European Review for Medical and Pharmacological Sciences 2020; 24: 10069-10077

Lipopolysaccharide-stimulated bone marrow mesenchymal stem cells-derived exosomes inhibit H_2O_2-induced cardiomyocyte inflammation and oxidative stress *via* regulating miR-181a-5p/ATF2 axis

H.-Y. LIU[1], L.-F. YU[2], T.-G. ZHOU[2], Y.-D. WANG[2], D.-H. SUN[2], H.-R. CHEN[2], Y.-F. HOU[2]

[1]Department of Gastroenterology, The First Affiliated Hospital of Shandong First Medical University, Jinan, China
[2]Department of Intensive Care Unit, The First Affiliated Hospital of Shandong First Medical University, Jinan, China

Abstract. – OBJECTIVE: Myocardial infarction (MI) is a cardiovascular disease that seriously endangers human health. Exosomes secreted by stem cells have big potential for the treatment of many diseases. The purpose of this study was to study the therapeutic effects of exosomes derived from lipopolysaccharide (LPS)-stimulated bone marrow mesenchymal stem cells (BMSCs) on MI.

PATIENTS AND METHODS: The surface markers of BMSCs were detected by Western blot. After BMSCs were stimulated with LPS for 2 days, the exosomes secreted by BMSCs were extracted and observed by transmission electron microscopy (TEM), and their specific surface markers were detected by Western blot. H9c2 cells were co-cultured with exosomes for 24 hours, and then, treated with H2O2 for 4 hours. Next, H9c2 cells were transfected with miRNA-181a-5p mimic (MIM) or negative control (NC). Inflammation and oxidative stress of H9c2 cells were detected by Western blot, cell staining, reactive oxygen species (ROS) quantification, and SOD activity assay. At last, miR-181a-5p expression in BMSCs, exosomes, and H9c2 cells was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RESULTS: The results revealed that the expression of miR-181a-5p was increased in LPS-stimulated BMSCs (L-BMSC) and in their secreted exosomes. Besides, the expressions of TNF-α and IL-1β were decreased, while those of SOD1 and SOD2 were increased in H9c2 cells co-cultured with exosomes secreted by LPS-stimulated BMSCs (L-EXO) and transfected with MIM. Moreover, the fluorescence intensity of IL-1β immunofluorescence was decreased in H9c2 cells co-cultured with L-EXO or transfected with MIM. The level of ROS was also decreased in H9c2 cells co-cultured with L-EXO or transfected with MIM. Furthermore, miR-181a-5p was found to target ATF2 through target gene prediction databases and Western blot and Dual-Luciferase reporter assays.

CONCLUSIONS: LPS stimulation can increase the expression of miR-181a-5p in BMSCs, and miR-181a-5p inhibits myocardial inflammation and oxidative stress by targeting ATF2.

Key Words:
Myocardial infarction, MiR-181a-5p, ATF2, Bone marrow mesenchymal stem cells, Exosome.

Introduction

Myocardial infarction (MI) has become an important cause of increasing mortality in cardiovascular disease[1]. Heart failure is a common complication of myocardial infarction and the leading cause of death[2]. MI is generally considered to be a coronary artery disease that causes inflammation and oxidative stress of the heart muscle, which leads to fibrosis of the heart muscle and pathological remodeling of the heart, thus ultimately causing heart failure[3]. Therefore, inhibition of myocardial oxidative stress and inflammation at the early stage of myocardial infarction is the key to preventing myocardial fibrosis.

Bone marrow mesenchymal stem cells (BMSCs) are a subpopulation of cells that differentiate into osteochondral, adipose, and neural cells, as well as myoblasts in the bone marrow stroma[4]. Muller et al[5] have found that bone marrow-derived stem cells have an effect on myocardial

Corresponding Author: Yunfeng Hou, BM; e-mail: 258370615@qq.com

repair. After MI, the myocardium will mobilize stem cells from bone marrow to regenerate into new tissue through factor secretion or activate myocardial repair *via* the paracrine pathway[6]. As important paracrine factors, exosomes play an important role in myocardial repair, which has attracted extensive attention of scientists[7]. The therapeutic effect of exosomes in myocardial ischemia has been clearly demonstrated[8]. Exosomes secreted by mesenchymal stem cells can enhance myocardial repair[9]. Whether exosomes secreted by BMSCs, a precursor group of circulating immune cells, in normal physiological state and in the environment of myocardial inflammation play different roles in the process of myocardial repair has not yet been clear[10]. Hence, lipopolysaccharide (LPS) was used to stimulate BMSCs to mimic the inflammatory environment, and the effects of exosomes secreted by LPS-stimulated BMSCs on myocardium were studied.

MicroRNAs (miRNAs) are small RNAs of non-coding proteins produced by a class of organisms[11]. They are widely involved in life processes and disease development by regulating the expression of gene transcription and translation[12]. Bernardo et al[13] have found that miRNAs are closely related to the pathophysiology of cardiovascular diseases, such as myocardial inflammation, oxidative stress, fibrosis, and cardiac hypertrophy. MiR-181a-5p has been extensively investigated in many diseases. Xue et al[14] showed that oxidative stress in osteoarthritis can be inhibited by miR-181a-5p. Su et al[15] believed that vascular inflammation could be inhibited by miR-181a-5p. However, miR-181a-5p has not been studied in myocardial inflammation and oxidative stress. It has been detected that exosomes contain a large number of miRNAs, and exosomes can exert different functions by transferring miRNAs between cells[16]. Therefore, this article aims to investigate the effect of LPS-stimulated BMSCs-derived exosomal miR-181a-5p on myocardial inflammation and oxidative stress.

Patients and Methods

BMSCs Isolation and Culture

After approval by the Local Medical Ethics Committee, BMSCs were extracted from the bone marrow of 4 donors from The First Affiliated Hospital of Shandong First Medical University by gradient centrifugation. Dulbecco's Modified Eagle's Medium (DMEM) low-glucose medium (Life Technology, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Life Technology, Gaithersburg, MD, USA) was used for primary culture in a 25 cm^2 culture bottle. After cell density reached 90%, the BMSCs were digested and subcultured with 0.25% trypsin containing ethylenediaminetetraacetic acid (EDTA; Life Technology, Gaithersburg, MD, USA).

Exosomes Isolation and Identification

BMSCs were subcultured in a culture plate with a diameter of 10 cm. When the cell density was about 80%, DMEM low-glucose medium containing 10% exosome free FBS (Life Technology, Gaithersburg, MD, USA) was used for culture. LPS (0.5 mg/L) was added in experimental group but not in the control group. The cell culture supernatant was collected after 48 h, and 2 mL of exosome extraction reagents (RiboBio, Guangzhou, China) were added to the collected cell culture supernatant at a ratio of 5:1 (cell supernatant: extract). After standing at 4°C for more than 12 h, centrifugation was conducted at 1500 g for 30 min, and then, the supernatant was discarded. Then, 1 mL of liquid was obtained and centrifuged again at 1500 g for 5 min, followed by complete discarding of the supernatant, and the resulting precipitate was the exosomes derived from BMSCs.

Western blot was utilized to detected surface markers of exosomes (Tsg101, CD63, CD81). The morphology of exosomes was observed by TEM (Olympus, Tokyo, Japan).

H9c2 Cells Culture and Transfection

H9c2 cells purchased from Procell (Wuhan, China) were cultured in DMEM containing 10% FBS with exosomes for 24 hours. Then, H_2O_2 (100 μM) was utilized to treat the H9c2 cells for 4 hours.

MIM or NC (RiboBio, Guangzhou, China) was transfected into H9c2 cells according to the protocols. After 24 hours, the H9c2 cells were also treated with H_2O_2 (100 μM, 4 h).

Western Blot

The total protein was obtained using a protein extraction kit (KeyGen, Shanghai, China). The concentration of protein was measured by bicinchoninic acid (BCA) protein quantitative analysis kit (Thermo Fisher, Waltham, MA, USA). The loading buffer (EpiZyme, Shanghai, China) was added to the total protein we bioled the mixture for 10 minutes. Sodium dodecyl sulphate-poly-

acrylamide gel electrophoresis (SDS-PAGE) gel preparation kit (Beyotime, Shanghai, China) was used to dispense gel. 30 μg of total proteins were taken for electrophoresis. After electrophoresis, the protein on the gel was transferred to polyvinylidene difluoride (PVDF; EpiZyme, Shanghai, China). Then, 5% skim milk was used to block the non-specific antigen on the protein band. After that, the primary antibodies (CD29, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; CD44, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; CD73, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; CD34, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; CD45, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; Tsg101, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; CD63, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; CD81, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; TNF-α, Abcam, Cambridge, MA, USA, Mouse, 1:1000; IL-1β, Abcam, Cambridge, MA, USA, Mouse, 1:1000; SOD1, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; SOD2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; ATF2, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; GAPDH, ProteinTech, Rosemont, IL, USA, 1:1000) were used to incubate the bands overnight at 4°C. The next day, the bands were washed with Tris Buffered Saline and Tween-20 (TBST) for half an hour, followed by incubation with the secondary antibody for 1.5 hours and washing with TBST for 1 hour. Image Lab™ Software (Bio-Rad, Hercules, CA, USA) was used to expose the bands.

RNA Extraction and Real Time-PCR

TRIzol reagent (MCE, Nanjing, China) was utilized to extract the total RNA of BMSCs and H9c2 cells, and exosomal RNA extraction kit (EZBioscience, Shanghai, China) was used for extraction of total RNA from exosomes. The cDNA was synthesized using Reverse transcription kit (Roche, Basel, Switzerland). SYBR Green qPCR Master Mix (MCE, Nanjing, China) was used for qRT-PCR. Prism 7900 System (Applied Biosystems, Foster City, CA, USA) was utilized to perform qRT-PCR. MiR-181a-5p expression in cells and exosomes was normalized using U6 and cel-miR-39 separately. All the primers were listed in Table I.

Superoxide Dismutase (SOD) Activity Assay

H9c2 cells were lysed with lysate, collected, and centrifuged to remove the supernatant. Detection of SOD levels in cells was performed by the SOD assay kit (KeyGen, Shanghai, China) in accordance with the protocol.

IL-1β Immunofluorescence

H9c2 cells were placed in a 24-well plate, fixed with 4% paraformaldehyde after different treatment, then, added with goat serum and incubated for 1 h. After that, an appropriate amount of primary antibody IL-1β was added for incubation overnight at 4°C. On the next day, the fluorescent secondary antibody was added in for 1 hour in the dark. DAPI (KeyGen, Shanghai, China) was added to stain the nucleus. Finally, it was observed by a Confocal Laser Scanning Microscope (CLSM) (Leica, Wetzlar, Germany).

ROS Quantification

DHR-ROS test kit (KeyGen, Shanghai, China) was used to detect the expression of ROS in accordance with the protocol.

Luciferase Activity Assay

The plasmids which contained wild-type (WT) or mutant (MUT) 3′-UTR of ATF2 were co-transfected with MIM or NC into HEK293T cells. After two days, the Luciferase reagent (RiboBio, Guangzhou, China) was added according to the protocols and the luciferase activity was detected using Dual-Glo® Luciferase Assay System.

Statistical Analysis

Data were expressed as $\bar{\chi}\pm s$. All data were plotted using GraphPad Prism5 software (La Jolla, CA, USA). The differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Test level $\alpha=0.05$.

Results

Identification of BMSCs and Exosomes

Western blot was used to detect some surface markers of BMSCs (Figure 1A). The expressions of stem cell-specific surface markers CD29, CD44, CD73 were increased, and the expressions of hematopoietic cells-specific surface markers CD34, CD45 were decreased in MSCs. These illustrated the successful separation of BMSCs. The expressions of surface markers of exosomes derived from BMSCs were also detected by Western blot (Figure 1B). It can

Table I. Real time-PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
MiR-181a-5p	GCAACATTCAACGCTGTCG	CGGCCCAGTGTTCAGACTAC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
Cel-miR-39	GCGGTCACCGGGTGTAAATC	GTGCAGGGTCCGAGGT

qRT-PCR, quantitative reverse-transcription polymerase chain reaction.

be seen that Tsg101, CD63, and CD81 were all positive. Under TEM, cystic vesicles of varying diameters were observed to be round or oval in shape (Figure 1C).

L-EXO Inhibited H_2O_2-Induced Inflammation and Oxidative Stress of H9c2 Cells

H9c2 cells were co-cultured with L-EXO for 24 hours, and then, treated with H_2O_2 for 4 h. Compared with exosomes derived from BMSCs without LPS stimulation (N-EXO), L-EXO could significantly reduce the expression of TNF-α and IL-1β (Figure 2A-2C). Immunofluorescence showed that IL-1β expression in the H_2O_2+L-EXO group was significantly decreased compared with the H_2O_2+N-EXO group (Figure 2D). The expressions of SOD1 and SOD2 were markedly increased after the treatment with L-EXO compared with the treatment with N-EXO (Figure 2E-2G). At the same time, the SOD activity assay was used to detect the level of SOD. The results showed that L-EXO could markedly reverse the decrease of SOD levels induced by H_2O_2 (Figure 2H). Finally, the level of ROS production was also detected by DHR dye assay. It could be seen from Figure 2I that the treatment of H_2O_2 increased the level of ROS production, while in the H_2O_2+L-EXO group, the level of ROS production was significantly decreased. These results indicate that L-EXO can significantly inhibit the inflammation and oxidative stress of cardiomyocytes compared with N-EXO.

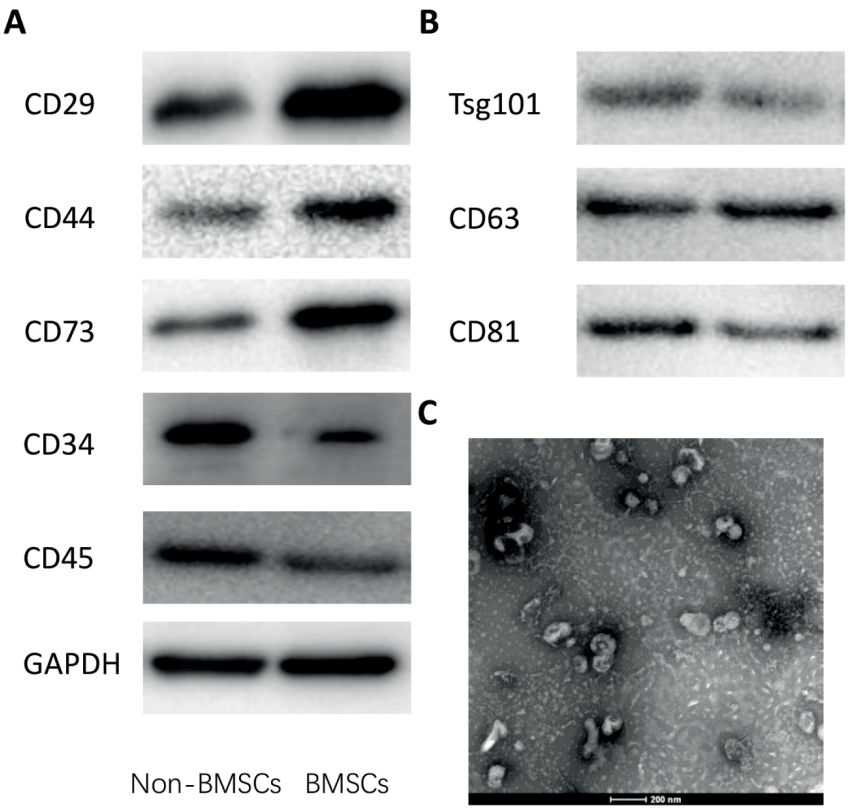

Figure 1. Identification of BMSCs and exosomes. **A,** Western blot analysis of the surface markers in BMSCs. **B,** Western blot analysis of the surface markers in exosomes. **C,** Ultrastructure of exosomes analyzed by TEM (magnification: 49,000x).

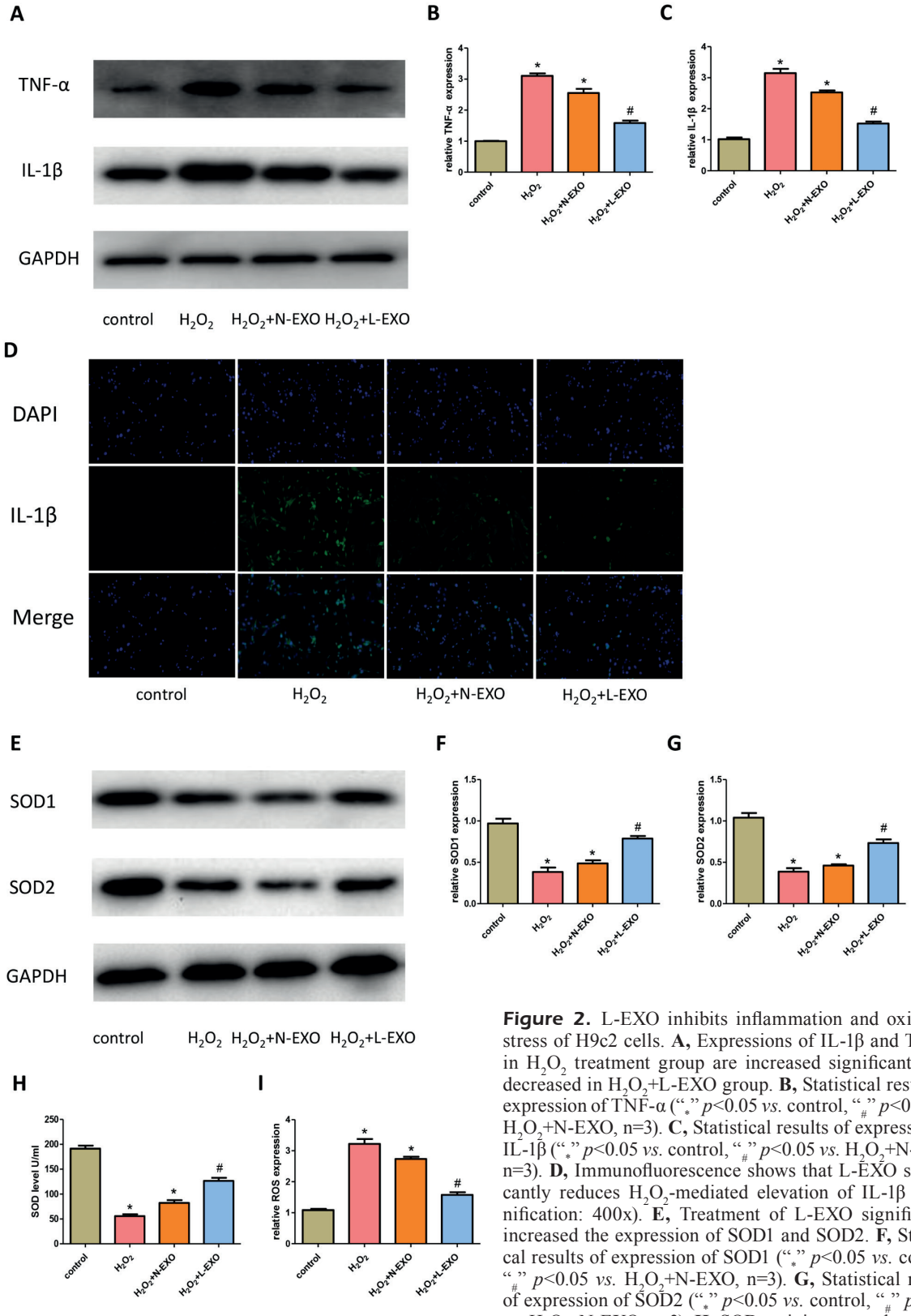

Figure 2. L-EXO inhibits inflammation and oxidative stress of H9c2 cells. **A,** Expressions of IL-1β and TNF-α in H_2O_2 treatment group are increased significantly but decreased in H_2O_2+L-EXO group. **B,** Statistical results of expression of TNF-α ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* H_2O_2+N-EXO, n=3). **C,** Statistical results of expression of IL-1β ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* H_2O_2+N-EXO, n=3). **D,** Immunofluorescence shows that L-EXO significantly reduces H_2O_2-mediated elevation of IL-1β (magnification: 400x). **E,** Treatment of L-EXO significantly increased the expression of SOD1 and SOD2. **F,** Statistical results of expression of SOD1 ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* H_2O_2+N-EXO, n=3). **G,** Statistical results of expression of SOD2 ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* H_2O_2+N-EXO, n=3). **H,** SOD activity assay shows that H_2O_2 can significantly reduce SOD levels, while L-EXO can reverse SOD levels ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* H_2O_2+N-EXO, n=3). **I,** Expression of ROS is increased in the H_2O_2 treatment group but decreased significantly in the H_2O_2+L-EXO group ("$_*$" $p<0.05$ *vs.* control, "$_\#$" $p<0.05$ *vs.* H_2O_2+N-EXO, n=3).

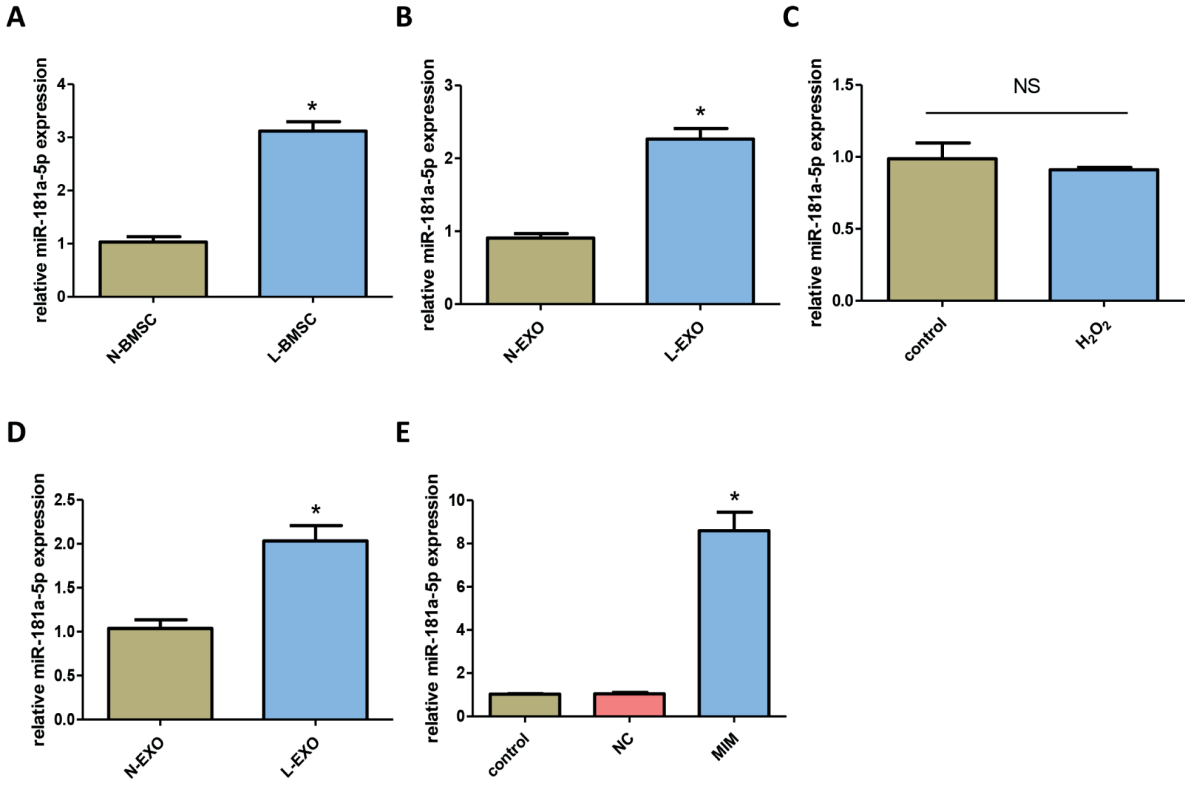

Figure 3. The expression of miR-181a-5p is increased in L-BMSC and L-EXO. **A,** The stimulation of LPS increases the expression of miR-181a-5p in L-BMSC ("*" $p<0.05$ *vs.* N-BMSC, n=3). **B,** The expression of miR-181a-5p in L-EXO is also increased ("*" $p<0.05$ *vs.* N-EXO, n=3). **C,** H_2O_2 treatment does not change the expression of miR-181a-5p in H9c2 cells. **D,** Co-culture with L-EXO increases the expression of miR-181a-5p in H9c2 cells ("*" $p<0.05$ *vs.* N-EXO, n=3). **E,** MIM transfection significantly increases the expression of miR-181a-5p ("*" $p<0.05$ *vs.* NC, n=3).

The Expression of MiR-181a-5p Increased In L-BMSC and L-EXO

First, qRT-PCR was used to detect the expression of miR-181a-5p in BMSCs. Compared with BMSCs without stimulation of LPS (N-BMSC), stimulation of BMSCs with LPS greatly increased miR-181a-5p expression (Figure 3A). Similar to L-BMSC, miR-181a-5p expression in L-EXO was also increased (Figure 3B). Then, the expression of miR-181a-5p in H9c2 cells was detected, and it was found that H_2O_2 treatment did not alter its expression (Figure 3C). When H9c2 cells were co-cultured with L-EXO, miR-181a-5p expression was markedly increased (Figure 3D). Therefore, it was believed that H9c2 cells took up exosomes and upregulated miR-181a-5p. To explore the function, MIM or NC was transfected into H9c2 cells. MIM greatly increased miR-181a-5p expression (Figure 3E).

MiR-181a-5p Overexpression Inhibited H2O2-Induced Inflammation and Oxidative Stress of H9c2 Cells

MIM was transfected into H9c2 cells for 24 hours and then H9c2 cells were treated with H_2O_2 for 4 hours. Similarly, the expressions of TNF-α, IL-1β, SOD1, and SOD2 were detected by Western blot. Overexpression of miR-181a-5p markedly reduced the expressions of TNF-α and IL-1β (Figure 4A-4C) but increased the expressions of SOD1 and SOD2 (Figure 4E-4G). Immunofluorescence showed that IL-1β expression was greatly reduced in MIM group (Figure 4D). Using SOD activity assay, the level of SOD was greatly increased after treatment with MIM (Figure 4H). Using DHR-ROS test kit, the expression of ROS was found to be reduced after treated with MIM (Figure 4I).

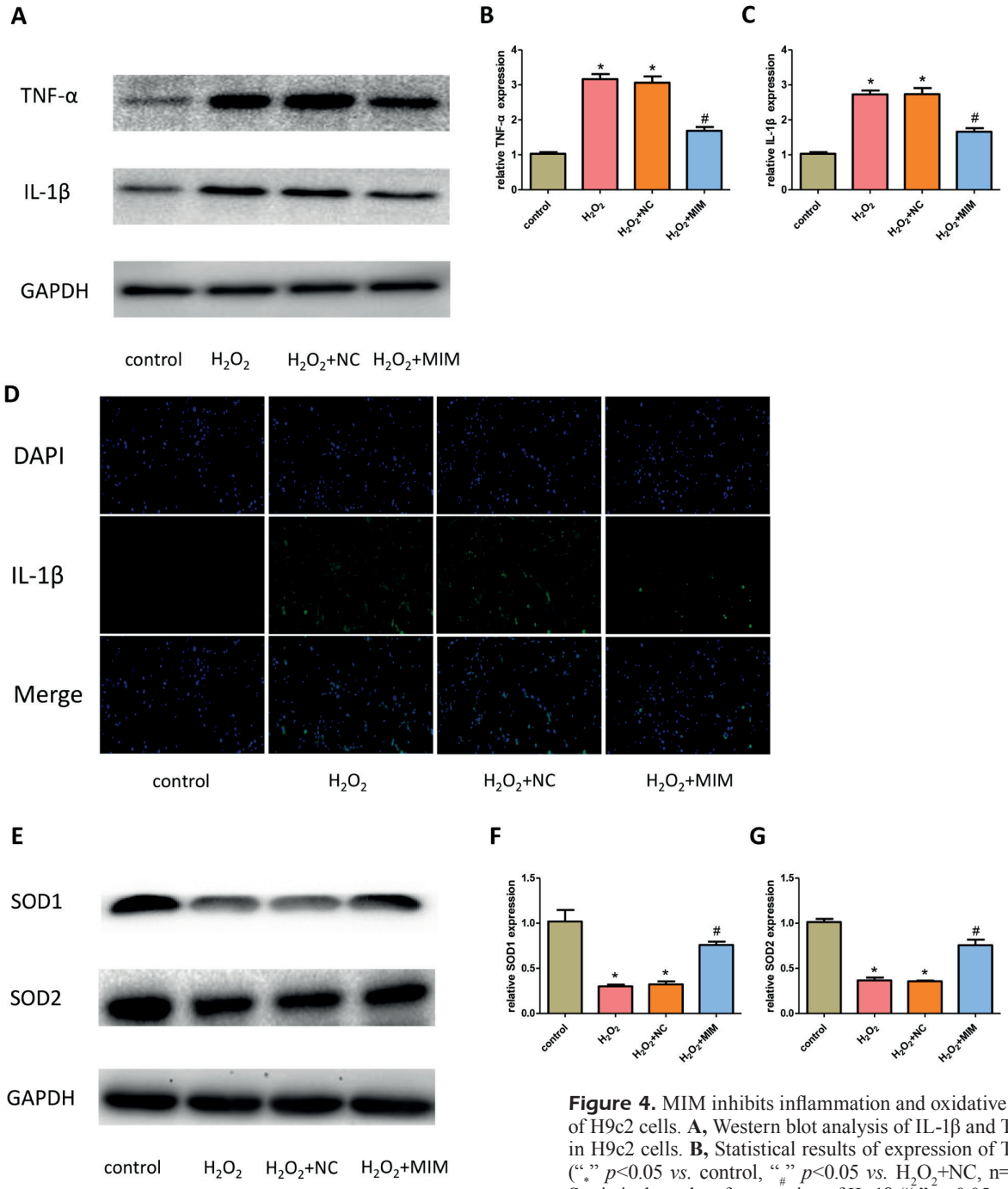

Figure 4. MIM inhibits inflammation and oxidative stress of H9c2 cells. **A,** Western blot analysis of IL-1β and TNF-α in H9c2 cells. **B,** Statistical results of expression of TNF-α ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **C,** Statistical results of expression of IL-1β ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **D,** Immunofluorescence shows that MIM significantly reduces H_2O_2-mediated elevation of IL-1β (magnification: 400x). **E,** Western blot analysis of SOD1 and SOD2 in H9c2 cells. **F,** Statistical results of expression of SOD1 ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **G,** Statistical results of expression of SOD2 ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **H,** SOD activity assay shows that MIM can increase SOD levels ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **I,** The expression of ROS is increased in the H_2O_2 treatment group, but decreased significantly in the H_2O_2+NC group ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3).

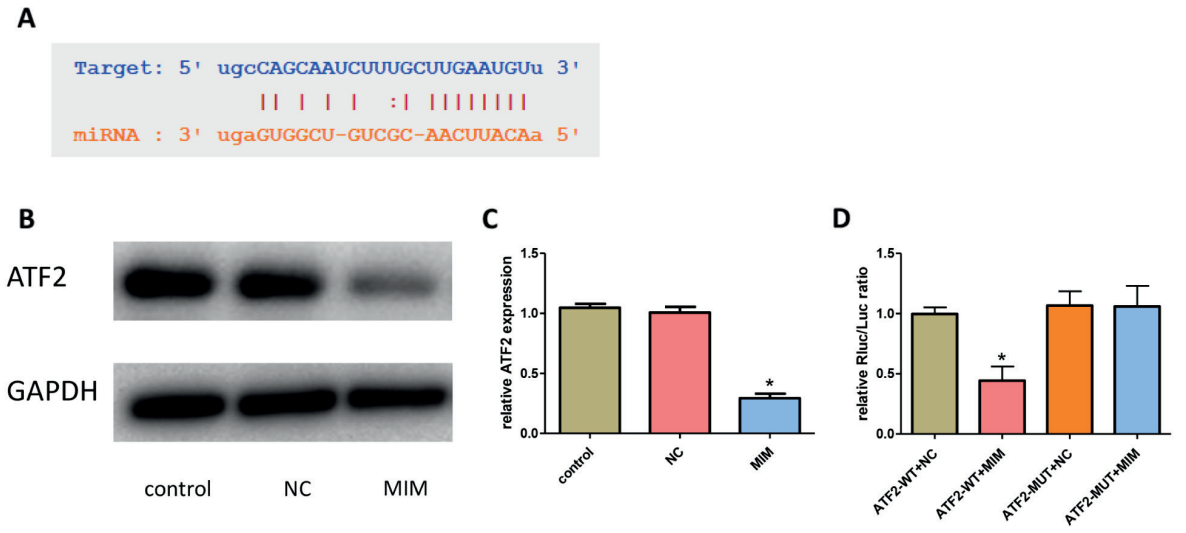

Figure 5. MiR-181a-5p directly targets ATF2. **A,** Binding site predicted by the StarBase database. **B,** Western blot shows that the expression of ATF2 is decreased in H9c2 cells transfected with MIM. **C,** Statistical results of expression of ATF2 ("*" $p<0.05$ *vs.* NC, n=3). **D,** MIM significantly decreases the relative Luciferase activity in WT group but does not decrease the relative luciferase activity in MUT group ("*" $p<0.05$ *vs.* ATF2-WT+NC, n = 3).

MiR-181a-5p Inhibited H2O2-Induced Inflammation and Oxidative Stress by Targeting ATF2

To study the potential mechanism by which miR-181a-5p can inhibit inflammation and oxidative stress, the target gene of miR-181a-5p was predicted through databases. ATF2 was predicted to have a binding site with miR-181a-5p (Figure 5A). MIM could markedly reduce the expression of ATF2 (Figure 5B and 5C). Besides, miR-181a-5p overexpression greatly inhibited the Luciferase activity in WT group but failed in mutant (MUT) group (Figure 5D). These demonstrate that miR-181a-5p can target ATF2 to reduce ATF2 expression.

Discussion

Inflammatory response and oxidative stress after MI are key processes leading to cardiac damage, repair, and remodeling[17]. How to effectively improve myocardial inflammation and oxidative stress after MI has become an urgent problem to be solved.

MSCs have the ability of self-renewal, self-replication, etc., and have broad clinical application background in inflammation and immune regulation[18]. Ischemic myocardium secreting chemokines and cytokines after MI mobilize stem cells and progenitor cells in the bone marrow to enrich the myocardial tissue[19]. The mechanism by which BMSCs repair myocardial damage is not fully understood. In recent years, the paracrine effect of BMSCs has received extensive attention, especially exosomes. They are special paracrine factors in a nanoscale size and abundant content (lipids, proteins, mRNA, miRNAs), enabling them to mediate information exchange between cells and tissues at close and long distances[6]. The strategy of pretreatment of BMSCs by LPS was used in this study for two reasons. One was to simulate the effect of inflammatory environment after MI on the secretion of exosomes by BMSCs *in vitro*. Second, some pretreatment, such as LPS, can improve the biological and functional characteristics of BMSCs exosomes to some extent[20]. The results of this study also showed that although normal BMSCs-derived exosomes can also reduce myocardial inflammatory factor levels and oxidative stress levels, LPS-stimulated BMSCs-derived exosomes had a more significant inhibitory effect on inflammation and oxidative stress because the stimulation of LPS changed the contents of the exosomes, including miR-181a-5p.

To further observe the function of miR-181a-5p, miR-181a-5p was overexpressed *in vitro*. MiR-181a-5p overexpression was also found to inhibit myocardial inflammation and oxidative stress. Further studies have shown that miR-181a-5p can target ATF2. ATF2 is an active transcription fac-

tor of the ATF/CREB family[21], which plays a role in various physiological and pathological processes, such as inflammation, proliferation, apoptosis, etc. In this study, it was found that LPS stimulation could greatly upregulate miR-181a-5p expression in BMSCs and their secreted exosomes, and exosomes can transfer miR-181a-5p to H9c2 cells, so as to improve the expression of miR-181a-5p in myocardial cells, and then, inhibit the expression of ATF2, so as to play a role in inhibiting inflammation and oxidative stress.

Conclusions

Our results showed that LPS stimulation can increase the expression of miR-181a-5p in BMSCs and exosomes secreted by BMSCs, and miR-181a-5p inhibits myocardial inflammation and oxidative stress by targeting ATF2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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