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ORIGINAL ARTICLE

Erectile Function

Combination of low-energy shock-wave therapy and bone marrow mesenchymal stem cell transplantation to improve the erectile function of diabetic rats

Hai-Tao Shan^{1,2}, Hai-Bo Zhang¹, Wen-Tao Chen³, Feng-Zhi Chen¹, Tao Wang⁴, Jin-Tai Luo¹, Min Yue⁵, Ji-Hong Lin⁵, An-Yang Wei¹

Stem cell transplantation and low-energy shock-wave therapy (LESWT) have emerged as potential and effective treatment protocols for diabetic erectile dysfunction. During the tracking of transplanted stem cells in diabetic erectile dysfunction models, the number of visible stem cells was rather low and decreased quickly. LESWT could recruit endogenous stem cells to the cavernous body and improve the microenvironment in diabetic cavernous tissue. Thus, we deduced that LESWT might benefit transplanted stem cell survival and improve the effects of stem cell transplantation. In this research, 42 streptozotocin-induced diabetic rats were randomized into four groups: the diabetic group ($n = 6$), the LESWT group ($n = 6$), the bone marrow-derived mesenchymal stem cell (BMSC) transplantation group ($n = 15$), and the combination of LESWT and BMSC transplantation group ($n = 15$). One and three days after BMSC transplantation, three rats were randomly chosen to observe the survival numbers of BMSCs in the cavernous body. Four weeks after BMSC transplantation, the following parameters were assessed: the surviving number of transplanted BMSCs in the cavernous tissue, erectile function, real-time polymerase chain reaction, and penile immunohistochemical assessment. Our research found that LESWT favored the survival of transplanted BMSCs in the cavernous body, which might be related to increased stromal cell-derived factor-1 expression and the enhancement of angiogenesis in the diabetic cavernous tissue. The combination of LESWT and BMSC transplantation could improve the erectile function of diabetic erectile function rats more effectively than LESWT or BMSC transplantation performed alone.

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INTRODUCTION

Erectile dysfunction (ED) has been defined as the persistent inability to attain and maintain an erection sufficient to permit satisfactory sexual performance. ED affects physical and psychological health and has a significant impact on the life quality of sufferers and their partners.¹ ED is one of the major complications of diabetes mellitus (DM).² The pathogenesis of diabetic ED is multifactorial and complicated, involving persistent damage to the vascular endothelium and smooth muscle and cavernous fibrosis.^{3,4}

Today, oral phosphodiesterase-5 inhibitors (PDE5i) are generally considered the first-line choice for ED patients. However, approximately 35% of patients with ED still fail to respond to this treatment. In these patients, DM is one of the most common causes of failure to respond.⁵ In diabetic men, the efficacy of PDE5i is considerably <70%.⁶ For the purpose of exploring more effective therapeutic strategies for restoration of the diabetic cavernous body, regenerative medicine is an inevitable subject. Recently, stem cell transplantation and low-energy shock-wave therapy (LESWT) have emerged as potentially effective treatment protocols for diabetic ED.^{7,8}

Bone marrow mesenchymal stem cells (BMSCs) are among the common stem cells transplanted in diabetic ED rats. These cells could improve erectile function, increase the content of endothelium and smooth muscle, and exert anti-fibrotic effects.^{9–11} On tracking transplanted stem cells in diabetic ED rat models, the number of visible BMSCs was rather low and decreased rapidly in the subsequent period.⁹ Similar results were achieved with adipose-derived mesenchymal stem cell transplantation in diabetic rats¹² and different types stem cell transplantation in rat models of cavernous nerve injury.¹³ The low survival number of intracavernosally injected stem cells limited the therapeutic effects of BMSC transplantation.

The small visible number of transplanted stem cells might be related to the migration of intracavernosally injected stem cells because research has confirmed that intracavernosally injected stem cells exited the penis within days and migrated preferentially to the bone marrow.¹⁴ In addition, the poor viability of transplanted BMSCs might be related to the hostile survival microenvironment.¹⁵ The vasculature plays an instructive role in the stem cell niche¹⁶ whereas a growing body of evidence has indicated that diabetes impacts angiogenesis

¹Department of Urology, Medical Center for Overseas Patients, Nanfang Hospital, Southern Medical University, Guangzhou, China; ²Department of Urology, Shawan People's Hospital, Panyu District, Guangzhou, China; ³Shenzhen Hyde Medical Equipment Co., Ltd., Shenzhen, China; ⁴Department of Urology, Longjiang Hospital, Shunde District, Foshan, China; ⁵Laboratory Animals Center, Southern Medical University, Guangzhou, China.

Correspondence: Dr. AY Wei (profwei@126.com)

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through a variety of mechanisms, including vascular endothelial growth factor (VEGF)/phosphoinositide 3' kinase/AKT/endothelial nitric oxide synthase (eNOS) proangiogenic pathway abrogation and vessel (CD31⁺) number reduction.^{17,18}

LESWT, which is typically defined as an energy flux density <0.1 mJ mm⁻², can improve the erectile function of diabetic rats by promoting the regeneration of endothelial cells, smooth muscle cells, and exerting anti-fibrotic effects.¹⁹⁻²¹ In addition to improving angiogenesis, the mechanism of action of LESWT might be related to the recruitment of endogenous stem cells.²² Intraperitoneally injected EdU-labeled mesenchymal stem cells (MSCs) are recruited by LESWT and migrate to the cavernous body.¹⁹

If LESWT can inhibit the migration of intracavernosally injected stem cells leaving the cavernous body and create a beneficial microenvironment for an exogenous stem cell niche to be established in the diabetic cavernous body, the survival of transplanted BMSCs would increase. Naturally, the combination of LESWT and BMSC transplantation should improve the erectile function of diabetic rats more effectively.

MATERIALS AND METHODS

Study design

Forty-eight diabetic rats were randomized into four groups. The DM group ($n = 6$) was diabetic control group. In the LESWT group ($n = 6$), the rats received a course of LESWT for 3 weeks. In the LESWT + BMSC group ($n = 15$), the rats received BMSC transplantation 1 day after 3-week course of LESWT. In the BMSC group ($n = 15$), the rats only received BMSC transplantation at the same time as the LESWT + BMSC group but without LESWT. Another six healthy adult rats that did not receive streptozotocin injections were included as normal group (normal group).

Before and at the end of the 3-week course of LESWT, the circulating endothelial progenitor cell (EPC) markers (CD31, CD34) of the groups ($n = 12$) that did or did not receive LESWT were evaluated by flow cytometry. At 1 day and 3 days after transplantation, three rats were chosen randomly from both BMSC group and LESWT + BMSC group separately to observe the number of labeled BMSCs. Four weeks after BMSC transplantation, intracavernous pressure (ICP)/mean arterial pressure (MAP) measurements, reverse transcription-polymerase chain reaction (RT-PCR) of stromal cell-derived factor-1 (SDF-1), and VEGF and penile histological assessment were performed.

Establishment of diabetic rat model

The establishment of the diabetic rat model was based on the process described before.⁹ Healthy adult male Sprague-Dawley rats (about 200 g and 8 weeks old) were intraperitoneally injected with 1% streptozotocin solution (65 mg kg⁻¹). Diabetes was confirmed by measuring tail vein random blood glucose levels 72 h after injection. Rats with random blood glucose concentrations >16.7 mmol l⁻¹ were diagnosed as diabetic. Random blood glucose from the tail vein blood was measured using a blood glucose meter (Roche, Basel, Switzerland) every week. The weight was also measured every week. The protocols were approved by the Committee of Ethics in Animal Experimentation of Southern Medical University. All the rats were maintained in a conventional temperature-controlled animal house with a 12 h light-dark cycle and with a continuous supply of food and water.

BMSC isolation culture, labeling, and cavernous injection

Isolation and expansion of BMSCs were performed according to a previous description.⁹ Male Sprague-Dawley rats (4 weeks old)

were sacrificed after anesthesia, and bone marrow was harvested by flushing the femoral and tibial cavities with phosphate-buffered saline (PBS). The collected cells were seeded in culture medium at a density of 1×10^6 cells per ml Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin and 100 mg ml⁻¹ streptomycin in culture flasks. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Two days later, nonadherent cells were removed and fresh culture medium was added. The culture medium was changed every 3 days. Cells were passed when they reached approximately 90% confluence. The third-passage BMSC phenotype was identified by flow cytometry analysis.

The cultured third-passage BMSCs were labeled with the green fluorescent lipophilic dye cell marker-DiO (CM-DiO Vybrant™, Berkshire, UK) for cell tracking, according to the manufacturer's instructions. After labeling, BMSCs were suspended in PBS at a concentration of 2000 cells per µl for intracavernosal transplantation.

One day after 3-week course of LESWT, 1×10^6 BMSCs dissolved in 500 µl of PBS were injected into the cavernous bodies of the rats in the BMSC and LESWT + BMSC groups. Rats in the DM and LESWT groups received an injection of 500 µl of PBS.

LESWT treatment

LESWT was modified and performed according to previously published studies.¹⁹ The rats were anesthetized with pentobarbital. The penis was drawn out of the prepuce, and the ultrasonic gel was applied to the penis. Then, the penis was placed on a shock-wave applicator (Shenzhen Hyde Medical Equipment Co., Ltd., Shenzhen, China). A total of 300 shocks were delivered at an energy level of 11.08 MPa (approximately 0.082 mJ mm⁻²) at a frequency of 60 per min. The rats from the LESWT and LESWT + BMSC groups received LESWT treatment three times weekly for 2 weeks with a 1-week interval.

Flow cytometry for BMSC identification and circulating EPC counting

Passage three BMSCs were prepared for flow cytometry analysis. In brief, 1×10^5 BMSCs were harvested and suspended in 500 µl of PBS followed by incubation with monoclonal mouse IgG to CD45 (eBioscience, San Diego, CA, USA), monoclonal mouse IgG to CD34 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), monoclonal mouse IgG to CD44 (eBioscience, San Diego, CA, USA), monoclonal Armenian hamster IgG to CD29 (Biolegend, San Diego, CA, USA) and monoclonal mouse IgG to CD90 (Biolegend, San Diego, CA, USA). After washing with PBS, the BMSCs were analyzed using the FACS Fortessa (BD Biosciences, Franklin Lakes, NJ, USA).

Before and at the end of the 3-week course of LESWT, peripheral blood samples from the tail veins of the groups receiving LESWT (LESWT and LESWT + BMSC groups, $n = 12$) or not (DM and BMSC groups, $n = 12$) were obtained to detect CD31 (eBioscience, San Diego, CA, USA) and CD34 expression levels. The results were expressed as the percentage of the total mononuclear cell number.

Evaluation of erectile function

Four weeks after intracavernous transplantation of BMSCs, ICP/MAP response to electric stimulation of the cavernous nerve was measured as previously described.⁹ After administration of pentobarbital for anesthesia, a lower abdominal midline incision was performed and the cavernous nerve was exposed. A bipolar electrode connected to an electrical pulse stimulator was connected to the cavernous nerves. A 23-gauge needle connected to a tube filled with heparin was inserted into the penis crus for the measurement of ICP. The carotid artery was

cannulated to record the MAP. The nerve was stimulated at a frequency of 10 Hz with a pulse width of 5 ms. Stimulation was performed at 10 V for 60 s with resting periods of 5 min between subsequent stimulations. Data were acquired using an MP150 physiological recorder (BIOPAC, Goleta, CA, USA).

RT-PCR of SDF-1 and VEGF expression

After erectile function evaluation, the rats were sacrificed and their penises were harvested. Part of the penis was chosen randomly for RNA isolation. Snap-frozen tissue was pulverized and then homogenized in TriZol (Invitrogen, Cergy-Pontoise, France). Primers were designed using Primer Premier software, version 5.0 (Primer, Palo Alto, CA, USA), and their sequences are listed as follows. VEGF primers: upstream primer 5'-CAATGATGAAGCCCTGGAGT-3', downstream primer 5'-TATGTGCTGGCTTTGGTGAG-3'; and SDF-1 primers: upstream primer 5'-GCTCTGCATCAGTGACGGTA-3', downstream primer 5'-GATGTTTGACGTTGGCTCTG-3'.

Reverse transcription was performed in strict accordance with the TaKaRa PrimeScript II 1st Strand cDNA short Kit (TaKaRa Bio Inc., Dalian, China) instructions. RT-PCR was performed according to the TaKaRa SYBR[®] Premix Ex Taq[™] II and Bio-Rad CFX96 Real-Time PCR System operating directions (Bio-Rad, Hercules, CA, USA). Data analysis was performed with Bio-Rad CFX Manager software, version 1.6 (Bio-Rad, Hercules, CA, USA). Expression of the gene of interest was computed relative to the expression of the internal standard β -actin.

Fluorescence microscopy and immunohistochemistry

At 1 day and 3 days after BMSC transplantation, freshly dissected tissues of the cavernous bodies of three rats from the LESWT + BMSC and BMSC groups were harvested to evaluate the numbers of transplanted BMSCs. Four weeks after transplantation and immediately after ICP/MAP evaluation, part of the freshly dissected tissue from the remainder of the cavernous bodies was harvested to evaluate the transplanted BMSC survival numbers. The samples were fixed and detected by fluorescence microscopy. We randomly chose five slices from each cavernous body for calculations.

Tissue from every group was fixed in 4% paraformaldehyde in PBS overnight, dehydrated by an ethanol gradient, and then embedded in paraffin. Paraffin-embedded sections 5 μ m in size were prepared. The tissue was immune-stained with anti- α -smooth muscle actin (α -SMA; Bioworld, Saint Louis Park, MN, USA), anti-CD31 (Bioworld, Saint Louis Park, MN, USA), or anti-eNOS (Bioworld, Saint Louis Park, MN, USA). Masson Trichrome staining was performed and expressed as the ratio of smooth muscle/collagen. Images with $\times 200$ magnification were obtained.

For tissue specimens, data were averaged from six fields of each section. Images were obtained using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and an Olympus DP71 camera (Olympus, Tokyo, Japan). Quantitative image analysis was conducted through computerized densitometry utilizing the ImagePro computer program version 6.0 (Media Cybernetics, Rockville, MD, USA). The resulting data were expressed as the ratio of the average stained area relative to the DM group.

Statistical analysis

The outcomes are shown as the mean \pm standard error. Statistical comparisons among the groups were performed with one-way ANOVA. The unpaired *t*-test was performed between two groups using SPSS software, version 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Characterization of BMSCs

After three passages, the BMSCs were identified by flow cytometry analysis, demonstrating that cultured cells positively expressed CD44, CD90, and CD29 but failed to express CD34 and CD45. These results indicate that the cultured cells were of mesenchymal origin with high purity (Figure 1).

CD31/CD34 cell marker expression

Before LESWT, no difference in the number of circulating CD31/CD34-positive cells (expressed as percentage) was noted between the rats receiving LESWT and those that did not (rats with LESWT vs rats without LESWT: 5.93 ± 0.66 vs 6.66 ± 0.88 , $P > 0.05$). At the end of the 3-week course of LESWT, the number of circulating CD31/CD34-positive cells (expressed as percentage) in the rats that received LESWT increased compared with the rats that did not receive LESWT (rats with LESWT vs rats without LESWT: 13.59 ± 1.38 vs 4.03 ± 0.98 , $P < 0.001$; Figure 2).

Survival of transplanted BMSCs in cavernous bodies

One day after BMSC transplantation, the numbers of stem cells implanted in the cavernous bodies in both the BMSC group and the LESWT + BMSC group were similar (BMSC group vs LESWT + BMSC group: 30.13 ± 2.25 vs 34.53 ± 1.85 , $P > 0.05$, per $\times 200$ magnification). Three days after transplantation, in the BMSC group, the stem cells disappeared quickly. However, in the LESWT + BMSC group, most of the stem cells remained in the cavernous body (BMSC group vs LESWT + BMSC group: 4.00 ± 0.60 vs 23.00 ± 2.02 , $P < 0.001$, per $\times 200$ magnification). Four weeks after BMSC transplantation, almost no CM-DiO labeled BMSCs were identified in the cavernous body per field (0.20 ± 0.07 , per $\times 200$ magnification) in the BMSC group. In contrast, in the LESWT + BMSC group, the number of CM-DiO labeled BMSCs in the specimens per field were increased approximately 10-fold (7.63 ± 0.58 per $\times 200$ magnification, $P < 0.001$; Figure 3).

Erectile function

Four weeks after transplantation, the mean of ICP/MAP in the DM group was reduced compared with the normal group. Both LESWT and BMSC transplantation could improve the ICP/MAP ratio whereas

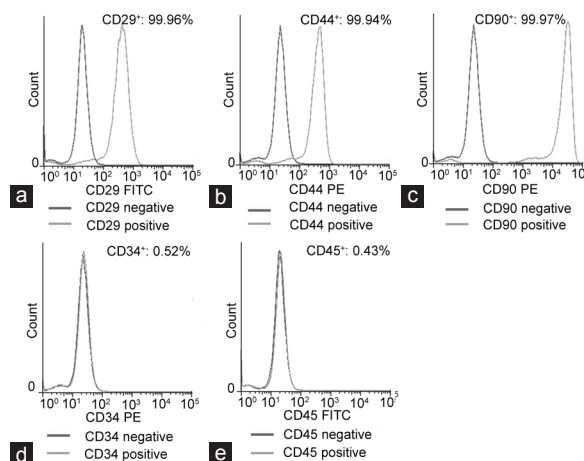


Figure 1: Flow cytometric identification of BMSCs: flow cytometric analysis revealed that cultured BMSCs highly expressed CD29 (a), CD44 (b), and CD90 (c) but did not express CD34 (d) and CD45 (e). BMSC: bone marrow mesenchymal stem cell; PE: phycoerythrin; FITC: fluorescein isothiocyanate.

the combination of LESWT and BMSC transplantation improved the ICP/MAP ratio more effectively than LESWT or BMSC transplantation performed alone (Figure 4 and Table 1).

RT-PCR of SDF-1 and VEGF expression

Both LESWT and BMSC transplantation improve SDF-1 and VEGF expression. The combination of LESWT and BMSC transplantation improved SDF-1 and VEGF expression more than LESWT or BMSC transplantation alone (Figure 5 and Table 1).

Endothelial marker (CD31) expression in cavernous tissues

To understand the impacts of different treatments on angiogenesis, immunohistochemical analysis was performed using the vascular endothelial marker CD31. Positive areas of CD31 expression were considerably reduced within the cavernous bodies of the diabetic rats compared with normal rats. Four weeks after LESWT, improvement in CD31 expression was noted in both the LESWT group and the BMSC group. The combination of LESWT and BMSC transplantation achieved better effects than LESWT alone (Figure 6a–6f and Table 1).

eNOS expression in cavernous tissues

To understand the impacts of different treatments on endothelial functions, immunohistochemical analysis was performed using eNOS. The positive area of eNOS expressed within the cavernous tissue significantly decreased in the DM group compared with the normal group. eNOS expression in the LESWT group or the BMSC group was increased compared with the DM group. The combination of LESWT and BMSC transplantation was more effective than LESWT alone (Figure 6g–6l and Table 1).

α -SMA expression in cavernous tissues

Immunohistochemical analysis was performed using α -SMA to reveal the impacts of different treatments on muscle restoration.

Compared with the normal group, α -SMA expression in the cavernous bodies of the diabetic group was reduced. Both LESWT and BMSC transplantation could increase α -SMA expression levels, but the combination of LESWT and BMSC transplantation increased α -SMA expression levels more than BMSC transplantation alone (Figure 6m–6r and Table 1).

Masson's trichrome staining of cavernous tissues

The fibrosis degree was evaluated by Masson's trichrome staining. The smooth muscle/collagen ratio in the DM group was reduced compared with the normal group. The smooth muscle/collagen ratio

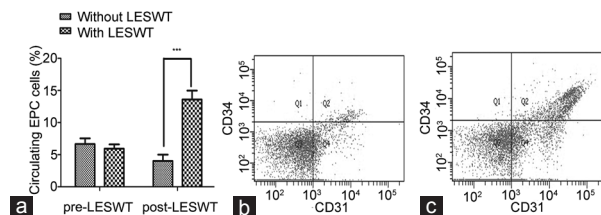


Figure 2: Flow cytometric analysis of circulating EPCs. (a) Impact of LESWT on percentage of circulating EPC. (b) Percentage of circulating EPC of rats without LESWT. (c) Percentage of circulating EPC of rats with LESWT. Flow cytometric analysis of peripheral blood revealed significantly increased number of circulating CD31/CD34-positive endothelial progenitor cells at the end of a 3-week course of LESWT (** $P < 0.001$). EPC: endothelial progenitor cell; LESWT: low-energy shock-wave therapy.

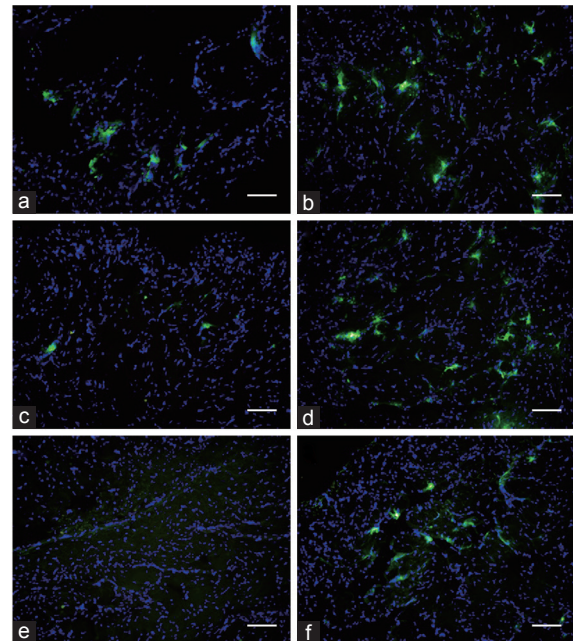


Figure 3: Survival of transplanted BMSCs in cavernous tissue: green fluorescent light CM-DiO labeled BMSCs implanted in the cavernous body were similar in the BMSC and LESWT + BMSC groups 1 day after transplantation. The stem cells in the BMSC group disappeared very quickly, and almost no transplanted stem cells remained at the end. Three days after transplantation, the number of CM-DiO-labeled BMSCs that survived in the cavernous body exhibited minimal change in the LESWT + BMSC group. Four weeks after BMSC transplantation, more CM-DiO labeled BMSCs could be observed in the LESWT + BMSC group compared with the BMSC group. (a and b) One day after transplantation. (c and d) Three days after transplantation. (e and f) Four weeks after transplantation. (a, c and e) BMSC group. (b, d and f) LESWT + BMSC group. Scale bar: 75 μ m. BMSC: bone marrow mesenchymal stem cell; LESWT: low-energy shock-wave therapy; CM-DiO: cell marker-DiO.

Table 1: Results of erectile function, PCR, immunohistochemistry, blood glucose and body weight

Group	DM	LESWT	LESWT+BMSC	BMSC	Normal
ICP/MAP	0.183 \pm 0.016	0.297 \pm 0.022	0.519 \pm 0.033	0.304 \pm 0.031	0.720 \pm 0.055
SDF-1	1.082 \pm 0.182	1.753 \pm 0.191	3.464 \pm 0.191	1.892 \pm 0.129	1.552 \pm 0.281
VEGF	1.070 \pm 0.197	2.089 \pm 0.304	3.338 \pm 0.274	2.397 \pm 0.297	3.077 \pm 0.347
CD31	310.625 \pm 53.645	584.792 \pm 109.490	1094.090 \pm 138.337	997.667 \pm 182.923	3099.790 \pm 417.566
eNOS	49 853.553 \pm 5777.078	68 441.185 \pm 5927.341	95 107.090 \pm 7458.898	78 376.604 \pm 9795.103	134 798.408 \pm 19 647.451
α -SMA	2126.933 \pm 394.238	7658.667 \pm 931.802	9227.600 \pm 1155.291	5307.750 \pm 1060.618	9527.803 \pm 1062.436
α -SMA/collagen	0.026 \pm 0.005	0.063 \pm 0.008	0.128 \pm 0.018	0.063 \pm 0.009	0.144 \pm 0.013
Blood glucose (mmol l ⁻¹)	22.083 \pm 3.710	20.189 \pm 3.089	18.011 \pm 2.386	15.300 \pm 2.926	5.434 \pm 0.878
Body weight (g)	294.333 \pm 19.238	249.333 \pm 38.724	281.222 \pm 27.110	298.556 \pm 16.843	432.441 \pm 35.458

Data are presented as mean \pm s.d. ICP/MAP: intracavernosal pressure/mean arterial pressure; SDF-1: stromal cell-derived factor-1; VEGF: vascular endothelial growth factor; eNOS: endothelial nitric oxide synthases; α -SMA: α -smooth muscle actin; DM: diabetes mellitus; LESWT: low-energy shock-wave therapy; BMSC: bone marrow mesenchymal stem cell; s.d.: standard deviation; PCR: polymerase chain reaction

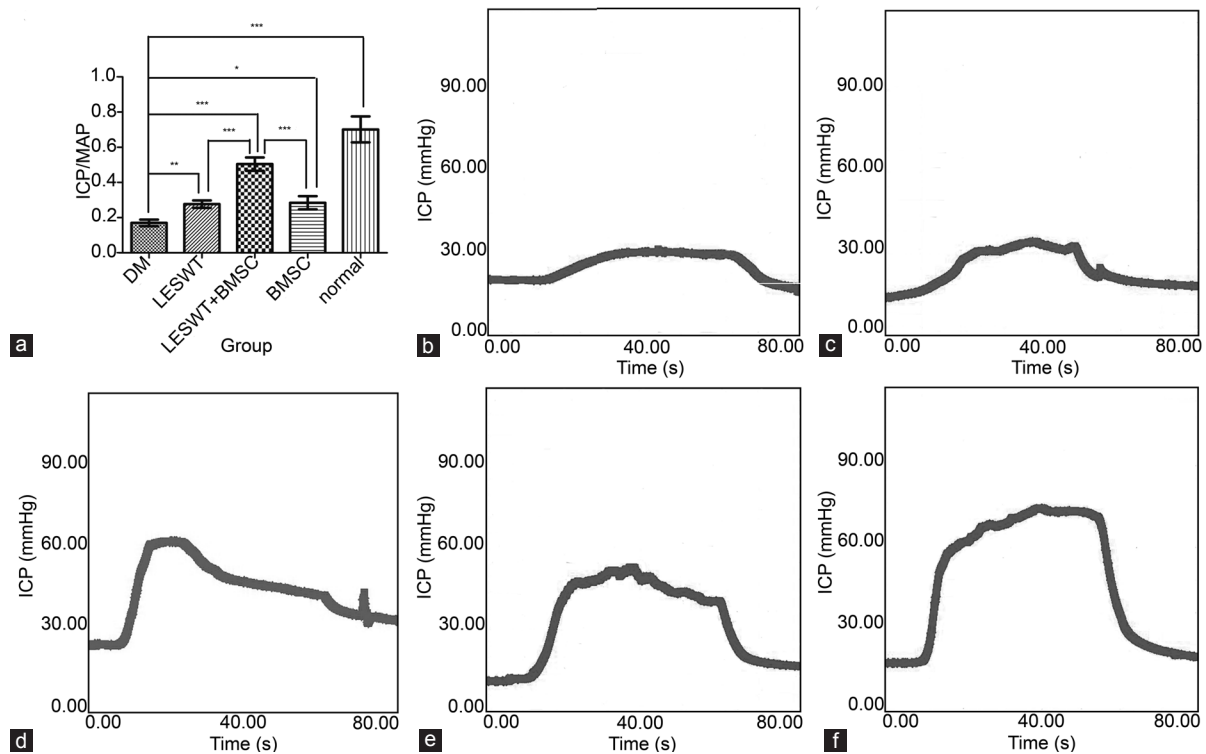


Figure 4: Measurement of ICP/MAP. Both LESWT and BMSC transplantation significantly improved the ICP/MAP of diabetic rats. The combination of LESWT and BMSC transplantation was more effective in improving the erectile function of diabetic rats than LESWT or BMSC transplantation alone. (a) Results of erectile function expressed as the ratio of ICP/MAP. (b) DM group. (c) LESWT group. (d) LESWT + BMSC group. (e) BMSC group. (f) Normal group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ICP: intracavernosal pressure; MAP: mean arterial pressure; LESWT: low-energy shock-wave therapy; BMSC: bone marrow mesenchymal stem cell; DM: diabetes mellitus.

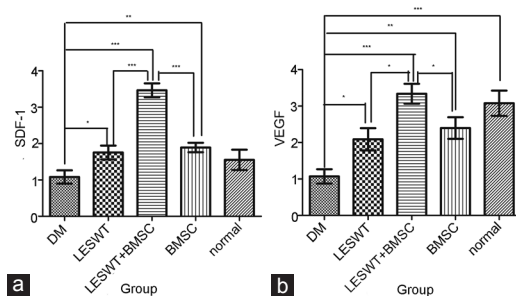


Figure 5: SDF-1 and VEGF expression in the cavernous tissue. SDF-1 and VEGF gene expression in the LESWT group, BMSC group and LESWT + BMSC group was significantly increased compared with the DM group, whereas increased SDF-1 and VEGF expression in the LESWT + BMSC group was more remarkable. (a) SDF-1 expression in the cavernous tissue. (b) VEGF expression in the cavernous tissue. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. SDF-1: stromal cell-derived factor-1; VEGF: vascular endothelial growth factor; LESWT: low-energy shock-wave therapy; BMSC: bone marrow mesenchymal stem cell; DM: diabetes mellitus.

was increased significantly in both the LESWT group and the BMSC group, whereas the LESWT + BMSC group outperformed the LESWT and BMSC groups (Figure 6s–6x and Table 1).

Serum glucose and body weight

Four weeks after BMSC transplantation, no remarkable differences in blood glucose or body weight were noted among the four diabetic groups (Table 1).

DISCUSSION

BMSC transplantation holds great promise for the future of regenerative medicine for diabetic ED due to its multilineage differentiation and self-renewal capacity.^{9–11,23,24} The low BMSC survival number in the cavernous body after transplantation caused paracrine to be the main mechanism of transplanted BMSCs used currently.^{9,23} Using a combination of LESWT and BMSC transplantation, we recruited more transplanted BMSCs to the cavernous body and improved the erectile function of diabetic rats more effectively than BMSC transplantation alone. We believe that the mechanism of action might be related to increased SDF-1 expression and enhanced angiogenesis in the cavernous body through LESWT.

One characteristic of BMSC migration is that it occurs against the blood concentration gradient of SDF-1.²⁵ In our research, SDF-1 gene expression in rats receiving LESWT was increased compared with rats that did not receive LESWT, indicating that the SDF-1 expression level in the cavernous tissue was increased by LESWT. In early stages (3 days) after BMSC transplantation, more labeled BMSCs were noted in the LESWT + BMSC group, indicating that the migration of exogenous BMSCs was inhibited by the high level of SDF-1 expression in the cavernous body induced by LESWT. LESWT improved SDF-1 expression levels, and SDF-1 mediated the migration of BMSCs, as demonstrated in hind limb ischemia rats, chronic spinal cord injury rats, acute myocardial infarction pig models,^{26–28} heart myocardial infarction rats and liver failure mouse models.^{29,30}

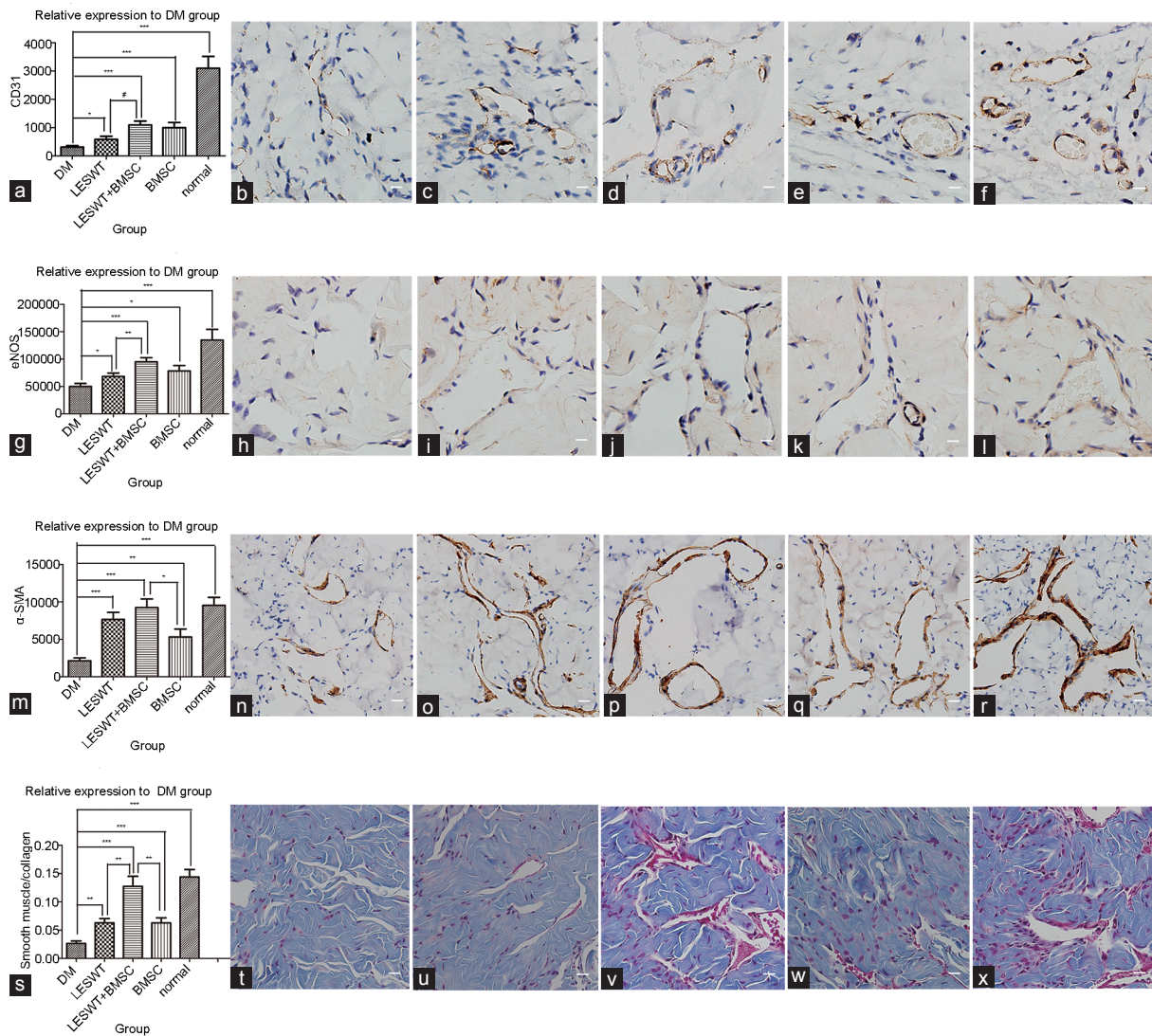


Figure 6: Immunohistochemistry of the cavernous tissue. Brown dye indicated the target factor-positive expression area. CD31, eNOS, or α -SMA-positive stained cells identified in the cavernous body appeared mostly on the inner wall surface of the vasculature or inside the vessel walls. The average stained area was expressed relative to the DM group. Compared with the normal group, a significant decrease in the positively stained area of CD31, eNOS, α -SMA or smooth muscle/collagen ratio was observed in the DM group, suggesting serious cell damage and fibrosis in penile tissue. After diabetic rats were treated by LESWT or BMSC transplantation, CD31-, eNOS-, and α -SMA-positive stained areas and smooth muscle/collagen ratio increased significantly. The combination of LESWT and BMSC transplantation increased CD31 and eNOS expression more effectively than LESWT, increased α -SMA expression more effectively than BMSC transplantation, and increased the smooth muscle/collagen ratio more effectively than LESWT and BMSC transplantation performed alone. (a–f) CD31 expression in the cavernous tissue. (g–l) eNOS expression in the cavernous tissue. (m–r) α -SMA expression in the cavernous tissue. (s–x) Smooth muscle/collagen ratio in the cavernous tissue. (a, g, m and s) Cell markers relative expression to DM group. (b, h, n and t) DM group. (c, i, o and u) LESWT group. (d, j, p and v) LESWT + BMSC group. (e, k, q and w) BMSC group. (f, l, r and x) Normal group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Scale bar: 25 μ m. eNOS: endothelial nitric oxide synthase; α -SMA: α -smooth muscle actin; DM: diabetes mellitus; LESWT: low-energy shock-wave therapy; BMSC: bone marrow mesenchymal stem cell.

MSCs were located around the capillaries in a variety of tissues,³¹ and the vasculature played an instructive role in the stem cell niche;¹⁶ in particular, CD31⁺ cells, which proved to be EPCs, might be an important component of the BMSC niche.³² CD31 expressed on vascular endothelial cells has been considered an angiogenesis cell marker of diabetic ED rats.^{9,33,34} An endothelial marker (CD31) and a progenitor cell marker (CD34) were described to evaluate the effects of LESWT on the circulating EPC count.³⁵ In streptozotocin-induced DM rats, the vasculature was impaired, and both cavernous CD31⁺ cells³⁶ and circulating EPCs decreased dramatically.^{37–39} The reduction in circulating EPCs decreased the number of cells that could migrate to

sites of damaged endothelium and differentiate into endothelial cells thereafter.⁴⁰

In this research, SDF-1 and VEGF gene expression, circulating CD31/CD34-positive EPC numbers and the CD31⁺ expression in cavernous tissue were increased in the LESWT group compared with the DM group. Angiogenesis in the cavernous body induced by LESWT might be related to two sources of endogenous stem cell activation.⁴¹ On the one hand, both SDF-1 and VEGF could recruit endogenous EPCs,^{42,43} and LESWT could mobilize more EPCs enter in circulating and recruit to the cavernous tissue. On the other hand, LESWT could enhance stem cell proliferation, differentiation and

paracrine.^{44–47} In addition, the types of stem cells detected around the cavernous vasculature, including perisinusoidal and subtunic penile progenitor cells⁴⁸ and cavernous smooth muscle-derived stem cells,^{49,50} could be induced to activate. Thus, LESWT induced angiogenesis and created a beneficial situation for the establishment of the stem cell niche. Four weeks after BMSC transplantation, more surviving BMSCs were noted in the LESWT + BMSC group compared with the BMSC group.

Endothelial cell dysfunction reduces eNOS bioavailability. Smooth muscle loss and fibrosis caused by phenotype modulation⁵¹ and apoptosis⁵² reflect a decrease in the α -SMA or smooth muscle/collagen ratio. In our research, both LESWT and BMSC transplantation increase the CD31, eNOS, α -SMA and smooth muscle/collagen ratios of the diabetic rats, indicating that both of these treatments increase the content of endothelium and smooth muscle. The addition of LESWT could benefit implantation and increase the number of surviving transplanted BMSCs. The combination of LESWT and BMSC transplantation could enhance erectile function restoration more effectively. Similar conclusions have been achieved both in critical limb ischemia rats⁵³ and myocardial infarction swine.⁵⁴

In brief, LESWT could increase SDF-1 levels, which could subsequently inhibit the migration of transplanted BMSCs and facilitate the implantation of transplanted BMSCs in the cavernous body. LESWT could also enhance revascularization in the cavernous body, which might create beneficial conditions for transplanted BMSCs to survive. The combination of LESWT and BMSC transplantation was more effective in restoring erectile function in diabetic rats than LESWT or BMSC transplantation alone.

AUTHOR CONTRIBUTIONS

HTS presented the study design and participated in BMSC preparing, DM rat model building, LWSWT, FCM, ICP/MAP assessment, statistical analysis and drafted the manuscript. HBZ participated in BMSC preparing, ICP/MAP assessment, RT-PCR and immunohistochemical assessment. WTC participated in study design, supplied LESWT device and performed energy measurement. FZC participated in BMSC preparing, RT-PCR and immunohistochemical assessment. TW participated in study design and statistical analysis. JTL participated in ICP/MAP assessment. MY participated in DM rat model building. JHL participated in DM rat model building. AYW conceived of the study, and participated in its design and coordination and helped to draft. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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