

## Original Article

# Correction of diabetes mellitus-induced erectile dysfunction with adipose tissue-derived stem cells modified with the DDAH2 gene in a rat model

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**Abstract:** Objective: This study was designed to investigate the effect of DDAH2 transfected with adipose tissue-derived stem cells (ADSCs) on improving erectile function in diabetic rats. Methods: Forty-eight male Sprague-Dawley rats were injected with streptozotocin to establish diabetes mellitus-induced erectile dysfunction (DMED). DMED rats were randomly divided into four groups: DMED+PBS group, rats received intracavernous injection of phosphate buffer solution; DMED+ADSCs group, rats received intracavernous injection of nontransfected ADSCs; DMED+null-ADSCs group, rats received intracavernous injection of empty vector transfected ADSCs; DMED+DDAH2-ADSCs group, rats received intracavernous injection of Ad-DDAH2-transfected ADSCs. Before injection, high levels of DDAH2 expression were confirmed in rats in the DDAH2-ADSCs group by PCR and western blot. Four weeks after the injection, erectile function was ascertained by measuring intracavernous pressure (ICP). The pathological structure of penile tissues was evaluated by immunohistochemical analysis. Results: Compared with the other groups, ICP significantly increased after papaverine injection in rats in the DMED+DDAH2-ADSCs group. Diffused fibrosis and impairment of endothelial cells were observed in the corpus cavernosum of rats in the DMED+DDAH2-ADSCs group. Conclusion: Rats in the DMED+DDAH2-ADSCs group displayed a significant improvement of erectile function. DDAH2 was able to enhance the effect of ADSCs in the treatment of diabetes-associated erectile dysfunction.

**Keywords:** Diabetes mellitus-induced erectile dysfunction, gene therapy, DDAH2, adipose tissue, derived stem cells

## Introduction

Erectile dysfunction (ED) is a prevailing health problem that seriously impacts quality of life. Approximately 52% of men between the age of 40 and 70 years may suffer from erectile dysfunction. ED is a common complication of diabetes mellitus (DM). The incidence rate of erectile dysfunction may reach up to 75% in diabetic patients [1]. Phosphodiesterase type-5 (PDE5) inhibitors are the primary means of treatment for ED [2]. However, PDE5 inhibitors are strictly contraindicated with concomitant nitrates due to the danger of synergistic hypotensive effects. PDE5 inhibitors are known to cause a variety of adverse side effects that may reduce its suitability for some patients. More importantly, PDE5 inhibitors are only partially effective in treating certain types of ED,

including those associated with DM. Thus, there is a need to conduct research on effective treatments for DMED [3].

Stem cell therapy is one of the strategies for ED treatment, which is currently being investigated [4]. Adipose tissue-derived stem cells (ADSCs) are easy to harvest, culture and expand *in vitro*, making them a desirable cell type for cell transplantation therapy [5]. Studies have revealed that nitric oxide (NO) is a critical chemical component in penile erection, and is generated by nitric oxide synthase (NOS) [6]. Asymmetric dimethylarginine (ADMA), a naturally occurring L-arginine analogue, is a powerful inhibitor of NOS, which competes with L-arginine to bind to the active site of NOS [7]. In addition, the majority of ADMA is degraded by dimethylarginine dimethylaminohydrolase (DD-

AH) [8]. There are two DDAH isoforms with different tissue distributions, DDAH-1 and DDAH-2. DDAH2 predominates in penile tissue eNOS expression [9]. Based on the above-mentioned research findings, it is inferred that the upregulation of DDAH2 could lead to the degradation of ADMA, leading to increased NOS activity and reduced NO synthesis. The aim of this study was to determine the contribution of DDAH2 to DMED in a rat model.

### Materials and methods

#### *Establishment of type-1 diabetic rats*

After an overnight fast, 48 Sprague-Dawley rats (10 weeks old) were intraperitoneally injected with streptozotocin (STZ, 60 mg/kg; Sigma, St. Louis, Missouri) dissolved in citrate acid buffer solution (pH 4.0) to induce type-1 diabetes according to the protocol previously described. Blood samples were obtained by tail prick for blood glucose measurement. Blood glucose levels were measured 72 hours later. Rats that had a blood glucose level higher than 200 mg/dL were selected as diabetic rats.

#### *Animal groups and study design*

Eight weeks after the establishment of type-1 diabetes, diabetic rats were randomly divided into four groups: DMED+PBS group ( $n=12$ ), diabetic rats injected with phosphate-buffered saline; DMED+ADSCs group ( $n=12$ ), diabetic rats injected with ADSCs; DMED+null-ADSCs group ( $n=12$ ), diabetic rats injected with empty vector transfected ADSCs; DMED+DDAH2-ADSCs group ( $n=12$ ), diabetic rats injected with Ad-DDAH2-transfected ADSCs. Four weeks later, all rats were sacrificed, and penile tissues were obtained for immunohistochemical analysis after undergoing an erectile function test. All procedures were approved by the Institutional Animal Care and Use Committee of Shandong University.

#### *Isolation and culture of ADSCs*

Adipose-derived stem cells were harvested from inguinal fat tissues of adult male Sprague-Dawley rats (350-450 g). A lower midline abdominal skin incision was performed. Then, fat pads around the spermatic cord were excised, finely minced and washed three times with PBS-containing penicillin (100 units/mL) and

streptomycin (100  $\mu\text{g/mL}$ ). After centrifugation (500 $\times$  g, five minutes), the tissue samples were digested with 0.2% collagenase type-1 (Invitrogen, Carlsbad, CA, USA) by agitation at 37°C for 90 minutes. After filtration using a 60- $\mu\text{m}$  nylon mesh (Millipore, Billerica, MA, USA), ADSCs were resuspended and cultured at a density of  $1 \times 10^5$  cells in DMEM-F12 medium supplemented with 10% FBS, antifungal agent and penicillin-streptomycin (Gibco-BRL, Carlsbad, CA, USA). Non-adherent cells were removed two days after culture, and the medium was changed every three days until 85-95% confluence was attained.

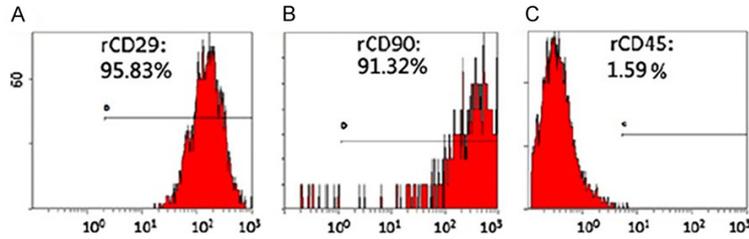
#### *Transfection of ADSCs with adenovirus*

The recombinant Ad-DDAH2 and empty vector adenovirus (containing EGFP) were obtained from Cyagen (Cyagen Biosciences Inc., Guangzhou, China). ADSCs were exposed to fetal bovine serum-free DMEM containing Ad-DDAH2 at a multiplicity of infection of 100 for 12 hours. After transfection, DMEM containing adenoviral particles was removed, and the ADSC culture medium was added. After 48 hours, the transfected cells were observed using fluorescence microscopy.

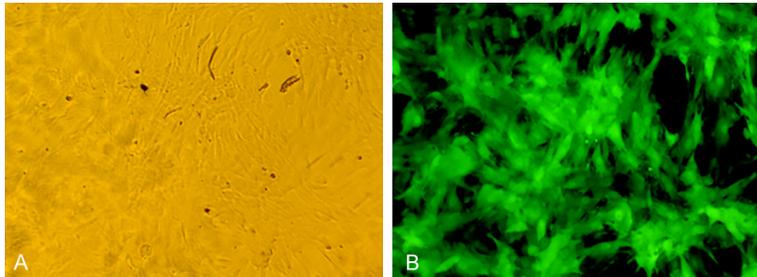
#### *Detection of DDAH2 expression in Ad-DDAH2-transfected ADSCs*

Forty-eight hours after transfection, the membrane and plasma proteins of the cells were extracted according to the instructions of the Membrane and Cytosol Protein Extraction Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Empty vector transfected cells and non-transfected cells were used as experimental controls. Protein concentrations of the recovered supernatants were analyzed using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). A total of 40  $\mu\text{g}$  of protein per well were subjected to SDS-PAGE, followed by electro-transferring of the gel to nitrocellulose membranes. The membranes were blocked in non-fat dry milk for 1.5 hours and probed with rabbit anti-DDAH2 and  $\beta$ -actin diluted at a ratio of 1:1,500. Then, membranes were incubated at 4°C overnight and rinsed three times for 10 minutes with TBST. The membranes were developed using ECL and imaged with the GEL imaging system. Protein indices were calculated as the percentage of DDAH2 scanning value over  $\beta$ -actin scanning value.

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**Figure 1.** Flow cytometric analysis of early passage rat ADSCs depicting positive expression for (A) CD29 (95.83%), (B) CD90 (91.32%), (C) CD45 (1.59%).



**Figure 2.** Adenovirus transfected into ADSCs. A: Adenovirus transfected cells under light microscopy; B: Adenovirus transfected cells under fluorescence microscopy. Under fluorescence microscopy, the cells emit green light. This indicates that the intended gene was successfully transfected into and expressed in the ADSCs.

### *Intracavernous injection of ADSCs*

Eight weeks after STZ injection, diabetic rats were anesthetized with ketamine (30 mg/kg) and xylazine (4 mg/kg). A skin incision was made to expose the penis, and 200 mL of PBS (PBS groups), or  $5 \times 10^5$  (Bivalacqua *et al.*, 2007) ADSCs dissolved in 200 mL of PBS (ADSCs group), or  $5 \times 10^5$  Ad-null-transfected ADSCs dissolved in 200 mL of PBS (null-ADSCs), or  $5 \times 10^5$  Ad-DDAH2-transfected ADSCs dissolved in 200 mL of PBS (DDAH2-ADSCs group) was injected into the middle of the left corpus cavernosum. An elastic band was placed at the base of the penis immediately before ADSC injection, and was removed three minutes after the injection.

### *Evaluation of erectile function*

Next, rats were anaesthetized with sodium pentobarbital (1 ml/kg, by intraperitoneal injection). The skin overlying the penis was denuded to the base to expose the penis. Two 25-gauge needles filled with heparinised saline (100 U/ml) and papaverine ( $3 \text{ g ml}^{-1}$ ) were carefully inserted into the penis on each side of the rats in both groups to measure intracavernous pres-

sure (ICP) and the drug injected, respectively. The needles were inserted at the junction of the glans and penile shaft, pointing towards its base. The heparinised saline-filled needle was connected to a pressure transducer, while another needle was connected to an injection syringe filled with papaverine, which was injected at a dose of 2 mg/kg. Real-time ICP values were displayed on the BL-420 biological and functional experimental system.

### *Pathology evaluation of the corpus cavernosum*

At four weeks after the cells were transplanted into the penis, the penile tissues were cut and washed repeatedly with PBS, fixed in 4% formaldehyde for 24 hours, and was prepared for 4- $\mu\text{m}$  paraffin slicing. Three non-consecutive

slices were observed by inverted fluorescence microscopy, in order to visualize ADSCs in the penile tissue.

### *Statistical analysis*

Quantitative data were presented as mean  $\pm$  standard deviation (SD). Statistical analysis was carried out by Student's *t*-test using SPSS 17.0. Differences were considered statistically significant when the *P*-value was  $<0.05$ .

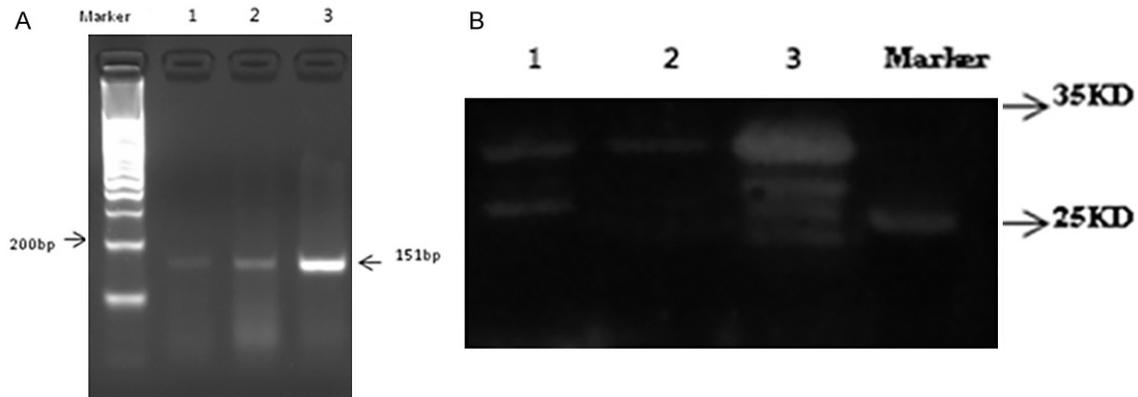
## **Results**

**Establishment of the rat model of DMED** Eight weeks after the start of the experiment, 48 rats had blood glucose levels higher than 16.7 mM. These 48 rats were considered diabetic, and were injected with APO. Rats in no penile erection within 30 minutes were diagnosed with ED.

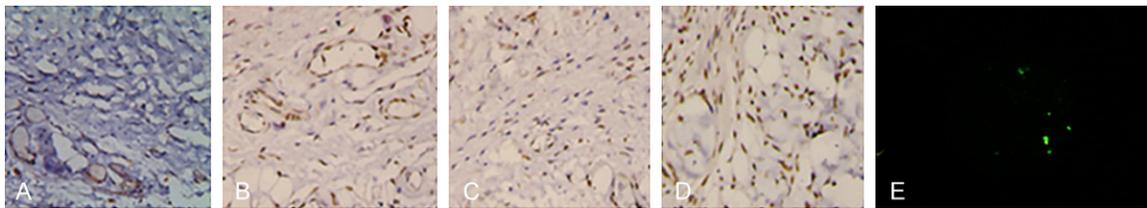
### *DDAH2 expression increased after Ad-DDAH2 transfection into ADSCs*

The successful transfection of the adenovirus into ADSCs was confirmed, and a green EGFP expression was displayed by fluorescent mi-

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**Figure 3.** mRNA and protein levels of DDAH2 in the three groups cells. A: RT-PCR results; B: Western blotting results. 1: nontransfected group; 2: empty vector transfected group; 3: DDAH2 transfected group; The brightness of lane 1 and lane 2 was not significantly different, lane 3 was significantly brighter than lane 1 and 2.



**Figure 4.** Expression of DDAH2 in penile tissues by immunoenzymatic staining. A: DMED; B: DMED+PBS; C: DMED+null-ADSCs; D: DMED+DDAH2-ADSCs. ADSCs transplanted into penile tissues. E: 14 days after ADSCs transplantation, a few number of transplanted cells with green fluorescence.

scopy (**Figure 1**). RT-PCR and western blot results revealed that the DDAH2 mRNA and protein levels in non-transfected cells and the empty vector transfected cells were not significantly different ( $P>0.05$ ), while the DDAH2 mRNA and protein levels in DDAH2 transfected cells were significantly higher than the non-transfected and empty vector transfected cells ( $P<0.05$ , **Figures 2 and 3**).

### *DDAH-2 expression increased after transfection of Ad-DDAH2*

As shown in **Figure 2**, DDAH-2 levels in the supernatant of transfected ADSCs significantly increased and reached a peak at day five, compared with the level of non-transfected ADSCs.

### *Erectile function increased after the transplantation of ADSCs-DDAH2*

Results revealed that the peak ICP and MAP values in the DDAH2-ADSCs group were significantly higher than in the other groups ( $P<0.05$ ), while the difference between the DMED+ADSCs group and DMED+null-ADSCs group was not statistically significant ( $P>0.05$ ).

### *DDAH2 expression in penile tissues in each group*

Yellow or brownish cells indicate the positive expression of DDAH2 (**Figure 4**). DDAH2 expression level was highest in the DMED+DDAH2-ADSCs group and lowest in the DMED+PBS group. The grey value in the DMED+DDAH2-ADSCs group was  $141.62 \pm 14.42$ , which was significantly higher than the grey value in the other groups ( $P<0.05$ ). The grey values in the DM+ADSCs and DMED+null-ADSCs groups were  $120.62 \pm 11.85$  and  $117.18 \pm 8.96$ , respectively; and the difference was not statistically significant ( $P>0.05$ ). Moreover, the grey values in the DMED+PBS group was  $67.18 \pm 5.88$ , which was significantly lower than the grey values in other groups ( $P<0.05$ ).

### **Discussion**

ED can have various causes such as vascular disease, neuropathy, and neurotransmitter changes [10]. DMED is a type of organic ED, which mainly involves neural, vascular and smooth muscle lesions. The treatment with PDE-V inhibitor alone rarely achieves satisfac-

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tory results. Technical advances have enabled the use of ADSCs in autologous transplantation for the clinical treatment of related diseases due to the high amount of ADSCs in adipose tissues, its low immunogenicity, and its long-term survival in the host. Song found that ADSCs transplanted into rat penile tissues can be converted into endothelial cells and smooth muscle cells, improving erectile function [11]. One of the pathogenic factors of DMED is the structural dysfunction and reduction of endothelial cells and smooth muscle cells. DDAH2 is involved in the electrical activity of cell excitement, neurotransmitter release, hormone secretion, and the contraction of blood vessels, trachea, the uterus, the gastrointestinal tract, the bladder and corpus cavernosum smooth muscle tissue, as well as in the regulation of many other important physiological activities [12].

Christ *et al.* injected DDAH2 plasmids into the penile tissues of atherosclerotic macaques, and observed an improvement in erectile and sexual functions. Therefore, we predicted that the implantation ADSCs modified by DDAH2 into penile tissues would improve erectile function in DMED rats, and that the effects of such treatment would last longer, compared to DDAH2 or ADSCs therapy alone. In this study, RT-PCR and western blot revealed that the level of DDAH2 was significantly higher in the DMED+DDAH2-ADSCs group than in the DMED+null-ADSCs group and DMED+ADSCs group. This demonstrates that adenovirus containing DDAH2 transfected successfully into ADSCs and transfected cells strongly expressed DDAH2. Thus, after cells were injected into the corpus cavernosum, these cells were likely to differentiate into endothelial cells and smooth muscle cells.

Two weeks later, green fluorescence was observed in the vascular endothelium and sinusoids edge. This indicates that ADSCs had colonized the penile tissue vascular endothelial and sinusoids, and integrated into the host penile tissue. The ratio of ICP/MAP increased in the DMED+ADSCs group and DMED+DDAH2-ADSCs group relative to the DMED and DMED+PBS groups. After cavernous nerve stimulation, the increase in ICP was highest in the DMED+DDAH2-ADSCs group. This demonstrates that simple ADSC transplantation can improve erectile function in DMED rats, and that DDAH2 in

combination with ADSCs exhibited a greater increase in ICP than either treatment alone. DDAH2 expression in penile tissues was strong in the DMED+ADSCs group, but not as strong as that in the DMED+DDAH2-ADSCs group. Furthermore, DDAH2 expression in penile tissues was very weak in the DMED+PBS group, with only a few cells observable in the field of view. The increased expression of DDAH2 can improve the erectile function of the penis. This may explain why the ICP/MAP ratio was higher in the DMED+DDAH2-ADSCs group than in the DMED+ADSCs group. The improvement of erectile function by DDAH2 may be *via* the induction of membrane hyperpolarization of the corpus cavernosum vascular endothelial and smooth muscle cells, which can reduce cell excitability and contractility. Thus, more blood will enter the corpus cavernosum during vasodilation, resulting in cavernous tissue congestion. This would maintain the intracavernous pressure plateau longer, thereby improving erectile function.

In conclusion, the transplantation of ADSCs-containing DDAH2 into penile tissues of DMED rats can improve erectile function. The mechanism of this improvement may be *via* the increased DDAH2 expression in ADSCs, with transplanted ADSCs differentiating into endothelial cells and smooth muscle cells, and the secretion through these cells of many kinds of cytokines and other effects. However, the type-1 diabetes model used in the experiment was somewhat metabolically different from clinical type-2 diabetes. After ADSCs transplantation, some cells left the graft site due to blood circulation. Hence, the treatment effect may have been weakened due to the loss of transplanted cells.

### Acknowledgements

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### Disclosure of conflict of interest

None.

### Abbreviations

DMED+PBS, rats that received an intracavernous injection of phosphate-buffered saline;

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DMED+ADSCs, rats that received an intracavernous injection of ADSCs; DMED+null-ADSCs, rats that received an intracavernous injection of empty vector transfected ADSCs; DM+DDAH2-ADSCs, rats that received an intracavernous injection of DDAH2 adenovirus-transfected ADSCs; ICP, intracavernous pressure; MAP, mean arterial pressure.

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