Plasma exosomal miR-142-3p induced by acupuncture protects against heart injury in rat with myocardial infarction by targeting Cofilin-2

Yue Zhao
Shanghai University of Traditional Chinese Medicine

Ying Wang
Shanghai University of Traditional Chinese Medicine

Haidong Guo
Shanghai University of Traditional Chinese Medicine

Chunrong Guo
Shanghai University of Traditional Chinese Medicine

Qiyu Sun
Shanghai University of Traditional Chinese Medicine

Pingping Lu
Shanghai University of Traditional Chinese Medicine

Yang Shen
Shanghai University of Traditional Chinese Medicine

Shuijin Shao
Shanghai University of Traditional Chinese Medicine

Rong Lu
Shanghai University of Traditional Chinese Medicine

Qiangli Wang (wlei810@163.com)
Shanghai University of Traditional Chinese Medicine

Research Article

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Abstract

Purpose

The protective effects of acupuncture on myocardial injury have been identified in clinical trials, but the underlying mechanisms are still not completely understood. This study aimed to investigate the roles of exosomes induced by acupuncture in cardioprotection.

Materials and methods

Masson's-trichrome staining was used to observe the size of infarcted myocardium region. Western blot was used to detect protein expression. Ultracentrifugation methods were used to isolate plasma exosomes of rats. Small RNA sequencing combined with real-time PCR were performed to analysis expression miRNAs. Bioinformatics-based analysis and dual luciferase reporter assay were used to identify target genes of miRNAs.

Results

Acupuncture thickened the infarct wall of the left ventricle, and inhibited apoptosis of infarcted myocardium by upregulation of Bcl-2 and down-regulation of BAX and cleaved caspase 3. These effects of acupuncture were significantly reversed by inhibiting exosome secretion. Plasma exosomes induced by acupuncture increased the viability of cardiomyocytes in H2O2-induced injury. Five miRNAs from the plasma exosomes were increased by acupuncture, and the unique miRNA (miR-142-3p) among these miRNAs was upregulated in myocardial tissue. These changes in miRNAs induced by acupuncture were similarly inversed when exosome secretion was inhibited by GW4869. Overexpressing miR-142-3p retarded oxidative damage of H9c2 by anti-apoptosis. miR-142-3p directly targeted and downregulated the expression of Cofilin-2 (CFL2). In vivo, CFL2 expression was downregulated in the infarcted myocardium by acupuncture and upregulated by GW4869 injection.

Conclusion

Acupuncture-derived circulating exosomes transfer cardio-protective signals to the injured heart and confer cardioprotective effects, and miR-142-3p is a major exosomal miRNA in the inhibition of myocardial apoptosis by targeting CFL2.

Introduction

Cardiovascular disease (CVD) accounts for 37% of deaths in noncommunicable diseases. As a major manifestation of coronary heart disease, the prevalence of myocardial infarction (MI) among US adults
≥ 20 years of age is 3.1% [1]. In China, MI mortality is reported at 5.869 per million in urban areas and 7.472 per million in rural areas in 2016 [2]. Severe MI kills nearly a billion of cardiomyocytes due to prolonged ischemia, which leads to heart failure (HF) and even sudden cardiac death [3]. Inhibiting cardiomyocyte death has been proposed as one of therapeutic strategies for protecting MI survivors against HF [4]. However, many randomized clinical trials demonstrated that conditioning or cardioprotective interventions exhibit equivocal clinical effects on infarct size and the clinical outcome of patients [5]. Alternative therapies for transitory treatments with cardioprotective agents have been investigated by an increasing number of clinicians and pharmaceutical companies. Acupuncture has been utilized as a nonpharmacologic treatment for more than 2000 years, and its efficacy on myocardial ischemia and heart failure has been confirmed by many randomized clinical trials and animal experiments [6–8]. The bilateral Neiguan (PC6), as a commonly used acupoints for the treatment of cardiovascular disease in Chinese medicine, has been proven to effectively protect ischemic heart through multiple pathways, such as activating adenosine receptor, alleviating mitochondrial damage, and attenuating the autophagy of ischemic heart [9–11]. However, the mechanism of Neiguan (PC6) triggering for remote cardioprotection is still unclear.

Exosomes are nanoscale lipid bilayer vesicles secreted by most types of cells, with a diameter ranging from 30 nm to 100 nm. Recently, the critical roles of exosomes have been uncovered in ferrying many signaling molecules, such as proteins, mRNAs, and miRNAs, to mediate communication messages between cells or even organs [12]. A number of studies showed that the cardioprotective effect of exosomes could be achieved by delivering miRNAs. For instance, exosomes induced by transient hindlimb ischemia could transfer miR-24 against apoptosis to offer cardioprotection on a rat acute ischemia/reperfusion (I/R) model [13]. Plasma exosomes isolated from long-term exercised humans protected the heart via transmitting miR-342-5p against I/R injury [14]. These findings suggested that the release of exosomes from limbal tissues into circulation by mechanical stimulation of Neiguan (PC6) may contribute to the remote acupuncture-conferred cardiovascular benefits.

In this study, exosomes were first confirmed to be involved in the cardioprotective effect of acupuncture at Neiguan (PC6) in a rat model of MI. On this basis, changes in miRNAs at the tissue of Neiguan (PC6) after acupuncture were detected by small RNA sequencing, and then the expression of these miRNAs in plasma exosomes and infarcted myocardial tissue was investigated. Finally, exosomal miR-142-3p was identified as a novel cardioprotective molecule by downregulating the expression of Cofilin-2 (CFL2).

**Materials and Methods**

**Rat care and AMI surgery**

All animal experiments in the study were approved by the Institutional Animal Care and Use Committee at Shanghai University of Traditional Chinese Medicine (SHUTCM). Eight-week-old male Sprague–Dawley (SD) rats purchased from Slaccas Laboratory Animal Corporation (Shanghai, China) were maintained in a specific-pathogen-free facility of the animal experiment center of SHUTCM, with free access to food
and water before use. After 1 week of acclimating to environments, the rats were anesthetized with isoflurane (5% induction and 2% maintenance), and endotracheal intubation connecting to the rodent ventilator (model UGO BASILE 7025, Milan, Italy) was performed. Once signs of discomfort or pain was no longer observed, the chest of rats was opened between the left fourth and fifth intercostal spaces by thoracotomy. After the heart was clearly exposed by a dilator, the left anterior descending coronary artery (LAD) was ligated 2mm below the left auricle to induce AMI as previously described [15]. The appearance of the left anterior myocardium converting from sanguine to paleness indicated successful establishment of an AMI model. The same procedures were performed in the sham-operated animals except LAD ligation.

Acupuncture intervention

Neiguan (PC6) was located at the distal 1/6 point on the imaginary midline connecting the palm crease and cubital crease of inner forelimb [16]. On the second day after AMI, the rats were randomly assigned into three groups: sham, control, and Acu (n = 6 per group). All the animals were separately restricted in special cylindrical devices with limbs stretching out. For the rats in the Acu group, sterilized Huatuo needles (0.25 mm×13 mm; Suzhou Medical Appliance Manufactory, Jiangsu, China) were perpendicularly inserted in bilateral PC6 at a depth of 2–3 mm and manually rotated back and forth in situ for 5 min, with a frequency of 120 times/min. For the control group, similar operations were performed at the tail non-acupoint of the rats. The sham-operated animals were only restrained without acupuncture intervention. Acupuncture or sham treatment was performed once daily for 6 days.

GW4869 treatment in acupuncture-intervened rat

GW4869 was used to inhibit exosome secretion to clarify the roles of exosomes in the effects of acupuncture. It was dissolved in DMSO (0.005%). On the second day after AMI, the rats were randomly assigned into four groups: sham, control, Acu, and Acu plus GW4869 (n = 6 per group). The first three groups were treated as above. The rats in the Acu plus GW4869 group were intraperitoneally injected with GW4869 at one dose of 1.5 mg/kg 1 h before daily acupuncture.

Histological examination

Seven days after AMI, all the animals were anesthetized with isoflurane as described above. The rats were transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde. The hearts were then cut transversely at the widest parts of the infarcted regions. Tissues were embedded in Tissue Tek OCT compound (Sakura, Tokyo, Japan), snap-frozen with liquid nitrogen, and stored at −80°C. The blocks were serially sliced into 5µm sections by cryostat microtome (Thermo Scientific, USA). Ten continuous sections from each block were stained using Masson's trichrome (Masson). Images were acquired using a light microscope (Olympus, Japan). The infarct size and left ventricular wall thickness were analyzed as previously described [15].

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay
The TUNEL method was used to identify apoptotic cells in the infarcted tissues in accordance with the instructions of the manufacturer (Beyotime, Shanghai, China). Sections were washed thrice with PBS and then incubated with 0.5% TritonX-100 for 5 min at room temperature. The TUNEL reaction mixture containing TdT and FITC-dUTP was added to the specimens and reacted for 60 min in a dark humidified atmosphere. After the nuclei were stained by DAPI, the results of TUNEL assay were observed under the fluorescence microscope (Olympus IX50, Japan).

Western blot analysis

Frozen tissues of the left ventricle anterior wall were homogenized in ice-cold RIPA lysis buffer that contained a protease inhibitor cocktail and a phosphatase inhibitor cocktail. The tissue lysates were centrifuged at 12,000×g for 20 min at 4°C. After the protein concentration of the supernatants was determined by BCA analysis (Beyotime, Shanghai, China), proteins (40 µg) were separated on a 12% SDS-polyacrylamide gel and transferred on NC membrane (Merck, Darmstadt, Germany). The membrane was incubated with primary antibodies, such as anti-CC3 antibody (Cell Signaling Technology, USA), anti-Bcl-2 antibody (Abcam, USA), or anti-BAX antibody (Cell Signaling Technology, USA), at 4°C overnight and incubated with secondary antibodies conjugated with peroxidase for 1 h at room temperature. Afterwards, the bands on the membrane were visualized using enhanced chemiluminescence Western blot detection reagents (NCM Biotech, Suzhou, China). Meanwhile, images were acquired using the Molecular Imager system (Bio-Rad, California, USA). The relative value of band density was analyzed by ImageJ software.

MiRNA sequencing and analysis of PC6 acupoint

The rats were sacrificed by euthanasia on the seventh day after MI to clarify the changes in the miRNA expression of PC6 acupoint. The PC6 tissues, including skin, subcutaneous tissue, and muscle tissue, were harvested. A total of nine samples from the sham, control, and Acu group were immediately frozen in liquid nitrogen and transported to Shanghai Majorbio Bio-pharm Technology Co., Ltd. for miRNA sequencing. The bioinformatics data were analyzed through the cloud platform of Majorbio Bio-Pharm Technology Co., Ltd.

Plasma exosome isolation and identification

Exosome was isolated from rat plasma as described [17]. Blood sample was collected from the abdominal aorta of the anesthetized rats before they were sacrificed and then centrifuged for 30 min at 1000 g in a tube with anticoagulant added. The plasma was then transferred to a new tube and centrifuged at 2000 g for 15 min at 4°C to deplete cell debris. Thereafter, it was centrifuged at 10,000 g for 30 min at 4°C to remove large micro vesicles. Anequal volume of 16% polyethylene glycol (PEG) with Mn of 6000 (Sigma, USA) was added to the supernatant and let stand at 4°C overnight after mixing thoroughly. On the next day, the samples were centrifuged at 16,000 g for 1 h at 4°C. The precipitations were suspended in 1 mL PBS and ultracentrifuged (100,000 g) for 70 min to wash the particles of the
contaminated protein and PEG. The resulting pellet was suspended in 50 µL of particle-free PBS (pH 7.4) and stored at – 80°C for further use.

The morphology of exosomes was observed using the Tecnai G2 Spirit Biotwin transmission electron microscope (FEI, Hillsboro, USA). The size distribution and concentration of exosomes were measured by nanoparticle tracking analysis (NTA) with Zeta View Particle Metrix 110 (Particle Metrix, Meerbusch, Germany) after diluting 10 µL exosomes in 1 mL PBS. The marker proteins of exosomes (CD9, CD63, CD81, and Alix) were detected by Western blot. The quantity of exosomes was determined using a BCA kit (YEASEN, Shanghai, China) for measurement of total protein. A PKH26 red fluorescent labeling kit (Umibio, Shanghai, China) was used in accordance with the manufacturer’s instructions to label the purified exosomes.

RT-PCR detection

The RNAiso for Small RNA kit (Takara, Dalian, China) was utilized to extract the total small RNA from exosomes and tissues. First-strand cDNA was reverse-transcribed with Mir-X miRNA First-Strand Synthesis Kit (Takara, Dalian, China). For miRNA quantification, TB Green Advantage RT-PCR Premix and mRQ 3’ Primer (Takara, Dalian, China) were used in real-time RT-PCR, and the delta-delta Ct method was applied to determine the relative expression of each miRNA to the level of U6 snRNA. All specimens were analyzed in triplicate. The following forward primers were used for the miRNAs of interest: miR-21-5p 5′ ACGTTGTGTAGCTTATCAGACTG 3′; miR-21-3p 5′ TGCGCCAACAGCAGTCGATGGG 3′; miR-27-5p 5′ GCGGCGGAGGGCTTAGCTGCTTG 3′; miR-31-5p 5′ CGGCGGAGGCAAGATGCTGGCA 3′; miR-142-5p 5′ GCCCCATAAAGTAGAAAGC 3′; miR-142-3p 5′ CTCCGTAGTGTTTCTTAC 3′; miR-223-5p 5′ TCGCGTGTATTTGACAAGCTGAGTTG 3′; miR-223-3p 5′ GAAGTTCTCGCTGCTAGTTTGTCA 3′.

Cell culture

H9c2 cells (Coboer, Nanjing, China), derived from embryonic rat cardiomyoblast, were cultured in complete DMEM containing 10% exosome-depleted FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

H₂O₂ treatment

H9c2 cells were seeded in a 96-well plate at a density of 1×10⁴ per well and pretreated with exosomes (40µg/mL) isolated from the sham, control, and Acu groups for 24 h. After the complete culture medium was replaced by serum-free medium, the plate was incubated with 400 µM H₂O₂ for 2h. The cell viability of H9c2 cells was detected by CCK-8 assay (YEASEN, Shanghai, China) in accordance with the manufacturer’s instruction. The H9c2 cells were seeded in a six-well plate, with 5×10⁵ cells per well, and similar experiments were performed as described above to investigate the protein expression these cells treated with H₂O₂.

Target gene prediction and identification
The online tools (miRanda, miRDB and TargetScan) were used to predict the target genes of rat miR-142-3p. Among the possible miR-142-3p target genes, this study focused on CFL2, which is involved in MI and the apoptosis pathway. Dual-luciferase reporter gene assay was performed to identify the target gene in 293 T cells. In brief, 3’ UTR and its site-specific mutated forms of CFL2 were cloned into the XbaI/XbaI site of the GV272 vector (GeneChem Biotechnology Company, Shanghai, China), which contains firefly luciferase reporter genes. Then, 1×10⁵ 293 T cells were seeded in a 24-well plate. The recombinant plasmid constructs (CFL23'-UTR and CFL23'-UTR-mut, 0.1 µg) were separately transfected into the 293 T cells with Renilla luciferase internal control plasma (pRL-TK, 0.02 µg) and miR-142-3p (0.4 µg) or the negative control of mimic plasma (NC mimic, 0.4 µg) by X-tremegene HP DNA transfection reagents (1 µl, ROCHE, Basel, Switzerland) as follows: Luc-CFL23'-UTR-NC + miR-142-3p-NC, Luc-CFL23'-UTR-NC + miR-142-3p-NC, Luc-CFL23'-UTR-NC + miR-142-3p, Luc-CFL23'-UTR-Mut + miR142-3p-NC, and Luc-CFL23'-UTR-Mut + miR142-3p. The firefly luciferase activity and Renilla luciferase activity were examined at 48 h post-transfection by using the dual-luciferase reporter assay system (Promega, Madison, USA) in accordance with the manufacturer's instructions. Each treatment was performed in triplicate at least three times.

Lentivirus preparation and transfection

The miRNA-142-3p mimic, miR-142-3p sponge, and scrambled control lentiviruses, which all carried Cherry red expression constructs, were designed and synthesized by GeneChem Biotechnology Company (Shanghai, China). H9c2 cells were seeded in 24-well plates with a density of 5×10⁴ cells per well. When the cells reached 70% confluence, lentiviral vectors were added to culture medium at an MOI of 50, followed by 5 µg/mL polybrene. The miRNA-142-3p-overexpressing cells, miR-142-3p sponge-expressing cell, and scrambled control cells were selected using either puromycin or neomycin.

Statistical analysis

Each experiment was repeated at least three times independently, and all derived statistical values were presented as the mean ± SEM. The statistical significance between sets of data was determined using GraphPad Prism 6 (GraphPad, San Diego, CA). Student's t-test was applied to compare two groups. For comparison of multiple groups, ANOVA followed by Tukey's multiple comparison test was used. Differences were considered statically significant at a P value of < 0.05.

Results

Acupuncture at Neiguan (P6) attenuates heart injury after MI

Masson's trichrome staining was performed to examine the histological changes in the infarcted heart. Seven days after MI, a decrease in left ventricular wall thickness was apparent in the control group (0.90 ± 0.12) compared with the sham group (2.34 ± 0.16). Acupuncture increased left ventricular wall thickness (1.69 ± 0.19), with more cardiocytes survived compared with the control group (Figs. 1A and B). However, no obvious difference in the infarct size was observed in the control and Acu groups (Fig. 1C).
The expression of cleaved caspase 3 (CC3) was examined by Western blot to investigate apoptosis of the infarcted heart. The results showed that CC3 was upregulated in the control group and downregulated in the Acu group (Figs. 1D and E). These data indicated that acupuncture at Neiguan (P6) could improve the survival of cardiac cells after MI through anti-apoptosis.

Inhibition of exosome secretion dampens the protective effect of acupuncture on myocardium

For clarification of the role of exosomes in acupunctural cardioprotection, its secretion was blocked by intraperitoneal injection of GW4869 (1.5 mg/kg, qd) 1 h before daily acupuncture. Seven days after MI, Masson's trichrome staining showed that the left ventricular wall thickness in the Acu plus GW4869 group obviously decreased (1.23 ± 0.13) compared with that in the Acu group (1.75 ± 0.15), and the survived cardiocytes in the infarcted region of the Acu plus GW4869 group was less than those in the Acu group (Figs. 2A and B). Similar to the above results, no difference in the infarct size was observed in the control, Acu, and Acu plus GW4869 groups (Fig. 2C). The results of TUNEL staining showed less apoptotic cells in the Acu group than in the control group and more apoptotic cells in the Acu plus GW4869 group (Fig. 2D). Western blot was performed to examine the changes in apoptosis-associated protein. The results showed overexpression of Bcl-2 and down-expression of BAX and CC3 in the Acu group compared with the control group, and GW4869 injection significantly reversed the expression of these proteins induced by acupuncture (Figs. 2E–G). Collectively, these data indicated that inhibition of exosome generation with GW4869 diminished acupuncture-induced myocardial protection.

Plasma exosomes from acupuncture ameliorate H9c2 injury induced by H$_2$O$_2$

Several studies have exhibited that circulating exosomes induced by remote ischemic preconditioning or exercise afford protective effects on injured heart [13, 14]. In the present study, to investigate whether circulating exosomes contribute to the beneficial effects of acupuncture treatment, the plasma exosomes of MI rats were isolated with or without acupuncture (Exo-Con and Exo-Acu) at Neiguan (PC6) by using ultracentrifugation methods. Transmission electron microscopy (TEM) analysis of isolated pellets revealed typical rounded bilayer-membrane structure with diameters of ~ 100 nm (Fig. 3A). Western blot analysis of Exo-Con and Exo-Acu confirmed the presence of exosome markers Alix, CD63, CD81, and CD9 (Fig. 3B). Nanoparticle tracking analysis (NTA) was used to determine the size of pelleted structures, and the results showed that exosomes (Exo-Con and Exo-Acu) of nearly 98% were about 90 nm and the rest of them ranging from 30 nm to 250 nm. Furthermore, the plasma concentration of Exo-Con was almost as much as that of Exo-Acu from the same amount of plasma (3.6×10$^{11}$ versus 4.2×10$^{11}$/mL, Fig. 3C). Exo-Con and Exo-Acu were labelled with PKH26 dye and incubated with cultured H9c2 cells separately to conform whether exosomes could be internalized through endocytosis. Six h later, a strong red fluorescence was observed in the H9c2 cells’ cytoplasm of the two groups, indicating the similar entry of Exo-Con and Exo-Acu to H9c2 cells (Fig. 3D). For investigation of the cardioprotective effects of plasma exosomes, the H9c2 cells were pretreated with phosphate-buffered saline (PBS), Exo-Con, or Exo-Acu for 24 h and subjected to H$_2$O$_2$ (0.4 mM) or PBS treatment for 2 h. The viability of cells was assessed using CCK-8 assay (Fig. 3E). The results showed obvious increment of cell viability in the Exo-Acu-treated H9c2
cells. Notably, Exo-Con did not benefit from the viability of $H_2O_2$-treated cells, similar with PBS. These results suggested that circulating exosomes after acupuncture could be internalized through cardiocytic endocytosis, affording cardioprotective effects.

MiR-142-3p is a key component harbored in circulating exosomes after acupuncture

This study focused on miRNAs for their dramatic effects in minute quantities to further clarify the molecular mediator in exosome-induced cardio protection post-MI. The changes of miRNAs in the tissue of Neiguan (PC6) were tested by small RNA sequencing, and eight differentially up-expressed miRNAs (miR-21-5p, miR-21-3p, miR-27-5p, miR-31-5p, miR-142-5p, miR-142-3p, miR-223-5p, and miR-223-3p; fold change $>2.0$; $P<0.05$; Fig. 4A) were detected in the acupunctured rats. Among them, six (miR-21-5p, miR-21-3p, miR-27-5p, miR-142-3p, miR-223-5p, and miR-223-3p) were identifiably upregulated in the Acu group compared with the control group according to real-time polymerase chain reaction (RT-PCR) analysis (Fig. 4B). The changes of these eight miRNAs in plasma exosomes were also investigated by RT-PCR analysis, and five of them (miR-21-5p, miR-21-3p, miR-27-5p, miR-142-5p, and miR-142-3p) were upregulated in the Acu group compared with the control group (Fig. 4C). Finally, the differential expression of eight miRNAs in heart was verified. The results showed that only miR-142-3p increased in the Acu group compared with the control group (Fig. 4D). Intriguingly, GW4869 reversed the miRNA changes in these tissues induced by acupuncture (Figs. 4B–D). Taken together, these data indicated that miR-142-3p, which was secreted at Neiguan (PC6) and transferred into the injured heart by exosomes, is indispensable in distal acupuncture-conferred distant cardio protection.

CFL2 is a target gene of miR-142-3p in acupuncture-induced cardioprotection

MiR-142-3p has been proven to inhibit cardiomyocytic apoptosis after heart injury through multiple signaling pathways [18, 19]. In the present study, the possible miR-142-3p target genes were predicted using bioinformatics-based analysis (miRanda, miRDB, and TargetScan) to clarify the target genes of miR-142-3p in acupuncture-induced cardioprotection; this study focused on CFL2, which is translocated to the mitochondria to induce massive apoptosis and involved in the response of myocardiocytes to oxidative stress (Fig. 5A) [20, 21]. According to the bioinformatics analysis, miR-142-3p was predicted to bind the CFL2 3′-untranslated region (UTR), which contains a highly conserved binding site (652–658, Fig. 5B). A dual luciferase reporter plasmid containing wild type of CFL2 3′-UTR or mutant of CFL2 3′-UTR was co-transfected with miR-142-3p mimic or a scrambling miRNA. The results of dual luciferase reporter assay indicated that the miR-142-3p mimic specifically suppressed the activity of CFL2 3′-UTR luciferase, whereas no inhibition in the mutant construct was observed (Fig. 5C). Meanwhile, H9c2 cells were infected with lentiviral vector to express miRNA-142-3p mimic or miR-142-3p sponge or their respective controls to further confirm the inhibition of CFL2 expression by miR-142-3p. The RT-PCR results showed a 100-fold increase in miRNAs induced by infection. The RT-PCR and Western blot results revealed a significant decrease in CFL2 induced by miR-142-3p mimic lentivirus and an increase in CFL2 induced by miR-142-3p sponge lentivirus in H9c2 cells (Figs. 5D–G). The expression of CFL2 was also investigated in vivo after acupuncture treatment, and the Western blot results showed downregulation of CFL2 in the
Acu group compared with the control group and upregulation of CFL2 in the Acu plus GW4869 group compared with the Acu group (Figs. 5H and I). Taken together, the data indicated that CFL2 is a downstream target gene of miR-142-3p in acupuncture-induced cardioprotection.

Overexpression of miR-142-3p attenuates $\text{H}_2\text{O}_2$-induced apoptosis of H9c2

H9c2 cells overexpressing miR-142-3p mimic or sponge induced by lentivirus were incubated with 0.4 mM $\text{H}_2\text{O}_2$ for 2 h to further examine the functional effect of miR-142-3p in cardioprotection. Distinctive morphological changes in the H9c2 cells were observed in the control, mimic NC, and sponge NC groups, showing shrunken, round, and distorted cell shapes. These morphological changes were partly ameliorated in the miR-142-3p mimic group and worsened in the miR-142-3p sponge group (Fig. 6A). The results of CCK-8 showed that miR-142-3p mimic increased the viability of H9c2 cells, whereas miR-142-3p sponge aggravated it (Fig. 6B). Meanwhile, miR-142-3p overexpression reduced CC3 expression at protein levels, whereas miR-142-3p sponge reversed this effect (Figs. 6C and D). These data indicated that miR-142-3p possesses a protective effect on $\text{H}_2\text{O}_2$-induced injuries in H9c2 cells via anti-apoptosis.

Discussion

This study revealed two major findings. First, acupuncture-derived circulating exosomes transfer cardioprotective signals to injured heart and confer cardioprotective effects. Second, miR-142-3p is a major exosomal miRNA in inhibiting apoptotic signal by targeting CFL2.

The acupoint of Neiguan (PC6) is the most advised for the treatment of heart diseases in the theory of Chinese acupuncture. A wealth of evidence showed the benefits of Neiguan (PC6) acupuncture for attenuating cardiac injury via multi-pathways, such as inhibiting apoptosis, alleviating mitochondrial damage, and decreasing the degree of inflammation [10, 22, 23]. In the present study, acupuncture at Neiguan (PC6) thickened the left ventricular wall in a rat model of MI and downregulated the expression of CC3. These results indicated that ischemic cardiocytes could benefit from the remote stimulation of acupuncture and survive from severe damage by anti-apoptosis of acupuncture. However, the mechanism of distant acupuncture associated with protection for injured heart is still unclear.

Recent studies discovered that circulating exosomes could transfer their signal molecules to recipient cells of the infarction area and enhance the function of heart [13, 14, 24]. Circulating exosomes represent a potentially central role of distance communication from Neiguan (PC6) to the injured heart. For confirmation of this assumption, exosome secretion of MI rat was inhibited by consecutive intraperitoneal injection of GW4869 before each needling. The results of Masson’s trichrome staining showed that GW4869 obviously weakened the protective effect of acupuncture on the infarcted heart, with thinner left ventricular wall and less cardiocytes than the Acu group without GW4869 injection. Meanwhile, with the reduction in exosome secretion by GW4869, the acupuncture inhibition of apoptosis in injured cells was attenuated, specifically manifested as more TUNEL positive cells, up-expression of Bax and CC3, and down-expression of Bcl-2. Furthermore, $\text{H}_2\text{O}_2$-induced apoptosis was used to determine
the cryoprotection of plasmatic exosomes on apoptosis in H9c2 cells. Consistent with the results in vivo, the H9c2 cells incubated with exosomes from acupuncture rats exhibited higher viability than those incubated with exosomes from model rats. These results demonstrated the anti-apoptosis effects of Acu-Exo on myocardial tissue after MI.

MiRNAs have received more interest for their dramatic effects with minute quantities than proteins and mRNAs harbored in exosome [25]. First, eight differently expressed miRNAs were found in the tissue of acupuncture Neiguan (P6) by using next-generation sequencing. Among them, six miRNAs were verified by RT-PCR. Then, RT-PCR was performed to further explore whether these miRNAs were harbored in circular exosomes. The results showed that five miRNAs were differently expressed in the exosome, and miR-142-3p was significantly increased among these miRNAs. Interestingly, miR-142-3p was found to be the unique upregulating miRNA in the myocardial tissue of the Acu group. In addition, these miRNA changes induced by acupuncture were reversed by GW4869 injection. The results demonstrated that acupuncture affects the quality of circulating exosomes, and exosomes with miR-142-3p enrichment induced by acupuncture may exert cardioprotective effect.

Theoretically, a miRNA could target many different mRNAs. In this study, the results of bioinformatics analysis showed that miR-142-3p targeted CFL2 mRNA, as verified by the dual luciferase reporter system. CFL2 is the major form of actin-binding protein family in differentiated cardiocytes [26]. A recent study indicated that CFL2 promotes apoptosis by translocation to the mitochondria, which leads to cytochrome c release and caspase activation [27]. In the present study, the expression of CFL2 was confirmed to be downregulated by miR-142-3p mimic and upregulated by miR-142-3p sponge in H9c2 cells. Moreover, acupuncture at Neiguan (PC6) decreased CFL2 expression in infarcted myocardium, and GW4869 injection reversed the suppression effect of acupuncture on CFL2. MiR-142-3p exhibits pleiotropic roles in cardiovascular diseases, and it was proven to be an important regulator for inhibiting inflammation, apoptosis, and autophagy in injured cardiomyocytes [18, 28]. Through up- and down-expression of miR-142-3p, miR-142-3p was proven to protect H9c2 cells treated with H2O2 and downregulate the expression of CC3. These results suggested that miR-142-3p possesses anti-apoptotic effects, partly through downregulation of CFL2 and the following inhibition of caspase 3 in MI hearts.

Conclusions

In summary, circulating exosomes are distinctively ferrying carrier for remote cardioprotection of acupuncture at Neiguan (PC6). Exosomal miR-142-3p was identified as a critical cardioprotective molecule for the inhibition of cardiocytic apoptosis. Moreover, CFL2 was found to be a target gene of miR-142-3p. These findings presented a novel cardioprotective mechanism of remote acupuncture by secreting exosomal miR-142-3p, revealing the therapeutic potential of miR-142-3p in the prevention and restoration of ischemic heart disease.

Abbreviations
CC3, cleaved caspase 3; CFL2, Cofilin-2; CVD, cardiovascular disease; I/R, ischemia/reperfusion; LAD, left anterior descending coronary artery; MI, myocardial infarction; NTA, nanoparticle tracking analysis; RT-PCR, real-time polymerase chain reaction; SHUTCM, Shanghai University of Traditional Chinese Medicine; TEM, transmission electron microscopy; TUNEL, Terminal Deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labeling; UTR, 3'-untranslated regions

Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Shanghai University of Traditional Chinese Medicine.

Consent for publication

Not applicable.

Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

WQ and LR planned and supervised the project. WQ, ZY, WY, and GH designed the experiments. ZY, WY, GH, GC, SQ, LP, and SY performed the experiments. WQ, ZY, and SS analyzed the data. WQ and LR validated the data, wrote the original draft, and revised the manuscript.

Acknowledgements

Not applicable.

References


Figures
Figure 1

Acupuncture at Neiguan (P6) thickened the infarcted left ventricular wall and inhibited apoptosis of infarcted heart. A Photographs of the heart transverse section at the widest parts of the infarcted regions (bar = 5 mm). The second row is the partial enlarged view of infarcted regions in the first row (bar = 100 μm). B and C Statistical analysis of the left ventricular wall thickness and scar size of infarcted hearts from various groups of rats (*p < 0.05; ***p < 0.001 versus sham group; ##p < 0.01 versus control group,
n=6). D and E Western blot and quantified data for the expression of cleaved caspase 3 in infarcted cardiac tissue from various groups of rats. (**p< 0.001 versus sham group; ##p< 0.01 versus control group, n=6).

Figure 2

GW4869 weakened the cardioprotective effects of acupuncture. A Photographs of the heart transverse section at the widest parts of the infarcted regions (bar = 5 mm). The second row is the partial enlarged view of infarcted regions in the first row (bar = 100 μm). B and C Statistical analysis of LV wall thickness and scar size of infarcted hearts from various groups of rats (**p< 0.001 versus sham group; ##p< 0.01 versus control group; #p< 0.05 versus acupuncture group, n=6). D TUNEL staining of the heart cross section from various groups of rats. The third row is the partial enlarged view of infarcted regions in the box of second row (bar = 100 μm). E–G Western blot and quantified data for the expression of Bcl-2, BAX, and cleaved caspase 3 in infarcted cardiac tissue from various groups of rats (**p< 0.001 versus control group; ###p< 0.001 versus acupuncture group, n=6).
Figure 3

Characterization and functional validation of plasma exosomes derived from MI rats with and without acupuncture treatment. A Transmission electron micrograph of plasma exosomes. B Western blot assessing the presence of Alix, CD63, CD81, and CD9 in plasma exosomes. C Nanoparticle trafficking analysis of the diameters and concentration of plasma exosomes. D Uptake of PKH26-labeled plasma exosomes (red spots) by H9c2 cells. E CCK-8 assay for detecting the viability of H9c2 cells incubated with 0.4mM H$_2$O$_2$ for 2 h after pretreatment of Exo-Con or Exo-Acu (* $p$ < 0.05; ** $p$ < 0.001, n=6).
Figure 4

MiR-142-3p is a crucial exosomal miRNA induced by acupuncture. A Expression heatmap showing the relative abundance of eight miRNAs in the tissue of Neiguan (PC6) treated with acupuncture (n=3). B RT-PCR analysis of eight differentially expressed miRNAs in the tissue of Neiguan (PC6) from various groups of rats (n=6). C RT-PCR analysis of eight differentially expressed miRNAs in exosomes from various groups of rats (n=6). D RT-PCR analysis of eight differentially expressed miRNAs in the infarcted region of hearts from various groups of rats (n=6). ***p< 0.001 versus control group; ####p< 0.001 versus acupuncture group.
Figure 5

CFL2 is a target gene of miR-142-3p in cardioprotection of acupuncture. A Screening scheme for putative target genes that may contribute to the antiapoptotic effects of miR-342-5p. B Luciferase reporter constructs containing wild-type or mutated 3′-UTR of rat CFL2 mRNA. The sequence in red indicates the predicted binding site for miR-142-3p, aligning with miR-142-3p seed sequence. C Relative luciferase activity normalized to negative control of miR-142-3p mimic (NC mimic) (CFL2, **p < 0.01, n=3). D RT-PCR analysis of miR-142-3p and miR-142-3p sponge expression in H9c2 infected by lentiviral-vector expressing miRNA-142-3p mimic or miR-142-3p sponge (***p < 0.001 versus negative control, n=3). E RT-PCR analysis of CFL2 expression in H9c2 infected by lentiviral-vector expressing miRNA-142-3p mimic or miR-142-3p sponge (*p < 0.05; **p < 0.01, n=3). F and G Western blot and quantified data for the expression of CFL2 in H9c2 cells infected by lentiviral-vector expressing miRNA-142-3p mimic or miR-142-3p sponge (*p < 0.05; ***p < 0.001, n=3). H and I Western blot and quantified data for the expression of CFL2 in infarcted cardiac tissues from various groups of rats (**p < 0.01, n=6).
Overexpressing miR-142-3p retarded oxidative damage of H9c2 by anti-apoptosis. H9c2 cells were infected by lentiviral-vector expressing miRNA-142-3p mimic or miR-142-3p sponge. A Morphological change in H9c2 cells incubated with 0.4mM H$_2$O$_2$ for 2 h. B CCK-8 assay assessing the viability of H$_2$O$_2$-incubated H9c2 cells (*p< 0.05, n=6). C and D Western blot and quantified data for the expression of cleaved caspase 3 (CC3) in H$_2$O$_2$-incubated H9c2 cells (**p< 0.01, n=6).