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Original Article

Endothelial-derived exosomes induced by lipopolysaccharide alleviate rat cardiomyocytes injury and apoptosis

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Abstract: Sepsis-induced myocardial dysfunction (SIMD) is one of the leading causes of death in sepsis. We hypothesized that exosomes released from ECs exposed to bacterial lipopolysaccharides (LPS) have some regulatory effect on cardiomyocytes (CMs). In this study, cultured rat ECs were exposed to 0.5 μg/ml of LPS, and exosomes were isolated from the conditioned medium through ultra-high-speed centrifugation. The exosomes were given to the cultured neonatal rat CMs to test the potential effects and proceed to small RNA sequencing to identify their miRNA expression. We found exosomes from ECs under LPS stimulation (^{LPS}-EC-Exo) enhanced the cell viability and attenuated the injury of CMs. The RNA sequencing depicted the expression of several miRNAs increased in ^{LPS}-EC-Exo compared with the exosomes from the control ECs (^{NC}-EC-Exo). Further analysis showed that some miRNAs could promote the survival of CMs by down-regulating the expression of apoptosis-related proteins such as BAK1, P53, and PTEN. This study showed that ^{LPS}-EC-Exo has a cardiac protective effect on CMs, which miRNAs may achieve.

Keywords: Sepsis-induced myocardial dysfunction, endothelial cells, exosome, miRNA, cardiomyocytes

Introduction

Sepsis is a critical life-threatening situation that resulted from the severe response of the body to an infection. In sepsis, chemicals such as cytokines are released from the host body to confrontation the infection. When the body's reaction to the chemicals is out of balance, multiple organ dysfunction could happen.

The heart is one of the most vulnerable target organs for sepsis. Even in the early stages of sepsis, tissue injury occurs in the heart, manifested as microvascular rupture, inflammatory cell infiltration, and focal myocardial necrosis [1, 2]. Therefore, sepsis can lead to myocardial dysfunction (SIMD) [3], which is manifested by contractile dysfunction, dilated cardiomyopathy, and decreased ejection fraction [4]. SIMD leads to a significant elevation in mortality [4]. The pathogenesis of SIMD includes myocardial inhibitors, myocardial energy metabolism disorders, oxygen-free radicals damage, cardiomyocyte apoptosis, abnormal gene regulation, cardiac renin-angiotensin system disorder, and autonomic nerve dysfunction, etc. [5].

Endothelial cells (ECs) are located on the blood vessel's inner side and contact the blood. In sepsis, ECs directly expose to bacterial metabolites or lysates and play a crucial role in the body's inflammatory response. ECs regulate adjacent cells' function through paracrine, and exosome transportation is one of the essential paracrine pathways. Exosomes are extracellular vesicles (EVs) with a diameter of 30 to 150 nm [6-8], which contain molecular components of the cells of their origin, including proteins and RNAs [9]. We isolated exosomes from bacterial lipopolysaccharide (LPS)-stimulated rat

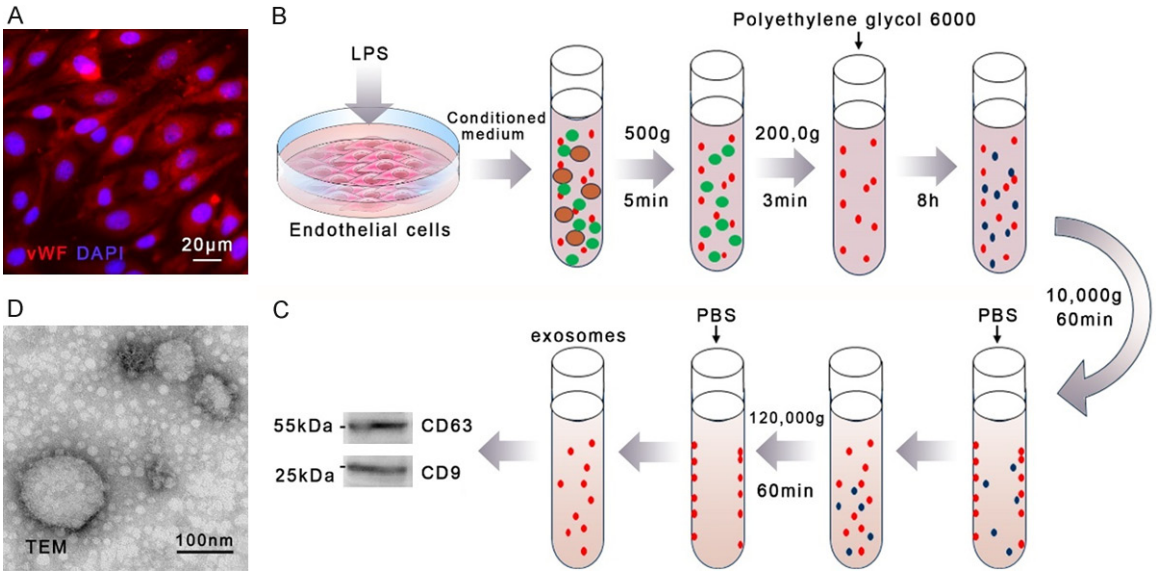

Figure 1. The isolation and identification of rat endothelial cell-derived exosomes. A. Rat endothelial cells (ECs) were isolated from the thoracic aorta and identified with the von Willebrand factor (VWF) antibody. B. ECs were stimulated with 0.5 μg/ml of lipopolysaccharides (LPS) for 6 h. The conditioned medium of ECs was harvested and proceeded to isolated exosomes following the steps demonstrated in the illustration. C. Vesicles were identified with the CD63 and CD9 antibodies. D. The shape and size of endothelial cell-derived exosomes were observed through a transmission electron microscope (TEM). *n*=5.

ECs and used to culture neonatal rat myocardial cells (CMs) to study the biological effects of EC-derived exosomes on CMs.

Material and methods

Ethical approval

The experimental animal procedures conformed to the Guide and Care and Use of Laboratory Animals (the US National Institutes of Health, 1996). The Animal Care and Use Committee of Southwest Medical University approved the experiment.

Primary endothelial cell isolation and cell culture

Male Sprague Dawley rats (8-week-old, bodyweight between 250-300 g) were anesthetized with 120 mg/kg of sodium pentobarbital and sacrificed. The thoracic aorta was harvested under a sterile circumstance, and the adventitia was removed. The artery was cut open, and the vessel's inner side was digested with 0.1% type I collagenase. Endothelial cells (ECs) were isolated from the cell suspension and cultured in EC medium (ScienCell, Carlsbad, CA, USA) for 5-7 d to obtain primary cells. The anti-von Willebrand factor antibody (Abcam, Cambridge, UK) was used to identify ECs (**Figure 1A**), and ECs of passage 4-7 were used in subsequent experiments.

Endothelial derived-exosomes isolation and identification

ECs were seeded in the 100 mm cell culture dish to a confluency of 70% to 80%. Cells were synchronized in serum-free M199 for 12 h. Lipopolysaccharides (LPS) (MilliporeSigma, Burlington, MA, USA) solution was added into the experimental group's EC culture medium to a final concentration of 0.5 μg/ml. An equal volume of PBS was added into the medium of the control group as a negative control. After 6 hours, the conditioned medium of ECs was collected. The conditioned medium was centrifuged at 500 g for 5 min at 4°C and 2000 g for 30 min to remove cell debris, larger microvesicles, and apoptotic bodies. Polyethylene glycol 6000 (PEG) was added into the supernatant to a final concentration of 8%. The mixture was well mixed to enrich the vesicle and stood for 8 h at 4°C. The hybrid solution was centrifuged at 10,000 g for 60 min at 4°C, and the supernatant containing excess PEG was discarded. Rinse the tube wall with PBS to resuspend exo-

somes. Centrifuge the suspension again at 120,000 g for 60 min at 4°C and discard the supernatant to further remove the PEG. Finally, the tube's wall was rinsed with 100 ul of PBS to resuspend the exosomes (**Figure 1B**). A transmission electron microscope (Hitachi H-9500) was used to detect the shape and size of exosomes. The anti-CD63 antibody (R&D Systems) and anti-CD9 antibody (Abcam) were used to identify the vesicles and their origin of ECs.

Primary cardiomyocytes isolation and cell culture

The heart of the neonatal Sprague Dawley rat was removed in a sterile environment and cut into small pieces. The tissue mass was digested with 0.25% trypsin and 0.1% type II collagenase successively, and the supernatant was collected. The isolated cell collected by centrifuging the cell suspension then cultured in DMEM Complete Medium for 1 d to harvest primary cells. The anti-alpha-actin antibody (Abcam, Cambridge, UK) was used to identify cardiomyocytes (CMs) (Cell Signaling Technology, Danvers, MA, USA) (**Figure 2A**). Primary CMs were used in subsequent experiments.

Incubation of rat cardiomyocytes with endothelial cell-derived exosomes

We seeded 1×10^5 CMs in the 15.6 mm culture plate with 400 ul DMEM Complete Medium. 1×10^6 ECs were cultured in the 100 mm culture dish with 5 ml of culture medium, and endothelial-derived exosomes (EC-Exo) were collected as previously described. Then the collected exosomes were resuspended in 100 ul PBS. Finally, the exosomes-containing resuspension was added to the culture medium of CMs and incubated for 24 h (**Figure 2B**).

Cell counting kit-8 (CCK-8), LDH-cytotoxicity, reactive oxygen species (ROS) and superoxide dismutase (SOD) activity assay of cardiomyocytes

CMs incubated with EC-Exo were tested for CCK8 cell viability, LDH-cytotoxicity, ROS, and SOD activity according to the manufacturer's instructions (Boster Biological Technology, China).

Western blot

RIPA buffer was used to lyse cardiomyocytes to extract total protein. The total protein was separated by 10% SDS-PAGE electrophoresis. The levels of PCNA, Bcl2, Bax, cleaved caspase-3, BAK1, P53, and PTEN were detected by western blot with the anti-PCNA antibody (Cell Signaling Technology), anti-Bcl2 antibody (Abcam), anti-Bax antibody (Abcam), anti-caspase-3 antibody (Cell Signaling Technology), anti-BAK1-antibody (Cell Signaling Technology), anti-P53-antibody (Cell Signaling Technology), and anti-PTEN-antibody (Cell Signaling Technology). GAPDH was used as an internal reference. Images were analyzed by Quantity One 4.6 (Bio-Rad, Hercules, CA, USA).

5-ethynyl-20-deoxyuridine (EdU) incorporation assay

Neonatal rat CMs were cultured in 96-well plates to 70% confluency. According to the manufacturer's instructions, an EdU kit (Ribio, Guangzhou, China) was used to detect the level of nucleic synthesis in CMs. Observe the number of fluorescently labeled CMs under a fluorescence microscope (Olympus TH-4-200) to calculate the proliferation rate.

microRNA sequencing of exosomes and analysis of results

Exosomes extracted from the conditioned medium of ECs were subjected to the miRNA sequencing (Kangchen Biotech, Shanghai, China). The miRNAs with the absolute value of expression fold change >2 and p value <0.05 between the exposed group (^{LPS}-EC-Exo) and the control group (^{NC}-EC-Exo) were considered as differentially expressed miRNAs (DE miRNAs). The expressions of DE miRNAs in the exosomes were validated by quantitative real-time PCR using a miScript II RT Kit, a miScript SYBR PCR kit (Qiagen, Hilden, Germany), and synthetic primers (Ribio). The targets of the DE miRNAs were predicted with TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org) database. The functional clustering of target genes was performed using DAVID v6.8 (Visualization and Integrated Discovery, https://david.ncifcrf.gov/). The signal pathways of target genes may participate in were forecasted using the KEGG Pathway (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg/).

Statistical analysis

All data were expressed as the mean ± SEM. The results were processed with GraphPad

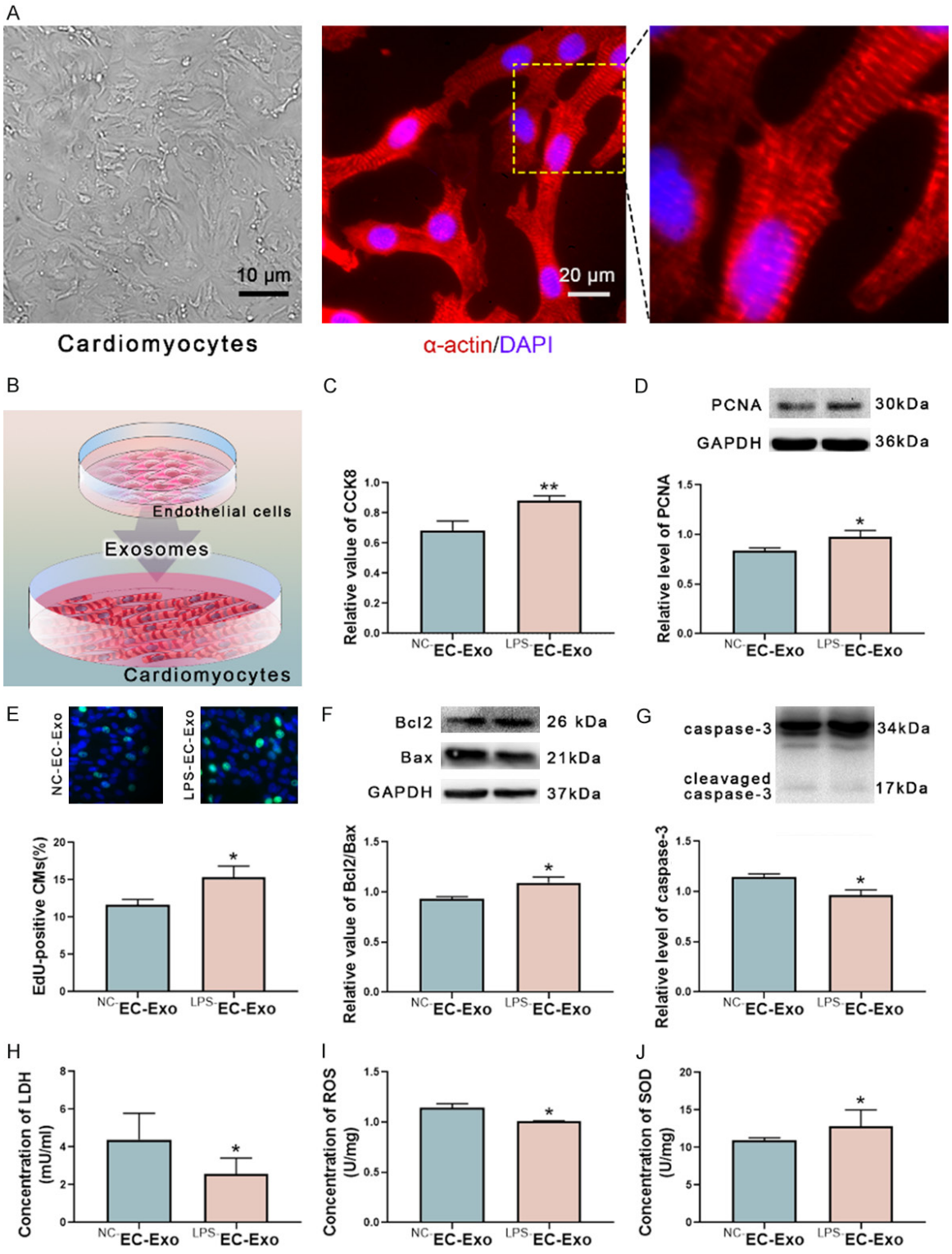

Figure 2. Endothelial cell-derived exosomes alleviate rat cardiomyocytes injury and apoptosis. A. The CMs were harvested from neonatal rats and identified with the α-actin antibody. B. The exosomes derived from ECs (EC-Exo) were isolated from the conditioned medium and added into the medium of CMs to test the biological effects. C. The exosomes from LPS-stimulated ECs (^{LPS}EC-Exo) increased the cell viability of CMs compared to the exosomes from normal ECs (^{NC}EC-Exo). D. ^{LPS}EC-Exo elevated the level of proliferating cell nuclear antigen (PCNA) of CMs. E. EdU (5-ethynyl-2'-deoxyuridine) incorporation assay demonstrated that the cell viability of CMs was up-regulated under

the $^{LPS-}$EC-Exo. F. The ratio of Bcl2 and Bax was raised under the induction of $^{LPS-}$EC-Exo. G. $^{LPS-}$EC-Exo reduced the level of cleaved caspase-3 of CMs. H. $^{LPS-}$EC-Exo down-regulated the level of lactate dehydrogenase (LDH) of CMs. I. $^{LPS-}$EC-Exo decreased the level of reactive oxygen species (ROS) of CMs. J. $^{LPS-}$EC-Exo enhanced the level of superoxide dismutase (SOD) of CMs. n=7, *P<0.05, **P<0.01.

Prism 8.0.1. Two-tailed Student's t-test was used to analyze the data from two groups. Bonferroni-corrected p-value <0.05 was statistically significant.

Results

Morphology and origin identification of endothelial cell-derived exosomes

ECs in the exposed group were stimulated with 0.5 μg/ml of LPS, while ECs in the control group were given an equal volume of PBS. Exosomes were isolated from the conditioned medium of ECs by polyethylene glycol (PEG) separation and ultracentrifugation. The extracted vesicles expressed CD63 and CD9 (**Figure 1C**). The transmission electron microscope showed that the extract contained many round vesicle structures with a diameter of 30-150 nm (**Figure 1D**). It is worth noting that when the LPS concentration was gradually increased to 10 μg/ml, exosomes produced by ECs decreased significantly, while the number of apoptotic bodies (ABs) with larger diameters increased markedly.

Exosomes from LPS-exposed ECs increased the viability and proliferation of neonatal rat cardiomyocytes

Cell Counting Kit-8 (CCK-8) assay showed that compared with the exosomes from the normal ECs ($^{NC-}$EC-Exo), the exosomes from LPS-exposed ECs ($^{LPS-}$EC-Exo) increased the viability of neonatal rat CMs (**Figure 2C**). Detection of the level of proliferating cell nuclear antigen (PCNA) and 5-ethynyl-20-deoxyuridine (EdU) incorporation assay showed that $^{LPS-}$EC-Exo elevated the proliferation of CMs (**Figure 2D** and **2E**).

$^{LPS-}$EC-Exo alleviated the apoptosis of cardiomyocytes

Western blot showed that the ratio of Bcl2 and Bax (Bcl2/Bax) of CMs raised and the level of cleaved caspase-3 dropped under the action of $^{LPS-}$EC-Exo, which indicated that $^{LPS-}$EC-Exo reduced the apoptosis of CMs (**Figure 2F** and **2G**).

$^{LPS-}$EC-Exo decreased the level of LDH and ROS in cardiomyocytes, while inflated the level of SOD

Compared with $^{NC-}$EC-Exo, $^{LPS-}$EC-Exo decreased the levels of lactate dehydrogenase (LDH) and reactive oxygen species (ROS) while increased the level of superoxide dismutases (SOD) in CMs (**Figure 2H-J**). These indicated that $^{LPS-}$EC-Exo resisted oxidative stress to CMs.

miRNAs expression difference between $^{LPS-}$EC-Exo and $^{NC-}$EC-Exo

Detected and compared the miRNA expression profiles of $^{NC-}$EC-Exo and $^{LPS-}$EC-Exo by small RNA sequencing. miRNAs with an absolute expression fold change >2 and p-value <0.05 between two groups were considered as the differential expressed miRNAs. In $^{LPS-}$EC-Exo, 15 miRNA expression levels are higher than in $^{NC-}$EC-Exo, and 4 miRNA expression levels are lower than in $^{NC-}$EC-Exo (**Figure 3A**, **3B**; **Table 1**). The top 8 highly expressed miRNAs in $^{LPS-}$EC-Exo (miR-221-3p, miR-222-3p, miR-221-5p, miR-155-5p, miR-1247-3p, mir-129-5p, miR-148a-5p, and miR-222-5p) were detected by real-time quantitative PCR to confirm their expression differences between the groups. The PCR results were consistent with the sequencing data (**Figure 3C**).

Bioinformatic analysis of the differential expressed miRNAs

TargetScan and miRanda databases were used for the bioinformatics analysis to the top 8 miRNAs (miR-221-3p, miR-222-3p, miR-221-5p, miR-155-5p, miR-1247-3p, mir-129-5p, miR-148a-5p and miR-222-5p).

Gene Ontology (GO) analysis was performed with DAVID 6.8 to forecast the biological process (BP), cellular component (CC), and molecular function (MF) that the DE miRNAs may participate in. The result showed that the DE miRNAs had a high possibility of affecting cell

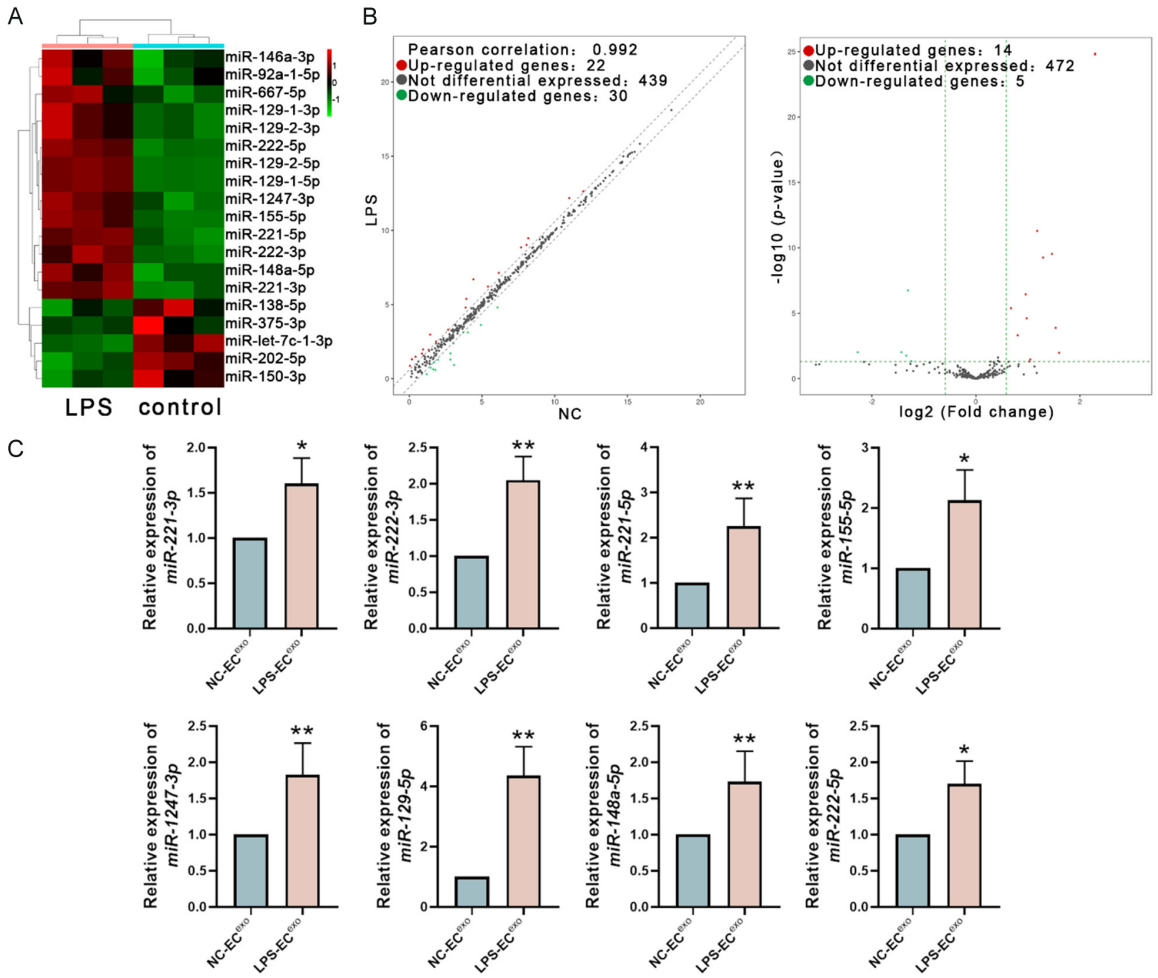

Figure 3. The analysis of miRNAs Carried by EC-Exo. A. The endothelial cell-derived exosomes were subjected to miRNA sequencing. The differential expressed miRNAs between ^{NC}EC-Exo and ^{LPS}EC-Exo (absolute value of the expression fold change >2.0, $P<0.05$) were identified. B. The differential expression of miRNAs in ^{NC}EC-Exo and ^{LPS}EC-Exo was shown in the volcano plots. C. The expression of the 8 miRNAs up-regulated in ^{LPS}EC-Exo was verified with real-time quantification PCR. The results of qPCR confirmed the data of miRNA sequencing. $n=7$, $*P<0.05$, $**P<0.01$.

metabolism. We evaluated the potential target genes of the DE miRNAs. We found that many of them were related to cardiomyocytes' function, such as regulating heart contraction, modulating heart rate, and cell communication involved in cardiac conduction (**Figure 4A**). Pathway analysis was performed on KEGG to predict the signal pathways that the DE miRNAs may participate in (**Figure 4B**). Twenty-one genes regulate cardiomyocytes' biological behavior through four pathways, including Adrenergic Signaling in Cardiomyocytes, Hypertrophic Cardiomyopathy, Arrhythmogenic Right Ventricular Cardiomyopathy, and Dilated Cardiomyopathy (**Table 2**).

The miRNAs highly expressed in ^{LPS}EC-Exo have extensive regulatory effects on cardiomyocytes. They play essential roles in physiological processes such as cardiac growth induced by physical exercise [10-13], embryonic-stem-cell-derived CMs maturation [14, 15]. They also take vital roles in pathophysiological processes such as viral myocarditis [16, 17], myocardial hypertrophy [18-20], and dilated cardiomyopathy [21] (**Table 3A**). Notably, several miRNAs have been reported to have anti-apoptotic effects on cardiomyocytes, including miR-221-3p, miR-221-5p, miR-155-5p, and miR-222-5p (**Figure 5A**).

Expression of apoptosis-related genes in CMs stimulated by ^{LPS}EC-Exo

We detected the potential targets of the differentially expressed miRNAs shown in **Figure 5A**

Table 1. The detailed information of the differential expressed miRNAs

Annotation					DE Statistic		Counts per million reads (CPM)	
Mature_ID	Length	Seed	Mature_Seq	Regulation	Fold_Change	*p*_value	LPS	NC
rno-miR-221-3p	23	GCUACA	AGCUACAUUGUCUGCUGGGUUUC	up	1.595974069	4.12268E-06	12.6296558	11.95522
rno-miR-222-3p	21	GCUACA	AGCUACAUCUGGCUACUGGGU	up	2.262590717	5.09634E-12	12.17364154	10.99567
rno-miR-221-5p	23	CCUGGC	ACCUGGCAUACAAUGUAGAUUUC	up	2.44701923	5.54174E-10	9.466560617	8.175535
rno-miR-155-5p	23	UAAUGC	UUAAUGCUAAUUGUGAUAGGGGU	up	1.941562851	3.60596E-07	9.020271954	8.063054
rno-miR-1247-3p	21	GGGAAC	CGGGAACGUCGAGACUGGAGC	up	1.965058526	2.4416E-05	7.129555063	6.154983
rno-miR-129-5p	21	UUUUUG	CUUUUUGCGGUCUGGGCUUGC	up	4.887359275	1.66167E-25	6.698536894	4.409482
rno-miR-148a-5p	23	AAGUUC	AAAGUUCUGAGACACUCUGACUC	up	1.744113276	0.000486805	6.207993957	5.4055
rno-miR-222-5p	21	GCUCAG	GGCUCAGUAGCCAGUGUAGAU	up	2.75305286	2.93239E-10	5.381283427	3.920251
rno-miR-129-1-3p	19	AGCCCU	AAGCCCUUACCCCAAAAAG	up	2.893477567	0.000131722	2.970139124	1.437335
rno-miR-129-2-3p	22	AGCCCU	AAGCCCUUACCCCAAAAAGCAU	up	2.893477567	0.000131722	2.970139124	1.437335
rno-miR-92a-1-5p	23	GGUUGG	AGGUUGGGAUUUGUCGCAAUGCU	up	2.035078562	0.049602557	1.971381937	0.946297
rno-miR-146a-3p	21	CCUGUG	ACCUGUGAAGUUCAGUUCUUU	up	2.062720704	0.034621139	1.477210296	0.432662
rno-miR-667-5p	24	GGUGCU	CGGUGCUGGUGGAGCAGUGAGCAC	up	3.029066665	0.010661818	0.548503655	-1.05037
rno-let-7c-1-3p	22	UGUACA	CUGUACAACCUUCUAGCUUUCC	down	0.404485731	1.79452E-07	3.61831496	4.924154
rno-miR-138-5p	23	GCUGGU	AGCUGGUGUUGUGAAUCAGGCCG	down	0.395627314	0.01767291	-0.363997435	0.973789
rno-miR-375-3p	22	UUGUUC	UUUGUUCGUUCGGCUCGCGUGA	down	0.207440959	0.009642982	-0.727851487	1.541376
rno-miR-202-5p	19	UCCUAU	UUCCUAUGCAUAUACUUCU	down	0.371070492	0.009487856	-0.730950453	0.699284
rno-miR-150-3p	19	UGGUAC	CUGGUACAGGCCUGGGGGA	down	0.423606483	0.046385033	-1.217325179	0.021878

MiRNAs in the pink background were up-regulated under the induction of LPS, and the miRNAs in the green were down-regulated.

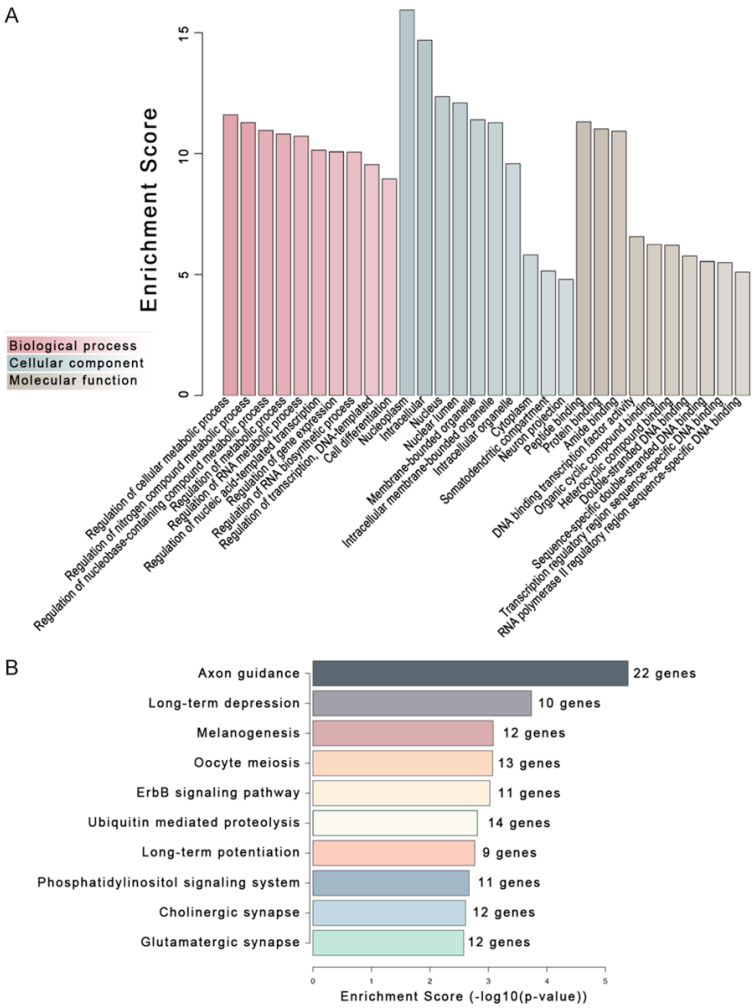

Figure 4. Eight miRNAs up-regulated in the exosomes from LPS-stimulated ECs were subjected to gene ontology (GO) and Pathway analysis to predict their roles and signaling pathways they potentially involved. A. The top 10 entries in the biological process, cellular component, and molecular function according to GO analysis. B. The top 10 signaling pathways predicted by KEGG analysis.

after stimulating cardiomyocytes with EC-derived exosomes. Compared with ^{NC}EC-Exo, ^{LPS}EC-Exo down-modulated the level of BAK1, P35, and PTEN, which are apoptosis-related genes in cardiomyocytes (**Figure 5B**).

Discussion

Sepsis is a life-threatening host response imbalance caused by an infection. Sepsis-induced myocardial dysfunction (SIMD) is one of the critical mortality causes of septic shock. SIMD is often difficult to correct and ultimately leads patients to death. In this study, we found that exosomes released by LPS-stimulated endothelial cells exerted cardiac protective effects.

We found that the exosomes from LPS (0.5 μg/ml) stimulated ECs (^{LPS}EC-Exo) enhanced neonatal rat cardiomyocytes' viability and reduced the apoptosis. Meanwhile, ^{LPS}EC-Exo led a decline in LDH and ROS, and a rise in SOD in CMs. These phenomena indicated that ^{LPS}EC-Exo possessed cardiac protective substances. Notably, we also found that in the presence of a higher concentration (10 μg/ml) of LPS, microvesicles (MVs) produced by endothelial cells are mainly apoptotic bodies, while exosomes are markedly reduced. A similar phenomenon has been observed when adipocytes were stimulated with LPS [22]. We deem that at high concentrations of LPS, due to the apoptosis of endothelial cells, the protective effect of EC-derived exosomes on cardiomyocytes weakens or disappears. Therefore, the protective effect of EC-Exo on cardiomyocytes may only exist in the early stages of sepsis.

We detected the miRNAs carried by exosomes and found that the expression profiles of miRNAs of ^{LPS}EC-Exo and ^{NC}EC-Exo were significantly different. The top 8 miRNAs highly expressed in ^{LPS}EC-Exo (miR-221-3p, miR-222-3p, miR-221-5p, miR-155-5p, miR-1247-3p, mir-129-5p, miR-148a-5p, and miR-222-5p) were analyzed with bioinformatic tools for target gene prediction, functional enrichment, and pathway forecasting. Some predicted target genes were marked in databases (TargetScan and miRanda) as involved in cardiomyocyte function regulation.

Most DE miRNAs on ^{LPS}EC-Exo are beneficial for the survival of cardiomyocytes (**Table 3A**). It can explain the reason why ^{LPS}EC-Exo improved the vitality of CMs and reduces myocardial inju-

Table 2. Bioinformatics analysis showed the candidate genes may regulate the biological behavior of cardiomyocytes in four pathways

ID	Term	Count	Genes
rno04261	Adrenergic signaling in cardiomyocytes	14	Atp1b1, Atp1b4, Atp2b2, Atp2b4, Cacnb4, Creb3l2, Gnai2, Gnai3, Mapk3, Myl3, Plcb1, Ppp2r5e, Ryr2, Scn4b
rno05410	Hypertrophic cardiomyopathy (HCM)	8	Cacnb4, Igf1, Itga6, Itgb1, Lmna, Myl3, Prkaa1, Ryr2
rno05412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	7	Cacnb4, Ctnna3, Itga6, Itgb1, Lmna, Ryr2, Tcf7
rno05414	Dilated cardiomyopathy	7	Cacnb4, Igf1, Itga6, Itgb1, Lmna, Myl3, Ryr2

Table 3. Cardiac effects of miRNAs highly expressed in $^{LPS-}$EC-Exo

A. Cardioprotective miRNAs	
DE miRNA	Effects
miR-221-5p	Attenuates LPS induced CM injury [24]
	Reduces hypoxia-induced CM apoptosis [25, 26]
	Enhances CM survival and improves cardiac function after MI [27]
miR-222-5p	Improves myocardial survival [28]
	Necessary for cardiac growth induced by physical exercise [10-13]
	Decreases virus-induced CM apoptosis [16]
miR-221/222 clusters	Induce embryonic-stem-cell-derived CMs maturation [14, 15]
	Balance inflammatory response of viral myocarditis [23]
miRNA-155-5p	Improves myocardial survival [28]
	Improves the survival rate of mice with acute viral myocarditis [17]
miRNA-148a-5p	Prevents the occurrence of dilated cardiomyopathy [21]
	Improves the survival rate of mice with viral myocarditis [17]
B. Pathological Cardiac miRNAs	
miRNA-155-5p	Induces pathological myocardial hypertrophy [18-20]
	Induces CM apoptosis in MI [29-32]
miRNA-221-3p	Participates in H_2O_2 induced myocardial injury [33]
miRNA-129-5p	Inhibits CM proliferation [34]

Abbreviations and acronyms: CM cardiomyocyte, MI myocardial infarction.

ry markers. Some miRNAs highly expressed in $^{LPS-}$EC-Exo possess anti-apoptosis roles in cardiomyocytes (**Figure 5A**). For example, miRNA-221/222 clusters help cardiomyocyte survival and differentiation in most cases. In a viral myocarditis model, miRNA-221/222 clusters play an antiviral role and balance inflammatory response [23]. The clusters also induce the maturation of embryonic-stem-cell-derived cardiomyocytes [14, 15]. miRNA-221-5p attenuates LPS induced cardiomyocyte damage by regulating the NF-κB and JNK pathways [24]. Under hypoxic conditions, miRNA-221-5p protects the heart muscle by inhibiting apoptosis [25, 26]. In a rat model of myocardial infarction (MI), miRNA-221-5p enhances myocardial cell survival and improves cardiac function after MI [27]. miRNA-222-5p also improves myocardial survival [28]. miRNA-222-5p is necessary for cardiac growth induced by exercise [10-13]. In viral myocarditis, miRNA-222-5p exerts myocardial protection by inhibiting apoptosis [16]. miRNA-155-5p has a double-sided effect on the heart. In some cases, it is beneficial for cardiac survival [28]. It improves the survival rate of mice with acute viral myocarditis [17]. miRNA-148a-5p also has myocardial protective effects. It prevents dilated cardiomyopathy [21] and enhances mice's survival rate with viral myocarditis [17]. In this study, we found the exosomes from LPS-stimulating ECs ($^{LPS-}$EC-Exo) attenuated the level of BAK1, P53, and PTEN in rat cardiomyocytes. These proteins are essential to initiate apoptosis. Their reduction can explain the mechanism of the cardioprotective effect of $^{LPS-}$EC-Exo.

However, a few DE miRNAs have been reported to be associated with myocardial injury (**Table 3B**). miRNA-155-5p induces pathological myocardial hypertrophy [18-20]. In the myocardial infarction model of mice, miRNA-155-5p in-

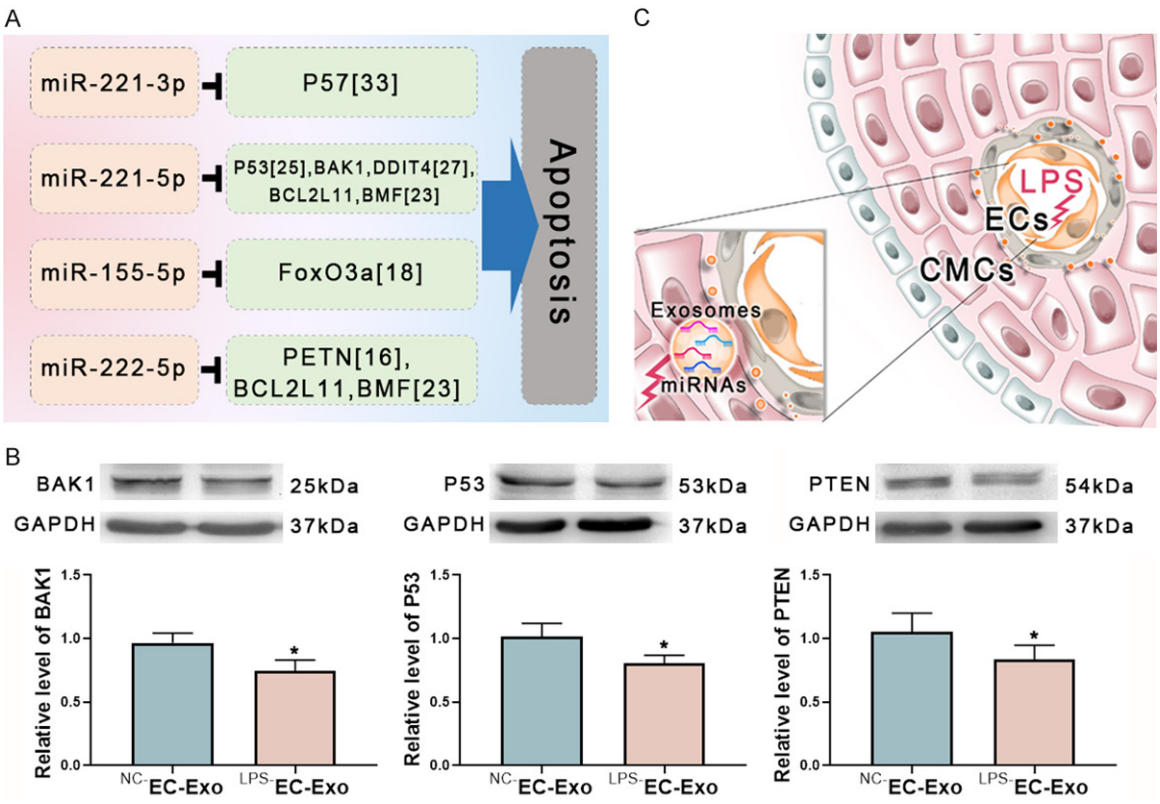

Figure 5. miRNAs prevent cardiomyocytes injury through the predicted targets. A. Several differentially expressed miRNAs in $^{LPS-}$EC-Exo have shown anti-apoptosis roles in myocardiocytes by targeting cell death-related genes. B. The presumed apoptosis-related genes were tested in the cardiomyocytes stimulated with EC-Exo. $^{LPS-}$EC-Exo attenuated the level of BAK1, P53, and PTEN. C. The possible mechanism of $^{LPS-}$EC-Exo protecting cardiomyocytes. In the early sepsis stage, LPS enters the blood circulation and stimulates ECs to express specific miRNAs. Then the differential expressed miRNAs are delivered outside ECs through exosomes and exert a cardiac protective effect. EC-Exo may play an important role in preventing myocardial dysfunction in the early stages of sepsis. n=7, *P<0.05.

duces cardiomyocyte apoptosis [29-32]. miRNA-221-3p participates in H_2O_2 induced myocardial damage by inhibiting p57 [33]. miRNA-129-5p inhibits cardiomyocyte proliferation by inhibiting CDK6 [34]. The roles of these miRNAs in myocardial injury caused by LPS needs further study.

In brief, this study found that the exosomes produced by the appropriate concentration of LPS-stimulated ECs to have protective effects on neonatal rat cardiomyocytes. $^{LPS-}$EC-Exo reduced the apoptosis and injury of CMs (**Figure 5C**). These protective effects may be the body's response to avoid sepsis-induced cardiomyocyte dysfunction in the early stages of sepsis and may be achieved by miRNAs carried on exosomes.

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Disclosure of conflict of interest

None.

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