

A total of 1 ml of protein extraction reagent (Beyotime, Nanjing, China) were used to lyse BMSCs, which were treated with a certain dose of ANGPTL4. The levels of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) were detected by enzyme-linked immunosorbent assay (ELISA) Kit produced by Roche (Basel, Switzerland), according to instructions.

2.10 | Determination of biochemical index

The kits for malondialdehyde (MDA), superoxide dismutase (SOD), adenosine triphosphate (ATP), and adenosine diphosphate (ADP) were purchased from Nanjing Institute of bioengineering. All the kits adopt standard colorimetric method and operate in strict accordance with the instructions (Wang & Zhang, 2019).

TABLE 1 Primer sequences

Primer name	(5′-3′)Primer sequences
F- VEGF	5′- GGCTCACTTCCAGAAACACG -3′
R- VEGF	5′- GTGCTCTTGCAGAATCTAGTGG-3′
F- bFGF	5′- GCGTGGACGGCGTCCGGGAG -3′
R- bFGF	5′- GGCCCCGTTTTGGATCCGAG-3′
F- HGF	5′- ATTGCCCTATTTCCCGTTGT-3′
R- HGF	5′- TTTCAAACTAACCATCCACCCT-3′
F- IGF	5′- GACCCGGGACGTACCAAAAT-3′
R- IGF	5′- GAACTGAAGAGCGTCCACCA-3′
F- TGF-β1	5′- CGAGCCTGAGGCCGACTAC-3′
R- TGF-β1	5′-AGATTTCGTTGTGGGTTTCCA-3′
F- CTGF	5′-GAAGCTGACCTGGAGGAAAA-3′
R- CTGF	5′-ACTGGCAGAGTGGTGGTTCT-3′
F- PDGF	5′-GTCCTAGAGCGTGTGG-3′
R- PDGF	5′-CGCCGTGCCTACTAGA −3′
F-β-actin	5′- CCCATCTATGAGGGTTACGC-3′
R-β-actin	5′- TTTAATGTCACGCACGATTTC-3′

Abbreviations: bFGF, basic fibroblast growth factor; CTGF, connective tissue growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor-beta 1; VEGF, vascular endothelial growth factor.

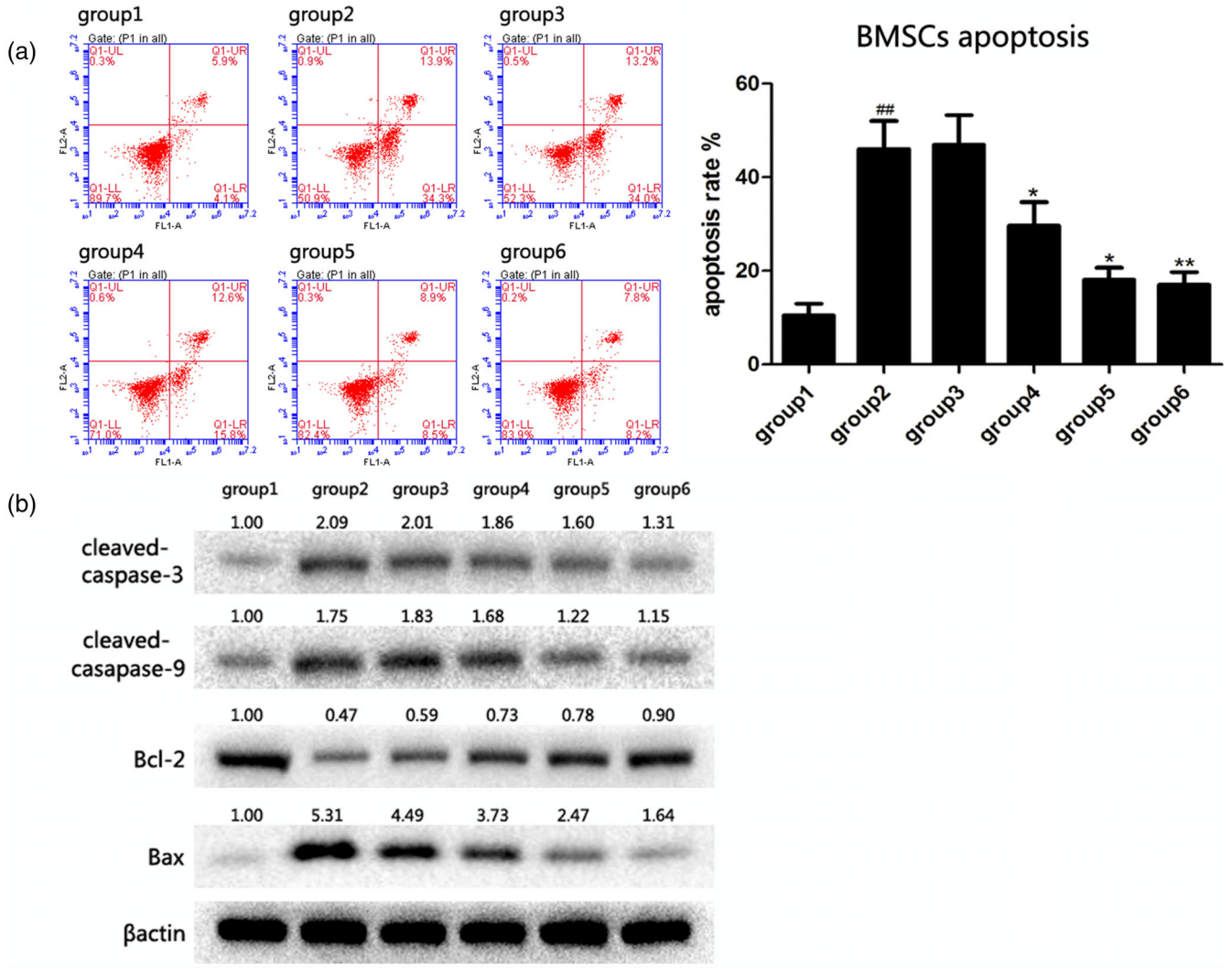

FIGURE 1 ANGPTL4 rescues BMSCs apoptosis induced by hypoxia/SD in a dose-dependent manner. BMSCs, which were inoculated into six-well plates at 1×10^4/well were randomly divided into six groups. Group 1, BMSCs were cultured by normoxia/serum supplement medium for 24 h. Group 2, BMSCs were cultured by hypoxia/SD medium for 24 h. Group 3, BMSCs treated with ANGPTL4 (1 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 4, BMSCs treated with ANGPTL4 (10 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 5, BMSCs treated with ANGPTL4 (100 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 6, BMSCs treated with ANGPTL4 (1000 ng/ml) were cultured by hypoxia/SD medium for 24 h. (a) Fluorescence-activated cell sorting (FACs) were performed to detect BMSCs apoptosis. (b) Western blot was conducted to measure the levels of apoptosis-related proteins, including Bax, Bcl-2, cleaved caspase-3, and cleaved caspase-9. Student's *t*-test or one-way analysis of variance is used for comparison between two groups, and Tukey posttest is used for comparison between multiple groups. ##$p < 0.01$ versus control group; *$p < 0.05$, **$p < 0.01$ versus sham group. Error bars represent SD. Data represent three independent experiments. Abbreviations: ANGPTL4, angiopoietin-like protein 4; BMSC, bone marrow-derived mesenchymal stem cell; SD, serum deprivation

2.11 | TTC staining

Four weeks after the establishment of the MI model, the infarct size was evaluated by 2,3,5-triphenyltetrazolium (TTC) staining. After anesthesia, the heart was cut into 1 mm thick slices. The sections were stained with 1% TTC (Sigma, USA) and fixed with 4% paraformaldehyde. The percentage of infarct size (white unstained necrotic tissue) to left ventricular area was calculated according to the previous description (Takagawa & Zhang, 2007). In short, tissue sections were placed in natural light and photographed with a Nikon COOLPIX A900 digital camera. Myocardial infarct size (%) was calculated by microphotographic color image processing system (DpxView Pro, Korea).

2.12 | Masson staining

Myocardial tissue was dehydrated with gradient alcohol and treated with xylene. Paraffin embedded sections (4 μm). The paraffin sections were stained with Masson and washed. Sections were stained with hematoxylin. The slices were washed and then restrained with Masson poceaux. The slices were treated with 2% glacial acetic acid and

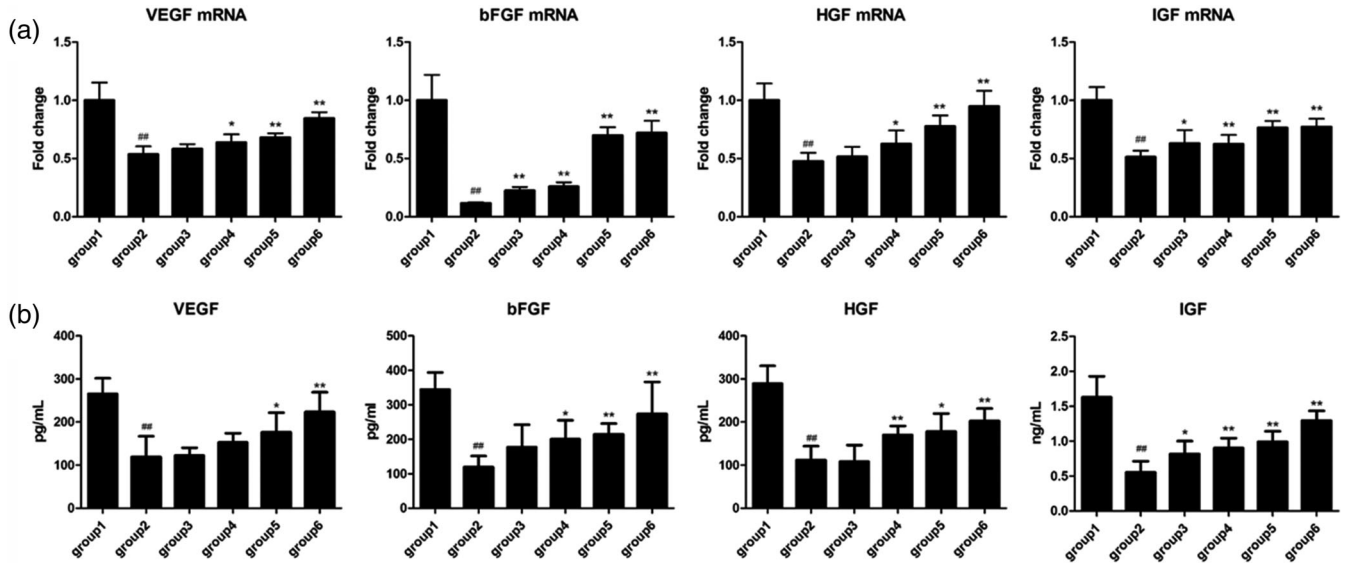

FIGURE 2 ANGPTL4 activates paracrine of BMSCs inhibited by hypoxia/SD in a dose-dependent manner. BMSCs, which were inoculated into six-well plates at 1×10^4/well were randomly divided into six groups. Group 1, BMSCs were cultured by normoxia/serum supplement medium for 24 h. Group 2, BMSCs were cultured by hypoxia/SD medium for 24 h. Group 3, BMSCs treated with ANGPTL4 (1 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 4, BMSCs treated with ANGPTL4 (10 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 5, BMSCs treated with ANGPTL4 (100 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 6, BMSCs treated with ANGPTL4 (1000 ng/ml) were cultured by hypoxia/SD medium for 24 h. (a) reverse transcription polymerase chain reaction was performed to assess the levels of VEGF, bFGF, HGF, and IGF in BMSCs. (b) enzyme-linked immunosorbent assay was conducted to detect the levels of VEGF, bFGF, HGF, and IGF in BMSCs. Student's *t*-test or one-way analysis of variance is used for comparison between two groups, and Tukey posttest is used for comparison between multiple groups. ##$p < 0.01$ versus control group; *$p < 0.05$, **$p < 0.01$ versus sham group. Error bars represent SD. Data represent three independent experiments. Abbreviations: ANGPTL4, angiopoietin-like protein 4; bFGF, basic fibroblast growth factor; BMSC, bone marrow-derived mesenchymal stem cell; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; SD, serum deprivation; VEGF, vascular endothelial growth factor

1% phosphomolybdic acid. Then, it was dyed with aniline blue and dehydrated. Finally, the sections were stained with resin.

2.13 | Immunofluorescence

Immunofluorescence is carried out as described, with slight modification. Bovine serum albumin (PBST) was used to block the sections, and 4% PBST was used to wash the sections. Subsequently, the heart sections were incubated with anti-CD31-PE antibody (Abcam, Cambridge, UK, 1:1000, ab215753) overnight at 4°C. After washing again, the sections were incubated with second antibody for 1 h, then stained with DAPI, sealed with cover glass, and finally observed with confocal fluorescence microscope (LSM880, Zeiss, Germany).

2.14 | Statistical analysis

Mean ± SD represents data from three independent experiments. GraphPad prism version 5.0 software (GraphPad Software, Inc.). All data were analyzed statistically. Unpaired Student's *t*-test was used for comparison between the two groups, and one-way analysis of variance and Turkey test were used for comparison between multiple groups. When $p < 0.05$, the difference was statistically significant.

3 | RESULTS

3.1 | ANGPTL4 alleviates BMSCs apoptosis induced by hypoxia/SD

It has been reported that BMSCs can effectively promote cardiac repair after MI (Xu & Zhang, 2019). To investigate specific molecular mechanisms, FACs and Western blot were performed to assess BMSCs apoptosis, following treating BMSCs with different doses of ANGPTL4. Studies have shown that ANGPTL4 plays an important role in the regulation of cell biological activity (Chiang & Shieh, 2020). Findings showed that compared with controls, hypoxia/SD significantly accelerated BMSCs apoptosis, whereas the apoptosis trend was alleviated by ANGPTL4 in a dose-dependent manner (Figure 1a). In addition, Western blot further confirmed this phenomenon, because hypoxia/SD can significantly promote the expression of proapoptotic protein Bax, cleavage caspase-3 and cleavage caspase-9, and inhibit the expression of antiapoptotic protein Bcl-2, whereas ANGPTL4 alleviated these trend in a dose-dependent manner (Figure 1b). Furthermore, under white light, we observed that ANGPTL4 had no effect on the morphology of BMSCs (Figure S1, bar = 100 μm). Similarly, CCK-8 assay showed that the proliferation of BMSCs had no change, following treated with ANGPTL4 at 0, 1, 10, 100, 1000 ng/ml (Figure S2). These findings revealed that ANGPTL4 alleviated BMSCs apoptosis induced

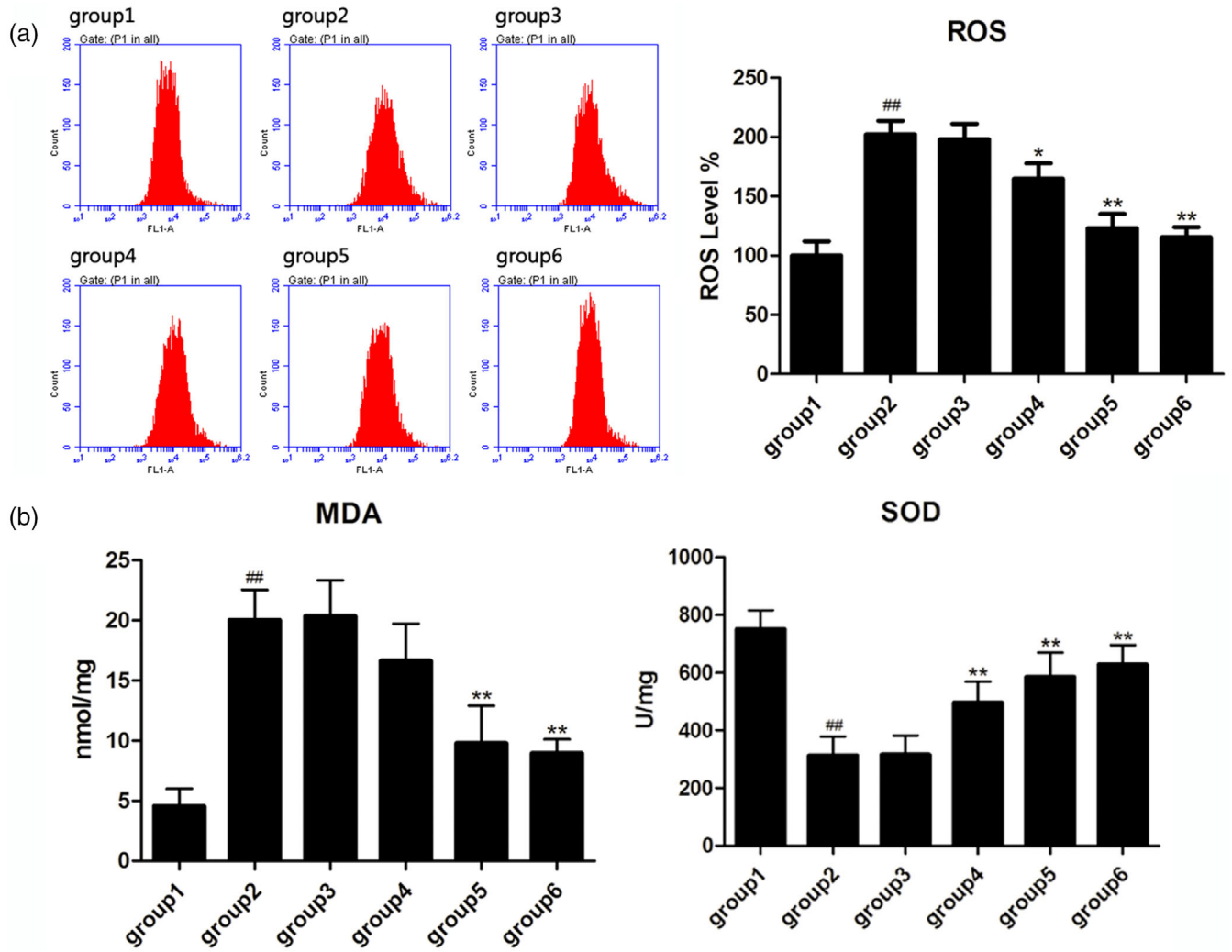

FIGURE 3 ANGPTL4 alleviates oxidative stress of BMSCs induced by hypoxia/SD in a dose-dependent manner. BMSCs, which were inoculated into six-well plates at 1×10^4/well were randomly divided into six groups. Group 1, BMSCs were cultured by normoxia/serum supplement medium for 24 h. Group 2, BMSCs were cultured by hypoxia/SD medium for 24 h. Group 3, BMSCs treated with ANGPTL4 (1 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 4, BMSCs treated with ANGPTL4 (10 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 5, BMSCs treated with ANGPTL4 (100 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 6, BMSCs treated with ANGPTL4 (1000 ng/ml) were cultured by hypoxia/SD medium for 24 h. (a) Fluorescence-activated cell sorting (FACs) were performed to detect ROS levels in BMSCs. (b) MDA Assay Kit (TBA) and SOD Assay Kit (WST-1) were applied to detect the levels of MDA and SOD in BMSCs, respectively. Student's *t*-test or one-way analysis of variance is used for comparison between two groups, and Tukey posttest is used for comparison between multiple groups. ##$p < 0.01$ versus Control group; *$p < 0.05$, **$p < 0.01$ versus Sham group. Error bars represent SD. Data represent three independent experiments. Abbreviations: ANGPTL4, angiopoietin-like protein 4; BMSC, bone marrow-derived mesenchymal stem cell; MDA, malondialdehyde; ROS, reactive oxygen species; SD, serum deprivation; SOD, superoxide dismutase

by hypoxia/SD, but had no effect on the morphology and proliferation of BMSCs.

3.2 | ANGPTL4 activates paracrine of BMSCs inhibited by hypoxia/SD

The reason BMSCs are important is that they participate in the regulation of bioactivities by secreting a variety of cytokines (He & Chen, 2018). To investigate the effect of ANGPTL4 on paracrine of BMSCs, following treating BMSCs with different doses of ANGPTL4, RT-qPCR and ELISA were performed to assess cytokines expression in BMSCs, including VEGF, bFGF, HGF, and IGF. Results showed that compared with control, hypoxia/SD significantly inhibited VEGF, bFGF, HGF, and IGF expression, whereas ANGPTL4 alleviated the inhibitory effect (Figure 2a,b). These findings revealed that ANGPTL4 activated paracrine of BMSCs inhibited by hypoxia/SD.

3.3 | ANGPTL4 alleviates oxidative stress of BMSCs induced by hypoxia/SD

The production of oxidative stress (OS) and ROS plays an important role in the infarcted cardiac dysfunction (Cho & Kang, 2019; Wang &

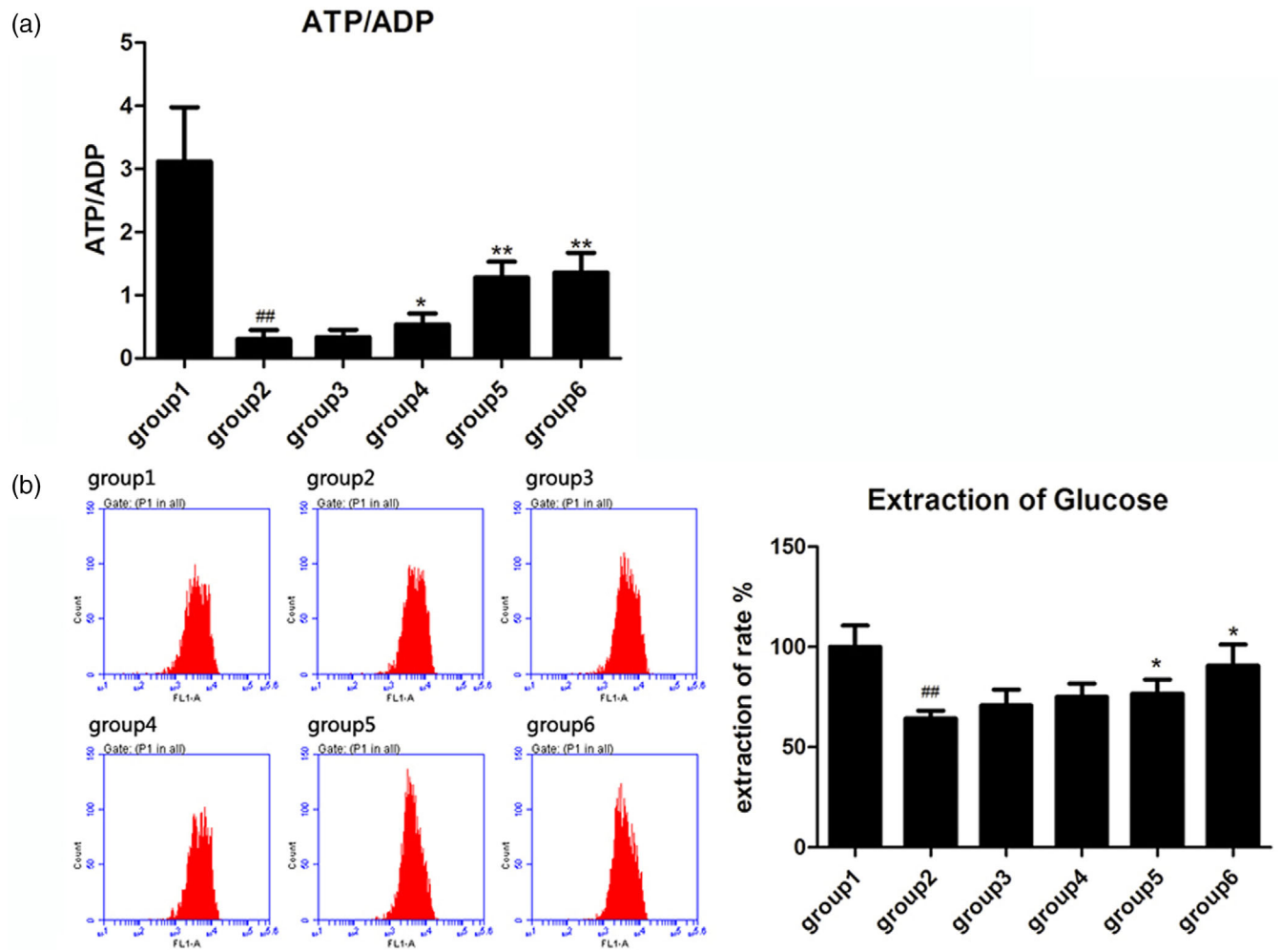

FIGURE 4 ANGPTL4 up-regulates metabolic energy of BMSCs inhibited by hypoxia/SD in a dose-dependent manner. BMSCs, which were inoculated into six-well plates at 1×10^4/well were randomly divided into six groups. Group 1, BMSCs were cultured by normoxia/serum supplement medium for 24 h. Group 2, BMSCs were cultured by hypoxia/SD medium for 24 h. Group 3, BMSCs treated with ANGPTL4 (1 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 4, BMSCs treated with ANGPTL4 (10 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 5, BMSCs treated with ANGPTL4 (100 ng/ml) were cultured by hypoxia/SD medium for 24 h. Group 6, BMSCs treated with ANGPTL4 (1000 ng/ml) were cultured by hypoxia/SD medium for 24 h. (a) ADP/ATP Ratio Assay Kit was applied to assess the levels of ATP/ADP in BMSCs. (b) Fluorescence-activated cell sorting (FACs) were conducted to assess the ability of glucose uptake of BMSCs. Student's *t*-test or one-way analysis of variance is used for comparison between two groups, and Tukey posttest is used for comparison between multiple groups. ##$p < 0.01$ versus Control group; *$p < 0.05$, **$p < 0.01$ versus Sham group. Error bars represent SD. Data represent three independent experiments. Abbreviations: ADP, adenosine diphosphate; ANGPTL4, angiopoietin-like protein 4; ATP, adenosine triphosphate; BMSC, bone marrow-derived mesenchymal stem cell; SD, serum deprivation

Wang, 2018). To investigate the effect of ANGPTL4 on OS of BMSCs, following treating BMSCs with different doses of ANGPTL4, FACs was performed to assess ROS levels in BMSCs. Findings showed that compared with control, hypoxia/SD significantly induced ROS production in BMSCs, whereas ANGPTL4 alleviated the promoted trend in a dose-dependent manner (Figure 3a). What is more, hypoxia/SD significantly down-regulated SOD levels in BMSCs, whereas the inhibitory effect was alleviated by ANGPTL4 in a dose-dependent manner. However, MDA expression showed the opposite effect (Figure 3b). These results revealed that ANGPTL4 alleviated OS of BMSCs induced by hypoxia/SD.

3.4 | ANGPTL4 up-regulates metabolic energy of BMSCs inhibited by hypoxia/SD

It is reported that ANGPTL4 plays an important role in energy metabolism (Aryal & Price, 2019). To investigate the roles of ANGPTL4 in energy metabolism of BMSCs, following treatment of BMSCs with different doses of ANGPTL4, ADP/ATP Ratio Assay Kit was applied to assess the levels of ATP/ADP in BMSCs. Findings showed that compared with controls, the rate of ATP/ADP in BMSCs was down-regulated by SD and hypoxia, whereas the inhibitory effect was alleviated by ANGPTL4 in a dose-dependent

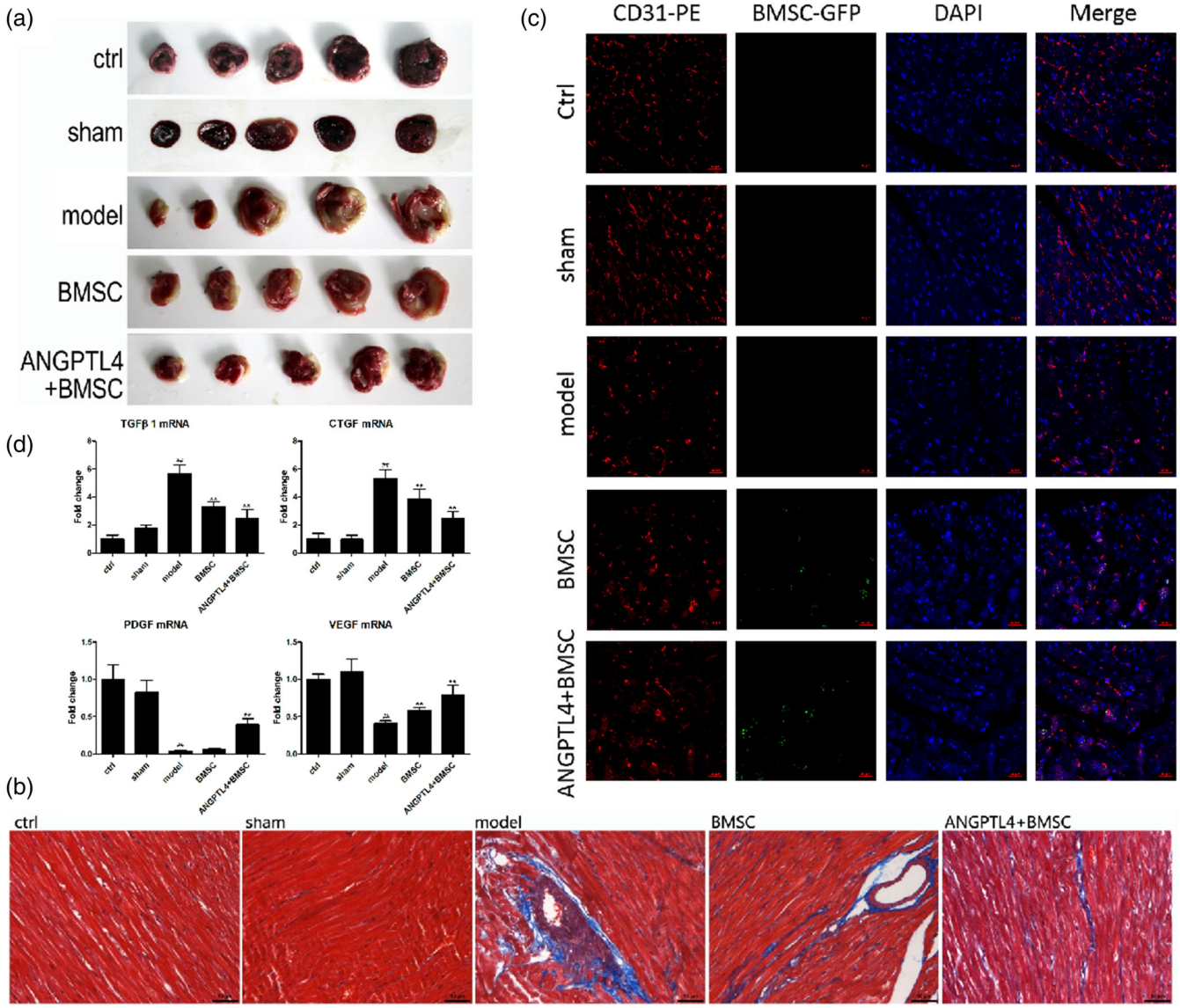

FIGURE 5 ANGPTL4 treated-BMSCs protect the cardiac function of rats with MI. Fifty males' rats were randomly divided into five groups, control group, sham group, MI model group (model group), model + BMSCs group, model + ANGPTL4 treated-BMSCs group, 10 in each group. (a) TTC staining was performed to assess MI area. (b) Masson staining was conducted to evaluate the degree of cardiac fibrosis. (c) CD31 levels were detected by immunofluorescence. (d) reverse transcription polymerase chain reaction was performed to detect the levels of TGF-β1, CTGF, PDGF, and VEGF. Student's *t*-test or one-way analysis of variance is used for comparison between two groups, and Tukey posttest is used for comparison between multiple groups. ##$p < 0.01$ versus Control group; **$p < 0.01$ versus Sham group. Error bars represent SD. Data represent three independent experiments; scale bar: 200 μm. Abbreviations: ANGPTL4, angiopoietin-like protein 4; BMSC, bone marrow-derived mesenchymal stem cell; CTGF, connective tissue growth factor; MI, myocardial infarction; PDFG, platelet-derived growth factor; SD, serum deprivation; TGF-β1, transforming growth factor-beta 1; VEGF, vascular endothelial growth factor

manner ($p < 0.05$; Figure 4a). Consistently, FACs analysis showed that compared with control, hypoxia/SD inhibited the glucose uptake ability of BMSCs, whereas ANGPTL4 alleviated this strengthening effect in a dose-dependent manner ($p < 0.05$; Figure 4b), indicating that, under the condition of SD and hypoxia, ANGPTL4 promotes the glucose utilization ability of BMSCs in a dose-dependent manner. These findings revealed that ANGPTL4 up-regulated metabolic energy of BMSCs inhibited by hypoxia/SD.

3.5 | ANGPTL4 treated-BMSCs alleviate myocardial injury of rats with MI

To investigate the roles of ANGPTL4 treated-BMSCs in myocardial protection of patients with MI, 4 weeks following MI model establishment, atrial tissues were separated to assess the degree of myocardial fibrosis. TTC and Masson staining analysis showed that in the MI model group, the degree of myocardial fibrosis was significantly higher than that in the control group and sham group, whereas injection of

exogenous BMSCs alleviated the degree of fibrosis, and ANGPTL4 enhanced the effect of BMSCs in a dose-dependent manner (Scale bar: 200 μm; Figure 5a,b). Besides, immunofluorescence analysis indicated that compared with controls, in the MI model, the levels of CD31 in atrial tissues was minimal, whereas injection of exogenous BMSCs up-regulated CD31 expression, and the promoting effect was enhanced by ANGPTL4 in a dose-dependent manner (Figure 5c). What is more, RT-qPCR analysis indicated that compared with control, transforming growth factor-beta 1 and connective tissue growth factor expression of atrial tissues in MI model group were significantly inhibited, whereas injection of exogenous BMSCs alleviated the inhibitory effect, and the effect of BMSCs was strengthened by ANGPTL4 in a dose-dependent manner. Inversely, the levels of platelet-derived growth factor and VEGF showed reverse trends ($p < 0.01$; Figure 5d). These findings revealed that ANGPTL4 treated-BMSCs alleviated myocardial injury of rats with MI.

4 | DISCUSSION

MI is caused by the formation of plaque on the inner wall of the artery, which reduces the blood flow to the heart and damages the heart muscle because of hypoxia (Lu & Liu, 2015). It has been reported that autologous BMSCs have great advantages in the recovery of cardiac function after transplantation into ischemic or infarcted heart for regeneration and reperfusion of injured myocardium (Hou & Cui, 2014). What is more, in the hypoxia/SD condition, ANGPTL4 has a strong antiapoptotic effect. Our findings indicated that ANGPTL4 alleviated apoptosis and OS of BMSCs induced by hypoxia/SD, ANGPTL4 activated paracrine and up-regulates metabolic energy of BMSCs, and ANGPTL4 treated-BMSCs alleviated myocardial injury of rats with MI.

ANGPTL4 is recognized as a hypoxia inducible gene, which has a wide range of functions and regulates a variety of biological processes, including cell biological activity, angiogenesis, inflammation, and wound healing (Camenisch & Pisabarro, 2002). Our findings showed that ANGPTL4 alleviated apoptosis of BMSCs induced by hypoxia/SD, which strongly supported the antiapoptotic effect of ANGPTL4. Besides, ANGPTL4, which belongs to the angiogenin-like protein family, shows angiogenic activity and is also involved in the regulation of plasma lipid levels (Belanger & Lu, 2002; Camenisch & Pisabarro, 2002). Our results indicated that ANGPTL4 activated paracrine of BMSCs inhibited by hypoxia/SD. Namely, in the hypoxia/SD condition, ANGPTL4 up-regulated VEGF, bFGF, HGF, and IGF levels in BMSCs, indicating ANGPTL4 might exert its angiogenic activity by promoting the paracrine of BMSCs. Consistently, findings show that in the hypoxia/SD condition, ANGPTL4 inhibited the ability of glucose uptake of BMSCs, and the rate of ATP/ADP is up-regulated by ANGPTL4, indicating that ANGPTL4 promotes the glucose utilization ability of BMSCs.

Hypoxia condition often leads to excessive OS, which is also the main reason for the aggravation of patients with MI (Shahzad & Hasan, 2018). Our findings showed that ANGPTL4 alleviated OS of BMSCs induced by hypoxia/SD, indicting in the hypoxia/SD condition, ANGPTL4 might play a role in protecting myocardial function by slowing down the OS response of BMSCs. Furthermore, functional researches further verified our conclusions in vitro. Namely, ANGPTL4 treated-BMSCs alleviated myocardial injury of rats with MI.

In summary, this manuscript illuminated that ANGPTL4 alleviated apoptosis and OS of BMSCs induced by hypoxia/SD; ANGPTL4 activated paracrine and up-regulated metabolic energy of BMSCs; and ANGPTL4 treated-BMSCs alleviated myocardial injury of rats with MI. These findings indicated that the combination therapy of ANGPTL4 and BMSCs alleviated myocardial injury in patients with MI. Our findings might provide a promising cell-free treatment strategy for MI.

ETHICAL APPROVAL

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the institutional animal protection and utilization committee of HB2002019.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Study design: Yang Dong. *Data collection*: Fen Zhang and Jie Wu. *Data analysis*: Fen Zhang, Jie Wu, and Yang Dong. *Manuscript writing*: Xingxing Li, Xuan Ying, and Wenbing Fang.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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