Exosomes derived from the blood of patients with sepsis regulate apoptosis and aerobic glycolysis in human myocardial cells via the hsa-miR-1262/SLC2A1 signaling pathway

FANGYUAN SUN*, HUAN GENG*, YUXIA SUN, WENTAO FENG, TIANNING TIAN, LIANG YE and MING LEI

Trauma Emergency Center, The Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai 200137, P.R. China

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Abstract. Myocardial injury occurs in the majority of patients with sepsis and is associated with early mortality. MicroRNAs (miRs) transported by exosomes have been implicated in numerous diseases, such as tumors, acute myocardial infarction and cardiovascular disease. Human serum albumin (hsa)-miR-1262 has been shown to serve a role in sepsis; however, its role in exosomes isolated from patients with sepsis and septic myocardial injury remains unclear. In the present study, serum exosomes were isolated via ultracentrifugation. Solute carrier family 2 member 1 (SLC2A1), an essential mediator in energy metabolism, was silenced and overexpressed in the human myocardial AC16 cell line using lentiviral plasmids containing either SLC2A1-targeting short interfering RNAs or SLC2A1 cDNA, respectively. Cell apoptosis was analyzed using flow cytometry, and the extracellular acidification rate and oxygen consumption rate of AC16 cells were determined using an XFe24 Extracellular Flux Analyzer. Furthermore, the dual-luciferase reporter assay was used to evaluate the interaction between hsa-miR-1262 and SLC2A1. Finally, reverse transcription-quantitative PCR and western blotting were used to evaluate gene and protein expression levels, respectively. Exosomes isolated from the blood of patients with sepsis (Sepsis-exo) markedly reduced aerobic glycolysis activity, but significantly promoted the apoptosis of human AC16 cells in a time-dependent manner. Moreover, Sepsis-exo significantly increased hsa-miR-1262 expression levels, but significantly decreased SLC2A1 mRNA expression levels in a time-dependent manner. Bioinformatics analysis indicated that hsa-miR-1262 bound to the 3' untranslated region of SLC2A1 to negatively regulate its expression. The silencing of SLC2A1 promoted apoptosis and suppressed glycolysis in AC16 cells, whereas SLC2A1 overexpression resulted in the opposite effects. Therefore, the present study demonstrated that exosomes derived from patients with sepsis may inhibit glycolysis and promote the apoptosis of human myocardial cells through exosomal hsa-miR-1262 via its target SLC2A1. These findings highlighted the importance of the hsa-miR-1262/SLC2A1 signaling pathway in septic myocardial injury and provided novel insights into therapeutic strategies for septic myocardial depression.

Correspondence to: Dr Liang Ye or Dr Ming Lei, Trauma Emergency Center, The Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine, 358 Datong Road, Pudong, Shanghai 200137, P.R. China
E-mail: yeliangemail@163.com
E-mail: leiming6891@163.com

*Contributed equally

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Introduction

Sepsis, the leading cause of death in critically ill patients worldwide, is a systemic inflammatory response to infection (1). The annual incidence of severe sepsis is increasing, with the mortality rate approaching 50% in the United States from 1993 to 2003 (2). Myocardial injury occurs in most patients with sepsis and is associated with early mortality (3). Moreover, septic cardiovascular dysfunction is associated with a significantly higher mortality rate than those who have sepsis without cardiovascular dysfunction (4); thus, infection management is key for the treatment of septic myocardial injury (1). A previous study has demonstrated that inflammatory mediators, hemostasis dysregulation, immunosuppression, and tissue and organ dysfunction are involved in the pathogenesis of septic myocardial injury (5). Despite the importance of myocardial depression in sepsis, its pathophysiology remains unclear.

Solute carrier family 2 member 1 (SLC2A1) serves an essential role in energy metabolism (6). Being present in nearly all mammalian cells, SLC2A1 transports glucose (7). Glucose binding causes a conformational change in SLC2A1 that results in the release of glucose into the cytoplasm (7), which is a key step in glycolysis (8). This process is dysregulated in numerous diseases, including sepsis. SLC2A1 is overexpressed in various types of cancer, including breast and lung cancer (9,10). In a murine model of sepsis, SLC2A1 was also overexpressed, leading to increased glucose uptake (11).

Vary *et al* (12) demonstrated that sepsis caused a 67% increase in glucose uptake compared with the control group, and determined that sepsis enhances glucose uptake secondary to increased SLC2A1 expression. However, the exact role of SLC2A1 and glucose uptake in septic myocardial injury remains unclear.

Exosomes are small membrane microvesicles that have attracted the attention of researchers for decades (13). Exosomes can deliver their cargo (proteins, lipids and nucleic acids) to target cells, resulting in metabolic reprogramming (14). MicroRNAs (miRNAs/miRs/mis) are small, non-coding RNAs that post-transcriptionally regulate gene expression (15). miRNAs, together with other types of nucleic acids, including mRNAs and other non-coding RNAs, have been identified in exosomes (16). Target cells absorb these exosomal miRNAs, which results in cell modulation (17). For example, human serum albumin (hsa)-miR-1262 has been revealed to enhance the anticancer effects of gefitinib on advanced non-small cell lung cancer cells (18). Furthermore, hsa-miR-1262 has been identified as one of the main differentially expressed microRNAs in the pathogenesis of sepsis (19). However, the role of exosomal hsa-miR-1262 in sepsis has not been fully elucidated.

Although previous studies have demonstrated that SLC2A1 actively participates in glycolysis, and that exosomal miRNAs are implicated in numerous diseases (20-22), the roles of hsa-miR-1262 and SLC2A1 in septic myocardial injury remain unclear. Therefore, the present study evaluated the role of the hsa-miR-1262/SLC2A1 signaling pathway in septic myocardial injury and explored the underlying mechanisms.

Materials and methods

Human serum samples. The present study was approved by the Ethics Committee of The Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine (approval no. 2021-AR-006). A total of 40 patients with sepsis (27 male patients and 13 female patients; age range, 37-67 years; mean age, 52.1±6.0 years) and 88 healthy controls (50 male patients and 38 female patients; age range, 37-67 years; mean age, 52.3±6.3 years). In all patients with sepsis included in the present study, sepsis was caused by bacterial infection. Patients with sepsis were diagnosed by blood test. All participants were enrolled at The Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine (Shanghai, China) between June 2017 and August 2019. Patients were all newly diagnosed cases and recurrent cases were excluded. Exclusion criteria included: i) Patients afflicted with other diseases, such as cancer and metabolic diseases; and ii) patients who were treated by any therapies prior to admission. Subsequently, serum samples were collected from patients with sepsis and healthy controls, which were then used for exosome isolation. Written informed consent was obtained from all participants. The present study was conducted in accordance with the Declaration of Helsinki.

Cell culture. The human myocardial AC16 cell line was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Beyotime Institute of Biotechnology). Cells were maintained at 37˚C with 5% CO_2. Cells were co-cultured with the Control-exo or sepsis-exosomes (50 μg/ml) at 37˚C with 5% CO_2 respectively.

Isolation and identification of exosomes. Exosomes were isolated as previously described (23). Briefly, the blood samples from sepsis patients and healthy donator were collected and centrifuged at 1,409 x g for 30 min at 4˚C. The supernatants were transferred to a fresh tube and centrifuged at 5,000 x g rpm for 30 min at 4˚C. The supernatant was concentrated, added to 30% sucrose/D_2O and centrifuged at 100,000 x g for 1 h at 4˚C. Pellets were diluted with PBS, filtered and maintained at -70˚C. Exosome concentration was measured using a bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.). The exosome pellets were suspended in PBS and fixed in 4% paraformaldehyde and 4% glutaraldehyde at 4˚C for 5 min. After adding a drop of the exosomal sample, the carbon-coated copper grid was immersed in a phosphotungstic acid solution (2%; pH 7.0) for 30 sec. The exosome pellets were fixed with 2.5% glutaraldehyde and thencentrifuged at 100,000 x g 4˚C for 5 min to remove the glutaraldehyde. Afterwards, the pellets were stained by 3% aqueous phosphotungstic acid and fixed on copper mesh formvar grids at 4˚C for 30 min. A transmission electron microscope (JEM-1200EX; Jeol, Ltd.; magnification, x100,000) was used to observe and assess the morphology and size of the exosomes. Western blotting was performed to examine the biomarkers of exosomes (CD9, CD63 and CD81).

Exosome endocytosis assay. AC16 cells ($5x10^4$ cells/well) were seeded into 24-well plates. A total of 250 μg Sepsis-exo and Control-exo were labeled using PKH Lipophilic Membrane Dyes (cat. no. PKH67GL; Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. PKH67-labeled exosomes were centrifuged (40,000 x g at 4˚C for 70 min) and suspended in PBS (50 μl). Cells were incubated with DMEM (Thermo Fisher Scientific, Inc.) or DMEM containing PKH-67-labeled exosomes (20 μg/ml) at 37˚C for 4 h. Subsequently, DAPI was used to stain the nucleus at 37˚C for 5 min. Cells were observed under a fluorescent microscope (Olympus IX71; Olympus Corporation; magnification, x400).

RT-qPCR. Total RNA from AC16 cells was extracted using TRIzol® reagent (cat. no. 1596-026; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA (cat. no. K1622; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The SYBR Green fluorochrome (cat. no. K0223; Thermo Fisher Scientific, Inc.) and following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 10 min; followed by 38 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 45 sec and extension at 72˚C for 30 sec; and final extension at 72˚C for 3 min.

The following primers were used for qPCR: SLC2A1 forward (F), 5'-TGCAGGAGATGAAGGAAG-3' and reverse (R), 5'-CAATGGTGGCATACACAG-3'; β-actin F, 5'-TGG CATCCACGAAACTAC-3' and R, 5'-CTTGATCTTCATGGT GCTG -3'; hsa-miR-1262 F, 5'-CGCGATGGGTGAATTTGT AG-3' and R, 5'-AGTGCAGGGTCCGAGGTATT-3'; U6 F, 5'-CTCGGCTTCGGCAGCACA-3' and R, 5'-AACGCTTCA CGAATTTGCGT-3'. mRNA and miRNA expression levels

were quantified using the $2^{-\Delta\Delta Cq}$ method (24) and normalized to the internal reference genes β-actin or U6, respectively. Each experiment was repeated three times.

Western blotting. Total protein from AC16 cells was extracted using radioimmunoprecipitation assay buffer with protease inhibitors (Beyotime Institute of Biotechnology). The protein levels were quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Proteins (20 μg/lane) were separated via 10% SDS-PAGE. The membranes were blocked using 5% skimmed milk at room temperature for 2 h, followed by incubation overnight at 4˚C with the following primary antibodies: Anti-SLC2A1 (1:1,000; cat. no. ab115730; Abcam), anti-SLC2A4 (1:1,000; cat. no. ab33780; Abcam), anti-CD9 (1:1,000; cat. no. ab92726; Abcam), anti-CD63 (1:1,000; cat. no. ab271286; Abcam), anti-CD81 (1:700; cat. no. ab109201; Abcam), anti-β-actin (1:3,000; cat. no. 66009-1-Ig; ProteinTech Group, Inc.). After washing with 0.1 M PBS, the membranes were incubated with the secondary antibody (HRP-labeled goat anti-rabbit IgG; cat. no. A16104; Thermo Fisher Scientific, Inc.) at 4˚C for 2 h. The Enhanced Chemiluminescence Detection kit (cat. no. WBKLS0100; MilliporeSigma) was used for signal detection.

Knockdown and overexpression of SLC2A1. Short interfering RNAs (sis/siRNAs) targeting human SLC2A1 (siSLC2A1-1, 5'-GCCCAUGUAUGUGGGUGAATT-3'; siSLC2A1-2, 5'-GCCUGUGUAUGCCACCAUUTT-3'; and siSLC2A1-3, 5'-GCUACCCUGGAUGUCCUAUTT-3') and a scrambled siRNA negative control (siNC; 5'-CAGUACUUUUGUGUAGUACAA-3') were synthesized (Beyotime Institute of Biotechnology) and inserted into the pLKO.1 vector (Enable, Biotech). AC16 cells ($5x10^5$ cells/well) were transfected with 100 nm SLC2A1 siRNAs or siNC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. For SCL2A1 overexpression, pLVX-puro lentiviral plasmid (5.94 μg) containing SLC2A1 (NM_006516.4; NCBI database) cDNA or an empty control plasmid were transfected into AC16 cells ($5x10^5$) as indicated above. After incubation for 48 h at 37˚C, cells were used for subsequent experiments.

Glycolysis and mitochondrial respiration assay. The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were determined every 7 min for 77 min using a Seahorse XFe96 analyzer (Agilent Technologies, Inc.). Cells ($1x10^4$/well) were seeded into Seahorse XFe96 plates. After measuring the basal ECAR, the cells were incubated with 10 mM glucose/well to test the capacity of glycolysis, followed by the addition of 1 μm oligomycin for inhibition of oxidative phosphorylation to inspect the maximum glycolytic ability of the cells. Finally, the glycolysis inhibitor 2-DG (50 mM) was added to determine acid production by non-glycolytic pathways. All reagents were added at 0 min and the incubation temperature was maintained at 38.5˚C. Cells were detected every 7 min following continuous administration of 10 mM glucose and inhibitors (1 μm oligomycin and 50 mM 2-DG). For the OCR examination, the basal OCR was first evaluated, after which the oxygen consumption for ATP synthesis was assessed after exposure to 2 μm oligo, an ATP synthase inhibitor. The maximum oxygen consumption capacity of the cells was assessed after cells were given 2 μm mitochondrial uncoupler (FCCP), and the cells were then treated with mitochondrial respiratory chain inhibitors antimycin A (0.5 μm) and oligomycin to prevent oxygen consumption by the mitochondria. All reagents were added at 0 min and the incubation temperature was maintained at 38.5˚C. Cells were measured every 7 min following continuous administration of 2.0 oligomycin, 2.0 FCCP and 0.5 μm antimycin A. All reagents in this experiment were purchased from Sigma-Aldrich (Merck KGaA).

Flow cytometry. Human AC16 cells ($1x10^5$ cells/well) were harvested 48 h after transfection, stained with Annexin V-FITC (Beyotime Institute of Biotechnology) and PI (Invitrogen; Thermo Fisher Scientific, Inc.) for 20 min at 37˚C in the dark and analyzed by flow cytometry (Beckman Coulter, Inc.). CytExpert software 2.0 (Beckman Coulter, Inc.) was used for analysis. Annexin V-FITC$^+$ and PI$^-$ populations indicated apoptosis. Experiments were conducted in triplicate. Annexin V$^-$ and PI$^-$ populations were healthy cells that were considered negatively stained, Annexin V$^+$ and PI$^-$ cells indicated cells in early apoptosis, and Annexin V$^+$ and PI$^+$ staining indicated cells in necrosis (post-apoptotic necrosis or late apoptosis). Vehicle cells were treated with PBS.

Dual-luciferase reporter assay. The interaction between hsa-miR-1262 and SLC2A1 was analyzed using TargetScan (http://www.targetscan.org/vert_72/) and starBase 2.0 (http://starbase.sysu.edu.cn/). Wild-type (WT) and mutant (Mut) SLC2A1 3' untranslated regions (UTRs) were synthesized and ligated into pGL3 vectors. AC16 cells ($1x10^6$) were transfected with the WT or Mut construct (50 μg, cat. no. E1910; Promega Corporation), and co-transfected with hsa-miR-1262 mimic (5'-AUGGGUGAAUUUGUAGAAGGAU-3'; Shanghai Meiji Biomedical Technology Co., Ltd.), hsa-miR-1262 inhibitor (5'-AUCCUUCUACAAAUUCACCCAU-3'; Shanghai Meiji Biomedical Technology Co., Ltd.), miNC-mimic (5'-CAGUACUUUUGUGUAGUACAA-3'; Shanghai Meiji Biomedical Technology Co., Ltd.) or miNC-inhibitor (5'-UUCUCCGAACGUGUCACGUTT-3'; Shanghai Meiji Biomedical Technology Co., Ltd.). Following incubation at 37˚C for 48 h, the firefly and *Renilla* luciferase activities were detected using a Dual-Luciferase Reporter assay system (Promega Corporation) according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis. Statistical analyses were performed using Prism 7.0 (GraphPad Software, Inc.). Data are presented as the mean ± standard deviation of three repeats. Comparisons among multiple independent groups were analyzed using the Kruskal-Wallis test followed by Dunn's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Exosomes isolated from the serum of patients with sepsis reduce the aerobic glycolysis activity of AC16 cells. To study

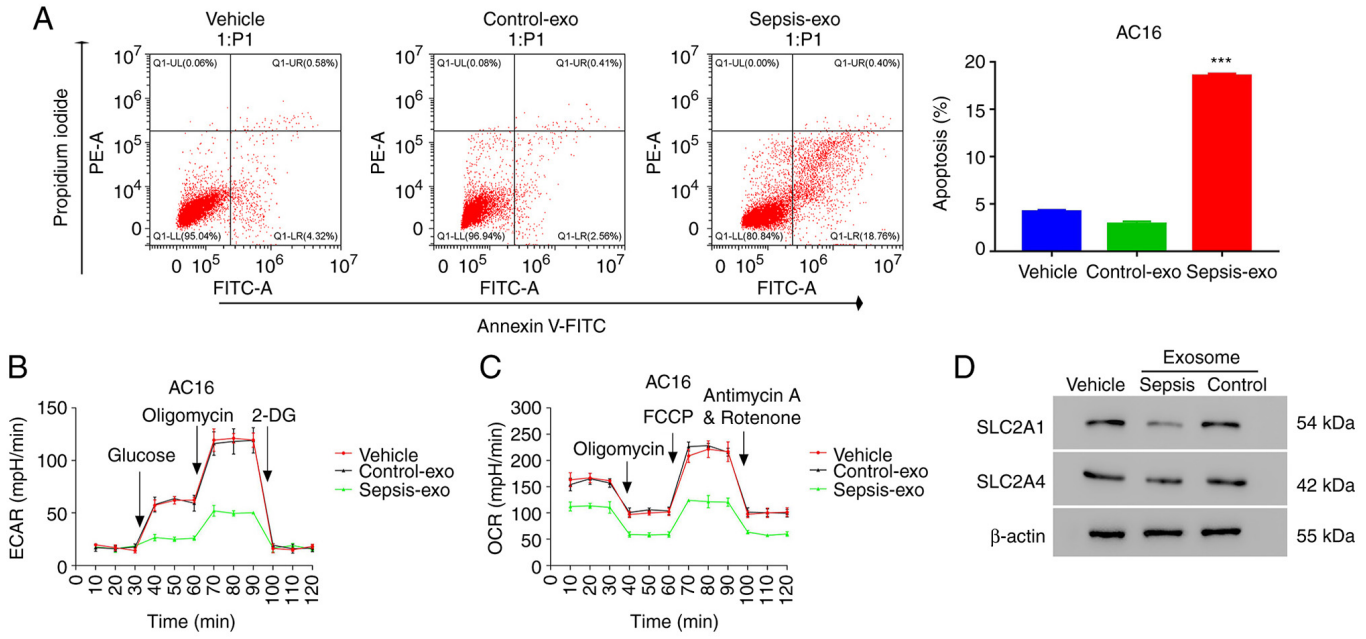

Figure 1. Aerobic glycolysis of human myocardial AC16 cells is downregulated by exosomes isolated from the blood of patients with sepsis. (A) AC16 cell apoptosis was promoted by exosomes isolated from the blood of patients with sepsis. (B) ECAR and (C) OCR of AC16 cells were notably suppressed by Sepsis-exo treatment. (D) Western blotting analysis of SLC2A1 and SLC2A4 in AC16 cells following Control-exo or Sepsis-exo treatment. ***P<0.001 vs. Control-exo. ECAR, extracellular acidification rate; OCR, oxygen consumption rate; SLC2A, solute carrier family 2 member; PE, phycoerythrin; 2-DG, 2-deoxy-D-glucose; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; exo, exosome.

the role of serum exosomes, exosomes were isolated from the serum of healthy individuals (Control-exo) and patients with sepsis (Sepsis-exo; Fig. S1A), and characterized by the exosomal markers CD9, CD63 and CD81 (Fig. S1B). PKH-67 staining revealed that exosomes were endocytosed by AC16 cells (Fig. S1C). These results indicated that exosomes had been successfully isolated and could be used in further experiments. Then, we examined the apoptosis of AC16 cells co-cultured with Sepsis-exo. Co-culture results demonstrated that Sepsis-exo treatment significantly increased the apoptosis of AC16 cells compared with that in the Control-exo treatment group (Fig. 1A). Furthermore, Sepsis-exo treatment resulted in marked suppression of ECAR (Fig. 1B) and OCR (Fig. 1C) compared with that in the Control-exo group. The western blotting results demonstrated that Sepsis-exo treatment notably decreased SLC2A1 protein expression levels in AC16 cells compared with those in the Control-exo treatment group (Fig. 1D). No significant change in SLC2A4 protein expression levels was observed in the Sepsis-exo treatment group compared with those in the Control-exo treatment group. These data indicated that Sepsis-exo treatment decreased aerobic glycolysis activity and increased apoptosis in AC16 cells.

Sepsis-exo time-dependently promotes apoptosis and inhibits aerobic glycolysis activity in AC16 cells. To further understand the effect of Sepsis-exo on apoptosis and glycolysis, a time-course study was performed. The flow cytometry results demonstrated that Sepsis-exo significantly increased AC16 cell apoptosis in a time-dependent manner (Fig. 2A). Furthermore, Sepsis-exo treatment markedly suppressed ECAR (Fig. 2B) and OCR (Fig. 2C) in a time-dependent manner. The RT-qPCR results revealed that Sepsis-exo significantly increased hsa-miR-1262 expression levels, but significantly decreased SLC2A1 mRNA expression levels in a time-dependent manner (Fig. 2D). Moreover, the western blotting results demonstrated that Sepsis-exo markedly decreased SLC2A1 protein expression in a time-dependent manner (Fig. 2D). These results indicated that Sepsis-exo time-dependently inhibited glycolysis and promoted apoptosis in AC16 cells.

hsa-miR-1262 inhibits SLC2A1 transcription by binding to its 3'UTR. To understand how hsa-miR-1262 regulated SLC2A1 expression, hsa-miR-1262 was silenced or overexpressed in AC16 cells (Fig. 3A). Bioinformatics analysis indicated a potential binding site of hsa-miR-1262 in the 3'UTR of SLC2A1 (Fig. 3B). Therefore, SLC2A1 WT 3'UTR + hsa-miR-1262 inhibitor, WT 3'UTR + hsa-miR-1262 mimic, Mut 3'UTR + hsa-miR-1262 inhibitor and Mut 3'UTR + hsa-miR-1262 mimic were co-transfected into AC16 cells. The dual-luciferase reporter assay results demonstrated that hsa-miR-1262 silencing significantly increased SLC2A1 promoter activity compared with that of the miNC-inhibitor group, whereas SLC2A1 promoter activity was significantly inhibited by hsa-miR-1262 overexpression compared with that of the miNC-mimic group. Furthermore, a mutation in the hsa-miR-1262 binding site of SLC2A1 blocked the effect of hsa-miR-1262 on the SLC2A1 promoter (Fig. 3C). These results indicated that hsa-miR-1262 bound to the 3'UTR of SLC2A1 to negatively regulate its expression.

hsa-miR-1262 mimic and Sepsis-exo exhibit similar effects on AC16 cells. Both Sepsis-exo and hsa-miR-1262 mimic were used to treat overexpression (oe)SLC2A1-AC16 cells (Fig. S2C and D) to further investigate their roles. Sepsis-exo

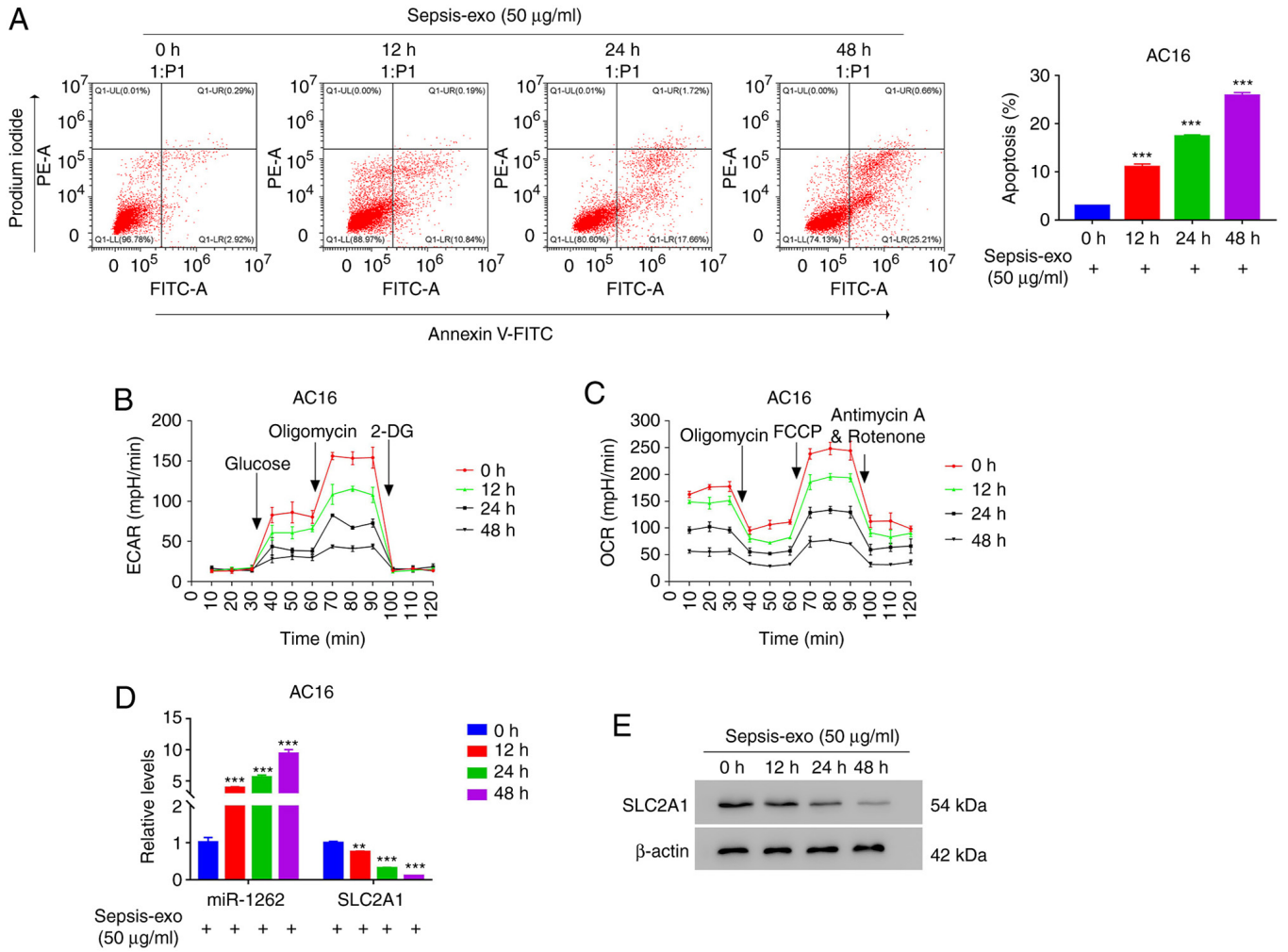

Figure 2. Sepsis-exo promotes apoptosis and inhibits glycolysis in AC16 cells in a time-dependent manner. AC16 cells were co-cultured with exosomes for 0, 12, 24 or 48 h. (A) Flow cytometry analysis of AC16 cell apoptosis. Analysis of (B) ECAR and (C) OCR in AC16 cells. (D) Reverse transcription-quantitative PCR analysis of hsa-miR-1262 and SLC2A1 expression levels under Sepsis-exo treatment. (E) Western blotting analysis of SLC2A1 in AC16 cells. **P<0.01 and ***P<0.001 vs. 0 h. ECAR, extracellular acidification rate; OCR, oxygen consumption rate; SLC2A, solute carrier family 2 member; hsa, human serum albumin; miR, microRNA; PE, phycoerythrin; 2-DG, 2-deoxy-D-glucose; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; exo, exosome.

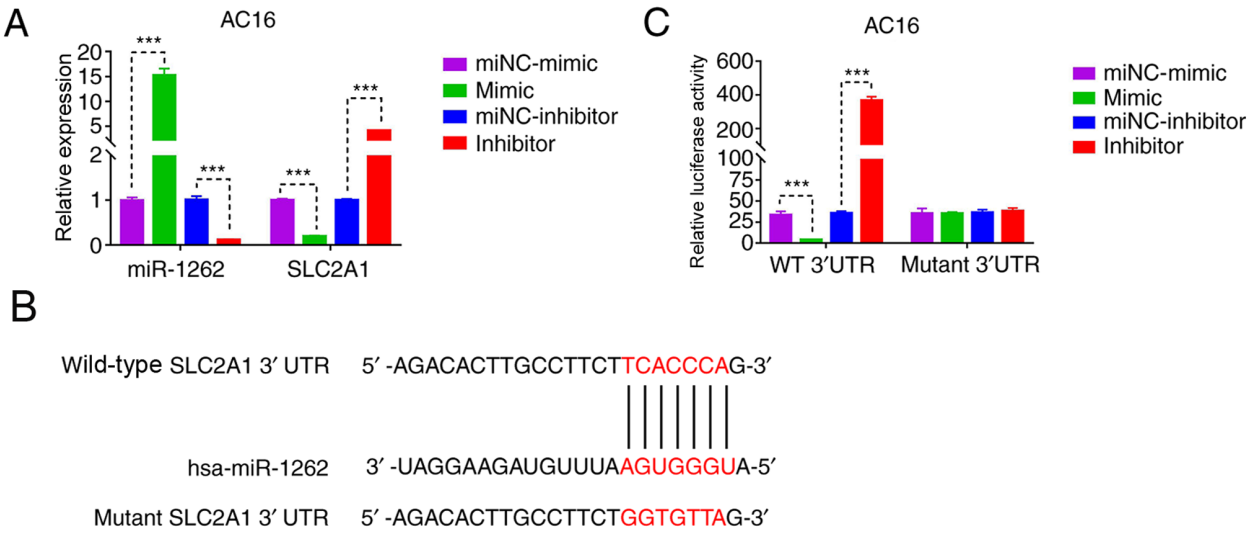

Figure 3. hsa-miR-1262 inhibits the transcription of SLC2A1 by binding to the 3'UTR. (A) Relative expression levels of hsa-miR-1262 and SLC2A1 were examined in AC16 cells in the presence of hsa-miR-1262 mimic and inhibitor using reverse transcription-quantitative PCR. (B) Binding sites of hsa-miR-1262 in the 3'UTR of SLC2A1 and corresponding mutant sites. (C) Dual-luciferase reporter assay was used to verify the binding of hsa-miR-1262 and SLC2A1. ***P<0.001. hsa, human serum albumin; miR/mi, microRNA; SLC2A, solute carrier family 2 member; UTR, untranslated region; NC, negative control; WT, wild-type.

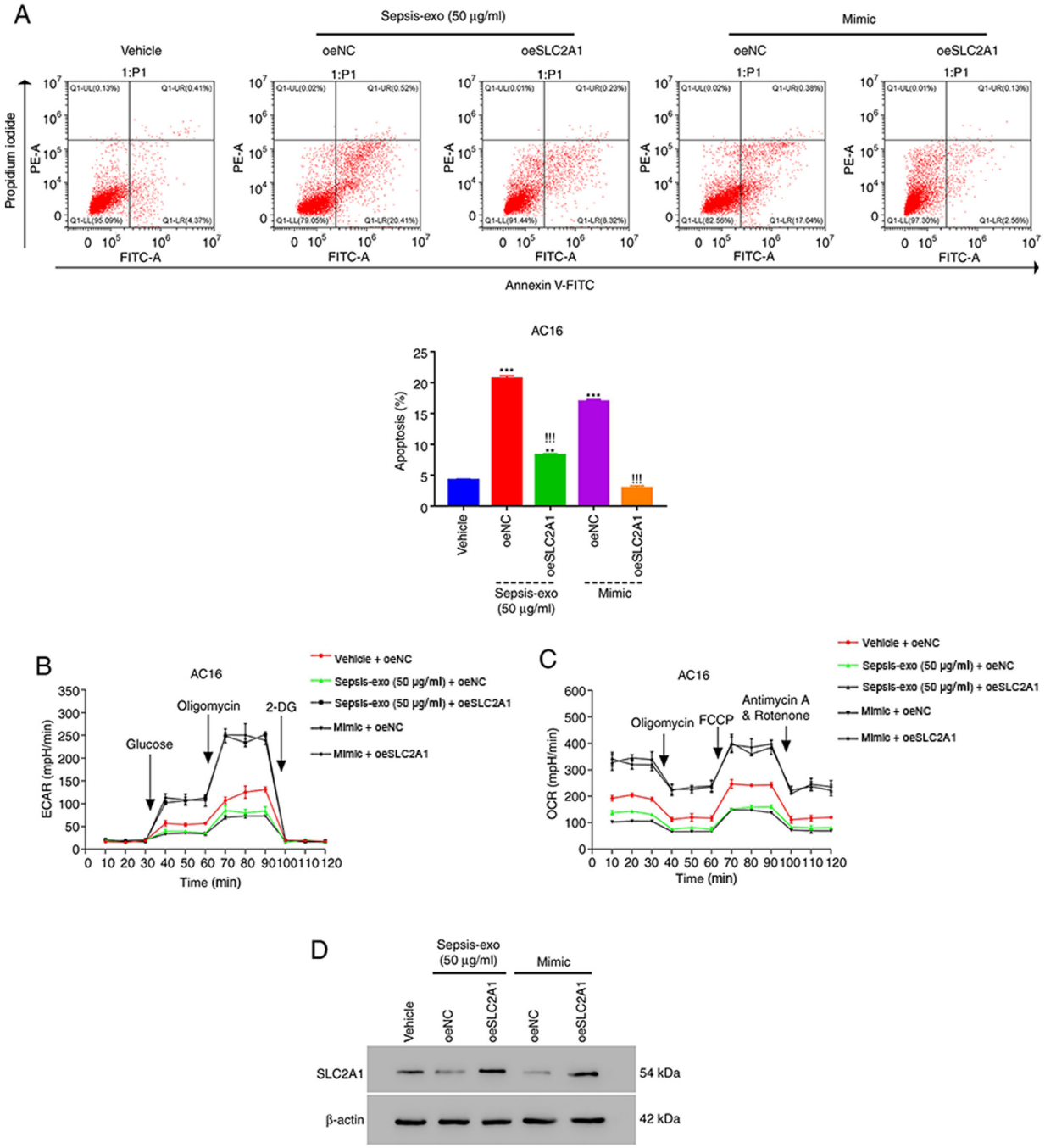

Figure 4. hsa-miR-1262 mimic and Sepsis-exo exhibit similar effects on AC16 cells. (A) Sepsis-exo and hsa-miR-1262 mimic significantly suppressed the apoptosis of oeSLC2A1-transfected AC16 cells. (B) ECAR and (C) OCR in oeSLC2A1-transfected AC16 cells were significantly suppressed by Sepsis-exo or hsa-miR-1262 mimic. (D) Western blotting analysis of SLC2A1. ***P<0.001 vs. vehicle; !!!P<0.001 vs. oeNC + Sepsis-exo; **P<0.01 vs. vehicle. hsa, human serum albumin; exo, exosome; miR, microRNA; SLC2A, solute carrier family 2 member; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; oe, overexpression; NC, negative control; PE, phycoerythrin; 2-DG, 2-deoxy-D-glucose; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.

and hsa-miR-1262 mimic significantly increased the apoptosis of oeNC-transfected AC16 cells compared with that in vehicle cells (Fig. 4A). Sepsis-exo and hsa-miR-1262 mimic-induced increases in apoptosis were significantly attenuated by SLC2A1 overexpression. Furthermore, SLC2A1 overexpression markedly diminished Sepsis-exo and hsa-miR-1262 mimic-mediated effects on ECAR (Fig. 4B) and OCR (Fig. 4C). The western blotting results demonstrated that both Sepsis-exo and hsa-miR-1262 mimic markedly decreased SLC2A1 protein expression levels compared with those in the vehicle group; however, these effects were abolished by SLC2A1 overexpression (Fig. 4D). Overall, these findings suggested that hsa-miR-1262 mimic and Sepsis-exo displayed similar effects on AC16 cells.

SLC2A1 silencing promotes apoptosis and suppresses glycolysis in AC16 cells. To further confirm the role of SLC2A1, SLC2A1 was silenced in AC16 cells (Fig. S2A and B), and then apoptosis and glycolysis were quantified. As shown in Fig. S2B, siSLC2A1-1 and siSLC2A1-2 were more effective

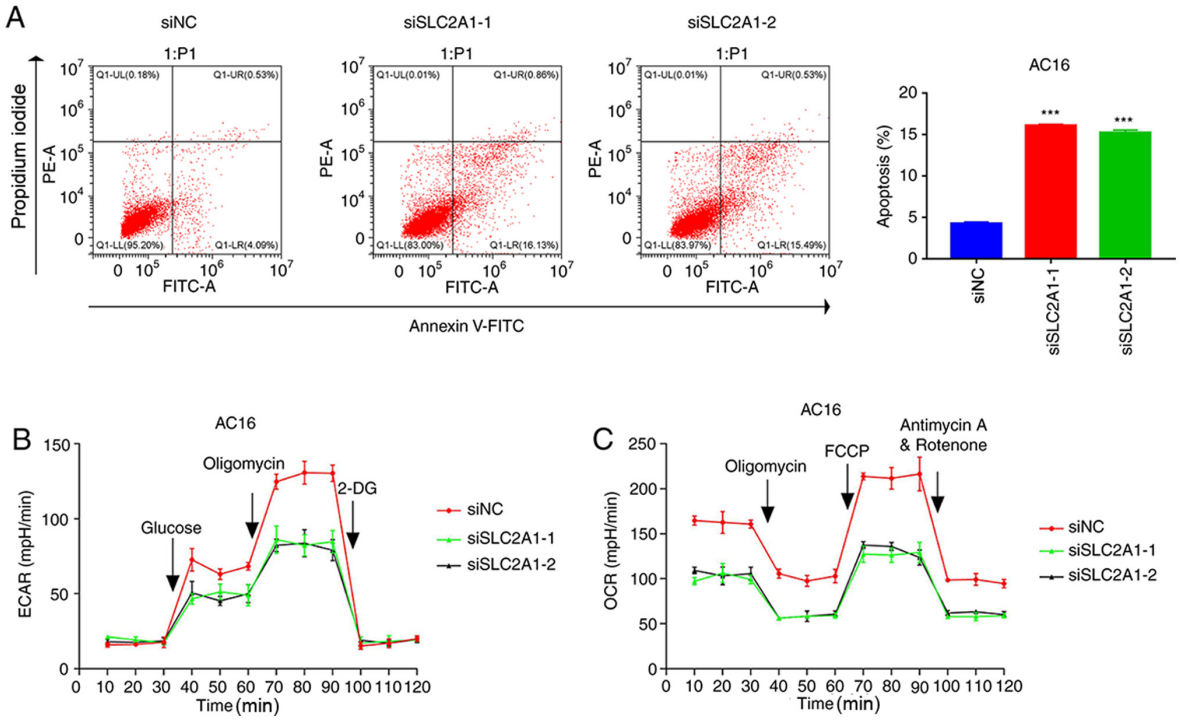

Figure 5. SLC2A1 silencing promotes apoptosis and suppresses glycolysis in AC16 cells. (A) AC16 cell apoptosis was significantly promoted by silencing SLC2A1. (B) ECAR and (C) OCR were significantly inhibited in AC16 cells by silencing SLC2A1. ***P<0.001 vs. siNC. SLC2A, solute carrier family 2 member; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; si, small interfering RNA; NC, negative control; PE, phycoerythrin; 2-DG, 2-deoxy-D-glucose; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.

compared with siSLC2A1-3. Therefore, SLC2A1-3 was not used in subsequent experiments. The flow cytometry results revealed that the silencing of SLC2A1 significantly promoted the apoptosis of AC16 cells compared with that in the siNC group (Fig. 5A). Furthermore, SLC2A1 silencing markedly inhibited ECAR (Fig. 5B) and OCR (Fig. 5C) in AC16 cells compared with that in siNC group. These data suggested that SLC2A1 silencing promoted apoptosis and suppressed glycolysis in AC16 cells. A graphical abstract summarizing the results of the present study is presented in Fig. S2E.

Discussion

To the best of our knowledge, the present study was the first to report that exosomes isolated from the blood of patients with sepsis reduced aerobic glycolysis activity and promoted apoptosis in human AC16 cells in a time-dependent manner. The results demonstrated that Sepsis-exo significantly increased hsa-miR-1262 expression levels, but significantly decreased SLC2A1 mRNA expression levels in a time-dependent manner. Moreover, the results indicated that Sepsis-exo exerted its effects via exosomal hsa-miR-1262. hsa-miR-1262 was also demonstrated to bind to SLC2A1 and negatively regulate its expression. Furthermore, SLC2A1 silencing promoted apoptosis and suppressed glycolysis in AC16 cells. The present study revealed that hsa-miR-1262 from Sepsis-exo inhibited glycolysis and promoted apoptosis in AC16 cells by targeting SLC2A1.

hsa-miR-1262 serves a role in numerous different diseases. For example, it has been reported that hsa-miR-1262 overexpression suppresses lung cancer cell proliferation by targeting Unc-51-like kinase 1 and Ras-related protein RAB3D (25). A recent study demonstrated that hsa-miR-1262 regulates tumor progression via low-density lipoprotein receptor-related protein 8 in breast cancer (26). hsa-miR-1262 has also been shown to serve a role in sepsis pathogenesis (19). The present study demonstrated that hsa-miR-1262 inhibited glycolysis and induced apoptosis, leading to suppression of AC16 cell proliferation. These results revealed a potential novel role for hsa-miR-1262 in sepsis and septic myocardial injury.

Among the 14 glucose transporter (GLUT) family members (27), SLC2A1 is the most extensively studied and has been shown to be overexpressed during oncogenesis to increase glucose uptake and glycolysis in tumor cells (28). Furthermore, it has been demonstrated that glucose uptake is dysregulated in sepsis (29). In a previous study, lipopolysaccharide, the cause of serious sequelae in patients with gram-negative bacterial sepsis, was attributed to the dysregulation of glucose uptake via SLC2A1, but not to other GLUT family members (30). It has also been demonstrated that dysregulated glucose uptake is associated with dysregulated SLC2A1 expression during septic shock (12). The present study reported that exosomal hsa-miR-1262 bound directly to the 3'UTR of the SLC2A1 promoter to negatively regulate its expression. The protection of AC16 cells by SLC2A1 overexpression against hsa-miR-1262 mimic- or Sepsis-exo-induced proliferation suppression further demonstrated that SLC2A1 was a target of hsa-miR-1262. These results not only indicated a role of hsa-miR-1262/SLC2A1 in septic cardiomyocyte injury, but may also improve the understanding of sepsis pathogenesis.

Glycolysis dysregulation has been demonstrated to serve a role in numerous diseases, including rheumatoid

arthritis and cancer (31). A previous study indicated that the dysregulation of glycolysis by inflammatory cytokines might be the molecular mechanism underlying severe sepsis (32). Zheng *et al* (33) reported that glycolytic metabolism serves a crucial role in septic cardiomyopathy, most likely by regulating inflammation and apoptosis. Yang *et al* (34) indicated that the inhibition of glycolysis protected against sepsis by partially suppressing lactate production and high mobility group box 1 release. Moreover, MacFarlane *et al* (35) reported that inhibiting glycolysis using 2-deoxyglucose potentiates TNF-related apoptosis-inducing ligand-induced cell apoptosis. Furthermore, Mason *et al* (36) determined that decreased glycolysis promoted cell apoptosis via the activation of p53 and induction of the proapoptotic protein p53 upregulated modulator of apoptosis. In the present study, SLC2A1 overexpression significantly diminished Sepsis-exo- and hsa-miR-1262 mimic-induced decreases ECAR and OCR, suggesting that glycolysis may serve an important role in Sepsis-exo-induced cardiomyocyte injury. Furthermore, in addition to glycolysis suppression, miR-1262 overexpression or Sepsis-exo administration significantly promoted the apoptosis of AC16 cells. Overall, these results suggested a role of hsa-miR-1262/SLC2A1/glycolysis in the pathogenesis of septic cardiomyocyte injury and sepsis. Although further studies are needed, the present study reported a potential mechanism underlying septic myocardial injury.

In conclusion, the present study indicated a novel role of the hsa-miR-1262/SLC2A1 signaling pathway, suggesting that serum exosomes isolated from patients with sepsis may cause cardiomyocyte proliferation suppression via hsa-miR-1262 and its target SLC2A1. These results may provide the foundations for the future development of novel therapeutic strategies for septic myocardial injury. A key limitation of the present study was that the results were not verified in vivo; thus, an animal model should be used in future studies.

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Availability of data and materials

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

Authors' contributions

ML designed the project and revised the manuscript. FS and HG performed the experiments and drafted the manuscript. YS and WF analyzed the data and edited the figures. TT and LY analyzed the data and confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Seventh People's Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai, China (approval no. 2021-AR-006). Written informed consent was obtained for all patients. The present study was performed in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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