RESEARCH ARTICLE

Adipose-derived stem cells modified by *BDNF* gene rescue erectile dysfunction after cavernous nerve injury

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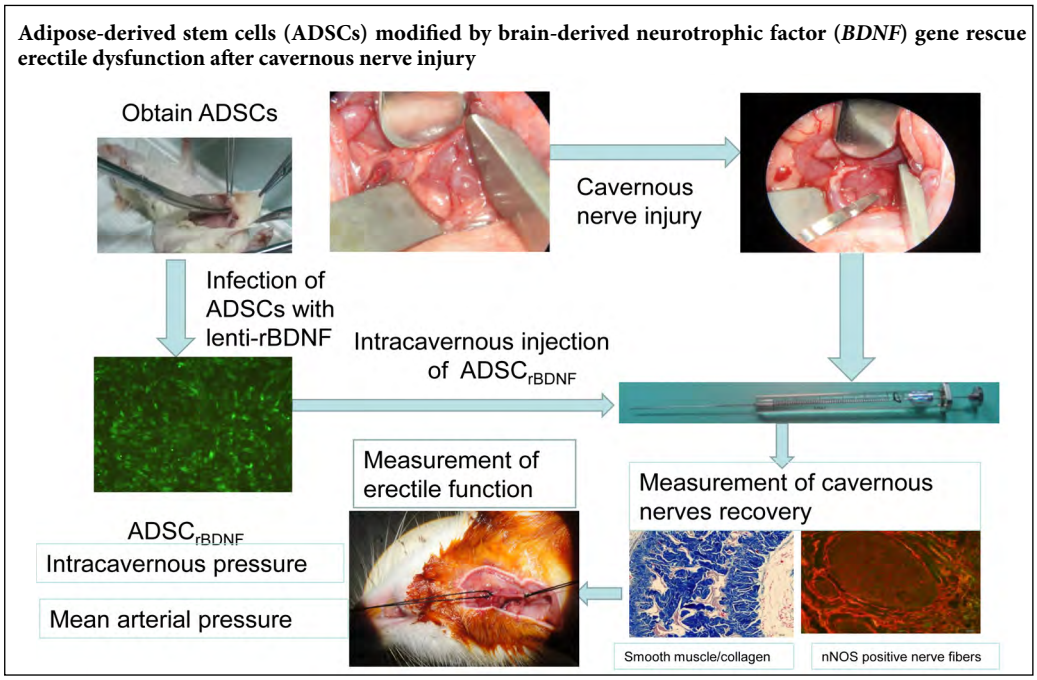
Graphical Abstract



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Abstract

Cavernous nerve injury is the main cause of erectile dysfunction following radical prostatectomy. The recovery of erectile function following radical prostatectomy remains challenging. Our previous studies found that injecting adipose-derived stem cells (ADSCs) into the cavernosa could repair the damaged cavernous nerves, but the erectile function of the treated rats could not be restored to a normal level. We evaluated the efficacy of ADSCs infected with a lentiviral vector encoding rat brain-derived neurotrophic factor (lenti-rBDNF) in a rat model of cavernous nerve injury. The rats were equally and randomly divided into four groups. In the control group, bilateral cavernous nerves were isolated but not injured. In the bilateral cavernous nerve injury group, bilateral cavernous nerves were isolated and injured with a hemostat clamp for 2 minutes. In the $ADSC_{GFP}$ and $ADSC_{rBDNF}$ groups, after injury with a hemostat clamp for 2 minutes, rats were injected with ADSCs infected with lenti-GFP (1×10^6 in 20 μL) and lenti-rBDNF (1×10^6 in 20 μL), respectively. Erectile function was assessed 4 weeks after injury by measuring intracavernosal pressures. Then, penile tissues were collected for histological detection and western blot assay. Results demonstrated that compared with the bilateral cavernous nerve injury group, erectile function was significantly recovered in the $ADSC_{GFP}$ and $ADSC_{rBDNF}$ groups, and to a greater degree in the $ADSC_{rBDNF}$ group. Neuronal nitric oxide synthase content in the dorsal nerves and the ratio of smooth muscle/collagen were significantly higher in the $ADSC_{rBDNF}$ and $ADSC_{GFP}$ groups than in the bilateral cavernous nerve injury group. Neuronal nitric oxide synthase expression was obviously higher in the $ADSC_{rBDNF}$ group than in the $ADSC_{GFP}$ group. These findings confirm that intracavernous injection with ADSCs infected with lenti-rBDNF can effectively improve erectile dysfunction caused by cavernous nerve injury. This study was approved by the Medical Animal Care and Welfare Committee of Wuhan University, China (approval No. 2017-1638) on June 20, 2017.

Key Words: *adipose-derived stem cells; brain-derived neurotrophic factor; cavernous nerve injury; erectile dysfunction; infection; intracavernous injection; lentiviral vector; neuronal nitric oxide synthase; radical prostatectomy*

Chinese Library Classification No. *R459.9; R363; R364*

Introduction

Recently, the detection of prostate-confined disease has been increasing because of earlier diagnosis by prostate-specific antigen screening, which has increased the number of radical prostatectomy procedures (Heidenreich et al., 2011; Martinez-Salamanca et al., 2015; Siegel et al., 2016). Radical prostatectomy is considered the gold standard for treatment of clinically localized prostate cancer (Heidenreich et al., 2011). Despite the use of nerve-sparing techniques, erectile dysfunction is a common complication of pelvic surgeries, such as radical prostatectomy, and it is associated with remarkably decreased quality of life (Burnett et al., 2008; Walz et al., 2010). It is very important to prevent this complication, because an increasing number of younger patients are undergoing radical prostatectomy procedures (Stephenson et al., 2005). To preserve potency in patients who undergo radical prostatectomy, research efforts have focused on pharmacological and gene- or stem cell-based therapies to improve neuroregeneration after cavernosal nerve injury (Bochiski et al., 2004; Albersen et al., 2008; Kato et al., 2009; Qiu et al., 2012; Hannan et al., 2013; May et al., 2013; Ying et al., 2013; You et al., 2013; Li et al., 2018). The primary etiology of erectile dysfunction following pelvic surgery is neurogenic and associated with iatrogenic trauma of the cavernous nerves, which provide autonomic regulation of penile erection. It is generally accepted that cavernous nerve injury is the main cause of erectile dysfunction (Sezen et al., 2009). Because the neuronal cell has poor ability to self-renew, it is difficult to return function to the damaged nerve. Thus, growth factors or stem cells represent a new approach in the treatment of erectile dysfunction (Harraz et al., 2010; Lin et al., 2016; Reed-Maldonado et al., 2016; Yiou et al., 2017; Wu et al., 2018; Zheng et al., 2018). Some studies have reported that growth factors and many neurotrophic factors, such as vascular endothelial growth factor and brain-derived neurotrophic factor (BDNF), play an important role in neural regeneration and recovery of erectile function after cavernous nerve injury (Lin et al., 2002; Hsieh et al., 2003). Studies have shown that BDNF, vascular endothelial growth factor, and the JAK/STAT signaling pathway are three important components in the regeneration of the cavernous nerve (Zhang et al., 2010; Luo et al., 2017; Wang et al., 2017). Among various neurotrophins, BDNF plays a specific and important role in penile nerve recovery after cavernous nerve injury (Bakircioglu et al., 2001). In addition, BDNF can promote neuronal survival during development and prevent neuronal death in experimental neuropathy models, and it is considered in the treatment of a variety of neurodegenerative diseases (Yan et al., 1997; Frostick et al., 1998; Apfel et al., 1999). The use of mesenchymal stem cells infected with recombinant adenoviruses expressing human BDNF (rAd/hBDNF) may have a better effect on erectile dysfunction caused by cavernous nerve injury compared with BDNF alone (Kim et al., 2012). However, the effects of combined therapies with BDNF and adipose-derived stem cells (ADSCs) have not yet been studied. Therefore, the aim of this study was to evaluate the effect of ADSCs infected with lentiviral vector expressing rat BDNF (lenti-rBDNF) on erectile function in a rat model of cavernous nerve injury.

Materials and Methods

Animals

Forty male specific-pathogen-free Sprague-Dawley rats weighing 250 to 300 g and aged 4 months with normal erectile function (verified by copulatory tests) were obtained from the Experimental Animal Center of Wuhan University, China (animal license No. SCXK (E) 2008-0004). This study was approved by the Medical Animal Care and Welfare Committee of Wuhan University, China (approval No. 2017-1638) on June 20, 2017 and treated in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

The rats were randomly divided into a control group (identification of the cavernous nerves bilaterally without further surgical manipulation; n = 10); a bilateral cavernous nerve injury (BCNI) group identification and injury of the cavernous nerves bilaterally by using a hemostat (clamp; n = 10); an ADSCGFP group (BCNI followed by injection with ADSCs via intracavernous injection with lenti-GFP; n = 10); and an ADSCrBDNF group (BCNI followed by intracavernous injection with ADSCs infected with lenti-rBDNF; n = 10).

Preparation of rat ADSCs

Isolation and culture of ADSCs were according to our previously published methods (Ying et al., 2012). Briefly, inguinal fat pads were collected from 1-month-old rats under anesthesia with intraperitoneal injection of 2% sodium pentobarbital 40 mg/kg. The tissues were washed in phosphate-buffered saline (PBS), cut into pieces, and digested with type I collagenase. Enzyme activity was neutralized with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The sections were centrifuged at 300 × g (1200 r/min) for 10 minutes to obtain high-density cellular particles. The stem cellular particles were collected following centrifugation and digested for 2 hours in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in 5% humidified CO_2 at 37°C. ADSCs were examined for surface markers (CD34, CD45, CD44) by flow cytometry (BD, Franklin, NJ, USA) as in our previous report (Ying et al., 2012).

Plasmid construction

pcDNA3.1 plasmid encoding rat BDNF (pcDNA3.1-rBDNF) was purchased from Shanghai Genechem Co. Ltd. (Shanghai, China). A third-generation self-inactivating lentiviral vector containing a CMV-driven enhanced green fluorescent protein (EGFP) reporter was purchased from Shanghai Genechem Co., Ltd. The lentiviral vector system had three parts before packaging: pGC-E1 vector, pHelper 1.0 (gag/pol element) vector, and pHelper 2.0 (VSVG element) vector. Primer pairs for amplifying rBDNF from pcDNA3.1-rBDNF and cloning into the AgeI site of pGC-E1vector are shown in **Table 1**.

The full-length cDNA of rBDNF was cloned into pGC-E1 by digesting with AgeI and ligating the resultant fragments

Table 1 Primer sequence

ID	Sequence (5′–3′)	Product size (bp)
BDNF(5096-4)-P1	GAG GAT CCC CGG GTA CCG GTC GCC ACC ATG ACC ATC CTT TTC CTT AC	791
BDNF(5096-4)-P2	TCC TTG TAG TCC ATA CCT CTT CCC CTT TTA ATG GTC	791

BDNF: Brain-derived neurotrophic factor.

into the AgeI site of the pGC-E1 vector (pGC-E1- rBDNF). PCR and DNA sequencing were used to confirm accurate insertion of rBDNF cDNA.

Production and titration of recombinant lentiviral vectors

To prepare pseudotyped lentiviral vectors, pHelper1.0 plasmid DNA (15 μg), pHelper 2.0 plasmid DNA (10 μg), and pGC-E1-rBDNF or pGC-E1-GFP plasmid DNA (20 μg) were co-transfected into subconfluent 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Infectious lentiviruses were harvested at 48 hours post-transfection. The recombinant lenti-rBDNF and lenti-GFP viral vector titers were assessed by using fluorescence-activated cell sorting analysis of GFP^+ 293T cells (Invitrogen). Virus titers were 10^9 transducing units/mL of medium.

Infection of ADSCs with lentiviral vectors

Third-passage cells were used for our study. On the day of infection, cells were plated at 4×10^3 cells/well in 96-well plates along with lenti-rBDNF at different multiplicities of infection (MOI) in serum-free growth medium containing 5 μg/mL Polybrene (Sigma-Aldrich, St. Louis, MO, USA) for 4 hours. Serum-containing growth medium was added for 48 hours and then replaced. At 4 and 5 days post-infection, reporter gene expression was examined using fluorescent microscopy. ADSCs were plated in T-75 flasks and infected at an MOI of 100 for transplantation. The $ADSC_{GFP}$ and $ADSC_{rBDNF}$ cells were passaged and prepared for cell transplantation.

Detection of BDNF expression

Total protein was extracted from either infected ADSCs or uninfected ADSCs. Lysate containing 20 μg of protein was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted. The membrane was sequentially incubated with monoclonal mouse anti-rat BDNF antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH antibody (1:1000; Santa Cruz Biotechnology) at 37°C for 24 hours. Following goat anti-mouse secondary antibody (Santa Cruz Biotechnology) incubations at the ambient temperature for 1 hour, signals were visualized by enhanced chemiluminescence (Eastman Kodak, Rochester, NY, USA).

Rat models of cavernous nerve injury and cell transplantation

The rat cavernous nerve injury model was performed as described by Fandel et al. (2012). After abdominal anesthesia with 2% pentobarbital (40 mg/kg; Shanghai Bi-Yun-Tian Biotechnology, Shanghai, China), body temperature was maintained isothermically with a heating pad at 37°C. A midline incision was made on the abdominal skin to expose the bladder and prostate. The bilateral major pelvic ganglion was dissected from the lateral areas of the prostate with the aid of a surgical microscope (SXP-1C; Medical Optical Instruments Factory of Shanghai Medical Instruments Co., Ltd., Shanghai, China). From the bilateral major pelvic ganglion, cavernous nerves were identified toward the corpus cavernosum. The other three groups underwent bilateral cavernous nerve-crushing injury by using a hemostat clamp (Cheng-He Microsurgical Instruments Factory, Ningbo, Zhejiang Province, China) for 2 minutes. In the two treatment groups, $ADSC_{GFP}$ (1×10^6 in 20 μL), infected with lenti-GFP, or $ADSC_{rBDNF}$ (1×10^6 in 20 μL), infected with lenti-rBDNF, was injected into the penile cavernous tissue after BCNI.

Measurement of erectile function

Four weeks after model establishment, all rats were assessed for erectile function. In brief, after abdominal anesthesia with 2% pentobarbital (40 mg/kg; Shanghai Bi-Yun-Tian Biotechnology), the cavernous nerves and major pelvic ganglion were exposed bilaterally via midline laparotomy using a 23-G needle. After cavernous nerves were heparinized (250 U/mL), the 23-G needle end was connected to pressure transducers. The proximal parts of the cavernous nerves were activated using platinum electrodes connected to a stimulator. The stimulus parameters were 20 Hz, duration of 50 seconds, 0.2-ms pulse width, and 1.5 mA. After carotid artery cannulation, mean arterial pressure (MAP) and intracavernous pressure (ICP) were recorded using a data acquisition system (Chengdu TME Technology Co., Ltd., Chengdu, China). Finally, the maximal ICP/MAP ratio was calculated. The penis and cavernous nerves were then harvested for histology.

Immunofluorescence staining

Freshly dissected penis tissue was fixed for 4 hours in cold 2% formaldehyde and 0.002% picric acid in 0.1 M phosphate buffer, followed by overnight immersion in buffer solution containing 30% sucrose. Tissues were frozen in optimum cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and stored at −80°C until use. Sections were cut at 5 mm, adhered to charged slides, air dried for 10 minutes, and rehydrated with PBS. Goat serum 3% in PBS was applied as blocking agent for 30 minutes. Sections were incubated overnight at 4°C with (1:400) rabbit anti-mouse monoclonal neuronal nitric oxide synthase (nNOS; Santa Cruz Biotechnology), followed by incubation with a 1:500 dilution of goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) conjugated with Alexa Fluor 488 (Invitrogen) for 1 hour at 37°C. To analyze the content of nNOS, the total areas of the dorsal nerves and the areas of nNOS-positive fibers in the dorsal nerves were evaluated at 200× magnification using a fluorescence microscope (Adobe, San Jose, CA, USA).

Masson's trichrome staining

Masson's trichrome staining was conducted according to our previous method (Ying et al., 2013). Briefly, the middle part of penile tissue was fixed in formalin (10%) overnight, stored, and washed at 4°C with 70% alcohol. Then, the penile tissue was sliced into 5-μm paraffin-embedded sections for Masson's trichrome staining. The color distribution of the muscle tissues was observed by using Adobe Photoshop CS 8.0 (Adobe). Using a light microscope (BX60; Olympus, Tokyo, Japan), we selected muscle tissues, which were stained red after all color distributions of the images were calculated.

Western blot assay

To conduct the western blot, the penis was excised, the urethra removed, and the corpus cavernosum homogenized in Tris-HCl buffer (pH 7.5). Cytosolic and membrane fractions were isolated to measure cytosolic nNOS protein content by western blot assay. Lysate containing 50 μg of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted. The membranes were reacted with blocking buffer (5% skim milk in Tris-buffered saline-Tween) for 30 minutes at 4°C and incubated with rabbit anti-mouse nNOS monoclonal antibody (1:1000; Santa Cruz Biotechnology) and goat anti-rabbit GAPDH monoclonal antibody (1:1000; Santa Cruz Biotechnology) for 24 hours. Following secondary antibody incubations at 4°C for 1 hour, signals were visualized by enhanced chemiluminescence. Finally, the protein bands were visualized using an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). GAPDH was used as an internal control for normalization to determine protein expression levels. The relative gray levels of nNOS to GAPDH were measured using an ultraviolet photometry imaging system (UVP LLC, Upland, CA, USA).

Statistical analysis

All data are reported as the mean ± SD. Statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was used for the first analysis of our results. A Student-Newman-Keuls *post hoc* test was conducted if differences were significant. A value of $P < 0.05$ was considered statistically significant.

Results

Morphology and phenotype of cultured ADSCs

ADSCs expressed CD44 and were negative for CD34 and CD45 on the third passage (**Figure 1A**).

Infection efficiency of lenti-rBDNF and rBDNF gene in ADSCs

The third-passage ADSCs were infected with lenti-rBDNF at different MOIs. Expression of GFP was examined 3 days after infection. The infection efficiency was –approximately 80% at an MOI of 100. (**Figure 1B**, panel a); therefore, an MOI of 100 was chosen for the study. To verify rBDNF gene expression in ADSCs, rBDNF protein levels were examined in infected ADSCs. The rBDNF protein was found in lenti-rBDNF-infected ADSCs, but not in ADSCs or lenti-GFP–infected ADSCs (**Figure 1B**, panel b).

Erectile function

The ICP and the ICP/MAP ratio of rats in the BCNI group were decreased compared with that of the control group ($P < 0.05$). The ICP of rats in the $ADSC_{rBDNF}$ group was much higher than that in rats in the $ADSC_{GFP}$ group ($P < 0.05$) but still lower than that of rats in the control group ($P < 0.05$). The ICP/MAP ratio was higher in the $ADSC_{rBDNF}$ group than in the BCNI group ($P < 0.05$) but lower than that in the control group ($P < 0.05$; **Figure 2**).

ADSCs infected with lenti-rBDNF increase the number of nNOS-positive nerve fibers in the penile tissue of a rat model of cavernous nerve injury

nNOS-positive nerve fibers were diffusely distributed throughout the penis and were usually observed on the periphery of dorsal nerves of the penis. In the dorsal nerves, the number of the nNOS-positive nerve fibers was significantly decreased in the BCNI group compared with the control group ($P < 0.05$). The number of nNOS-positive nerve fibers was significantly higher in the $ADSC_{rBDNF}$ and $ADSC_{GFP}$ groups than in the BCNI group ($P < 0.05$) but less than in the control group ($P < 0.05$; **Figure 3**).

ADSCs infected with lenti-rBDNF increase smooth muscle in the penile tissue of a rat model of cavernous nerve injury

Computerized histomorphometric analysis showed that the ratio of smooth muscle/collagen was lower in the BCNI group than in the control group ($P < 0.05$). The ratio of smooth muscle/collagen was almost fully preserved in the $ADSC_{rBDNF}$ group compared with the control group ($P > 0.05$) and much higher than that in the $ADSC_{GFP}$ group ($P < 0.05$; **Figure 4**).

Quantification of nNOS protein expression

Western blot assay results showed that nNOS expression was decreased in the BCNI group compared with the control group ($P < 0.05$). The expression of nNOS was significantly increased in the $ADSC_{rBDNF}$ and $ADSC_{GFP}$ groups compared with the BCNI group ($P < 0.05$; **Figure 5**).

Discussion

Our studies showed a measurable neurotrophic effect of ADSCs and lenti-rBDNF on cavernous nerve regeneration following crush injury in a rat model. The restoration of erectile function was confirmed by both functional and morphological studies. The ICP changes and ICP/MAP indicated that the erectile response was better in the $ADSC_{rBDNF}$ and $ADSC_{GFP}$ groups than in the BCNI group at 4 weeks.

The $ADSC_{rBDNF}$ group showed a significant increase in nNOS content in the dorsal nerves. The nNOS content was significantly higher in the $ADSC_{rBDNF}$ and $ADSC_{GFP}$ groups than in the BCNI group, which indicated the recovery of cavernous nerves directly. In addition, the western blot assay showed that expression of nNOS proteins was increased in

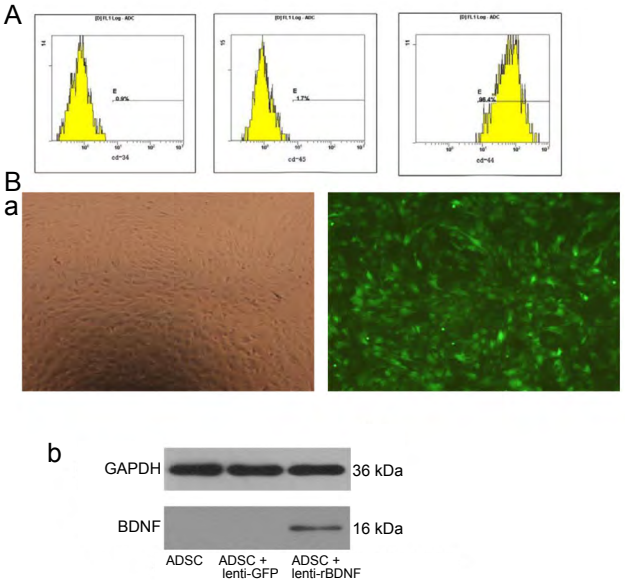

Figure 1 Identification and infection of ADSCs.
(A) Phenotypes of ADSCs at the third passage by flow cytometry; a: CD34 (0.9%), b: CD45 (1.7%), c: CD44 (96.4%). (B) Determination of lentiviral infection efficiency and rBDNF expression (arrows) in ADSCs. Panel (a) Infection efficiency 3 days after infection with lenti-rBDNF at an MOI of 100. GFP (green) was observed under light (left) or fluorescence (right) microscopy (original magnification, 100×). Panel (b) Representative immunoblot of rBDNF protein as measured by western blot assay. ADSCs were infected with control lentivirus (ADSC + lenti-GFP) or lenti-rBDNF (ADSC + lenti-rBDNF) at an MOI of 100. ADSCs: Adipose-derived stem cells; GFP: green fluorescent protein; MOI: multiplicity of infection; rBDNF: rat brain-derived neurotrophic factor.

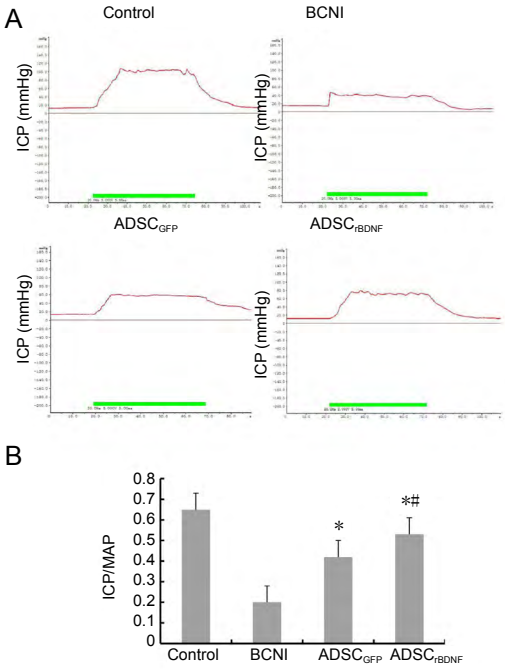

Figure 2 Effects of ADSCs infected with lenti-rBDNF on nerve conduction capacity.
(A) Representative recordings of ICP for each group in response to cavernous nerve stimulation. (B) ICP/MAP ratio was compared among each group of rats. Data are expressed as the mean ± SD (n = 10; one-way analysis of variance followed by the Student-Newman-Keuls *post hoc* test). *P < 0.05, *vs.* BCNI group; #P < 0.05, *vs.* ADSC$_{GFP}$ group. BCNI: Bilateral cavernous nerve injury; GFP: green fluorescent protein; ICP: intracavernosal pressure; MAP: mean arterial pressure; rBDNF: rat brain-derived neurotrophic factor.

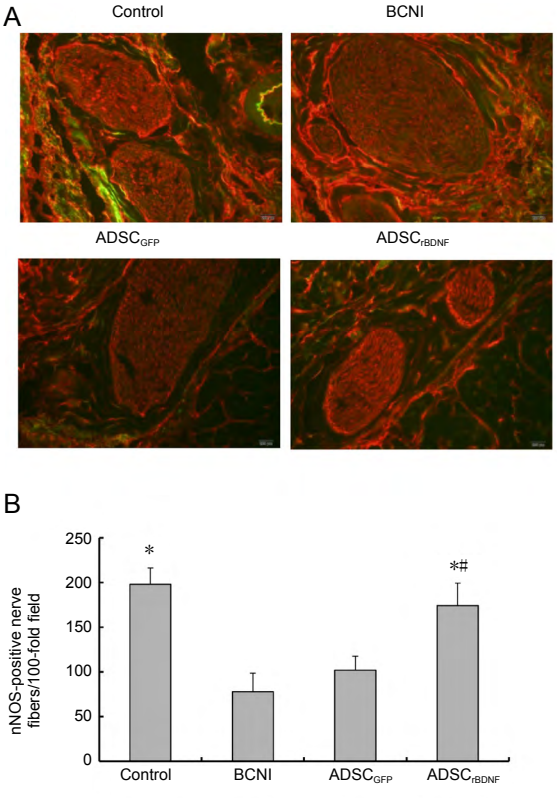

Figure 3 nNOS staining in a penile midshaft specimen.
(A) nNOS (red-stained fiber) in penile tissue (immunofluorescence microscope: 200×). Control group: Predominant red staining of nerve fibers; BCNI group: a paucity of red-stained fibers; ADSCr$_{BDNF}$ group: a significant increase in the number of red-stained nerve fibers compared with the ADSC$_{GFP}$ group. (B) nNOS-positive nerve fibers in penile midshaft sections. Data are expressed as the mean ± SD (n = 10; one-way analysis of variance followed by the Student-Newman-Keuls *post hoc* test). *P < 0.05, *vs.* BCNI group; #P < 0.05, *vs.* ADSC$_{GFP}$ group. ADSC: Adipose-derived stem cell; BCNI: bilateral cavernous nerve injury; GFP: green fluorescent protein; nNOS: neuronal nitric oxide synthase; rBDNF: rat brain-derived neurotrophic factor.

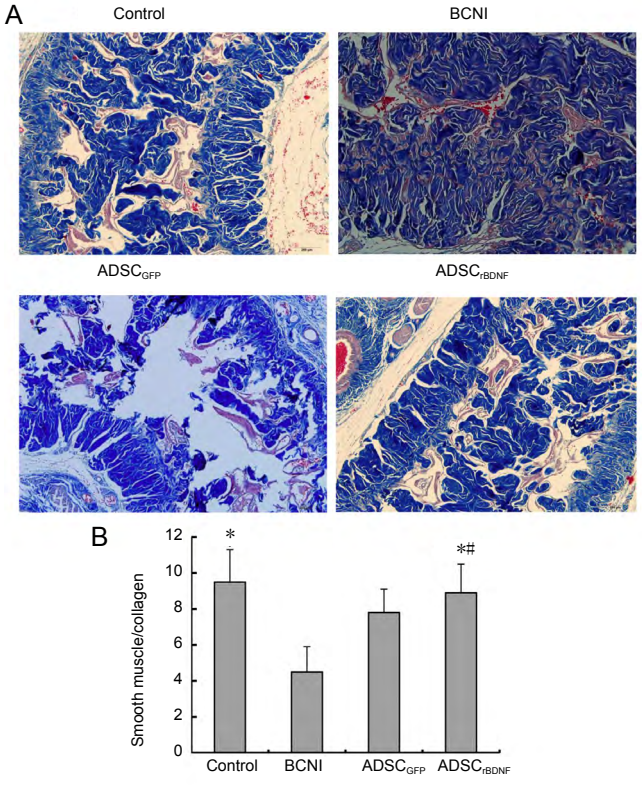

Figure 4 Effect of ADSC$_{rBDNF}$ on smooth muscle/collagen ratio in penile tissue of a rat model of cavernous nerve injury.
(A) Smooth muscle (red)/collagen (blue) changes in penile tissue with a light microscope (original magnification, 100×). (B) Ratio of smooth muscle/collagen. Data are expressed as the mean ± SD (n = 10; one-way analysis of variance followed by the Student-Newman-Keuls *post hoc* test). *P < 0.05, *vs.* BCNI group; #P < 0.05, *vs.* ADSC$_{GFP}$ group. ADSC: Adipose-derived stem cell; BCNI: bilateral cavernous nerve injury; GFP: green fluorescent protein; rBDNF: rat brain-derived neurotrophic factor.

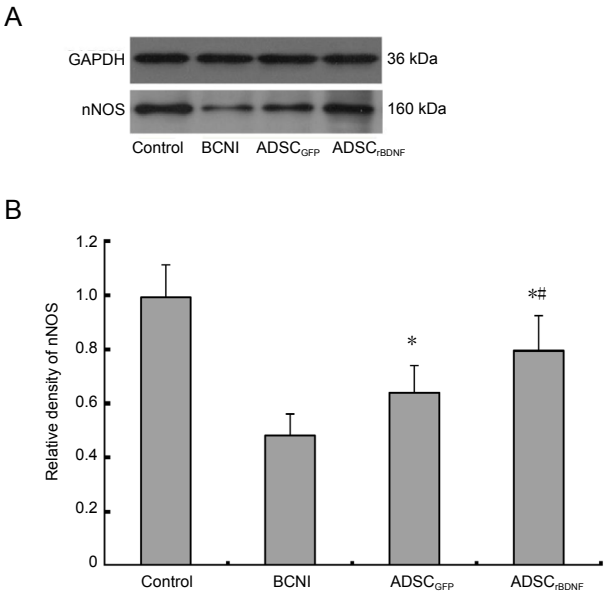

Figure 5 Effects of ADSC$_{rBDNF}$ on nNOS expression in the corpus cavernous tissue of rats after cavernous nerve crush injury.
(A) Western blot assay of nNOS expression in the corpus cavernosum at 4 weeks in the control group, BCNI group, ADSC$_{GFP}$ group, and ADSC$_{rBDNF}$ group. (B) Relative gray levels of nNOS to GAPDH. Data are expressed as the mean ± SD (n = 10; one-way analysis of variance followed by the Student-Newman-Keuls *post hoc* test). *P < 0.05, *vs.* BCNI group; #P < 0.05, *vs.* ADSC$_{GFP}$ group. ADSC: Adipose-derived stem cell; BCNI: bilateral cavernous nerve injury; GFP: green fluorescent protein; nNOS: neuronal nitric oxide synthase; rBDNF: rat brain-derived neurotrophic factor.

the ADSC-treated groups compared with the BCNI group.

Therapy using stem cells derived from adipose tissue has recently become an attractive treatment for tissue regeneration and engineering because of the differentiation potential of this cell type (An et al., 2010; Wu et al., 2010; Nagasaki et al., 2011; Ying et al., 2012; Jang et al., 2016; Li et al., 2017). ADSCs can be obtained from ubiquitously distributed adipose tissue with little harm to the patient and are expandable *in vitro* (Kim et al., 2008). In addition, ADSCs secrete neurotrophic factors that promote cavernous nerve regeneration (Albersen et al., 2010; Zhang et al., 2011). Therefore, many studies using ADSCs have reported restoration of erectile function following dysfunction caused by many factors, such as hypercholesterolemia, diabetes, and cavernous nerve injury (Wankhade et al., 2016; Zhu et al., 2017). In our study, intracavernous injection of ADSC$_{GFP}$ was expected to have a role in the restoration of erectile function. The ICP/MAP ratio was significantly higher in the ADSC$_{GFP}$ group than in the BCNI group. Furthermore, the preservation of cavernous smooth muscle was much better in the ADSC$_{rBDNF}$ group than in the BCNI group. These findings agree with those of other studies that evaluated the effect of various stem cells on neurogenic erectile dysfunction (Albersen et al., 2010; Harraz et al., 2010; Zhang et al., 2011). Considering these results, we propose that erectile function after cavernous nerve injury can be preserved by the regenerative effect of BDNF from ADSC$_{rBDNF}$ cells in the major pelvic ganglion.

Neurotrophins such as BDNF, growth differentiation factor-5, and neutrin act as neuromodulators in neuronal survival after cavernous nerve injury (Bella et al., 2009). BDNF belongs to the mammalian family of neurotrophins and can promote neuronal survival during development and prevent neuronal death in experimental neuropathy models (Yan et al., 1997; Frostick et al., 1998). BDNF provides neurotrophic support for a host of neuron types, such as peripheral sensory neurons and motor neurons (Sendtner et al., 1992). As we know, the JAK/STAT pathway modulates central and peripheral nerve regeneration by transducing cellular signals directly from the cell membrane to the nucleus. The mechanism by which BDNF promotes neurite growth in the major pelvic ganglion is through the JAK/STAT pathway (Bella et al., 2006; Lin et al., 2006).

Our results showed that intracavernous injection of ADSC$_{rBDNF}$ had a positive effect on cavernous nerve regeneration and functional recovery. Bakircioglu et al. (2001) found that intracavernous injection of adeno-associated virus (AAV)-BDNF may prevent the degeneration of nNOS-containing neurons in the major pelvic ganglion, facilitate the

regeneration of nNOS-containing nerve fibers in penile tissue, and enhance the recovery of erectile function after BCNI. Piao et al. (2012) used ADSCs with BDNF-membrane on the cavernous nerve to improve erectile function in a rat model of erectile dysfunction post-prostatectomy. Their results showed that ADSC/BDNF-membrane treatment increased smooth muscle/collagen ratio, nNOS content, phospho-eNOS protein expression, and cyclic guanosine monophosphate level compared with BCNI and other treatment groups. Jeong et al. (2013) showed that udenafil combined with ADSC/BDNF-membrane protected the cavernous nerve and improved angiogenesis in the corpus cavernosum after cavernous nerve injury, which further maintained erectile function in a rat model of erectile dysfunction post-prostatectomy. In the current study, results of Masson's trichrome staining showed that the muscle content was decreased and collagen content increased in the BCNI group. The smooth muscle/collagen ratio was higher in the $ADSC_{rBDNF}$ and $ADSC_{GFP}$ groups than in the BCNI group. The western blot assay and immunofluorescence showed that nNOS was observed in the corpus cavernosum of the $ADSC_{rBDNF}$ and $ADSC_{GFP}$ groups 4 weeks after injury. nNOS was remarkably elevated after intracavernous injection of ADSCs infected with lenti-rBDNF. These results are similar to those of previous studies (Bakircioglu et al., 2001; Piao et al., 2012; Jeong et al., 2013). Histologic evaluation of the dorsal nerves in the $ADSC_{rBDNF}$ group showed a dramatic increase in the number of myelinated axons and nNOS content, which could indicate regeneration of cavernous nerves.

Although our study demonstrated successful increase of erectile function following combined treatment with ADSCs and BDNF, further study is required. Our study had three important limitations. The first relates to clinical application: the safety of the lentivirus used as a vector for introduction of BDNF into ADSCs has not yet been established in humans. Second, our study lasted for 4 weeks, so we do not know whether the effect of $ADSC_{rBDNF}$ would persist up to 8 weeks or 3 months or longer. Third, the mechanism of preserving erectile function after cavernous nerve injury depends on whether ADSCs that differentiate into neural tissue have a regenerative effect in the major pelvic ganglion. Therefore, further investigations are needed.

In summary, functional and histological restoration of erectile dysfunction was better in the $ADSC_{rBDNF}$ group than in the $ADSC_{GFP}$ group after cavernous nerve injury. Injection of ADSCs infected with a lentiviral vector encoding rBDNF may be an effective method to treat erectile dysfunction following radical prostatectomy and deserves further clinical research.

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