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Original Article

Use of sodium butyrate combined with bone marrow mesenchymal stem cells improves acontractile detrusor in a cryoinjury rat model

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Abstract: Objective: To determine the feasibility of rat bone marrow-derived mesenchymal stem cells (BM-MSCs) combined with sodium butyrate in the treatment of acontractile detrusor (ACD) in a cryoinjury-induced ACD rat model. Methods: The ACD rats were given an injection of vehicle, sodium butyrate, BM-MSCs, or sodium butyrate combined with BM-MSCs in the bladder wall, respectively (n = 17 each group). Seventeen rats served as normal controls. Bladder micturition function, contractile tension, histological changes, homing features of BM-MSCs, and expressions of detrusor smooth muscle cell markers were assessed. Results: Rats with ACD showed abnormal urodynamic parameters, decreased contractile amplitude of detrusor strips, and reduced expression of α-smooth muscle actin (α-SMA), calponin, and smooth muscle myosin heavy chain (SM-MHC) at both the mRNA and protein levels. Treatment with BM-MSCs, but not sodium butyrate, could repair the impaired detrusor, improve detrusor contractibility, and partially restore the expression of α-SMA, calponin, and SM-MHC in ACD rats ($P < 0.01$). These effects could be further enhanced by the additional use of sodium butyrate. Moreover, sodium butyrate could promote the homing of BM-MSCs to the impaired detrusor layer. Conclusion: Sodium butyrate in combination with BM-MSCs may be a novel strategy for the treatment of ACD by repairing the impaired bladder and reconstructing bladder tissues.

Keywords: Acontractile detrusor, bone marrow, mesenchymal stem cell, sodium butyrate

Introduction

Acontractile detrusor (ACD) is characterized by symptoms of bladder dysfunction, including dysuria and urinary hesitancy, urgency, intermittency, or retention [1, 2]. It may simply be a result of aging or secondary to several conditions, such as spinal cord injury, interstitial cystitis, or diabetes [3]. Current treatment options are mainly restricted to conservative therapies that aim to relieve symptoms, and no optimal strategy exists to repair the impaired detrusor [4, 5]. Nowadays, more and more researchers are focusing on improvement of detrusor contractibility by tissue engineering techniques [6-8].

Mesenchymal stem cells (MSCs), derived from various sources such as bone marrow [9], adipose tissue [10], umbilical cord [11], endometrium [12], and urine [13], have been proven to be effective in the treatment of bladder dysfunction in the field of tissue engineering. Bone marrow MSCs (BM-MSCs) have been studied for nearly 50 years; they are characterized by their self-renewing capacity and multi-lineage differentiation potential. They act as a small cell population, exist in almost every type of tissue and organ, and are able to replace cells lost on a daily basis. BM-MSCs have the ability to differentiate into diverse cell types under different culture conditions, such as hepatocytes, gastrointestinal epithelial cells, and vascular endothelial cells [14]. BM-MSCs are one of most commonly used cell types for tissue regeneration, including bladder reconstruction. It has been reported that transplantation of homologous BM-MSCs improves bladder contractility and ameliorates bladder dysfunction that is caused by Parkinson's disease [15], partial

bladder outlet obstruction [16], and an impaired urethral sphincter muscle [17]. However, application of these cells faces several limitations, including uncertain homing or differentiation, poor survival, and immune rejection, which directly affect their therapeutic effects on tissue repair. Therefore, effort should be made to develop novel strategies to improve the therapeutic potential of BM-MSCs for cell therapy.

Sodium butyrate, a histone deacetylase inhibitor, may enhance histone acetylation, relax the DNA wrapped around the core histones, promote transcription of the genes involved in important cellular processes, and initiate the multilineage differentiation of stem cells. Sodium butyrate is reported to induce stem cell differentiation into various cell types, such as mature osteocytes [18] and bile duct cells from adipose-derived stem cells, or hepatocytes from embryonic stem cells [19].

However, limited data have been reported to evaluate whether a histone deacetylase inhibitor enhances the therapeutic effects of BM-MSCs in the treatment of ACD. In this study, we investigated the feasibility of rat BM-MSCs combined with sodium butyrate in the treatment of ACD in a cryoinjury-induced ACD rat model.

Material and methods

Experimental animals and grouping

Eighty-five female Sprague-Dawley (SD) rats, aged 8 weeks old, were purchased from the Da Ping Hospital of The Third Military Medical University. All animal care, treatment, and procedures were approved by the Research Council and Animal Care and Use Committee of The Third Military Medical University, China. Seventeen rats served as normal controls (group 1), and the ACD rat model was established in another 68 rats as reported previously [20]. Briefly, the rats were anesthetized with 5% chloral hydrate. A low midline incision was made to expose the bladder and urethra, and cryodamage was created with an aluminum rod (diameter of 8 mm) chilled on dry ice (-40°C). The chilled rod was placed against the serosal surface of the bladder wall for 30 s. Then, the rats were given an injection of 0.2 mL of phosphate-buffered saline (PBS; group 2, n = 17), 3 × 10^6 M sodium butyrate in 0.2 mL of PBS (group 3, n = 17), 1 × 10^6 BM-MSCs in 0.2 mL of PBS (group 4, n = 17), or 3 × 10^6 M sodium butyrate combined with 1 × 10^6 BM-MSCs in 0.2 mL of PBS (group 5, n = 17) in the bladder wall, respectively. The incision was closed after treatment.

Isolation and culture of rat BM-MSCs

BMSCs were isolated from the ilium and femurs of SD rats, according to a method described previously [21]. Briefly, the bone marrow was flushed using alpha-minimum essential media (α-MEM; Gibco, Grand Island, NY, USA). The cell layer of bone marrow was separated and centrifuged for 20 min at room temperature. Mononucleated cells isolated at this step were centrifuged again at 1,600 rpm for 5 min and suspended in α-MEM supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 μg/mL penicillin/streptomycin (Beyotime, Jiang Su, China), and 1% L-glutamine (Gibco, Grand Island, NY, USA) and then incubated at 37°C with 5% CO_2 and 95% O_2. The third generation of 1 × 10^6 BM-MSCs was collected into a conical tube containing 0.2 mL of PBS and used for an injection immediately after cryo-induced injury to the bladder.

Cystometry experiments in anesthetized rats

At 14 days after intervention, cystometry was carried out in the rats (n = 5 for each group) as described previously [22]. Briefly, the rats were prohibited from drinking water for 12 h in order to empty their bladders before cystometry. The rats were anesthetized with 1.2 g/kg urethane injected subcutaneously (Sigma, St. Louis, MO, USA). A catheter (PE-50) was inserted into the bladder for saline infusion (37°C) at a rate of 8 mL/h. The intravesical pressure was recorded continuously. After stabilization for approximately 30 min, urodynamic parameters, including basal pressure, maximum voiding pressure, micturition frequency, intercontraction interval, bladder capacity, and postvoid residual volume were evaluated.

Spontaneous phasic contractions of rat detrusor strips

Detrusor strips (2 mm × 8 mm) were obtained from freshly isolated bladders by making a longitudinal incision from the triangular base to the dome in rats of each group (n = 5). After

Table 1. Cystometric parameters of rats in each group (n = 5)

Group	Group 1	Group 2	Group 3	Group 4	Group 5
BP (cm H_2O)	10.83 ± 2.31	13.16 ± 1.72	13.83 ± 1.96	12.16 ± 1.94	13.88 ± 1.47
MVP (cm H_2O)	65.00 ± 2.19	36.33 ± 2.06[a]	35.33 ± 2.80	52.33 ± 2.42[b]	60.66 ± 1.75[b,c]
MF (Nu/h)	7.00 ± 0.89	17.67 ± 1.63[a]	18.50 ± 1.874	10.00 ± 1.41[b]	7.33 ± 0.51[b,c]
BC (mL)	1.16 ± 1.48	0.45 ± 0.04[a]	0.43 ± 0.04	0.81 ± 0.12[b]	1.09 ± 0.07[b,c]
ICI (min)	8.67 ± 1.125	3.47 ± 0.25[a]	3.24 ± 0.34	6.08 ± 0.88[b]	8.17 ± 0.52[b,c]
PRV (mL)	0.12 ± 0.02	0.85 ± 0.09[a]	0.83 ± 0.10	0.54 ± 0.08[b]	0.34 ± 0.07[b,c]

Data are presented as mean ± standard deviation. [a]P < 0.01, compared to group 1; [b]P < 0.01, compared to group 2; [c]P < 0.01, compared to group 4. BP, basal pressure; MVP, maximum voiding pressure; MF, micturition frequency; BC, bladder capacity; ICI, intercontraction interval; PRV, postvoid residual volume.

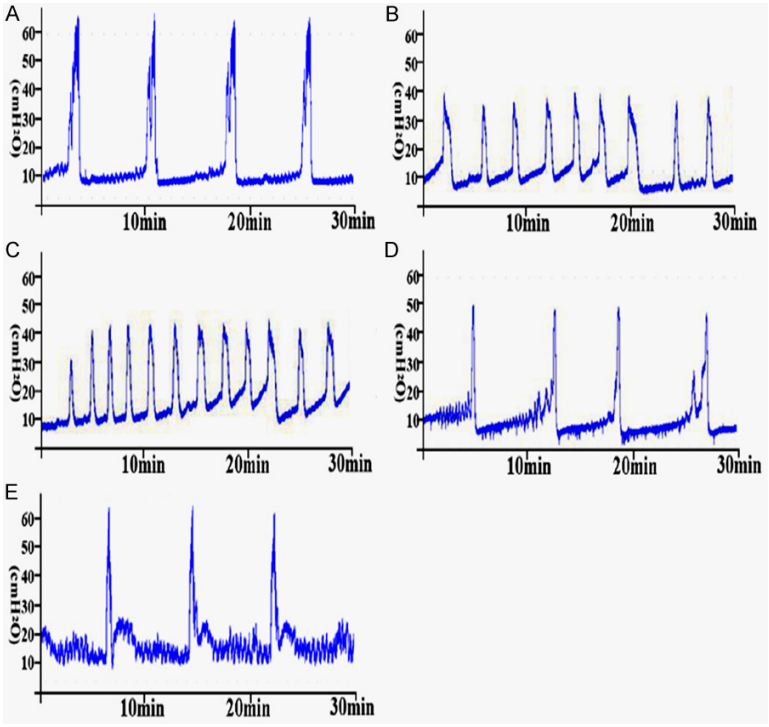

Figure 1. Micturition pressure recorded from cystometrograms (n = 5) in normal rats (A), acontractile detrusor (ACD) rats (B), ACD rats treated with 3 × 10^6 M sodium butyrate (C), ACD rats treated with 1 × 10^6 BM-MSCs (D), and ACD rats treated with 3 × 10^6 M sodium butyrate combined with 1 × 10^6 BM-MSCs (E).

removing the mucosa, the strip was suspended in an organ bath at 37°C containing Kreb's solution gassed with 95% O_2 and 5% CO_2. The tension loaded on the tissue was measured using isometric force transducers (Chengyi Co., Chengyi, China), which were connected to Powerlab data acquisition software (RM6280 USB4.8, Chengyi Co.). After equilibrating for 30 min, the strips were initially preloaded at 0.5 g of tension, and the continuous curve was recorded. The ratio of tissue weight (g) to amplitude (g) was calculated. The Kreb's solution contained 119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM glucose, and the pH was adjusted to 7.4 with NaOH.

Histological analysis

The bladders of rats in each group (n = 7) were washed with saline solution, immersed in 10% neutral buffered formalin, embedded in paraffin, and cut into 10-μm sections. The sections were then stained with hematoxylin and eosin (H&E) and were observed under a light microscope (Olympus CX22, Olympus Tokyo, Japan).

Immunofluorescence labeling of cells

Freshly isolated bladder tissues from rats in each group (n = 7) were washed in PBS at 4°C (pH = 7.4). Then, they were frozen and cut into 6 μm-thick sections, followed by fixation with 4% paraformaldehyde for 1 h. The sections were blocked in 3% bovine serum albumin for 2 h and permeabilized with 0.5% Triton X-100 for 10-15 min. The sections were then incubated with a rat monoclonal anti-CD44 antibody (1:200; Abcam, Cambridge, UK) at 4°C overnight. Immunoreactivity was visualized using a Fluor 555-conjugated, goat anti-ratIgG secondary antibody (1:500; Beyotime, Jiang Su, China). The cells were counterstained with DAPI (1:5; Beyotime, Jiang Su,

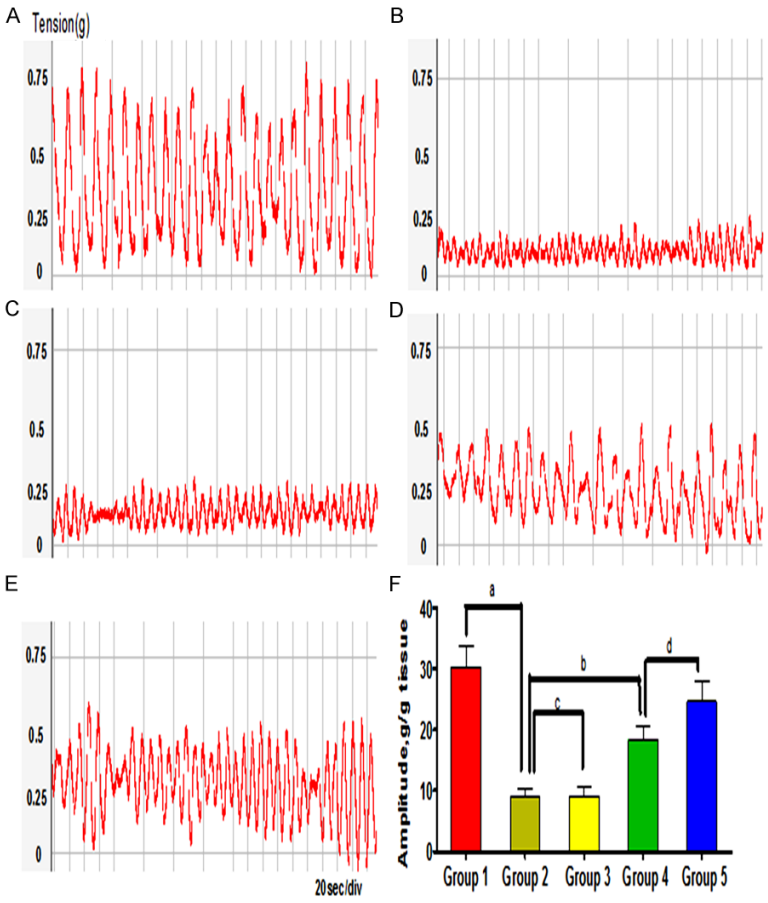

Figure 2. Contractile amplitudes of bladder detrusor strips in rats of each group (n = 5). The contractile tension (g) was normalized to the strip weight (g). The contractile amplitudes of the detrusor strips in normal rats (A, group 1), acontractile detrusor (ACD) rats (B, group 2), ACD rats treated with 3 × 10^6 M sodium butyrate (C, group 3), ACD rats treated with 1 × 10^6 BM-MSCs (D, group 4), and ACD rats treated with 3 × 10^6 M sodium butyrate combined with 1 × 10^6 BM-MSCs (E, group 5). (F) Quantification of the contractile amplitudes in the rats of each group. $^aP < 0.01$, $^bP < 0.01$, $^cP = 0.468$, $^dP < 0.01$.

China) for 10 min to visualize the cell nuclei. The average numbers of immunopositive cells were counted in 10 random microscopic visual fields.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

The mRNA expression levels of α-smooth muscle actin (α-SMA), calponin, and smooth muscle myosin heavy chain (SM-MHC) in the rat bladders of each group (n = 7) were analyzed using quantitative RT-PCR. The total RNA was isolated from 40 mg of bladder tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The concentration of RNA was estimated by measuring the absorbance at 260 nm. The ratio of the absorbance at 260 and 280 nm (A260/280) above 1.9 was used to evaluate the RNA purity. Complementary DNA was synthesized using a SYBR Green Real-time PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan). The sequences of the primers used were as follows: α-SMA forward, 5'-GGAGATGGCGTGACTCACAA-3', reverse, 5'-CGCTCAGCAGTAGTCACGAA-3'; calponin forward, 5'-GCCCAGAAATACGACCACCA-3', reverse, 5'-CCGGCTGGAGCTTGTTGATA-3'; SM-MHC forward, 5'-TAGCAGATCTGGGACCACCA-3', reverse, 5'-CGCCCTTCTCCTCCTTGATG-3'. The reactions were started at 95°C for 10 min, followed by 40 cycles of 10 s at 95°C, 20 s at 60°C, and 20 s at 72°C. A melting curve was obtained from 65°C to 95°C after amplification. The relative amount of mRNA of each sample was calculated using the $2^{-\Delta\Delta CT}$ method.

Western blotting analysis

Total proteins were extracted from rat bladders of each group (n = 7) using radio immunoprecipitation assay protein lysate buffer (containing 1 mM phenylmethanesulfonyl fluoride, Beyotime). Then, samples (100 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. They were probed with primary antibodies, including polyclonal rabbit anti-α-SMA (1:800, anti-acta2 antibody, Sigma), monoclonal rat anti-calponin (1:1000, anti-calponin antibody, Abcam, Cambridge, UK), or polyclonal rabbit anti-SM-MHC (1:1000, anti-calponin antibody, Abcam, Cambridge, UK), then incubated with anti-mouse or anti-rabbit secondary antibodies coupled to horseradish peroxidase (Zhongshan Golden Bridge Biotechnology, Beijing, China). The density of the corresponding bands was measured using the ChemiDoc XRS + Image System and analyzed using Image Lab (Bio-Rad, Hercules, CA, USA).

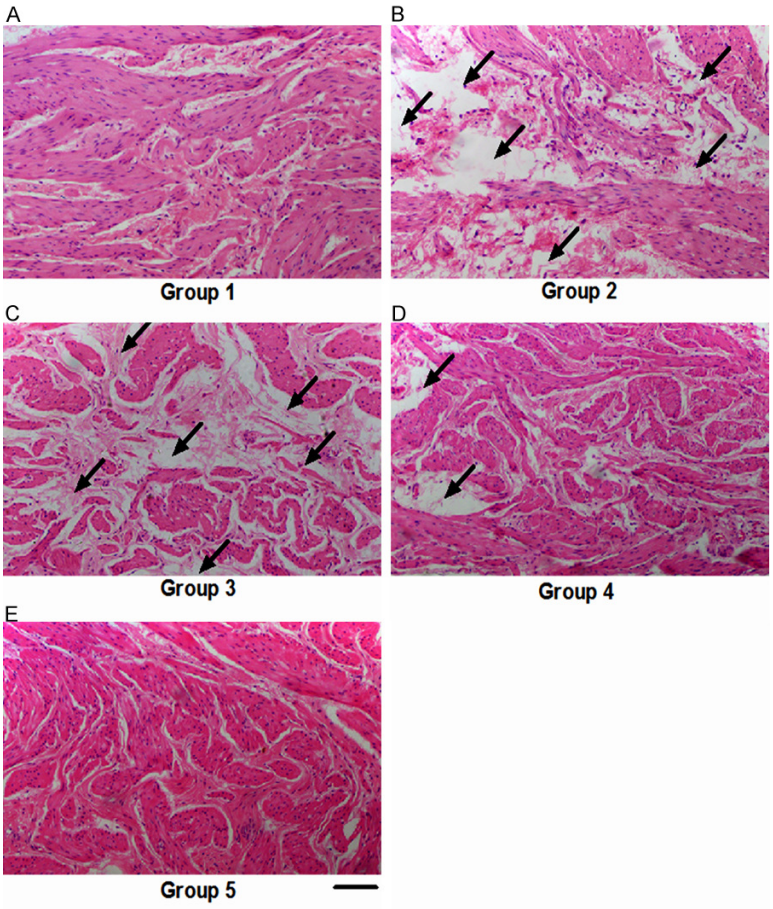

Figure 3. Histological findings of bladder tissues stained with H&E at 2 weeks after injection (n = 5). Frozen sections of bladder tissues from normal rats (A, group 1), acontractile detrusor (ACD) rats (B, group 2), ACD rats treated with 3 × 10^6 M sodium butyrate (C, group 3), ACD rats treated with 1 × 10^6 BM-MSCs (D, group 4), and ACD rats treated with 3 × 10^6 M sodium butyrate combined with 1 × 10^6 BM-MSCs (E, group 5). The black arrows indicate the lack of smooth muscle bundles. Scale bar = 100 μm.

Statistical analysis

Statistical analysis was performed using the SPSS software package (version 16.0; SPSS Inc., Chicago, IL, USA). Quantitative data were presented as the mean ± standard deviation. Analysis of variance and post hoc test were used for comparisons among the five groups. Differences with *P* values of less than 0.01 were considered significant.

Results

Changes in urodynamic parameters

Rats with cryoinjury-induced ACD (group 2) showed a significant decrease in the maximum voiding pressure, intercontraction interval, and bladder capacity, but an increase in micturition frequency and postvoid residual volume, compared with the normal rats ($P < 0.01$; **Table 1** and **Figure 1**). Acontractile detrusor rats treated with 3 × 10^6 M sodium butyrate (group 3) did not exhibit any changes in urodynamic parameters, as compared with the ACD rats. In contrast, treatment with 1 × 10^6 BM-MSCs partially reversed the abnormal urodynamic parameters in the ACD rats (in group 4; $P < 0.01$). Interestingly, treatment with 3 × 10^6 M sodium butyrate combined with 1 × 10^6 BM-MSCs led to a greater increase in the maximum voiding pressure, intercontraction interval, and bladder capacity in ACD rats as compared to treatment with only BM-MSCs ($P < 0.01$), suggesting that these treatments remarkably ameliorated the micturition dysfunction of ACD rats.

Evaluation of detrusor strips

There was a significant decrease in the contractile amplitude of the detrusor strips in the cryoinjury-induced ACD rats, compared to normal rats (8.973 ± 1.325 g vs. 30.213 ± 3.482 g, $P < 0.01$, **Figure 2**). Acontractile detrusor rats treated with BM-MSCs exhibited a significant increase in the contractile amplitude as compared with the ACD rats (18.302 ± 2.238 g vs. 8.973 ± 1.325 g, $P < 0.01$), while sodium butyrate had no such effects (9.109 ± 2.384 vs. 8.973 ± 1.325, $P = 0.468$). However, sodium butyrate seemed to enhance the effects of BM-MSCs on detrusor contraction and lead to an increased contractile amplitude (group 5, 24.683 ± 3.259 g, $P < 0.01$ compared to group 2).

Histological findings

Frozen sections stained with H&E showed damage in the detrusor myocytes and an increased number of apoptotic smooth muscle cells in the

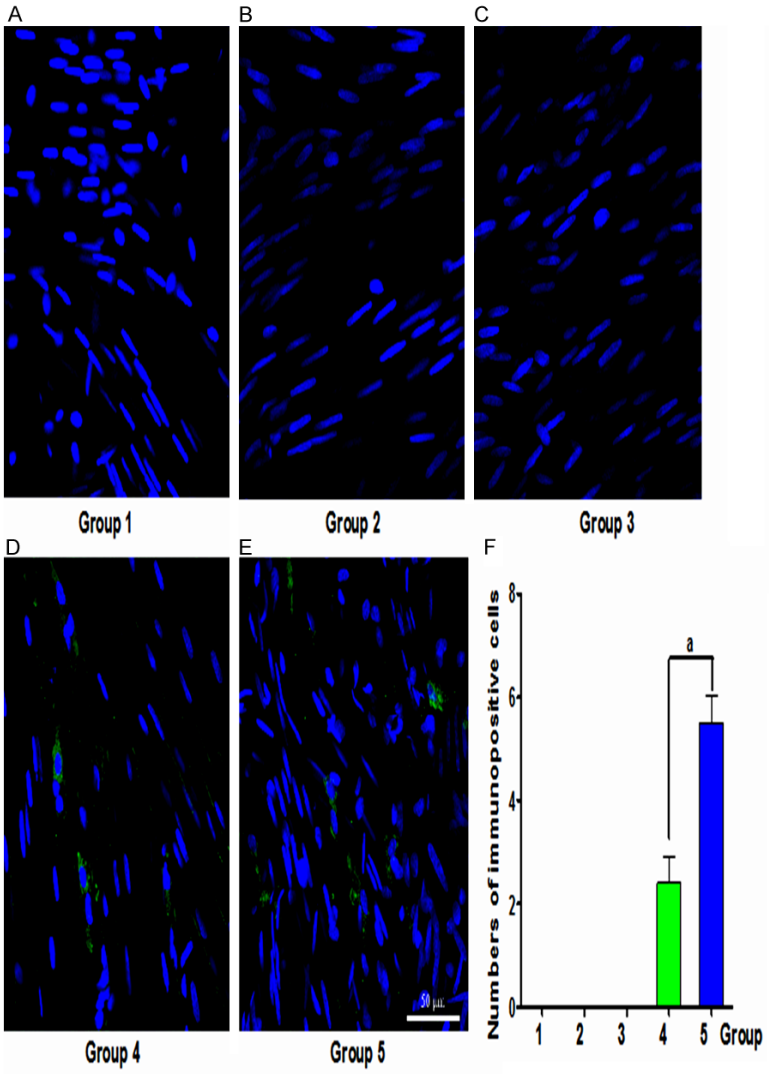

Figure 4. Immunofluorescence staining of rat bladders (n = 5 in each group). A BM-MSC marker, CD44, was used to label BM-MSCs (green); and the nuclei were colored with DAPI (blue). (A-E) Represent five different experimental groups: normal rats (A, group 1), acontractile detrusor (ACD) rats (B, group 2), ACD rats treated with 3×10^6 M sodium butyrate (C, group 3), ACD rats treated with 1×10^6 BM-MSCs (D, group 4), and ACD rats treated with 3×10^6 M sodium butyrate combined with 1×10^6 BM-MSCs (E, group 5). (F) Quantification of the CD44-positive cells in the rats of groups 4 and 5. Scale bar = 50 μm.

ACD rats. Treatment with BM-MSCs, but not sodium butyrate, could improve the impaired detrusor in the ACD rats. However, the detrusor injury was substantially improved after the combined treatment of BM-MSCs and sodium butyrate (**Figure 3**).

To verify the features of homing and differentiation of BM-MSCs, an immunofluorescence assay was performed by using the BM-MSC marker CD44. The injected BM-MSCs mainly homed to the detrusor layer of the bladder. More CD44-positive cells were detected in group 5, as compared with group 4 (5.5 ± 0.527 vs. 2.4 ± 0.516, $P < 0.01$, **Figure 4**).

Expression of α-SMA, calponin, and SM-MHC in bladders

In the bladder tissues of ACD rats, the RNA expression levels of detrusor smooth muscle cell markers, including α-SMA, calponin, and SM-MHC, were significantly lower than those of normal rats ($P < 0.01$, **Figure 5**). However, treatment with BM-MSCs could partially restore their mRNA expression in the ACD rats ($P < 0.01$). These effects could be further enhanced by the use of sodium butyrate, although sodium butyrate itself could not increase the mRNA expression of α-SMA, calponin, or SM-MHC in the ACD rats. Similar trends were found for the protein expression of α-SMA, calponin, and SM-MHC ($P < 0.01$).

Discussion

Acontractile detrusor is a common clinical syndrome that is caused by a variety of myogenic and neurogenic diseases, and it seriously affects a patient's quality of life. In this study, we successfully established a cryoinjury-induced ACD rat model as reported previously [20]. The ACD rats showed an impaired detrusor myocyte layer in the bladder, resulting in a reduced detrusor contractility, maximum voiding pressure, and bladder capacity as well as an increased micturition frequency [23].

Conventional therapies, including medication and electrical nerve stimulation, mainly target reducing the bladder outlet resistance and improving the bladder detrusor contractibility

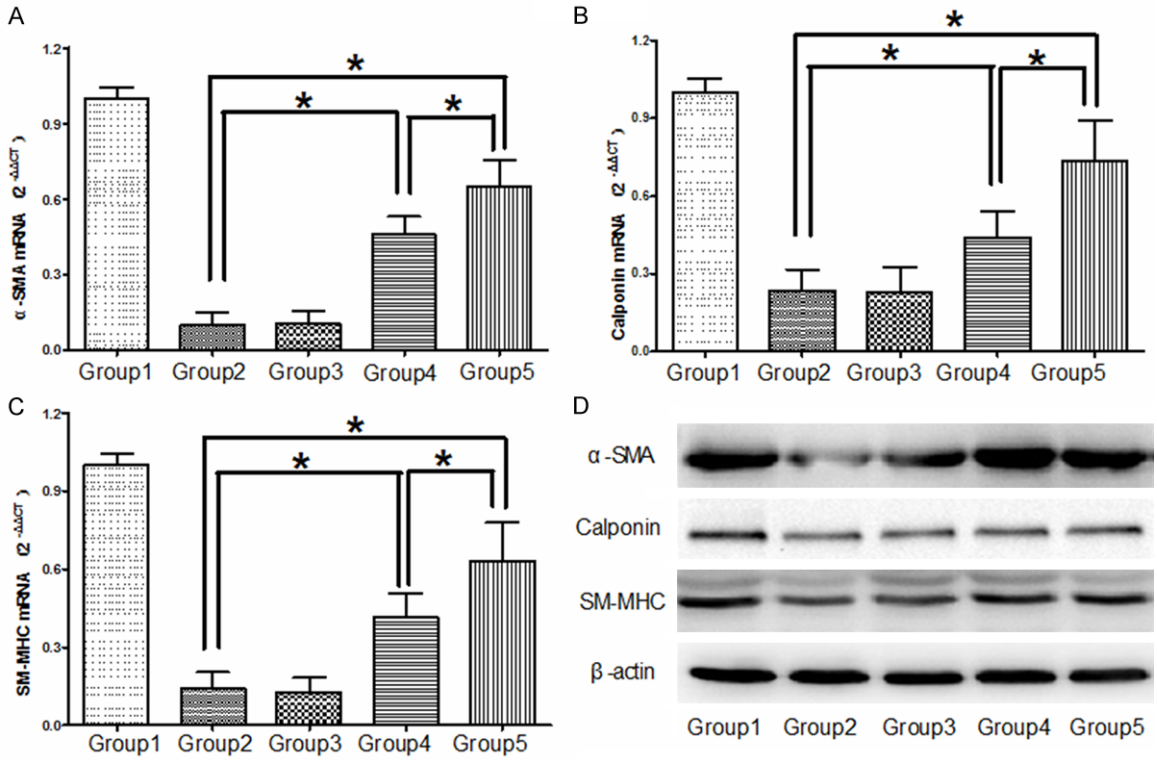

Figure 5. Expression of α-SMA, calponin, and SM-MHC at the mRNA and protein levels (n = 5). RT-PCR analysis showed the mRNA expression of α-SMA (A), calponin (B), and SM-MHC (C) in normal rats (group 1), acontractile detrusor (ACD) rats (group 2), ACD rats treated with 3 × 10^6 M sodium butyrate (group 3), ACD rats treated with 1 × 10^6 BM-MSCs (group 4), and ACD rats treated with 3 × 10^6 M sodium butyrate combined with 1 × 10^6 BM-MSCs (group 5). Western blotting analysis showed the protein expression of α-SMA, calponin, and SM-MHC (D). *P < 0.01.

[24]; however, they have yet to achieve a satisfactory curative effect [25]. Stem cell-based therapy is one of the most promising options for restoring the function of an impaired bladder. BM-MSCs are capable of differentiating into multiple bladder cell types, SMCs in particular, and thus have become an optimal alternative for cell-based therapy or tissue engineering of the bladder [26].

In this study, we implanted BM-MSCs into the bladders of ACD rats and evaluated their ability to repair the bladder micturition function and detrusor contractility. Treatment with BM-MSCs resulted in an improvement in the contractile amplitude of the detrusor strips, bladder capacity, and maximum voiding pressure in the ACD rats. However, these effects were not very satisfactory because of the low efficiency of the BM-MSC homing and differentiation. Thus, novel strategies are needed to enhance the therapeutic effects of BM-MSCs for ACD. Recently, sodium butyrate, as a histone deacetylase inhibitor, has been reported to inhibit the growth of various types of tumor cells by blocking G1-to-S cell cycle progression [27, 28]. In addition to promoting cell death and differentiation of tumor cells, sodium butyrate also induces stem cell differentiation *in vitro* and *in vivo* [19].

But whether sodium butyrate is involved in promoting BM-MSC differentiation into SMCs is not clearly known. In this study, the combined application of sodium butyrate and BM-MSCs achieved more satisfactory results in the treatment of ACD, as compared to treatment with BM-MSCs alone. Moreover, more CD44-positive cells homed into the impaired bladder when BM-MSCs were combined with sodium butyrate, and the expression of bladder contraction-related proteins at both the mRNA and protein levels was also higher than treatment with BM-MSCs alone (P < 0.01). In fact, BM-MSCs transplanted to the bladder might differentiate into bladder cells, such as SMCs, interstitial

cells, and urothelial cells, all of which play important roles in the repair process of the bladder. It has not been proven yet whether sodium butyrate promotes homing or multilineage differentiation of BM-MSCs by upregulation of their histone acetylation or secretion of a variety of cytokines. The exact mechanism by which sodium butyrate enhances the therapeutic effects of BM-MSCs in the treatment of ACD is not clearly known, and it requires further investigation.

An increasing number of studies have shown that the existence of material scaffolds is required for BMSCs seeded into the bladder to be functional [11, 29]. Various types of scaffolds coupled with trophic factors have been used as the structure to promote stem cell homing and growth [30]. The use of a histone deacetylase inhibitor, which is involved in the growth and differentiation process of stem cells, would be a promising strategy to treat ACD when used in combination with stem cell treatment.

Conclusion

In conclusion, sodium butyrate combined with BM-MSCs may reconstruct the detrusor smooth muscle layer as well as improve detrusor contractility and micturition function via their direct injection into the bladders of ACD rats. Our findings suggest that sodium butyrate in combination with BM-MSCs may be a novel strategy to treat ACD by repairing the impaired bladder and reconstructing bladder tissues.

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Disclosure of conflict of interest

None.

Authors' contribution

SPY carried out the molecular genetics studies, participated in the sequence alignment, and drafted the manuscript. ZSZ participated in the design of the study and performed the statistical analysis. JZ conceived of the study, participated in the design and coordination of the study, and helped to draft the manuscript. All authors read and approved the final manuscript.

Abbreviations

ACD, Acontractile detrusor; BM-MSCs, Bone marrow-derived mesenchymal stem cells; PBS, Phosphate-buffered saline; MSCs, Mesenchymal stem cells; SD, Sprague-Dawley; SMCs, Smooth muscle cells; PCR, Polymerase Chain Reaction; α-SMA, α-smooth muscle actin; SM-MHC, Smooth muscle myosin heavy chain.

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