Delivery of High Mobility Group Box-1 via Engineered Exosomes Improves Cavernosa Injury - Induced Erectile Dysfunction in Rats

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Abstract

Background

Cavernous is a vascular-rich tissue. Thus, vascular reconstruction is suggested to be essential for the treatment of erectile dysfunction (ED) caused by cavernosa injury. High mobility group box-1 (HMGB1) has been involved in the regulation of growth and differentiation of vascular endothelial cell. In this study, the therapeutic efficiency of engineering HMGB1/Exosomes (exos) derived from adipose tissue derived stem cells (ADSCs) were determined on injured cavernosa.

Methods

We constructed engineered HMGB1/exos and CD63-HMGB1/exos derived from ADSCs. MTT assay, migration assay, and angiogenesis assay were performed in vitro, and erectile and vascular function were detected in a mouse model.

Results

HMGB1 could be successfully delivered to human umbilical venous endothelial cells (HUVECs) via engineered CD63-HMGB1/exos. Treatment with CD63- HMGB1/exos resulted in a significant increase in the proliferation, migration, and angiogenesis of HUVECs. *In vivo* experiments showed that rats of CD63- HMGB1/exos group had the highest ICP/MAP values at 14 and 28 days after injection. Meanwhile, treatment of CD63- HMGB1/exos remarkably increased the content of smooth muscle. The expression of CD31 and vascular endothelial factors were increased after the treatment of CD63-HMGB1/exos, which indicating improved vascular function.

Conclusions

Engineering CD63- HMGB1/exos derived from ADSCs notably repaired injured cavernosa-induced ED via promoting vascular reconstruction.

1. Introduction

Erectile dysfunction (ED) refers to the inability of the corpus cavernosum to achieve or maintain a sustained erectile state, which can be divided into temporary ED and permanent ED according to the length of time [1, 2]. More than 50% of men over the age of 40 in the world suffer from ED [1]. Corpus cavernosum is a tissue rich in blood vessels [2]. Normal erectile function depends on the circulation and blood supply of cavernous sinus [2]. Therefore, the reconstruction of cavernous microvascular system is suggested to be critical to the treatment of ED. Studies have confirmed that the repair of the severed ischial cavernous vessels could improve the erectile function and restore fertility in rats [3, 4]. Previous treatments, such as allogeneic penis transplantation, acellular matrix replacement, prostate brachytherapy, all have defects [5, 6]. Therefore, more safer and effective treatment of ED caused by cavernous injury is worth exploring.

Adipose tissue derived stem cells (ADSCs) is an adult stem cell with an ability of multi differentiation and self-renewal, and has been widely used in many diseases treatments [7]. ADSCs have been reported to play an important role in angiogenesis [8]. Studies have also shown that the function of damaged corpus cavernosum is improved after implantation of ADSCs [9, 10]. However, ADSCs therapy might change the phenotype of the disease and produce uncontrolled transplanted cells [8]. Exosomes (Exos) are small (40–120 nm) membrane vesicles derived from endocytosis, which are released into the extracellular environment during the fusion of multiple vesicles with plasma membrane [11]. It has been found that Exos derived from MSCs could mimic the biological functions of MSCs and exert effects on the diagnosis and treatment of cardiovascular diseases, tumors and neurodegenerative diseases [11, 12]. In addition, Exos promote the effective delivery of biological drugs, such as the ability to deliver functional RNA and small molecule drugs to cells through a complete biological barrier (such as blood-brain barrier), and maintain their stability in the blood [13]. Increasing studies focus on engineering natural Exos to deliver drugs or molecules to specific target cells [14, 15]. However, the effect of ADSCs-derived Exos on ED induced by cavernous injury is still unclear.

High mobility group box-1 (HMGB1), a DNA binding protein in the nucleus, regulates a variety of biological functions [16]. In addition to inducing inflammatory response, HMGB1 also has the function similar to chemokines, acting on hematopoietic cells and endothelial cells [17]. HMGB1 is an important regulator of neovascularization based on its regulatory role in cell migration and proliferation [18]. Treutiger et al.'s study showed that HMGB1 increased the expression of ICAM-1, VCAM-1 and RAGE in a dose-dependent manner [19]. HMGB1 promotes angiogenesis by enhancing the mobilization and differentiation and migration of endothelial progenitor cells in bone marrow cells [20]. Therefore, HMGB1 might be a potential target for the treatment of cavernous injury.

In this study, engineering Exos were also used to successfully deliver HMGB1 to target cells. In order to promote the loading of HMGB1 into Exos, HMGB1 was fused with CD63, and then the fusion was transfected into ADSCs. The effect of CD63-HMGB1/Exos on vascular remodeling was investigated after the injection into cavernosal injury rat model.

2. Methods

2.1 Isolation of ADSCs and cell culture

Adipose tissue-derived stem cells (ADSCs) and human umbilical venous endothelial cells (HUVECs) were purchased from the Cell bank of Chinese Academy of Sciences (Shanghai, China). ADSCs were grown in DMEM-low glucose containing 10% fetal bovine serum (FBS). HUVECs were cultured ECM medium containing 5% FBS. All cells are cultured at 37 °C with 5% CO₂.

2.2 Extraction and characterization of ADSC Exos

After infection, ADSCs were cultured for 72h. Exos of ADSCs were extracted as follows: Medium were collected and centrifuged at 300×g and 2000×g for 10 minutes respectively to remove the cells and

apoptotic fragments; centrifuged at 10000×g for 30 minutes to remove the vacuoles. After washing with PBS with 10mins, pellet was centrifugated for 90 minutes at 100000×g, and then centrifuged at 100000×g for another 90 minutes. The morphology of extracted Exos was analyzed by transmission electron microscopy (TEM).

2.3 The uptake of Exos

The Exos were labeled with PKH67 (Sigma, USA) for 30 min at room temperature. Then, 2 µg of PKH67-stained Exos were co-cultured with HUVECs for 2h. After incubation for 24 hours, cells were fixed with 4% paraformaldehyde, and then were washing with PBS three times. Nuclei of Exos-labeling HUVECs was stained with DAPI for 5 min. The uptake of Exos was visualized by a Zeiss LSM 780 confocal microscope.

2.4 CCK-8 assay

The CCK-8 assay was performed to measure the effect of Exos on the HUVECs cell viability. HUVECs were cultured in 96-well plates (5×10^3 cells in 100 µL/well). After an overnight culture, cells were treated with ADSCs-exos for 24, 48, and 72 hours. Then 10uL/ well CCK-8 reagent (Biowater, China) was added. After 1 hour of incubation, OD values at 450 nm were determined using a microplate reader.

2.5 Migration assay

The upper chamber of the Transwell plates was coated and then placed at room temperature for 30 minutes. Cell suspension (5×10^5) was prepared from a serum-free medium containing BSA. 100 uL cell suspensions were planted in Transwell chambers and cultured for 24 h. After washing with PBS for 2 times, the cells were fixed with methanol for 30min. The cells were stained with 0.1% crystal violet for 20 min and then washed with PBS for 3 times. Cells were counted at random five fields under a 400x microscope.

2.6 Quantitative RT-PCR (qRT-PCR) analysis

Trizol reagent kit (Thermo Fisher Scientific) was used for extraction of total RNA from ADSCs and rats. Then, PrimeScript RT reagent kit (Biowater, china) was performed for the reverse transcription of RNA into cDNA, which would as the template of following amplification experiment by using the SYBR Premix kit (Biowater, china). β-actin was used as an internal control.

2.7 Immunofluorescence assay

ADSCs and slices of rat were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 for 10 minutes. Then they were stained with primary antibodies against α-SMA (Cell Signaling Technology) at 4 °C overnight. After washing with PBS three times for 5mins, cells and slices were incubated with FITC anti-mouse IgG (Proteintech, Rosemont, IL, USA) for 1 h. Then nucle were stained with DAPI for 30mins. Images were observed using a fluorescence microscope (Zeiss, Jena, Germany).

2.8 Establishment of mouse Cavernosa cavernosal injury model

Male Sprague-Dawley rats (n = 40) were randomly divided into 5 groups: PBS, Vector/exos, Control/exos, CD63-HMGB1/exos and HMGB1/exos. The establishment of the mouse cavernosa injury model is shown below: After the anaesthetization using with 1 % pentobarbital sodium (Biowater,china), rats were cut off a median incision of about 1 cm above the penis. In the middle of the penile dorsal, a gap of about 0.2 cm long and 0.1 cm deep was cut in the left or right corpus cavernosum. While the incision was closed, rats in the PBS group were injected with 200 μ L phosphate balanced saline (PBS); The other four groups were injected with corresponding Exos (Control/exos, CD63-HMGB1/exos and HMGB1/exos) extracted from ADSCs. Experiments were performed under a project licens (SYXK-2020-0233) granted by Third Affiliated Hospital of Guangzhou Medical University

2.9 Measurement of erectile function

We measured the erectile function of rats on the 14th and 28th day after treatment. Rats were anesthetized and dissected from the middle of the abdomen until the major pelvic ganglion (MPG) was exposed. The electrode was placed on the mpg for electrical stimulation (parameters: voltage 5 V, stimulation wave width 5 ms, frequency 20 Hz, duration 30 s each time). 23 g infusion needle was connected with PE50 catheter to puncture the left penile peduncle into the penile cavernous body. The PE50 catheter was connected with the pressure transducer. The changes of mICP and MAP were continuously monitored by PowerLab physiological recorder (AD Instruments, Australia), and the ratio of mICP / MAP was calculated.

2.10 Western blotting

The total proteins of cells and penis tissue were extracted and the protein concentration was determined by BCA method. The equivalent proteins were added to SDS-PAGE gel and separated by electrophoresis. The separated protein was transferred to PVDF membrane and sealed by 5% skim milk powder at room temperature for 1 h. These PVDF membranes were stained with Rabbit anti-CD9, CD63, TSG-101, HMGB1, CD31 and VEGF (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. After washing off the first antibody, goat anti rabbit IgG was added to incubate for 1 h. The images of bands were acquired by ECL quantification detection.

2.11 Statistical analysis

All data were presented as mean $\pm$ standard deviation (SD). SPSS (Version 22.0; IBM, Armonk, NY, USA) was used significant difference analysis. Statistical significance values of the multiple groups were analyzed by one-way analysis. The *P* values < 0.05 were considered significant.

3. Results

3.1 Engineered Exos enhance the loading of HMGB1

HMGB1 is involved in revascularization. In order to improve the effectiveness of the delivery of HMGB1 to target cells, HGMB1 was fused onto the C-terminal of CD63 and then subcloned into the PLVX-GFP vector (Fig. 1A). Figure 1B revealed the stable expression of CD63-HMGB1 in ADSCs. Then, cell viability assay suggested that lentivirus infection of HGMB1 and HGMB1-CD63 decreased the ADSCs viability (Fig. 1C). PCR assay was used to verify that HGMB1 and CD63-HMGB1 were successfully transcribed into ADSCs.

3.2 Exos containing CD63-HGMB1 promote the proliferation, migration, and angiogenesis of HUVECs.

Studies showed that Exos might be used as a valuable therapeutic vehicle for molecular delivery. Then, the effect of Exos containing HGMB1 and HGMB1-CD63 secreted by ADSCs on revascularization was examined. TEM was used for the identification of EXs secreted from ADSCs was verified by TEM (Fig. 2A). The levels of EXs markers (CD9, CD91, TSG-101) were measured by Western blotting, and showed that these markers were successfully expressed in all four groups (Fig. 2B). Additionally, Western blotting was performed to detect HGMB1 loading in EXs. Compared with the Control/exos and Vector/exos group, HMGB1 expression was increased significantly in the HMGB1/exos group. Interestingly, the expression of HMGB1 was greater in the CD63-HMGB1/exos group compared with the HMGB1/exos group (Fig. 2C). Then, Exos was labeled with PKH67 dye and co-cultured with HUVECs. As is shown in Fig. 2D, labeled Exos were taken up by HUVECs (Fig. 2D). Together, these results indicated that engineered Exos effectively increased the loading of HMGB1.

Next, we detected the effect of HMGB1-labeled Exos on angiogenesis of HUVECs. Figure 3A showed that the HMGB1/exos and CD63-HMGB1/exos group significantly increased the HUVECs activity. And CD63-HMGB1/exos group had higher cell viability of HUVECs than the HMGB1/exos group. CD63-HMGB1/exos promoted the migration of HUVECs, which was identified by the Transwell migration assay (Fig. 3B). More tube-like structures were formed in CD63-HMGB1/exos group compared to HMGB1/exos, Control/exos and Vector/exos group (Fig. 3C, $P < 0.05$). Moreover, the mRNA and protein expression of CD31 and vascular endothelial factors (VEGF) were remarkably increased in the CD63-HMGB1/exos group, which showing the enhanced vascular function (Fig. 3D and E, ALL $P < 0.05$). These results suggesting the positive effect of CD63-HMGB1/exos on promoting angiogenesis.

3.3 Exos containing CD63-HGMB1 improved erectile response in cavernosa injury rat model.

After the successful establishment of the cavernosa injury rat model, CD63-HGMB1/exos was injected into rats for 14 and 28 days. As was showed in Fig. 4A and B, after injection for 14 days, the ICP/MAP values in the CD63-HMGB1/exos group (79.25 ± 0.57) were notably higher than in the HMGB1/exos group (70.73 ± 0.03), as well as the PBS, Control/exos and Vector/exos group (38.41 ± 0.21 , 52.61 ± 0.05 ,

55.87±0.11, respectively). After injection for 28 days, compared with the ICP/MAP values of rats in the PBS (39.34±0.37), Control/exos (61.36±0.17), Vector/exos (62.09±0.10) and HMGB1/exos group (72.46 ± 0.46), the ICP/MAP values in the CD63-HMGB1/exos group (82.44 ± 0.45) also increased.

3.4 Exos containing CD63-HGMB1 enhanced smooth muscle content and vascular function in the penis tissues.

Then, we detected the effects of CD63-HGMB1/exos on enhancing revascularization. Figure 5A showed that rats injected with CD63-HGMB1/exos had a higher α -SMA levels than rats in the PBS, Control/exos, Vector/exos and HMGB1/exos group, which indicating increasing smooth muscle content. We also measured the expressions of CD31, and VEGF and indicated that the mRNA and protein expression of CD31 and VEGF in the CD63-HGMB1/exos group were higher than that of rats in the PBS, Control/exos, Vector/exos and HMGB1/exos group, which suggesting the improved vascular function (Fig. 5B and C). These findings showed that CD63-HGMB1/exos from ADSCs enhanced revascularization via increasing the contents of SMCs and improving vascular function.

4. Discussion

Revascularization is essential for the effective treatment of ED caused by cavernosa injury. In this study, we developed a strategy for the production of engineered Exos. It can improve the proliferation, migration and angiogenesis of HUVECs by accurate delivery of HMGB1 molecule, thus promoting vascular remodeling and providing a new therapeutic approach for the treatment of ED.

Vascular endothelial cells are the first barrier of vascular protection, which play an important role in wound healing, thrombosis and neovascularization [21]. Angiogenesis is a series of complex processes of proliferation, migration and tubular formation of vascular endothelial cells, in which chemokines, growth factors, adhesion molecules and extracellular matrix regulators are important regulators [22]. HMGB1 is a nuclear DNA-binding protein, named for its rapid migration during polyacrylamide gel electrophoresis [16]. It is widely distributed in brain, heart, liver, lymphoid tissue, kidney and other tissues [16]. In addition to mediating inflammation, the chemotaxis of HMGB1 has also become a new focus of research on its extracellular function [17]. Studies have shown that HMGB1 induces EPCs to migrate to the wound surface, increases the formation of new blood vessels in the wound granulation tissue, and thus promoting the wound healing [23]. Li et al. showed that HMGB1 can activate fibroblast proliferation and guide its migration [24]. HMGB1 also has chemotactic activity on fibroblasts, mesoblast transnerocytes and bone marrow derived stem cells, and promotes their transport across endothelial cell monolayer [25]. In this study, we also found that HMGB1 intervention promoted the proliferation, migration and angiogenesis of HUVECs.

HMGB1 regulates vascular growth *in vivo* and *in vitro* through a variety of mechanisms, including promoting the release of pro-angiogenic cytokines, the activation of fibroblast growth factors, and the activation of endothelial cells, macrophages, and endothelial progenitor cells [26, 27]. Therefore, HMGB1

has critical role in many angiogenesis-related diseases such as tumor, wound healing, and angiogenesis induced by ischemia and hypoxia [23, 28]. HMGB1 up-regulated the expression of vascular fibrosis factors (including VEGF, bFGF, TGF- β 2 and CTGF) in retinal pigment cells and promoted the occurrence of diabetic retinopathy [29]. In contrast, blocking HMGB1 activation prevented the occurrence of pathological neovasculation [30]. Schlueter et al. described HMGB1 as an "angiogenesis switch molecule" due to its induction of angiogenesis *in vitro* and *in vivo* [31]. In mouse models of embryonic wound healing, high levels of HMGB1 were also positively associated with increased angiogenesis and macrophage infiltration [32].

Exos are recognized as a valuable targeted delivery tool and play an important role in the diagnosis and treatment of cardiovascular diseases, oncology and neurodegenerative diseases [13]. Scientists have found that Exos carrying endothelial differentiation signals influence the formation of new blood vessels, demonstrating the effectiveness of Exos in the treatment of angiogenesis [33]. Exos derived from human umbilical cord mesenchymal stem cells (HUCMSCs) have been reported to promote cardiac repair after ischemic injury by protecting cardiomyocytes from apoptosis and promoting cell proliferation and angiogenesis [34]. Furthermore, HUCMSCs-derived Exos promoted wound healing *in vivo*, which was mediated by activation of Wnt4/ β -catenin in endothelial cells [35]. Nevertheless, cargo loading is a major challenge for Exos delivery. Exos of different cell types are drug/targeting selective, so overexpression of specific molecules alone may not increase load [36]. In view of the possibility that Exos membrane is derived from cell membrane, the co-overexpression of CD9, CD63, and LAMP2 might notably improve the delivery efficiency [37, 38]. Therefore, in this study, we found that the fusion of HMGB1 with CD63 remarkably increased HMGB1 levels in ADSCs-derived Exos. Compared with the HMGB1/exos group, the CD63-HMGB1/exos group promoted the proliferation, migration and angiogenesis of HUVECs.

5. Conclusion

In the present study, we demonstrated the effect of engineered CD63-HMGB1/exos derived from ADSCs in the treatment of cavernosa injury-induced ED in rats. CD63-HMGB1/exos remarkably promoted the revascularization via enhancing the proliferation, migration, and angiogenesis of HUVECs. Our findings supply a novel engineered Exos-based strategy for ED therapy.

Abbreviations

HMGB1 High mobility group box-1

ED Erectile dysfunction

Exos Exosomes

ADSCs Adipose tissue-derived stem cells

Declarations

Acknowledgments

Not applicable

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author [email: lanyu2024@126.com] upon reasonable request

Authors' contributions

(I) Conception and design: Yu Lan and Shiyu Zhou; (II) Administrative support: Wen Zhang; (III) Provision of study materials or patients: Xin Fu, Chen Liao, Tianwen Peng; (IV) Collection and assembly of data: Yu Lan and Shiyu Zhou; (V) Data analysis and interpretation: Yu Lan and Shiyu Zhou; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Consent for publication

No

Ethics approval

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Figures

Figure 1

Engineered ADSCs exosomes. (A) Schematic diagram of the construction of CD63-HMGB1-exos. (B) immunofluorescence images of HMGB1 in ADSCs transduced with different lentiviruses (100×). (C) CCK-

8 assay for cell viability of ADSCs. (D) The mRNA level of HMGB1. Data are shown as the means $\pm$ SD. *P < 0.05, ** P < 0.01.n = 3.Data represent the mean $\pm$ SD of three separate experiments; comparison was performed with one-way ANOVA followed by Tukey's post hoc test.

Figure 2

Characterization of engineered CD63-HMGB1/exos. (A)Transmission electron photomicrograph of exosomes. (B) Protein expression of CD9, CD63 and TSG-101. (C) Protein expression of HMGB1. (D) Confocal images of PKH67-labeled exosomes taken up by HUVECs. Data are shown as the means $\pm$ SD. *P < 0.05, ** P < 0.01.n = 3.Data represent the mean $\pm$ SD of three separate experiments; comparison was performed with one-way ANOVA followed by Tukey's post hoc test.

Figure 3

Exosomes derived from CD63-HMGB1-modified ADSCs the proliferation, migration, and angiogenesis of HUVECs. (A) CCK-8 assay for cell viability of HUVECs. (B) The Transwell assay determined the HUVECs migration. (C) Photomicrographs of tube-like structures and quantification of the tube number. (D) The mRNA level of VEGF and CD31. (E) Protein expression of CD31 and VEGF in HUVECs. Data are shown as the means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. $n = 3$. Data represent the mean $\pm$ SD of three separate experiments; comparison was performed with one-way ANOVA followed by Tukey's post hoc test.

Figure 4

The measurement of erectile response after injection with CD63-HMGB1/exos. (A) Intra-cavernous pressure (ICP). (B) The value of ICP/ mean arterial pressure (MAP). Data are shown as the means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. $n = 3$. Data represent the mean $\pm$ SD of three separate experiments; comparison was performed with one-way ANOVA followed by Tukey's post hoc test.

Figure 5

CD63-HMGB1/exos increases smooth muscle contents and improves vascular function in rats. (A) Immunofluorescence images of α -SMA in the penis tissues. (B) The mRNA level of VEGF and CD31. (C) Protein expression of CD31 and VEGF in rats. Data are shown as the means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$. $n = 3$. Data represent the mean $\pm$ SD of three separate experiments; comparison was performed with one-way ANOVA followed by Tukey's post hoc test.