



Original Article

Exosomes from adipose-derived stem cells protect against high glucose-induced erectile dysfunction by delivery of corin in a streptozotocin-induced diabetic rat model

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ABSTRACT

Introduction: Increasing study have found that stem cell transplantation have a therapeutical effect to diabetes mellitus (DM)-induced erectile dysfunction (ED). So, the aim of this study was to evaluate the beneficial effect of corin from adipose-derived stem cells (ADSCs) on DM-induced ED.

Methods: Exosomes were isolated from ADSCs (ADSC-EXOs) or from ADSCs in which corin gene expression was silenced by siRNA (siCorin). For *in vivo* studies, rats with streptozotocin-induced DM were intravenously injected with ADSC-EXOs or siCorin-ADSC-EXOs. Two weeks later, intracavernosal pressure (ICP) and mean arterial pressure (MAP) were measured to assess erectile function, and penile tissues were harvested for further evaluation of levels of inflammatory factors and expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and neuronal nitric oxide synthase (nNOS). We also evaluated the recovery of neurovascular function in penile tissues by immunofluorescence analysis.

Results: The results showed that ADSC-EXOs restored erectile function in diabetic rats, as determined by the ICP/MAP ratio. Exosomes from ADSCs also promoted neurovascular function and suppressed expression of inflammatory factors. In contrast, the decreased content of corin in exosomes after silencing corin in ADSCs reduced the therapeutic effect of exosomes on ED.

Conclusion: These findings demonstrated the therapeutic mechanism underlying the use of ADSC-EXOs for treating ED and the beneficial effect of corin.

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1. Introduction

Erectile dysfunction (ED) is a common chronic complication in diabetes mellitus (DM). Increasing attention has been focused on ED in men with diabetes due to its multifactorial pathophysiology and the concurrence of the same components present in vasculopathy, neuropathy and depression [1,2]. ED is defined as the

inability to achieve and/or maintain an erection sufficient to permit satisfactory sexual intercourse [3]. ED results from damage to nerves, blood vessels, corporal smooth muscle and/or endothelial cells [4,5]. The incidence of ED is three times higher among diabetic men compared with non-diabetic men, with up to 75% of men with DM experiencing ED [6]. This condition negatively affects the quality of life in affected individuals.

The proposed mechanisms causing diabetic ED include increased advanced glycation end-products, impaired synthesis of neuronal nitric oxide synthase (nNOS), elevated oxygen free radical levels, decreased cyclic guanosine monophosphate (cGMP)-dependent kinase-1 and nitric oxide (NO)-dependent selective nitergic nerve degeneration, largely resulting from endothelial dysfunction. Endothelial dysfunction refers to loss of the ability of endothelium to produce vasorelaxation messengers and to

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maintain vasodilation and vascular homeostasis functionally [7,8]. Thus, evidence is accumulating to consider ED as a vascular disorder [9].

Corin is a type-II transmembrane serine protease found mainly in the heart [10,11]. By converting natriuretic peptides to their active form [12,13], corin plays an essential role in the regulation of water and salt balance, especially under edematous disease states including heart failure. Corin converts pro-atrial natriuretic peptide (ANP) and pro-brain natriuretic peptide (BNP) to their active forms, with ANP being the main substrate. ANP and BNP induce the production of cGMP, which in turn promotes vasodilation. A previous study found that protein levels of corin, ANP, BNP, nNOS, and the level of cGMP were significantly downregulated in the corpus cavernosum of diabetic ED rats [14], suggesting that loss of corin contributes to the progression of diabetic ED. A previous study also demonstrated that both adipose-derived mesenchymal stem cells (ADSCs) and ADSC-derived exosomes (ADSC-EXOs) could ameliorate ED [15], but the role of corin in these effects was not delineated. Therefore, the aims of this study were to determine if transplantation of ADSC-EXOs could restore erectile function in diabetic rats and to evaluate the ability of corin to protect against ED induced by DM.

2. Materials and methods

2.1. Animals and ethics statement

All animal procedures were approved by the Animal Care and Utilization Committee of The Third Affiliated Hospital of Nantong University. Eight-week old male Sprague–Dawley rats were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The rats were individually housed in independent ventilated cages under constant ambient temperature (24–26 °C) and humidity under a 12 h light/dark cycle. All animal experiments were approved and performed following the guidelines of the Ethics Committee of the Third Affiliated Hospital of Nantong University of China. All surgical procedures were performed under anesthesia, and every effort was made to minimize suffering. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg).

2.2. Isolation, culture and identification of ADSCs

2.2.1. ADSC isolation

In brief, adipose tissue was harvested from normal rats. The tissue was washed with phosphate-buffered saline (PBS) and mechanically chopped before digestion with 0.2% collagenase I (Sigma–Aldrich, Milwaukee, WI, USA) for 1 h at 37 °C with intermittent shaking. The digested tissue was washed with Dulbecco's Modified Eagle's Medium (DMEM; Sigma–Aldrich, Milwaukee, WI, USA) containing 15% fetal bovine serum (FBS) and then centrifuged at 1000 g for 10 min to remove mature adipocytes. The cell pellet was resuspended in DMEM supplemented with 15% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin in a 37 °C incubator with 5% CO₂. ADSCs reaching 80%–90% confluency were detached with 0.02% EDTA/0.25% trypsin (Sigma–Aldrich, Milwaukee, WI, USA) for 5 min at room temperature and then replated. For phenotypic analysis, phycoerythrin was used. The expression of the following markers was investigated: CD29, CD90, CD44, CD105 and von Willebrand factor (vWF). An IgG-matched isotype served as the internal control for each antibody. ADSCs were cultured under normoxic conditions in 95% air (20% O₂) and 5% CO₂.

2.3. Multilineage differentiation of ADSCs

To identify the capacity of ADSCs for multilineage differentiation, third passage rat ADSCs were cultured under differentiation conditions. For adipocyte differentiation, ADSCs were cultured in adipogenic differentiation medium and Oil Red O staining was performed to assess adipocyte differentiation after 14 days. For osteoblast differentiation, ADSCs were cultured in osteogenic differentiation medium and cells were stained with alizarin red after 21 days to monitor osteogenesis.

2.4. Isolation and identification of ADSC-EXOs

Upon reaching 80–90% confluency, the ADSCs were rinsed with PBS and cultured in EGM-2MV media without FBS and supplemented with 1 × serum replacement solution (PeproTech, New Jersey, USA) for an additional 48 h. Conditioned medium from ADSCs was obtained and centrifuged at 300×g for 10 min and 2000×g for 10 min to remove dead cells and cellular debris. Finally, after centrifugation at 12,000×g for 30 min, the supernatant was filtered using a 0.22 µm filter (Millipore, Billerica, MA, USA) and 15 mL of supernatant was added to an Amicon Ultra-15 Centrifugal Filter Unit (100 kDa; Millipore, Billerica, MA, USA) and centrifuged at 4000×g to about 1 mL. The ultrafiltration liquid was washed twice with PBS and the ultracentrifugation was repeated at 4000×g to 1 mL. All procedures were performed at 4 °C. The protein content of exosomes was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). ADSC-EXOs were stored at –80 °C or used immediately for downstream experiments. Transmission electron microscopy and western blotting were used to identify the collected exosomes.

2.5. Rat model of streptozotocin-induced DM

Sprague–Dawley rats were administered a single intraperitoneal injection of streptozotocin (Sigma–Aldrich, Milwaukee, WI, USA) at a dose of 60 mg/kg in citrate buffer (50 mM sodium citrate, pH 4.5). Blood glucose levels in samples collected from the tail vein were measured at 72 h and every 2 weeks after the injection of streptozotocin with a blood glucose meter. Rats with a constant blood glucose level higher than 16.7 mmol/L were considered as diabetic models. After 12 weeks, evaluation of erectile function was performed on the diabetic rats and those with ED were selected for the following test. The ED group of diabetic rats was administered a single intravenous injection of 100 µL of exosomes (200 µg dissolved in 100 µL PBS) from ADSCs transfected with or without siRNA directed against corin. For the control group of rats and diabetic rats not administered exosomes, an equal volume of vehicle solution (normal saline) was injected. After 2 weeks, the rats in each group were analyzed for the restoration of erectile function before penile tissues were harvested.

2.6. Intracavernous pressure (ICP) and mean arterial pressure (MAP) measurements

After the 2-week treatment period, rats were anesthetized with 30 mg/kg sodium pentobarbital injected intraperitoneally. Then, the major pelvic ganglion, cavernous nerves and pelvic organs were exposed and a 23-gauge needle connected to a PE-50 tube containing 250 IU/mL heparinized saline was carefully inserted into the cavernous tissues. The other end of the PE-50 tube was connected to a pressure transducer (Statham P23 Gb; Waltham, MA, USA) integrated into a computerized data acquisition system (BioPac, Goleta, CA, USA) to measure ICP and MAP under electric stimulation at 20 Hz and 5 V for 60 s. A butterfly needle was inserted into the

aorta at the aortic bifurcation to determine the ICP/MAP ratio using the same equipment.

2.7. Construction and transfection of corin siRNA

Specific siRNA sequences targeting corin were synthesized (Genepharma, Shanghai, China) and transfections of ADSCs were performed with Lipofectamine 3000 (Invitrogen, California, USA) according to the manufacturer's instructions. The sense and antisense strands of the corin siRNA sequence were 5'-GCAGU-GUAAUGGCUACAAUTT-3' and 5'-AUUGUAGCCAUUACACUGCTT-3', respectively. The efficiency of corin silencing was assessed by qRT-PCR and Western blot analysis. Data were obtained from at least three independent experiments.

2.8. Quantitative reverse transcription-PCR (qRT-PCR)

The extraction of total RNA was performed using TRIzol according to the manufacturer's protocol and processed for cDNA synthesis using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA, USA). The following primers were used to amplify equal amounts of cDNA: corin, 5'-TGCCCAAGCGGAAGTGAG-3' and 5'-GACGGATGGTCCAGGTTGTTT-3'; β -actin, 5'-GTTGACATCCGTAAAGACC-3' and 5'-GACTCATCGTACTCCTGCT-3'. The ABI 7900 thermocycler (Applied Biosystems) was used to perform qRT-PCR. Each cDNA sample was examined in triplicate. Quantification of the relative expression of mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. Data were normalized to β -actin.

2.9. Immunofluorescence analysis

Cavernous tissue samples from each group were fixed in 10% formalin solution and embedded in paraffin. Sections (5 μ m) were stained with CD31 and beta-III tubulin antisera to evaluate histopathological changes in angiogenesis and nerves. Sections were examined using an Axiophot light microscope (Zeiss, Oberkochen, Germany) or fluorescence microscope (Nikon, Tokyo, Japan) and photographed with a digital camera.

2.10. Western blot analysis

The cavernous tissues were lysed, protease inhibitors were added to the lysates and the lysates were centrifuged at 12,000 g at 4 °C. The protein concentration was determined using the BCA kit (Thermo Fisher, MA, USA). Total proteins were resolved using 10% SDS-PAGE assays and transferred to PVDF membranes. Antibodies against the proteins listed below (all from Santa Cruz Biotechnology, Dallas, TX, USA) were used to determine protein expression: Corin (1:600), CD63 (1:600), CD81 (1:600), CD31 (1:1000), CD9 (1:500), ANP (1:500), BNP (1:600), nNOS (1:500) and Akt (1:500). Anti- β -actin (1:1000) was provided by Sigma-Aldrich (Milwaukee, WI, USA), and horseradish peroxidase-conjugated secondary antibody (1:1000) was from Sigma-Aldrich (Milwaukee, WI, USA). An ECL chemiluminescent kit (Millipore) was used to measure protein bands.

2.11. ELISA

To examine the amount of soluble IL-6, IL-1 β and TNF- α in the cavernous tissue of rats, commercially available enzyme-linked immunosorbent assay (ELISA) kits (Sen-Xiong Technology, Shanghai, China) were used. In accordance with the manufacturer's instructions, all supernatants were stored at -80 °C before measurements were taken and both the standards and samples were

run in triplicate. The OD₄₅₀ was calculated by subtracting the background value and standard curves were plotted.

2.12. Measurement of cGMP levels

Corpus cavernosum tissue was removed quickly and frozen in liquid nitrogen after measurement of MAP and ICP. A cGMP direct immunoassay kit (K272-100; BioVision, Edmonton, Canada) was used to measure the cavernous cGMP levels by reading the absorbance at 450 nm.

2.13. Statistical analysis

Continuous variables were expressed as means \pm standard deviation (SD). One-way analysis of variance was performed for multiple comparisons using GraphPad Prism software, version 5.0 (GraphPad, La Jolla, CA, USA). A value of $P \leq 0.05$ was assumed to indicate a statistically significant difference.

3. Results

3.1. Characterization of ADSC-Exos

Previous studies have shown that ADSC-EXOs and bone marrow-derived mesenchymal stem cells are effective treatments for ED [16–18], but the underlying mechanisms are still unknown. In this study, we isolated ADSCs from rat adipose tissue and showed that isolated ADSCs displayed a typical cobblestone-like morphology (Fig. 1A). Immunofluorescence detection demonstrated expression of ADSC cell surface markers CD29, CD90, CD44 and CD105, but the cells were negative for the endothelial marker vWF (Fig. 1B–G). The results also showed that ADSCs have the capacity to differentiate into adipocytes and osteoblasts as demonstrated by Oil Red O and alkaline phosphatase staining, respectively (Fig. 1H and I). These results suggested that the stem cells we isolated were ADSCs. We then isolated exosomes from ADSCs using an ultracentrifugation process. Transmission electron microscope detection found that the exosomes from ADSCs exhibited a cup- or sphere-shaped morphology approximately 100 nm in diameter (Fig. 1J). Western blot detection further confirmed expression of exosome markers CD63, CD81 CD31 and CD9 in ADSC-Exos (Fig. 1K), which suggested that these nanoparticles were in fact exosomes.

3.2. ADSC-EXOs improved ED in diabetic rats by delivery of corin

ED adversely affects the quality of life for many men. Diabetes mellitus (DM) induced ED has been shown to involve major changes to the neurovasculature of the penile tissue [19]. Corin is primarily expressed in cardiac tissue and acts as an enzyme in converting pro-ANP to biologically active ANP, a signaling peptide that has the ability to regulate blood pressure [20]. In order to determine if corin contained in ADSC-EXOs had a therapeutic effect on ED, we constructed siRNA against corin (siCorin) and negative control RNA (NC) and transfected them into ADSCs. After 48 h of transfection, we collected exosomes from the ADSCs and found that ADSC-EXOs derived from corin-silenced ADSCs showed low expression of corin at both the mRNA and protein level when compared with untreated cells or NC-transfected control cells (Fig. 2A and B).

Exosomes from siCorin-silenced ADSCs or from wild-type ADSCs were transplanted into diabetic rats to investigate their effect on erectile function. ICP was measured after cavernous nerve stimulation at 5 V for 1 min in normal rats, diabetic rats and exosome-transplanted diabetic rats (Fig. 3A and B). A significant drop in

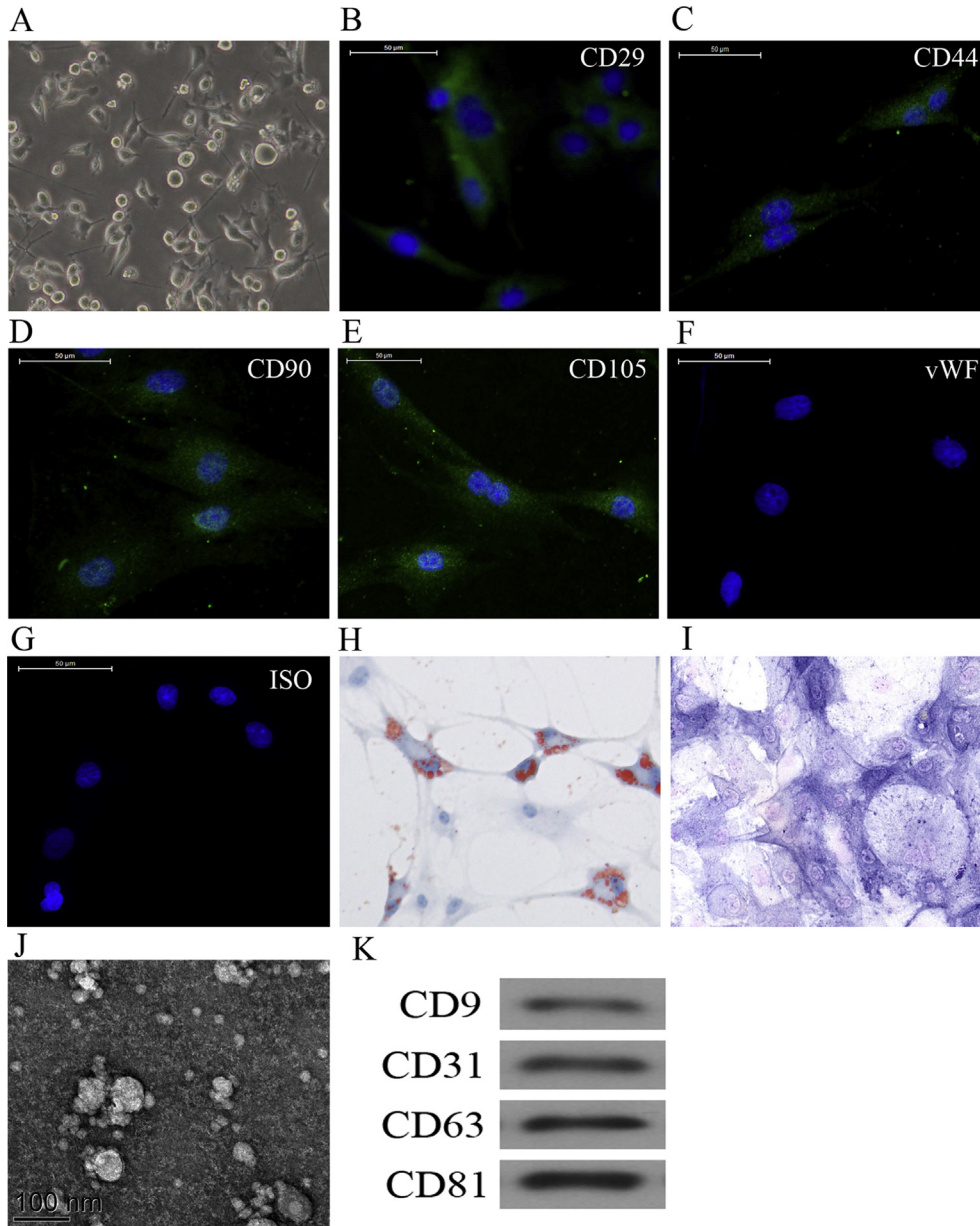


Fig. 1. Characterization of exosomes released by ADSCs. (A) ADSCs showed a typical cobblestone-like morphology. (B–G) Immunofluorescence staining of cell surface markers. (H and I) Differentiation potential of ADSCs assessed by oil red O (H) and alkaline phosphatase staining (I). (J) Transmission electron micrographs showing ADSC-EXO morphology. (K) Western blots of CD31, CD9, CD63 and CD81 expression in exosomes from ADSCs.

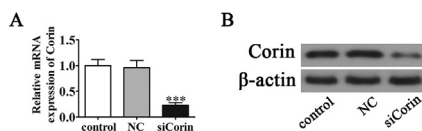


Fig. 2. The expression of corin in ADSC-EXOs. (A and B) qRT-PCR (A) and western blots (B) show that expression of corin in ADSC-Exos was reduced after transfection of ADSCs with siRNA against corin (siCorin) or negative control RNA (NC). Data are expressed as means \pm SD. *** $p \leq 0.001$ vs. control group.

ICP was evident in diabetic rats compared with normal rats, but restoration of ICP was observed in diabetic rats transplanted with exosomes from wild-type ADSCs. However, after silencing corin expression in ADSCs, the effect of ADSC-EXOs was suppressed,

suggesting that the therapeutic effect of exosomes on ED was mediated by corin.

3.3. ADSC-EXOs improved ED in diabetic rats by promotion of neurovascular function and suppression of inflammatory factor expression

The cGMP concentration in the corpus cavernosum was determined using a cGMP direct immunoassay kit (Fig. 4A). In diabetic rats, cGMP levels were restored by transplantation with exosomes derived from wild-type ADSCs. In contrast, downregulation of corin by siCorin suppressed the restorative effect of ADSC-EXOs on cGMP expression in rats with DM. Western blot detection found that the expression of ANP, BNP and nNOS were all downregulated in the DM group. In contrast, ADSC-EXO transplantation restored ANP,

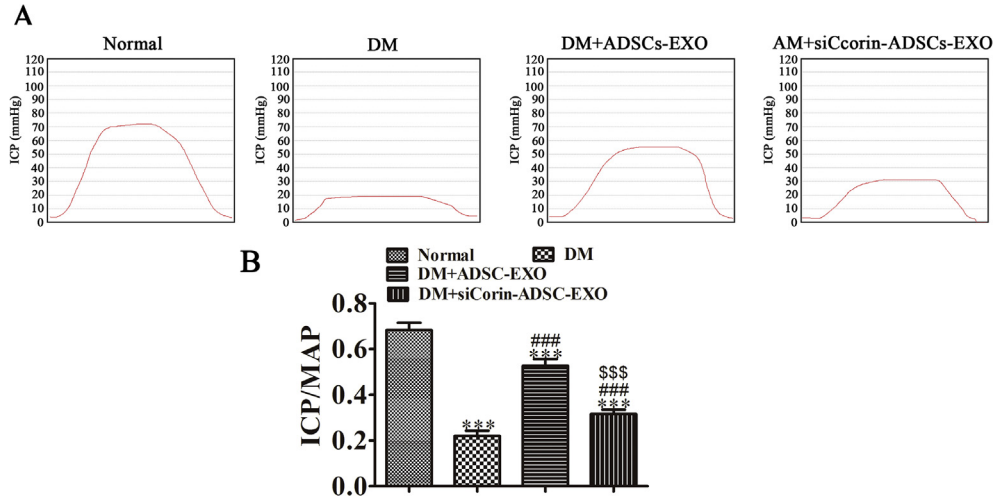


Fig. 3. ADSCs-EXOs restored erectile function in diabetic rats via delivery of corin. (A) Representative recordings of the maximal ICP induced by cavernous nerve stimulation at 5 V for 1 min in control rats, diabetic rats and rats transplanted with ADSCs or siCorin-transfected ADSCs. DM indicates rats with diabetes mellitus. (B) Maximal ICP values normalized to mean systemic arterial pressure (MAP), presented as the ICP/MAP ratio, for control rats and each of the treatment groups. Data are expressed as means \pm SD. *** $p \leq 0.001$ vs. normal group, ### $p \leq 0.001$ vs. DM group, \$\$\$ $p \leq 0.001$ vs. DM + ADSC-Exo group. DM, diabetes mellitus; EXO, exosome.

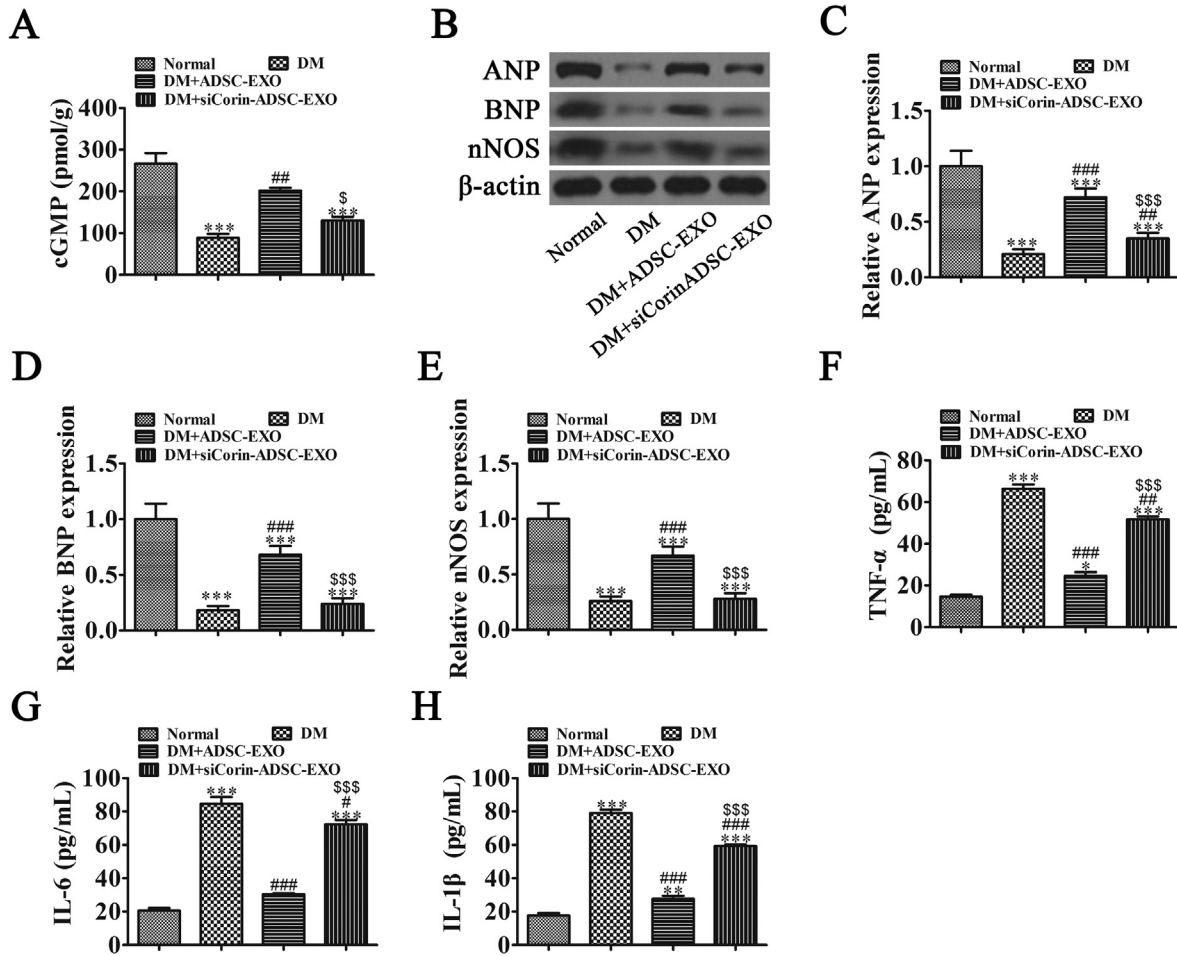


Fig. 4. Expression changes in neurotrophic factors, inflammatory factors and natriuretic peptides relative to protein in the corpus cavernosum in response to transplantation of ADSC-EXOs or siCorin-ADSC-EXOs in DM rats. (A) cGMP concentration in the corpus cavernosum determined using a cGMP direct immunoassay kit. Data are expressed as means \pm SD. *** $p \leq 0.001$ vs. normal group, ## $p \leq 0.01$ vs. DM group, * $p \leq 0.05$ vs. DM + ADSC-EXO group. (B–E) Western blot analysis showing the expression of ANP, BNP and nNOS. Data are expressed as means \pm SD. *** $p \leq 0.001$ vs. normal group, ## $p \leq 0.01$, ### $p \leq 0.001$ vs. DM group; \$\$\$ $p \leq 0.001$ vs. DM + ADSC-EXO group. (F–H) ELISAs show the expression of inflammatory factors TNF- α , IL-6 and IL-1 β . Data are expressed as means \pm SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ vs. normal group; # $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$ vs. DM group; \$\$\$ $p \leq 0.001$ vs. DM + ADSC-EXO group.

BNP and nNOS expression, while downregulation of corin eliminated the restorative effect of ADSC-EXOs on ANP, BNP and nNOS expression in DM rats (Fig. 4B–E). ELISA assays showed that expression of high glucose-induced inflammatory factors TNF- α , IL-6 and IL-1 β increased in the DM group. Exosome treatment suppressed the expression of these inflammatory factors, but after silencing corin, the inhibitory effect of ADSC-EXOs on expression of high glucose-induced inflammatory factors was reversed (Fig. 4F–H).

Immunofluorescence assays of beta-III tubulin (Fig. 5) and CD31 (Fig. 6) were performed to show nerve content and angiogenesis. We found that both nerve content and angiogenesis were decreased in the DM-induced ED group, while exosome treatment restored neurovascular function. However, after silencing corin, the neurovascular recovery stimulated by ADSC-EXOs was inhibited. These results indicated that ADSC-EXOs protected against high glucose-induced ED by delivery of corin in a streptozotocin-induced diabetic rat model.

4. Discussion

ED is a common complication of DM. In this study we found that exosomes from ADSCs have the capacity to repair damage caused by high glucose-induced ED, which was consistent with previous reports [21–23]. In this study, we successfully isolated exosomes from ADSCs, confirmed their diameters at about 100 nm and demonstrated protein expression of exosome markers CD9, CD31, CD81 and CD63. It has been shown that exosomes are secreted nanosize membrane vesicles with diameters of 40–100 nm. Exosomes contain functional proteins, mRNAs, microRNAs and tRNA species that play important roles in intercellular communication [24]. Mesenchymal stem cell-derived exosomes have been shown to act as therapeutic agents to reduce tissue injury and enhance tissue repair in cases of cardiovascular disease [25]. In our study we found that ADSC-derived exosomes restored the expression of cGMP. It is known that the NO-cGMP pathway plays a part in maintaining normal erectile function and that loss of cGMP results in ED [14]. Our data also demonstrated that exosome treatment enhanced nNOS, ANP and BNP expression, suggesting that activation of the ANP/NO/cGMP signaling pathway can reverse DM-induced ED.

Corin is primarily expressed in cardiac tissue and acts as an enzyme to convert proANP to biologically active ANP, a signaling peptide that has the ability to regulate blood pressure [20]. In order

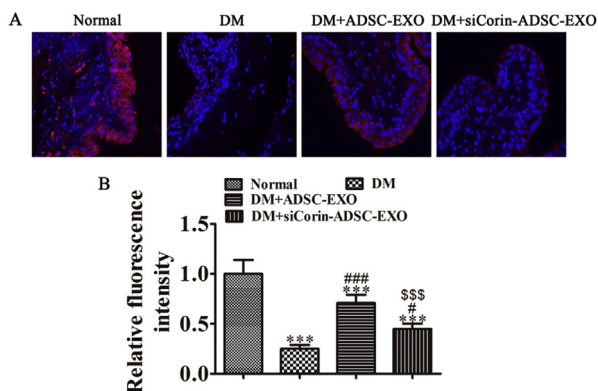


Fig. 5. Exosome treatment increased nerve content in the penis dorsal nerve bundle. (A) Penis sections were stained for beta-III tubulin (red) and DAPI (blue). (B) The relative fluorescence intensity of beta-III tubulin was calculated and expressed as means \pm SD. *** p \leq 0.001 vs. normal group; # p \leq 0.05, ### p \leq 0.001 vs. DM group; \$\$\$ p \leq 0.001 vs. DM + ADSC-EXO group.

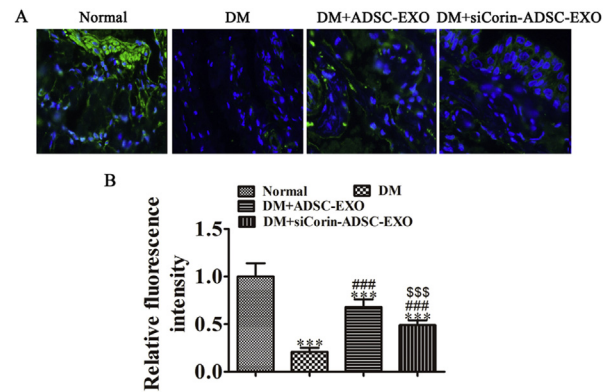


Fig. 6. Exosome treatment increased angiogenesis. (A) Immunofluorescence analysis shows angiogenesis in spongy tissue with CD31. (B) The relative fluorescence intensity of CD31 was calculated and expressed as means \pm SD. *** p \leq 0.001 vs. normal group, ### p \leq 0.001 vs. DM group, \$\$\$ p \leq 0.001 vs. DM + ADSC-EXO group.

to determine if corin plays a role in the exosome-mediated therapeutic effect on DM-induced ED, siRNA against corin was transfected into ADSCs from which we then isolated exosomes. The content of corin in exosomes from corin-silenced ADSCs was greatly reduced. Downregulating corin reversed the therapeutic effect of ADSC-EXOs on DM-induced ED based on ICP/MAP ratio measurements. ADSC-EXOs which induced cavernous cGMP, nNOS, ANP and BNP expression failed to do so after corin was silenced. Thus, corin plays an important role in activation of the ANP/NO/cGMP signal pathway after ADSC-EXO transplantation.

Immunohistochemical detection found that high glucose suppressed penile nerve function and blood vessel which was restored by ADSC-EXO treatment. Decreased content of corin in exosomes derived from corin-silenced ADSCs also resulted in the failure of exosomes to promote neuroangiogenesis.

5. Conclusions

In summary, our findings demonstrated the therapeutic benefits of corin and suggested a potential application for stem cell-derived exosomes in treating DM-induced ED in humans.

Ethical approval

All procedures performed were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the local ethical committee.

Authors' contributions

JW, YM, SW, XY and YH generated and analyzed the data. JZ and LZ designed the experiments and drafted the manuscript. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest. No Funding Source or industrial links and affiliations.

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