Original Paper

Therapeutic Potential of Human Adipose-Derived Stem Cell Exosomes in Stress Urinary Incontinence – An *in Vitro* and *in Vivo* Study

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Key Words

Stress urinary incontinence • Adipose-derived stem cell • Exosome • Therapy

Abstract

Background/Aims: To evaluate whether local injection of exosomes derived from human adipose-derived stem cells (hADSCs) facilitates recovery of stress urinary incontinence (SUI) in a rat model. **Methods:** For the *in vitro* study, a Cell Counting Kit-8 (CCK-8) array and proteomic analysis were performed. For the *in vivo* study, female rats were divided into four groups: sham, SUI, adipose-derived stem cell (ADSC), and exosomes ($n = 12$ each). The SUI model was generated by pudendal nerve transection and vaginal dilation. Vehicle, hADSCs, or exosomes were injected into the peripheral urethra. After 2, 4, and 8 weeks, the rats underwent cystometrography and leak point pressure (LPP) testing, and tissues were harvested for histochemical analyses. **Results:** The CCK-8 experiment demonstrated that ADSC-derived exosomes could enhance the growth of skeletal muscle and Schwann cell lines in a dose-dependent manner. Proteomic analysis revealed that ADSC-derived exosomes contained various proteins of different signaling pathways. Some of these proteins are associated with the PI3K-Akt, Jak-STAT, and Wnt pathways, which are related to skeletal muscle and nerve regeneration and proliferation. *In vivo* experiments illustrated that rats of the exosome group had higher bladder capacity and LPP, and had more striated muscle fibers and peripheral nerve fibers in the urethra than rats of the SUI group. Both urethral function and histology of

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rats in the exosome group were slightly better than those in the ADSC group. **Conclusions:** Local injection of hADSC-derived exosomes improved functional and histological recovery after SUI.

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Introduction

Stress urinary incontinence (SUI) is a common medical condition affecting approximately 30% of postpartum women and interfering with their quality of life [1, 2]. It is defined as a “complaint of involuntary loss of urine upon effort or physical exertion (e.g., sporting activities), or sneezing or coughing” according to the International Continence Society (ICS) guidelines [3]. SUI results from a combination of intrinsic urethral sphincter deficiency and an anatomic defect in urethral support that leads to insufficient urethral closure pressure [4]. The mechanism of SUI is multifactorial and complex [5].

Several treatments for SUI are currently available, of which mid-urethral sling surgery is the most common and effective therapeutic method [6]. Despite the common use of mid-urethral sling surgery, this procedure is associated with a high level of complications, and therefore there is a need for new medical treatment options [7].

Stem cell-based therapy has gained attention as a promising treatment for SUI in recent years [6-8]. Among the different types of stem cells, adipose-derived stem cells (ADSCs) have been widely used since they can be easily and abundantly obtained and because of their multiple differentiation potential and low immunogenicity. Several studies have reported the efficacy of ADSCs in improving SUI in rats and humans [9-12]. However, the underlying mechanisms remain controversial. Recently, an increasing number of studies have demonstrated that a paracrine effect rather than stem cell differentiation is the main factor resulting in their therapeutic effect [13]. Meanwhile, the direct use of stem cells remains restricted by issues such as potential chromosomal variation, tumorigenicity, thrombosis, immunological rejection, and ethical limitations [14-16]. Hence, a superior cell-free approach that can exert the same therapeutic effects of stem cells and avoid their adverse events is anticipated.

Exosomes are 40- to 150-nm-sized nanoparticles originating from multivesicular bodies and are important paracrine effectors in intercellular communication. They function by transferring proteins and genetic materials to target cells [17]. It has been demonstrated that exosomes exhibit functional properties similar to those of the cells from which they are derived, and direct treatment with these nanoparticles has no apparent adverse effects such as immunogenicity, tumorigenicity, or vascular obstruction [18, 19]. Recent studies have reported that exosomes secreted by ADSCs show potential in tissue regeneration and protection, including bone, muscle, skin, and brain tissue [20-23]. Thus far, there have been no studies to our knowledge on the application of exosomes released by stem cells in SUI therapy. We hypothesized that ADSC-derived exosomes could be effective in treating SUI by promoting tissue regeneration after injury.

In the present study, we used human ADSCs (hADSCs) as the origin of exosomes and investigated whether the early administration of these exosomes could treat SUI in both *in vitro* and *in vivo* studies.

Materials and Methods

Isolation and culture of hADSCs

hADSCs were isolated from the musculocutaneous flap of a patient as described previously [24], with patient consent in accordance with procedures approved by the Medical Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (approval number 2017-004). Briefly, the fresh fat specimen was washed with phosphate-buffered saline (PBS) and chopped using sterile surgical scissors. The chopped tissues were digested with 0.2% collagenase type I (Gibco, Thermo Fisher Scientific, Waltham,

MA, USA) at 37°C for 1.5 h. The suspension was neutralized with isometric culture media and centrifuged at 1500 rpm for 5 min. The cell pellet was then resuspended in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) (Gibco) supplemented with 10% fetal bovine serum (Gibco). The cells were plated at a density of 10^6 cells/ml and then maintained at 37°C in a humidified incubator supplemented with 5% CO₂, and the medium was changed every other day. The hADSCs at passage 4 were used for the subsequent experiments.

Characterization of hADSCs

The culture-expanded hADSCs were tested for their ability to differentiate into adipocytes and osteoblasts. Passage 4 hADSCs at a density of 5×10^3 cells/cm² were seeded into 6-well plates pre-coated with a cover glass and induced for 3 weeks with adipogenic (HUXMA-90031, Cyagen Biosciences, Inc., Guangzhou, China) or osteogenic (HUXMA-90021, Cyagen Biosciences Inc.) differentiation media. The cells were then fixed in 10% formalin and stained with Oil Red O or Alizarin red in accordance with the manufacturer's protocol (Cyagen Biosciences, Inc.). Stained cells were washed 3 times with PBS and detected using microscopy (Olympus, Tokyo, Japan).

To observe the phenotypes, flow cytometry was used simultaneously. Passage 4 hADSCs were used to prepare a single-cell suspension, and subsequently 2 µl of CD90-FITC, CD44-PE, CD73-APC, or CD105-PerCP-CP5.5 antibodies were added to the suspension. These cell aliquots were incubated for 30 min on ice protected from light, washed 3 times, and then detected by flow cytometry (BD FACS Aria III; BD Biosciences, San Jose, CA, USA).

Isolation of hADSC exosomes

Exosomes were isolated from ADSC supernatants by our novel ultrafiltration method, as previously described [25], and cultured in DMEM/F12 medium. After hADSCs reached 80% confluence, the conditioned medium was collected and filtered through a 0.22-µm Sterilize Steritop filter (Millipore, Burlington, MA, USA). Then, the conditioned medium was transferred to a 15-ml 100-kDa Amicon Ultra Centrifugal filter and centrifuged at $4000 \times g$ for 40 min at 4°C. The supernatants were then transferred to a 500-µl Amicon Ultra Centrifugal filter and ultracentrifuged at $14,000 \times g$ for 10 min. Then, the ultrafiltration liquid was resuspended in 450 µl PBS and ultrafiltrated again at $14,000 \times g$ for 10 min. Finally, the ultrafiltration liquid was centrifuged at $1000 \times g$ for 2 min to collect concentrated exosomes. Exosomes were stored in aliquots at -80°C or used for other downstream experiments. An equal volume of DMEM/F12 was obtained in the same procedure as the collection of exosomes for control medium.

Characterization of hADSC exosomes

The isolated exosomes were characterized morphologically by dynamic light scattering (DLS), scanning electron microscopy (SEM), and antibody array for the expression of surface markers. DLS measurements were performed by a Zetasizer Nano ZS90 (Malvern, Worcestershire, UK). The size distribution plot with an x axis showing the distribution of estimated particle radius (nm) and a y axis showing the relative intensity of the scattered light was analyzed with NS300 software. For SEM examination, the sample exosomes were fixed in 0.5% glutaraldehyde solution overnight and then dehydrated in absolute ethanol for 10 min and collected on formvar grids stabilized with carbon (TED PELLA, Inc., Redding, CA, USA). The grids were contrasted with 1% phosphotungstic acid for 1 min and then examined using a field-emission electron microscope (JEOL Ltd., Tokyo, Japan). Exosomal markers were detected using an Exo-Check antibody array pre-printed with 8 antibodies for exosomal markers (FLOT-1, ICAM, ALIX, CD81, CD63, EpCAM, ANXA5, and TSG101) (System Biosciences, Mountain View, CA, USA). Briefly, 300 µg of total protein extracted from exosomes was incubated with membrane overnight at 4°C. After washing, the labeled proteins were visualized with the ChemiDoc XRS imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell proliferation analysis

Cell proliferation was evaluated by the Cell Counting Kit-8 assay (CCK-8, Dojindo, Kumamoto, Japan). L6 rat skeletal muscle cells and RSC96 rat schwann cells, obtained from the cell bank of the Chinese Academy of Science (Shanghai, China), were seeded into a 96-well plate at a density of 4×10^3 cells per well and allowed to attach for 4 h. Then, to each well was added control culture medium and culture medium containing different concentrations of exosomes (0.05, 0.5, and 5 µg/ml). The concentration of the exosomes was

measured using a BCA Protein Assay Kit (Beyotime, Nantong, China). At 2, 24, 48, and 72 h, CCK-8 agent (10 μ l per well) was added to cells, followed by incubation at 37°C for 2 h. The growth curves were generated using absorbance values at 450 nm ($n = 6$) detected with a microplate reader (Bio-Rad).

Proteomic analysis of hADSC exosomes

For proteomic analysis, the collected exosomes were digested at 4°C for 20 h with 0.25% trypsin (proteomics grade, Sigma-Aldrich, St. Louis, MO, USA). The digestion was quenched by adding 2 μ l formic acid (30 min at 37°C), centrifuged at $17,000 \times g$ for 10 min, and then transferred into the micro-vial for analysis.

Mass spectrometric measurements were performed using a high-performance liquid chromatography system with a Q-Exactive mass spectrometer equipped with an Easy-nLC (Thermo Fisher Scientific). Ten microliters of deglycosylated peptides was loaded onto an EASY-Column (Thermo Fisher Scientific, 0.075 mm $\times$ 100 mm, 3- μ m particle size), and separation was performed at a flow rate of 250 nl/min using a gradient constructed from solution A (2% acetonitrile, 0.1% formic acid) and solution B (84% acetonitrile, 0.1% formic acid): 4–50% B for 50 min; 50–100% B for 4 min; and 100% B for 6 min. The mass/charge ratio was determined after full scan.

For peptide identification, all the spectra were analyzed with Proteome Discoverer (version 1.4.0.29, Thermo Fisher Scientific). For database searching, the Mascot search engine (version 2.3.02, Matrix Science, London, UK) was used to search against the appropriate database. Non-intact (>20 ppm) peptides and fragmented ions (0.6 Da) were removed, with allowance for one missed cleavage in trypsin digests. For the study of the biological functions of identified proteins, a Gene Ontology (GO) analysis was performed using GoMiner software (available at <http://discover.nci.nih.gov/gominer/>). To reveal which pathways were significantly represented by the identified proteins, pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (available at <http://www.genome.jp/kegg/pathway.html>).

Animal experiment design

This study was approved by the Institutional Animal Care and Use Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Forty-eight adult female Sprague-Dawley rats (250–300 g) were randomly divided into four groups: sham, SUI, SUI+ADSCs, and SUI+Exosomes. The sham group underwent sham injury with 50 μ l saline injected into the peripheral urethra. The SUI group underwent pudendal nerve transection (PNT) and vaginal dilation (VD) with 50 μ l saline injected into the peripheral urethra. The SUI+ADSCs group underwent PNT+VD with 2 million ADSCs suspended in 50 μ l saline injected into the peripheral urethra. The SUI+ADSCs group underwent PNT+VD with 50 μ g exosomes suspended in 50 μ l saline injected into the peripheral urethra. All treatments were administered 1 h after the end of PNT+VD. Four rats in each group underwent continuous cystometrography (CMG) and leak point pressure (LPP) testing and were then euthanized for anatomic assessment of the urethra at 2, 4, and 8 weeks after injury.

PNT and VD

Rats were anesthetized with 2% isoflurane and underwent PNT and VD according to the following procedure. The pudendal nerve (Fig. 1A) was isolated bilaterally and transected. A modified 10F Foley catheter (Fig. 1B) was inserted into the vagina, and the balloon was inflated with 3 ml water for 4 h. A sham injury was created by making an incision in the dorsal skin and inserting the Foley catheter for 4 h without inflation. Buprenorphine (0.1 mg/kg) was administered subcutaneously twice daily after PNT+VD or sham injury for 2 days for analgesia.

Fig. 1. Images of animal experiments. A: Anatomical labels show sciatic nerve, posterior cutaneous nerve, and pudendal nerve. B: Different sizes of urethral dilators. C: Mid-urethra was detected after a lower abdominal incision.

Peripheral urethra injection

One hour after the end of VD or sham VD, a lower cut was made, and the mid-urethra was exposed. Saline, ADSCs, or exosomes were injected into the peripheral urethra using a Hamilton microinjector (Fig. 1C).

CMG and LPP test

CMG and LPP were recorded simultaneously by an investigator blinded to the experimental group. Under urethane anesthesia (1.2 g/kg, intraperitoneal), a transvesical catheter was inserted into the bladder dome and fixed with a suture. The abdominal wall was closed, and the catheter was connected to a pressure transducer (MLT0380/D, AD Instruments Pty, Ltd., Sydney, Australia) and a syringe pump (WZ50CZ, Smiths Medical Instrument Zhejiang Co., Ltd, China) to fill the bladder (6 ml/h). The bladder was filled for three to four voiding cycles, and the mean filling time was calculated as the interval between the end of each void and the beginning of next void. The continuous CMG was recorded, and the bladder capacity (BC) was calculated. The bladder was then manually emptied and refilled until approximately half mean filling time, when a slowly increasing external pressure was applied directly to the bladder using a cotton-tipped swap. This externally applied pressure was rapidly removed at the first sign of leakage at the urethral meatus. It has been previously shown that LPP is not highly sensitive to bladder volume in rats, except at very low volumes [26]. Therefore, this estimate of half capacity was sufficient to ensure consistent results. This LPP test was repeated three to four times in each rat. LPP was calculated by subtracting baseline bladder pressure from peak bladder pressure during LPP testing.

Histology

To assess anatomical recovery of the urethra, the urethra was harvested en bloc with the anterior vagina 2, 4, or 8 weeks after injury and paraformaldehyde fixed, paraffin embedded, transversely sectioned (5 μ m), and stained with hematoxylin & eosin (HE) or Masson. For image analysis, 5 randomly selected sections per animal were photographed using NIS-Elements D 3.10 software (Nikon). The proportion of striated muscle in the urethra was quantified (20 $\times$ magnification).

Immunofluorescent staining

To investigate the effects of each treatment, the urethral sections were stained with anti-fast myosin skeletal heavy chain (anti-FMHC) rat polyclonal antibody (1:200, ab91506, Abcam, Cambridge, UK) and anti-neurofilament 200 mouse monoclonal antibody (1:100, N0142, Sigma-Aldrich) followed by Cy3-donkey anti-goat secondary antibody (1:300, No. GB21404, Servicebio, Wuhan, China) and FITC-donkey anti-rabbit secondary antibody (1:200, No. GB22403, Servicebio). Slides were coverslipped with mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Southfield, MI, USA) and imaged on a confocal microscope. For determining the mean intensity of FMHC and neurofilament 200 immunofluorescence, 5 random sections per animal were observed and analyzed using ImageJ software (NIH, USA).

Statistical analysis

Numerical data are presented as mean $\pm$ the standard deviation. All statistical analyses in this study were performed using SPSS 16.0 software (IBM Corp., Armonk, NY, USA). The significance of differences between two groups was analyzed by one-way analysis of variance (ANOVA) with the Tukey honest significant difference test for post hoc analysis, or the paired-samples *t* test, as appropriate. A *P* value < 0.05 was considered significant.

Results

Isolation and characterization of hADSCs

After four passages in culture, the expanded hADSC population became homogeneous, showing a monolayer of adherent cells and demonstrating a typical fibroblast-like and shuttle-shaped morphology (Fig. 2A). To characterize the phenotype of hADSCs, flow cytometric analysis was performed. The results revealed that more than 90% of the cells

Fig. 2. Characterization of human adipose-derived stem cells (hADSCs). A: Morphology of P0 and P5 hADSCs. B: Flow cytometry revealed that more than 90% of the cells strongly expressed surface antigens, such as CD90 (99.8%), CD44 (100%), CD105 (97.6%), and CD73 (97.1%) but were virtually negative for CD34, CD45, CD11b, CD19, and HLA-DR (all <3%). C: Differentiation assays revealed that ADSCs could differentiate into adipocytes and osteocytes, which were stained by Oil Red O and Alizarin red, respectively.

strongly expressed surface antigens such as CD90 (99.8%), CD44 (100%), CD105 (97.6%), and CD73 (97.1%) but were virtually negative for CD34, CD45, CD11b, CD19, and HLA-DR (all <3%) (Fig. 2B). To definitively characterize the multipotency of these hADSCs, adipogenic differentiation and osteogenic differentiation were performed. After culture in adipogenic induction media for 3 weeks, more than 80% of the cells differentiated into adipocytes that could be stained with Oil Red O. Similarly, after osteogenic induction, more than 70% of the cells differentiated into osteoblasts, as demonstrated by Alizarin red staining (Fig. 2C). The results indicated that the multipotency of cultured hADSCs was well maintained.

Isolation and identification of exosomes derived from hADSCs

DLS, SEM, and antibody array were used to characterize the particles secreted from ADSCs. The size profiles of exosomes were evaluated by NanoSight technology and SEM imaging. The results showed that most of these vesicles ranged from 30 to 150 nm in size (Fig. 3A, 3B). Brownian activity of these particles was recorded as Video S1. For all supplemental material see www.karger.com/doi/10.1159/000492298. We detected the surface markers of exosomes using the antibody array method, and observed that markers such as FLOT1, ICAM, ALIX, TSG101, ANXA5, EpCAM, CD63, and CD81 were positively expressed (Fig. 3C).

Fig. 3. Characterization of human adipose-derived stem cell exosomes. A: Under scanning electron microscopy (SEM), exosomes appear with a round shape and are approximately 30 to 150 nanometers in size. B: Dynamic light scattering also showed that most of these vesicles ranged from 30 to 150 nm in size. C: We detected the surface markers of exosomes using an antibody array method, and observed that markers such as FLOT1, ICAM, ALIX, TSG101, ANXA5, EpCAM, CD63, and CD81 are positively expressed.

Fig. 4. CCK-8 assay. Both cell lines treated with exosomes showed a significant increase in proliferation in a dose-dependent manner as compared with those treated with conditioned medium at day 3 (72 h). * $P < 0.05$.

Fig. 5. Proteomic analysis of human adipose-derived stem cell (hADSC) exosomes. A: Gene Ontology (GO) analysis of three ontologies (biological process, molecular function, and cellular components). The bar graph shows the relationship between each the three ontologies and the number and amount (percentage) of the proteins. The x axis represents the three ontologies, the left y axis represents the number of proteins, and the right y axis represents the percentage of the proteins. B: The proteins carried by the hADSC exosomes are involved in many different important biological processes. The FASTA protein sequences of differentially changed proteins were blasted against the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://geneontology.org/>), and were subsequently mapped to processes in KEGG. In A and B, GO and KEGG pathway enrichment analyses were applied based on Fisher's exact test, considering the whole quantified protein/phosphor proteins annotation as the background dataset. Benjamini-Hochberg correction for multiple testing was further applied to adjust derived P values, and only functional categories and pathways with P values under a threshold of 0.05 were considered significant.

Exosomes derived from ADSCs induced proliferation

To explore whether ADSC-derived exosomes could induce the regeneration of skeletal muscle and nerve fibers, both skeletal muscle cells and glial cells were incubated with control medium or medium containing exosomes at three different concentrations (0.05, 0.5, and 5 $\mu\text{g}/\text{ml}$) for 72 h. Both cell lines treated with exosomes, as measured by the CCK-8 assay, exhibited significantly increased proliferation in a dose-dependent manner as compared with cells treated with control medium at day 3 (Fig. 4A, 4B).

Proteomic analysis of hADSC exosomes

Proteomic analysis of the exosomes identified 1,466 proteins that are implicated in various cell functions and pathways.

Gene Ontology (GO) analysis

To obtain a functional overview of hADSC exosome proteins, we further analyzed the exosome proteome using the GoMiner program by searching against GO databases (January 2008 version). The results showed that 331 proteins mapped to specific biological processes, 341 were associated with diverse cellular components, and 336 had other molecular functions. The resulting graph shows the relationship between each of the three ontologies and the number and amount (percentage) of the associated proteins (Fig. 5A).

Table 1. Proteins of related signaling pathways

Protein_ID	KO	Name	Definition	Map_ID	Map_Name
P14136	K05640	GFAP	glial fibrillary acidic protein	map04630	Jak-STAT signaling pathway
ABK1Z4	K05058	LIFR, CD118	leukemia inhibitory factor receptor	map04630	Jak-STAT signaling pathway
P11047	K05635	LAMC1	laminin, gamma 1	map04151	PI3K-Akt signaling pathway
A0A0A0MQS9	K06241	LAMA4	laminin, alpha 4	map04151	PI3K-Akt signaling pathway
P55268	K06243	LAMB2	laminin, beta 2	map04151	PI3K-Akt signaling pathway
P07942	K05636	LAMB1	laminin, beta 1	map04151	PI3K-Akt signaling pathway
A4QPA9	K04368	MAP2K1, MEK1	mitogen-activated protein kinase kinase 1	map04151	PI3K-Akt signaling pathway
G5E9C7	K04369	MAP2K2, MEK2	mitogen-activated protein kinase kinase 2	map04151	PI3K-Akt signaling pathway
O15111	K04467	IKBKA, IKKA, CHUK	inhibitor of nuclear factor kappa-B kinase subunit alpha	map04151	PI3K-Akt signaling pathway
P04004	K06251	VTN	vitronectin	map04151	PI3K-Akt signaling pathway
P78509	K06249	RELN	reelin [EC:3.4.21.-]	map04151	PI3K-Akt signaling pathway
P36955	K19614	SERPINF1, PEDF	pigment epithelium-derived factor	map04310	Wnt signaling pathway
Q93097	K00182	WNT2	wingless-type MMTV integration site family, member 2	map04310	Wnt signaling pathway

KEGG pathway analysis

KEGG pathway analysis (September 2007 version) was performed on the identified exosome proteins to evaluate which pathways were significantly represented. The results showed that two hundred twenty-two different pathways were linked to the exosomes. The top 20 KEGG pathways are shown in Fig. 5B, and proteins of pathways related to muscle and nerve regeneration and proliferation, including PI3K-Akt, Jak-STAT, and Wnt, are shown in Table 1.

Assessment of urethral function

Functional evaluation of the urethra was assessed through CMG and LPP testing at 2, 4, and 8 weeks after injection. In the sham group, BCs were 0.525 ± 0.065 ml, 0.533 ± 0.043 ml, and 0.535 ± 0.044 ml respectively, while LPPs were 47.25 ± 2.22 cmH₂O, 46.25 ± 2.21 cmH₂O, and 47.5 ± 2.65 cmH₂O, respectively. In the SUI group, BCs were 0.275 ± 0.065 ml, 0.28 ± 0.044 ml, and 0.27 ± 0.026 ml, respectively, while LPPs were 22.75 ± 2.23 cmH₂O, 23.25 ± 2.20 cmH₂O, and 25.25 ± 2.5 cmH₂O, respectively. In the ADSC group, BCs were 0.4 ± 0.044 ml, 0.438 ± 0.035 ml, and 0.455 ± 0.034 ml, respectively, while LPPs were 32.5 ± 2.08 cmH₂O, 35.75 ± 1.71 cmH₂O, and 41.25 ± 1.71 cmH₂O, respectively. In the exosome group, BCs were 0.413 ± 0.03 ml, 0.455 ± 0.045 ml, and 0.478 ± 0.054 ml, respectively, while LPPs were 34.5 ± 2.1 cmH₂O, 37.5 ± 2.08 cmH₂O, and 42.8 ± 2.22 cmH₂O, respectively.

In the sham and SUI groups, BC and LPP remained stable at 2, 4, and 8 weeks after injection. In the ADSC and exosome groups, the BC and LPP values increased gradually at 2, 4, and 8 weeks after injection. At each time point, BCs and LPPs of both groups were significantly different compared with the SUI group ($P < 0.05$), and there was no statistically significant difference between the two groups (Fig. 6).

Histological changes in the urethra

The proportion of striated muscle in the urethra in each group was quantified to show the histological change in the urethra. In the sham group, the proportion of striated muscle was 0.304 ± 0.009 at 2 weeks, 0.301 ± 0.012 at 4 weeks, and 0.312 ± 0.01 at 8 weeks. In the SUI group, the proportion of striated muscle was 0.151 ± 0.01 at 2 weeks, 0.155 ± 0.008 at 4 weeks, and 0.157 ± 0.006 at 8 weeks. In the ADSC group, the proportion of striated muscle was 0.177 ± 0.007 at 2 weeks, 0.221 ± 0.012 at 4 weeks, and 0.248 ± 0.01 at 8 weeks. In the exosome group, the proportion of striated muscle was 0.181 ± 0.009 at 2 weeks, 0.225 ± 0.012 at 4 weeks, and 0.261 ± 0.01 at 8 weeks.

Fig. 6. Assessment of urethral function. A: Cystometrography (left) and leak point pressure (LPP) curves (right) of different groups at 8 weeks after injection. B and C: The bladder capacity and LPP of each group at 2, 4, and 8 weeks after injection. *** $P < 0.01$. IVP, intravesical pressure; NS, non-significance.

Because of the sphincter striated muscle degeneration and atrophy, the proportion of striated muscle in the urethra of the SUI group was significantly decreased compared to that in the sham group (Fig. 7). In the ADSC and exosome groups, the proportion of striated muscle increased continuously at 2, 4, and 8 weeks after injection. At each time point, the proportion of striated muscle of both groups was significantly different compared with the SUI group ($P < 0.05$), and there was no statistically significant difference between the two groups (Fig. 7).

Results of HE staining (Fig. 8) showed no marked difference in inflammatory cells around the injection site after 8-week hADSC and exosome injections compared with the sham group, which indicated that no detectable immune response was induced.

Immunofluorescent staining of urethra

Striated muscle was FMHC positive and stained red, while peripheral nerves located outside the striated muscle layers were neurofilament 200 positive and stained green (Fig. 9). Eight weeks after injection, striated muscle and peripheral nerves of SUI rats were significantly fewer than those of the sham group. In contrast, the ADSC and exosome groups showed similar amounts of striated muscle and peripheral nerves compared to the sham group, and the difference was insignificant.

Fig. 7. Histological changes in the urethra. A: Masson's trichrome staining of mid-urethra of all groups at each time point. B: The proportion of striated muscle in the urethra in each group was quantified to show the histological change in the urethras. Scale bar = 100 μ m. *** $P < 0.01$. NS, non-significance.

Fig. 8. HE staining of mid-urethra 8 weeks after injection showed no detectable increase in inflammatory cells around the injection site in the ADSC and exosome groups compared with sham. The results indicated that no significant immune response was induced. Scale bar = 100 μ m.

Fig. 9. Immunofluorescent staining of urethra at 8 weeks after injection. Striated muscle was fast myosin heavy chain (FMHC) positive and stained red, while peripheral nerves located outside of the striated muscle layers were neurofilament 200 positive and stained green. Bar graphs showing the mean immunofluorescence intensity of FMHC and neurofilament 200 of 4 groups. Scale bar = 100 μ m. *** $P < 0.01$. NS, non-significance.

Discussion

Animal models have been used to better understand the mechanisms underlying, and find a potential treatment for SUI. Bilateral PNT produces denervation of the external urethra sphincter (EUS) and significantly decreases LPP, EUS electromyogram, and striated muscle [27]. This model offers the advantage that it may be representative of neurogenic damage that occurs in radical prostate surgery or urethral denervation and/or pudendal nerve and muscular damage that probably occurs during vaginal delivery [28, 29]. VD, another common model, damages the muscular and neurologic structures that maintain urinary continence [30]. The main limitation of this model is the short durability of the functional aspect, with a recovery within 6 weeks in most cases [31]. In the current study, we expanded the investigation to a complex dual-injury model, which represents both neurogenic and myogenic injuries and could last for months. This model enabled studying the long-term effects of exosomes on either tissue simultaneously.

It is generally accepted that skeletal muscle and peripheral nerve tissue have a low capacity to regenerate once injured. Various approaches using cellular and biochemical components have been explored *in vitro* and *in vivo* to enhance the regenerative capacity of injured skeletal muscle and peripheral nerves. Among them, stem cell-based therapies have clear beneficial effects on skeletal muscle and peripheral nerve repair [32, 33]. Although the mechanisms by which stem cell therapy act are still unknown, recent evidence has suggested they might be related to long-distance cell-to-cell communication via secreted paracrine factors in the extracellular environment [34]. Recent studies have shown the therapeutic potential of exosomes derived from various cells with secretory capacity (e.g., stem cells and progenitor cells) in various injured tissues [35, 36], including skeletal muscle and peripheral nerves [37, 38]. Based on these considerations, the aim of this study was to investigate whether exosomes can be considered as effectors of ADSC functions in treating SUI and to identify their related molecular mechanisms.

In line with our hypothesis, our data showed that ADSCs exert their restorative properties via a paracrine mechanism that utilizes exosomes rather than by direct cellular interaction. The CCK-8 experiment demonstrated that ADSC-derived exosomes could enhance the growth of skeletal muscle and Schwann cell lines in a dose-dependent fashion. We found that the exosomes at 5 µg/ml have the strongest proliferation effect on both cell lines, and thus we used this concentration for *in vivo* study. *In vivo* experiments showed that ADSC-derived exosomes could improve urethral function and histology after SUI and performed slightly better than ADSCs. CMG and LPP tests demonstrated that both exosomes and ADSCs could accelerate the recovery of urethral function after SUI. Rats in both groups showed significantly higher BC and LPP compared with SUI rats. Moreover, we found that the restorative effects of exosomes and ADSCs were constant and could last for 8 weeks because the BC and LPP kept increasing over time. The simultaneous improvement of urethral histology could explain the functional change. By Masson's trichrome staining, we found that the striated muscle layer in rats of the exosome and ADSC groups were significantly thicker and denser than that of the SUI group. By HE staining, we demonstrated that no substantial immune response was induced around the injection site after 8-week injection of both hADSCs and exosomes. By immunofluorescence staining of FMHC and neurofilament 200, we found that rats of the exosome and ADSC groups showed significantly higher density of striated muscle fibers and peripheral nerve fibers than that of the SUI group. The pattern of histological improvement was similar to that of functional change. On the other hand, the exosome group showed slightly better results than the ADSC group, and the difference was not significant. Accordingly, we could speculate that the exosome is the main effector of ADSCs, and a higher concentration of exosomes has a higher restorative effect for SUI.

The next question is how the exosomes benefit the recovery of SUI. In our study, we performed proteomic analysis using GO and KEGG pathway analyses. According to these results, ADSC-derived exosomes contained various proteins from a variety of pathways. Some of these proteins are associated with PI3K-Akt, Jak-STAT, and Wnt pathways that are related

to skeletal muscle and nerve regeneration and proliferation. For instance, laminins critically contribute to cell attachment and differentiation, cell shape and movement, maintenance of tissue phenotype, and survival [39]. Reelin (RELN) is a large secreted extracellular matrix glycoprotein that helps regulate processes related to neuronal migration. Pigment epithelium-derived factor (PEDF) displays neurotrophic functions, and a previous study has reported that PEDF could protect against the neurogenic destruction of erectile function [40]. Therefore, these results support our hypothesis that the proteins contained in exosomes could effectively facilitate skeletal muscle and peripheral nerve regeneration by stimulating related signaling pathways.

To our knowledge, this study is the first in which SUI has been treated with exosomes and the first in which ADSC-derived exosomes were delivered into the pelvis and urethra. Stem cell-derived exosomes, as a cell-free therapy, have low immunogenic capacity and have no risks of infection, tumorigenicity, or thrombosis. Despite accumulating evidence of the therapeutic effects of exosomes, it remains unclear which factors in exosomes play key roles in controlling cell fate and promoting skeletal muscle and peripheral nerve regeneration. Further identification of the main molecules contained in exosomes that could facilitate the regeneration of skeletal muscle and peripheral nerves is required to elucidate the mechanisms underlying exosome-based therapeutics.

In conclusion, in the current research, we have demonstrated that rats receiving exosomes derived from ADSCs as a therapeutic strategy showed improved functional and histological recovery after SUI. More striated muscle fibers and peripheral nerve fibers were found in the urethra after exosome treatment. Proteomic analyses identified 1466 proteins, and some of these are associated with functions such as cell regeneration and migration. Thus, exosome treatment could be a new, clinically useful tool providing a cell-free therapeutic approach for treating SUI. However, future work will be necessary to confirm the implications and mechanisms.

Abbreviations

ADSC (adipose-derived stem cell); hADSC (human adipose-derived stem cell); SUI (stress urinary incontinence); CCK-8 (Cell Counting Kit-8); PNT (pudendal nerve transection); VD (vagina dilation); CMG (cystometrography); LPP (leak point pressure); BC (bladder capacity); DLS (dynamic light scattering); SEM (scanning electron microscopy); GO (Gene Ontology); KEGG (Kyoto Encyclopedia of Genes and Genomes); FMHC (fast myosin heavy chain); EUS (external urethral sphincter); PEDF (pigment epithelium-derived factor).

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Disclosure Statement

The authors declare that they have no competing interests.

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